Enzymatic Kinetic Resolution of Amines

Enzymatic Kinetic Resolution of Amines

Proefschrift

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Hilda ISMAIL

Magister Sains Farmasi
Universitas Gadjah Mada, Indonesia,
geboren te Yogyakarta, Indonesië

Dit proefschrift is goedgekeurd door de promotor:

Prof. dr. R. A. Sheldon

Toegevoegd promotor: Dr. ir. F. van Rantwijk

Samenstelling promotiecommissie:

Rector magnificus	Voorzitter
Prof. dr. R. A. Sheldon	Technische Universiteit Delft, promotor
Dr. ir. F. van Rantwijk	Technische Universiteit Delft, toegevoegd promotor
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to the memory of my beloved parents:
Ismail Isa and Zakiah Nawi

Contents

Chapter 1	Introduction	1
Chapter 2	(R)-Phenylglycine Ester as Acyl Donor for The Fully Enzymatic Resolution of Amine	27
Chapter 3	Effect of β-Heteroatom in The Acyl Donor	43
Chapter 4	Pyridylacetate Ester as Acyl Donor for The Fully Enzymatic Resolution	63
Chapter.5	Improved Acyl Donor: The Pyridyloxyacetate Ester	81
Chapter 6	Dynamic Kinetic Resolution of Amines	93

1

Introduction:

Preparation of Chiral Amines

Enantiomerically pure intermediates are gaining importance in the fine chemical and pharmaceutical industries. Especially in the pharmaceutical industry, the demand for enantiomerically pure intermediates has significantly increased since it became known that biological activities are often influenced by the molecular configuration[1]. The introduction of enantiopure compounds was also enforced by stricter regulations of the US Food & Drug Administration (FDA) and the European Committee for Proprietary Medicinal Products. Since 1992, the authorities have stipulated that the physiological action of each enantiomer of a pharmaceutical product must be individually characterized, followed by a program to shorten the registration time for single enantiomer drug, the so-called "*chiral switch*" program[2]. These developments have driven the pharmaceutical industry to change from racemic active compounds to enantiopure ones. Accordingly, it was reported that sales of enantiomerically pure intermediates are increasing about 7-8% annually, and nowadays about 80% of the active pharmaceutical ingredients (APIs) in the pipeline are chiral[3,4].

Chiral amines are highly valuable intermediates in the fine chemical and pharmaceutical industries. They are used as resolving agents, chiral auxiliaries, and chiral synthetic building blocks. A number of methods for the production of enantiopure chiral amines are known, and some of these will be briefly reviewed in this Chapter. In general the methods are grouped as in the following scheme:

1. Abiological methods

a. Crystallization of diastereomeric salts
b. Enantioselective imine or enamine reduction
c. Enantioselective olefins hydroamination
d. Asymmetric synthesis *via* a 2-methyl-2-propanesulfinamide intermediate
e. Separation of racemates by preparative HPLC

2. Biotechnological methods

a. Enzymatic kinetic resolution catalyzed by:
    1. Serine hydrolases: lipases, subtilisins, and penicillin acylases
    2. Monoamine oxidases
    3. Transaminases
b. Asymmetric synthesis catalyzed by transaminases

1. Abiological methods

1a. Crystallization of diastereomeric salts.

The crystallization of diastereomeric salts is still of considerable importance today in the isolation of enantiopure amines. The technique involves a reaction of the racemic amine with a chiral carboxylic acid, to give diastereomeric salt mixtures that are separated by crystallization (Figure 1).

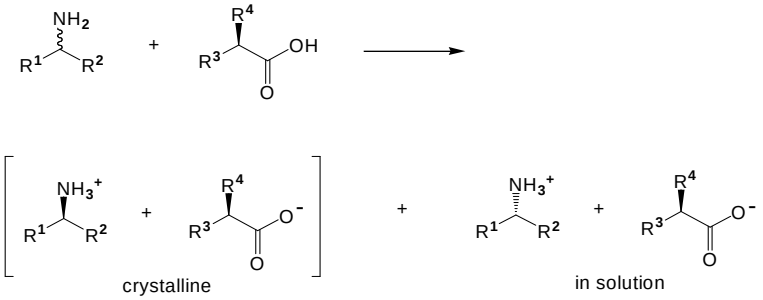

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Figure 1. Clasical resolution of racemate through fractional crystallization of salts of racemic amines with enantiopure carboxylic acid

Production of enantiopure (*R*) or (*S*)-1-phenylethylamine (>95% *ee*), for example, was accomplished on an industrial scale using this method[3] (Figure 2). Resolving agents were typically (*S*)-malic acid, (*R*)-mandelic acid, (*R,R*)-tartaric acid, or L-phenylcarbamoyl-lactic acid. The latter acid is known to be a versatile resolving agent, which can be prepared readily and cheaply from commercially available L-lactate.

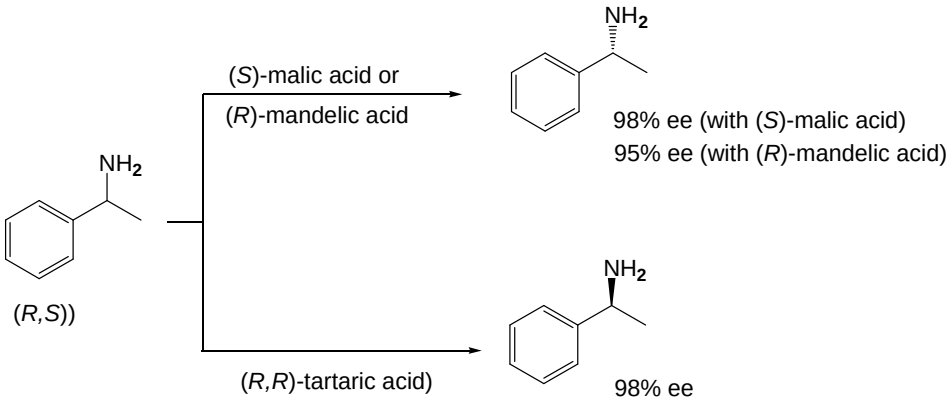


Figure 2. Resolution of (*R,S*)-1-phenylethylamine

The disadvantages of this method are the large amounts of solvents used, the requirement for recovery and recycling of the resolving agent, and the racemization of the unwanted amine enantiomer.

1.b. Enantioselective reduction of an imine or enamine

The method is based on the reduction of a prochiral precursor containing a C=N or C=C-N bond as illustrated in Figure 3.

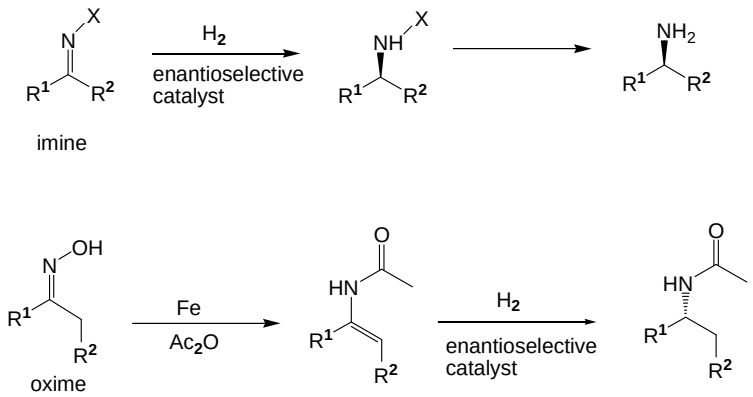


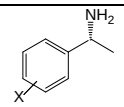
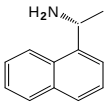
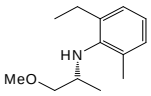
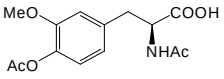
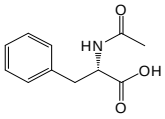
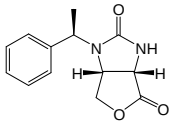
Figure 3. Asymmetric synthesis of chiral amines by the reduction of an imine and an enamine

Schiff's bases formed in a reaction of an amine with a carbonyl compound are used as the precursor, which is subsequently asymmetrically reduced to the amine. The outcome of this method is accordingly determined by the activity and enantioselectivity of the hydrogenation catalyst. Such enantioselective catalysts are usually a combination of transition metal catalysts with chiral ligands[5]. An extensive research effort has resulted in diverse sets of chiral ligands (*chiral toolbox*). The use of rhodium, ruthenium, and iridium in combination with certain chiral ligands are reported to catalyze the enantioselective hydrogenation of enamides and imine[6,7,8,9].

Major progress in the development of the enantioselective hydrogenation catalysts has led to their large-scale application in enantioselective hydrogenation of amine precursors[6]. *Ciba-Geigy/Novartis* is now producing an intermediate for the (*S*)-metolachlor herbicide on a scale of >10,000 t/a. In the reaction; a new iridium complex with ferrocenyl diphosphine ligands is used as the hydrogenation catalyst[10,11]. Other examples are presented in Table 1. The imine formation and enantioselective reduction were performed in one synthetic step in the synthesis of cyclic chiral amines, giving 85% yield with *ee* of 88% [12].

However, the need for large libraries of chiral ligands, modifiers and precursors, is a major drawback in the application of this method on industrial scale. Besides, it is known that the chiral ligands and metal precursors are usually expensive and not easily available[6].

Table 1. Production of chiral amines via imine reduction[5, 6]

	Chiral amine	Producer	Ligand/catalyst	ee / prod. scale
1		*Solvias and Avecia*	Rh / josiphos	99%
2		*Avecia*	Rh / CATHy	98%
3	 Intermediate for (*S*)-metolachlor herbicide	*Ciba-Geigy / Solvias*	Ir / josiphos4	80% 10000 t/a
4	 Intermediate for L-DOPA	*Monsanto*	Rh / Dipamp	95% 1 t/a
5	 Intermediate for aspartame	*Enichem / Anic*	Rh / Eniphos	83% 15 t/a
6	 Intermediate for biotin	*Lonza*	Rh / Josiphos2	*de* 99% multi ton/a

1.c. Hydroamination of allylic compounds.

A method to produce branched secondary or tertiary amines *via* amination of achiral allylic compounds has recently been developed[13]. The procedure involves enantioselective amination of achiral allyl carbonates or allyl acetate using an iridium phosphoramidite complex as the catalyst (Figure 4). The new method has been reported to give secondary or tertiary chiral amines in high yield and with excellent enantioselectivity[8,14]. Research on the improvement of this method is still in progress, and its application on industrial scale has not yet been reported.

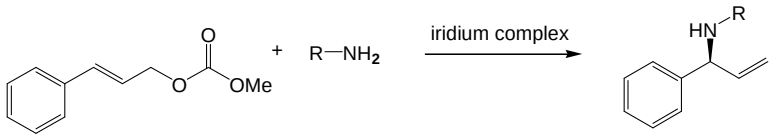


Figure 4. Hydroamination of allyl carbonate catalyzed by iridium complex.

1.d. Asymmetric synthesis *via* 2-methyl-2-propanesulfinamide.

A wide range of highly enantioenriched amines, including α-branched and α,α-dibranched amines were reported to be efficiently synthesized *via* the formation of 2-methyl-2-propanesulfinamide. This versatile reagent is prepared from *tert*-butyl disulfide by a two step asymmetric synthesis procedure in the presence of a chiral ligand and hydrogen peroxide as the oxidant (Figure 5).

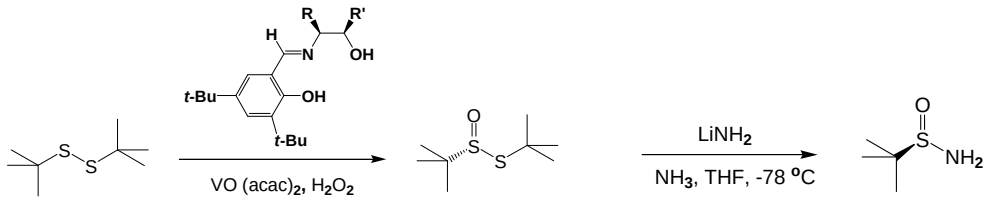


Figure 5. Preparation of enantiomerically pure 2-methyl-2-propanesulfinamide

The direct condensation of 2-methyl-2-propanesulfinamide with aldehydes and ketones provides the 2-methyl-2-propanesulfinyl in high yields, which upon further diastereoselective transformation leads to the desired amines after cleavage of the sulfinyl groups[15] (Figure 6).

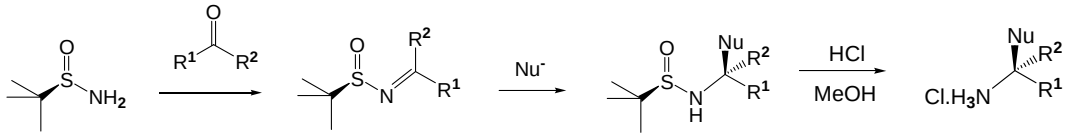


Figure 6. Synthesis of branched amines starting from 2-methyl-2-propane-sulfinamide.

The latter method is often applied in small-scale syntheses such as drug discovery, for example, but not yet on production scale[16].

1.e. Separation of racemates through HPLC

Separation by chiral HPLC using simulated moving bed technology is in an emerging stage. Compared to the diastereomeric crystallization methods that can be applied on any scale, HPLC is restricted to a relatively small scale and is probably most important in the early phase of product development[5]. This method is however considered as expensive, since large amounts of solvent are needed. Examples are for the resolution of levetiracetam and ethyl-2-oxo-1-pyrrolidineacetamide, which allowed the application on industrial scale[17, 18].

2. Biotechnological procedures

The industrial application of biological catalysts has expanded dramatically in the last twenty years[19], and enzymes are now used in very diverse industrial sectors. In recent years, biocatalysts have increasingly been adopted by the fine-chemicals industries, in particular for the manufacture of stereoisomerically pure products[20]. Some useful features of enzymes regarding their practical application are[21]:

1. The very high efficiency in catalyzing diverse and complex organic reactions (10^8 to 10^{12} time faster than the un-catalyzed reaction),
2. The capability to catalyze the reactions under mild reaction conditions
3. Their high substrate specificity
4. Their inherent enantioselectivity
5. Enzymes can be produced on a large scale, and can be directly applied as cell free extracts or as immobilized preparations.
6. Enzymes are biodegradable.

Enzymes are also known to have some disadvantages, such as their instability as protein molecules in aqueous medium, their inactivation by high temperatures, extreme pH, organic solvents, or proteases, and the

phenomenon of substrate/product inhibition. However, research on engineering of enzymes or engineering of biocatalytic processes has resulted in siginificant advancements and nowadays biocatalysis has become an established technology for industrial manufacture of fine chemicals.

Enzymatic Kinetic Resolution

Enzymatic kinetic resolution is a well-developed approach towards chiral amine resolution, and is now industrially practiced on a scale of more than 1000 t/a[22]. Mainly lipases are employed industrially, but penicillin acylases, transaminases and nitroreductases have also been studied.

Enzymatic kinetic resolution is based on the capability of the enzyme to discriminate the substrate enantiomers. When the catalyst is enantioselective, the two enantiomers are converted into the corresponding product enantiomers with different reaction rates. The ratio of the enantiomer reaction rates (v_A and v_B) is the *enantiomeric ratio* (*E*), which represents the enantioselectivity of the enzyme for the two enantiomers. Since the reaction rate is governed by the V_{max} and K_m, *E* can be expressed as equation (1):

$$E = \frac{v_A}{v_B} = \frac{V_{max}^A / K_m^A}{V_{max}^B / K_m^B} \qquad (1)$$

Enantiomeric purities are expressed as enantiomeric excess (*ee*). The slow reacting enantiomer accumulates in the unconverted substrate. As the reaction proceeds, the enantiomeric purity of the substrate increases, whereas that of the product decreases. The correlation between *ee* and the conversion is illustrated in Figure 7 [23].

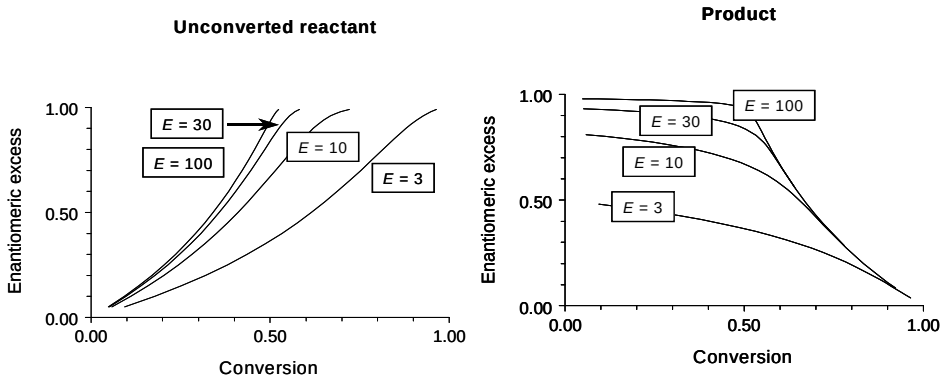


Figure 7. Plot of the enantiomeric excess of substrate or product as a function of the conversion for various enantiomeric ratios.

Several methods to determine *E* from the conversion and the enantiomeric excess of the substrate or product are presented in the equations (2), (3) and (4)[24]. Equations (2)-(4) represent the *E* determination based on the enantiomeric excess of substrate (ee_s) and conversion (C), on the enantiomeric excess of product (ee_p) and conversion, and on the enantiomeric excess of substrate and product, respectively.

$$E = \frac{\ln [(1- C) (1 - ee_s)]}{\ln [(1- C) (1 + ee_s)]} \qquad (2)$$

$$E = \frac{\ln [1- C (1 + ee_p)]}{\ln [1- C (1 - ee_p)]} \qquad (3)$$

$$E = \frac{\ln [(1- ee_s) / (1 + ee_s / ee_p)]}{\ln [(1+ ee_s) / (1 + ee_s / ee_p)]} \qquad (4)$$

In this thesis, *E* was calculated via numerical integration of equation (1) by fitting the experimental point sets of ee_s versus C.

2.1. Serine hydrolases as catalysts:

a. Lipase

Lipases are the most widely used biocatalysts in organic synthesis. The attractive feature of lipases is their high catalytic activity and stability in organic solvents[25]. In particular CaLB (lipase B from *Candida antarctica*), which seems to prefer anhydrous conditions, has been widely used in consequence. Lipases are serine hydrolases and possess the same catalytic machinery as the serine proteases, which consist of a catalytic triad and an oxyanion hole. The reaction mechanism can be characterized as a bi-bi ping-pong mechanism that takes place in two steps as illustrated in Figure 8 [26,27].

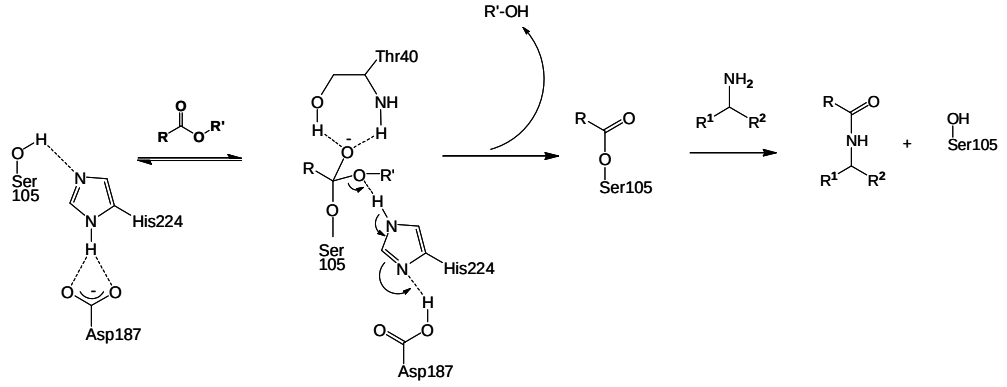


Figure 8. Reaction mechanism of lipase catalysis (numbering is for *C. antarctica* lipase B)

The catalytic triad of lipases consists of a serine residue that is activated by histidine and aspartate residues. In the first step, the ester reacts with the OH group of the catalytic serine residue to form a tetrahedral acyl-enzyme intermediate. The resulting excess of the negative charge on the carbonyl oxygen is stabilized by the oxyanion hole. In the second step, the enzyme-acyl intermediate collapses to the serinate ester with elimination of the alcohol. Subsequent reaction of the serinate ester with a nucleophile affords the product and free enzyme. In the case of hydrolysis the nucleophile is water, which in the amine acylation is replaced by the amine affording the corresponding amide as the product[28].

In most lipases, a mobile element that consists of one or two α-helices ('lid') covers the active site. In the active form, the 'lid' is moved away and makes the active site accessible for the substrate. CaLB is known to have a small lid, although do not show interfacial activation[29]. The catalytic machinery of lipases, as well as that of the other proteases, is located at the bottom of a binding pocket. The substrate specificity is reflected in the shape of the acyl binding site. The lipase B from *Candida antarctica* (CaLB) is known to have a small lid and a long, funnel-like binding site. This substrate-binding pocket is an elliptical, steep funnel of 9.5 x 4.5 Å[30]. The substrate is oriented parallel to its long axis, hence the chain length of the acyl donor was reported to strongly influence the enantioselectivity[31,32]. The kinetic resolution of chiral amines through lipase-catalyzed acylation is illustrated in Figure 9.

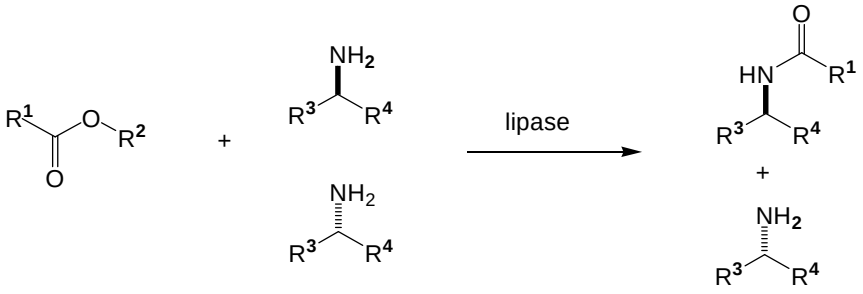


Figure 9. The lipase-catalyzed enantioselective acylation of a chiral amine.

The utilization of lipases in the resolution of chiral amines is rapidly increasing. For example, BASF applies lipase-catalyzed resolution of amines at a scale of over 1000 t/a[22]. Chiral amines that have been resolved *via* lipase-catalyzed kinetic resolution are presented in Figure 10 and Table 2.

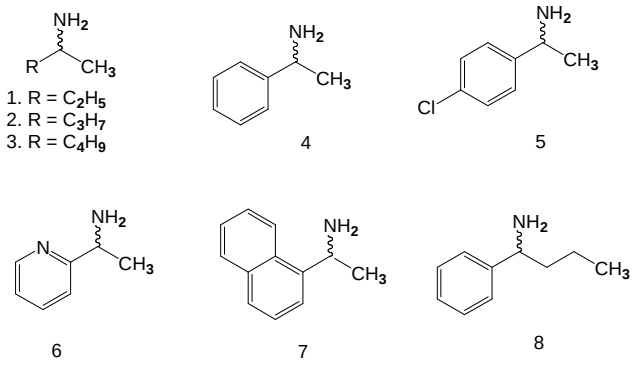


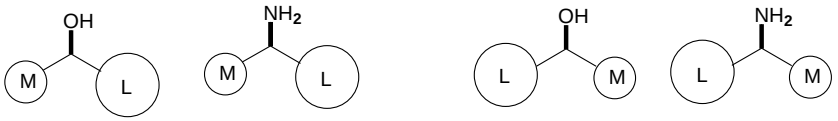
Figure 10. Examples of chiral alkyl- and arylalkylamine that can be resolved through lipase-catalyzed acylation

Table 2. Resolution of chiral alkyl- and arylalkylamines[28].

Amine	Acyl donor	Reaction conditions (solvent / temp. ^{o}C)	E
1	Dimethylsuccinate	Dioxane / 30	34
2	Ethyl acetate	Ethyl acetate / rt	>100
3	Ethyl acetate	Ethyl acetate / 21	> 31
4	Isopropyl methoxyacetate	MTBE / rt	> 1000
5	Methyl methoxyacetate	TAME / 40	> 458
6	Ethyl acetate	Ethyl acetate / 30	66
7	Isopropyl acetate	DME / rt	650
8	1-phenylethyl acetate	Dioxane	60

b. Subtilisin

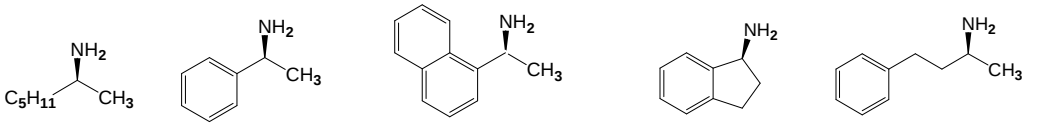
Subtilisin is a serine protease; the active site architecture is similar to that of lipases but as a mirror image. Hence, lipases and subtilisin have opposite enantioselectivity (Figure 11)[33].



lipases subtilisin

Figure 11. Empirical rule to predict the enantiopreference of lipases and subtilisin towards secondary alcohols and amines.
M = medium size substituent; L= large substituent.

Enantioselective acylation catalyzed by subtilisin has been applied to the resolution of several chiral amines (Figure 12). Due to the slow acylation rate, usually an activated acyl donor, for example 2,2,2-trifluoroethyl butyrate, is used. Similar to lipases, the enantioselectivity of subtilisin-catalyzed reactions is influenced by the solvent[34,35,36]. It would seem that the uncatalyzed background reaction accounts for some of the solvent effect[28].



(*S*)-alkylamine (E = 62) (*S*)-phenylethylamine (E= 19) (*S*)-1-(naphtyl)-ethylamine (E = 146) (*S*)-aminoindane (E > 21) (*S*)-1-phenylbutylamine (E = 33)

Figure 12. Preferred enantiomers of alkyl and arylalkylamines for the enantioselective acylation catalyzed by subtilisin

Like lipases, subtilisin is highly stable and maintains its activity in anhydrous media. Subtilisin has been immobilized through adsorption on glass beads or Accurel EP 100, which allows its application on a larger scale[37]. The potential of subtilisin in amine resolution is illustrated by the preparation of (*R*)-1-(naphthyl)ethylamine and (*R*)-1-aminoindane on a scale of 1.6 kg and 300 g; the amines were obtained with *ee* >90% and >95%, respectively[37].

c. Penicillin acylase

Penicillin acylase is a serine hydrolase with a catalytic mechanism similar to that of lipases and the serine proteases[38]. Structurally, penicillin acylase belongs to the class of *N*-terminal nucleophilic hydrolases that have no catalytic triad but an *N*-terminal serine that is activated by a water-bridging molecule (Figure 13)[39].

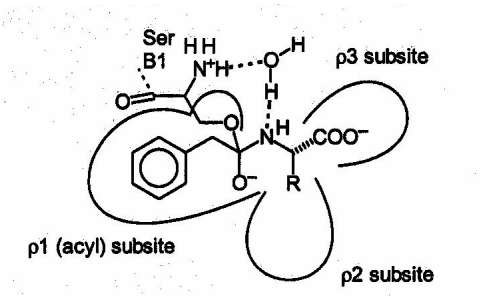


Figure 13. Schematic depiction of the active site of penicillin acylase;
--- denotes a hydrogen bond

Studies on penicillin acylase from *E. coli* revealed the acyl donor-binding site of penicillin acylase has a very high affinity for a phenylacetyl moiety (penicillin G acylase) or a phenoxyacetyl moiety (penicillin V acylase)[40,41,42], while the acyl acceptor binding site accepts a wide range of nucleophiles[43]. To apply this enzyme in the preparation of chiral amines, the choice of substrate is restricted by the substrate tolerance; accordingly simple esters of phenylacetic acid or phenylacetamide are obvious choices. Other acyl compounds that are more soluble in water, such as esters or amides of (L)-mandelic acid and (*R*)-phenylglycine have been investigated and were reported to be good acyl donors as well[44,28].

Penicillin acylases are fragile enzymes that lose their catalytic activity upon dehydration, and in consequence some water must be present during the reactions. Alternatively, the reaction can be carried out in an aqueous medium, and some examples of penicillin acylase in amine resolution concern enantioselective hydrolysis of the amide.

Method through enantioselective amine acylation in aqueous medium has also been demonstrated. In this latter reaction, the relatively unknown penicillin acylase from *A. faecalis* was reported to be more stable and showed higher enantioselectivity than that of *E. coli*[45]. The kinetic resolution via enantioselective acylation is hampered by the competing hydrolysis of the acyl donor. In an aqueous medium, three mechanisms contribute to the outcome of the reaction (Figure 14)[46].

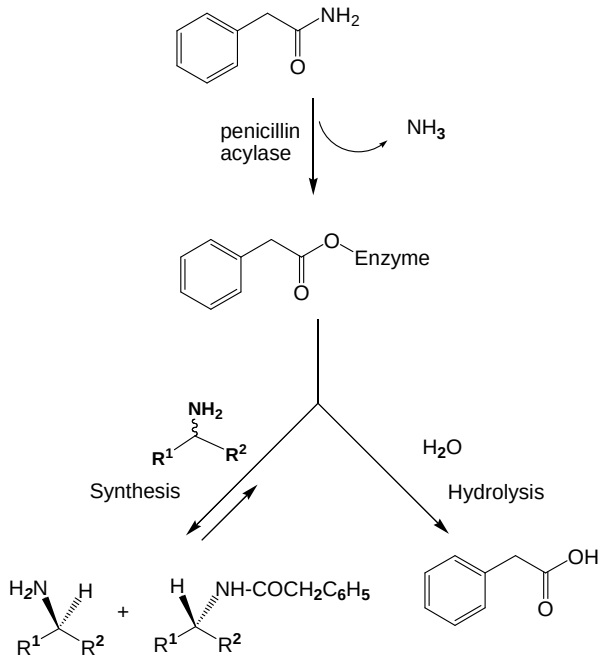


Figure 14. Enantioselective amine acylation in aqueous medium catalyzed by penicillin acylase

In the first step, water and amine compete for the acyl donor. Furthermore, the enantiomerically enriched acylated amine can be hydrolyzed, causing a rapid erosion of the product *ee*. Hence, the key to efficient amine acylation is maintaining a full kinetic control, by using an excess of acyl donor and/or by applying a highly activated acyl donor that monopolises the active site[28].

Some alkyl- and arylamines (Figure 15) have been prepared through the hydrolysis of the corresponding phenylacetamides, or through kinetically controlled enantioselective acylation in aqueous media[47].

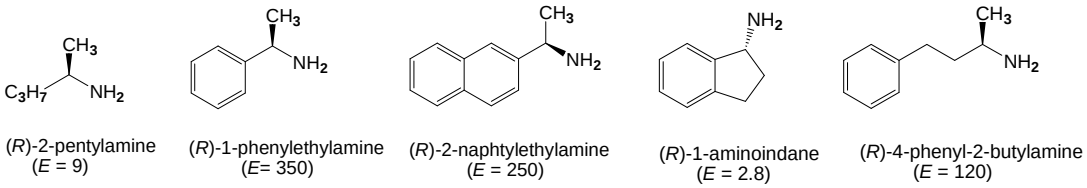


Figure 15. Preferred enantiomers of alkyl- and arylalkylamines for the enantioselective acylation in aqueous medium catalyzed by penicillin acylase *A. faecalis*[28]

2.2. Monoamine oxidase-mediated amine resolution

In this method, the resolution approach relies on the coupling of the enantioselective oxidation catalyzed by monoamine oxidase, followed by non-selective reduction of the imine to effect the inversion of (*S*) to (*R*)-enantiomer via an achiral imine[48] (Figure 16). In the process, the (*S*)-enantioselective monoamine oxidase oxidizes the *S*-amine to the corresponding imine, which is subsequently reduced *in situ* to give racemic amine again. The repeated cycles result in accumulation of the (*R*)-enantiomer in high yield and enantiomeric excess[49].

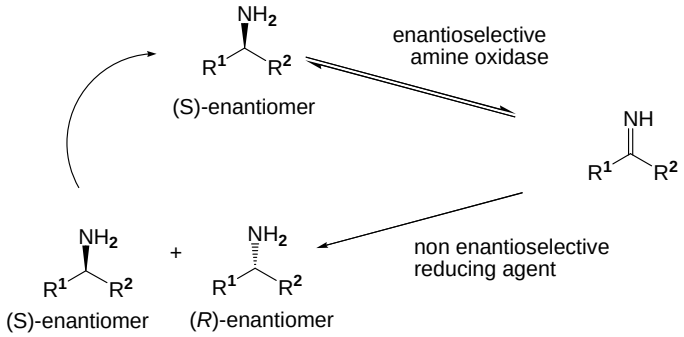


Figure 16. Deracemization of amine by applying enantioselective oxidation in combination with non-enantioselective reduction of the imine.

The choice of the reducing agent and the monoamine oxidase are the key factors that determine the efficiency. Regarding the reaction conditions, the ammonia borane complex ($NH_3.BH_3$) was found to be the appropriate reductant[50]. Enzyme tailoring through directed evolution has been studied to get the variant of enzyme with higher activity and broader substrate specificity. Some variants that are able to oxidize a wide range of chiral primary, secondary and tertiary amines with high enantioselectivity have been reported[48,51]. So far, the development of the method is still ongoing. One of the success examples is the preparation of (*R*)-N-methyl-2-phenylpyrrolidine on a preparative scale, in which 75% isolated yield was obtained after 24 h (*ee* 99%)[49]. However, this is not yet applied on an industrial scale.

2.3. Transaminase-mediated amine kinetic resolution

The process is based on the transamination reaction, in which an amino group is transferred from an amino donor to a carbonyl compound in the presence of transaminase. Since the enzymatic transamination was found to be enantioselective, this enzyme is applied in the resolution of chiral amines. One known example is the ω-transaminase from *B. thuringiensis* JS64, which is highly enantioselective for (*S*)-1-phenylethylamine. Here, the (*S*)-enantiomer will be enantioselectively transaminated, leaving the unreacted (*R*)-1-phenylethylamine with high enantiopurity [3] (Figure 17).

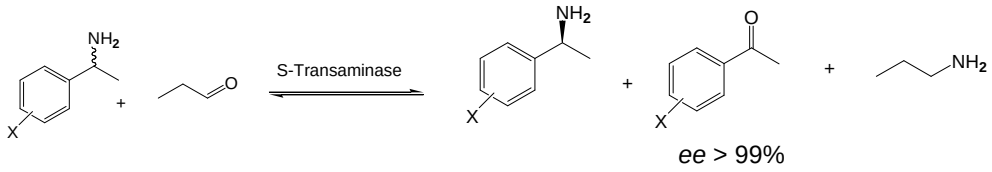


Figure 17. Kinetic resolution of substituted 1-phenylethylamine catalyzed by (S)-transaminase

The drawback of the enzymatic transamination is the low conversion of the reaction due to the reversible interconversion between substrate and product[52]. Besides, the side product ketone is known to inhibit the

transaminase[53]. Hence, in principle the reaction can only be carried out either in a biphasic system (water and organic solvent) or in an aqueous system, though in consequence, only low product concentration can usually be attained with hydrophobic substrates[3]. Some improvements have been sought by developing new enzymes[54], or by applying a enzyme membrane reactor system[55].

2.d. Transaminase-mediated asymmetric synthesis of amine

Another application of transaminases is to catalyze the asymmetric synthesis of chiral amines, which in theory takes place with 100% yield of enantiomerically pure product. Recently, researchers at Celgene have developed both *S*- and *R*-selective transaminases, which make both amine enantiomers accessible by the method[56]. An industrial application is the synthesis of metolachlor and imethenamide herbicide, in which the intermediate (*S*)-methoxyisopropylamine ((*S*)-MEOIPA) is synthesized *via* transamination of methoxyacetone with isopropylamine (Figure 18). By this method, 97% conversion was reached within 7 h with high enantioselectivity (*ee* >99%)[57,58]. Nowadays, this technology has already been used in industrial practice on a 2.5 m scale[3].

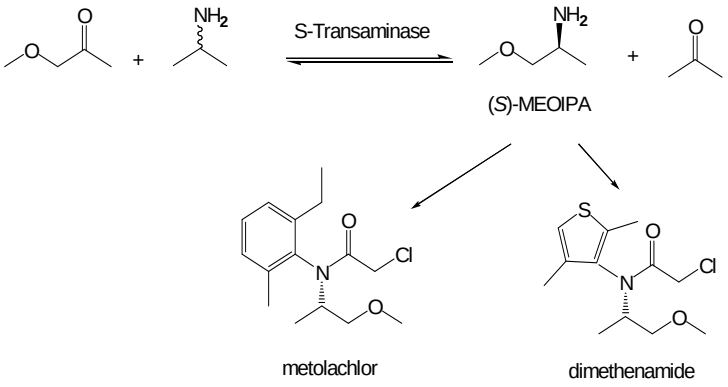


Figure 18. Synthesis of metolachlor and dimethenamide.

Dynamic Kinetic Resolution

Enzymatic kinetic resolution remains one of the easiest methods to produce enantiopure amines[3], however only 50% maximum yield can be obtained *via* this method. Dynamic kinetic resolution (DKR) is an approach to overcome the 50% yield limit, by recycling the unwanted enantiomer *in situ* through a racemization process (Figure 19).

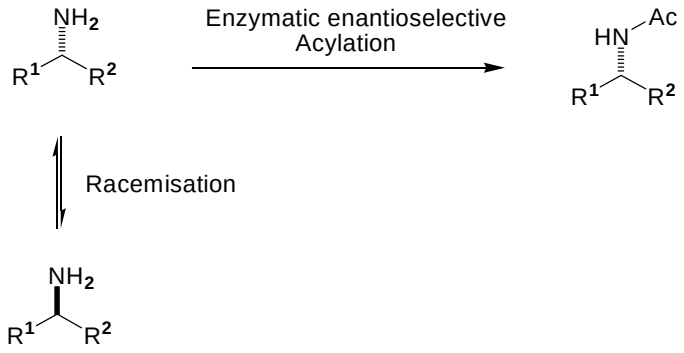


Figure 19. The dynamic kinetic resolution of amine

The amine racemization is now usually carried out in a separate step. Some known amine racemization methods are: thermal racemization, base- or acid-catalyzed racemization and oxidation-reduction based racemization[1]. The latter method is the most used one in the racemization of amines, which is achieved by performing the oxidation and reduction in separate steps. In the first step an imine or immonium species is generated, then reduction in the second step leads back to the amine[59] (Figure 20). Racemization of amine with some transition metal based has been reported[60,61,62].

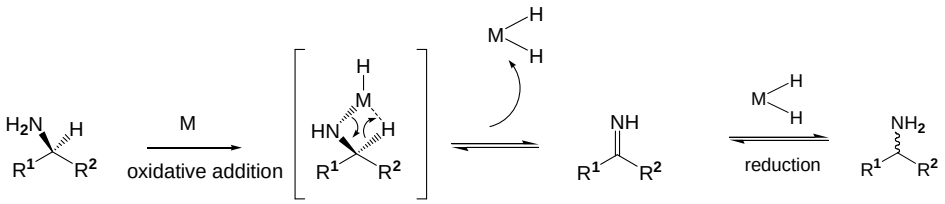


Figure 20. The mechanism of the metal catalyzed racemization of amine

Compared to the DKR of alcohols, which is already well developed, reports on the dynamic kinetic resolution of amines are still scarce. This is presumably because amine racemization requires harsh reaction conditions and accordingly is difficult to combine with the enzymatic resolution. Thus, for example, BASF disclosed racemization of the unwanted enantiomer *via* Schiff's base derivative after isolating the undesired enantiomer[3, 22].

Among those racemization methods, the redox racemization is the most readily compatible with the enzymatic acylation. Some known procedures that combine in situ redox racemization with the kinetic resolution generally involve a palladium[63,64,65], ruthenium[66], or iridium catalyst[67] in combination with lipase. However, a long reaction time, high temperature or excessive amounts of catalyst are needed, which revealed the difficulties in the racemization step.

Scope of the thesis: application of a fully enzymatic amine resolution strategy

Chiral amine resolution *via* enzymatic enantioselective acylation is increasing in significance, relative to other methodologies[29]. The method is nowadays industrially applied at a scale of several thousand t/a[22]. Nevertheless, the subsequent chemical deacylation of the enantiomerically enriched amide presently involves harsh reaction conditions[3], which are incompatible with sensitive functional groups. Hence, a strategy based on enzymatic deacylation at close to neutral pH and ambient temperature would be much preferred. In this respect, a fully enzymatic procedure employing enzymatic reactions in both acylation and deacylation is proposed to overcome these problems (Figure 21).

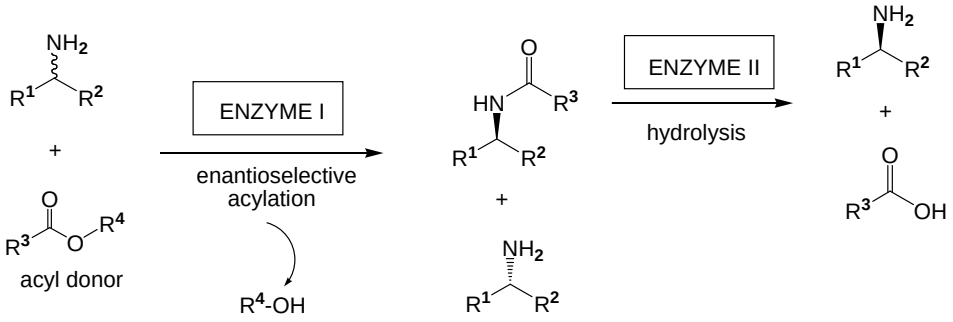


Figure 21. The fully enzymatic method in the resolution of chiral amines.

The proposed strategy involves enantioselective lipase-catalyzed amine acylation followed by penicillin acylase-catalyzed deacylation. Since most enzymes need a specific substrate to fit their active site, the choice of an acyl donor that is compatible with both enzymes becomes important.

This Thesis concerns investigations into the use of non-conventional acyl donors with the objective of complementing enzymatic acylation with enzymatic deacylation. In Chapter 2, an ester of the amino acid (*R)*-phenylglycine was employed as the acyl donor, on the basis of the consideration that the enzymatic deacylation would be quite facile due to the stability of the liberated zwitter ion. In Chapter 3, acyl donors derived from glycine esters substituted with a β-heteroatom were investigated, as regards their acceptability to both enzymes used in the fully enzymatic strategy.

Since the amide solubility in aqueous medium is often considered to be the cause of the low hydrolysis rate[68], we attempted to improve the amide solubility by applying pyridine derivatives as the acyl donor. Their application is discussed in Chapter 4 and Chapter 5.

Furthermore, a DKR method that allows *in-situ* recycling of the unwanted enantiomer, in parallel with the kinetic resolution, is presented in Chapter 6. Considering that the racemization rate is determining[69], a highly active racemization catalyst is needed to combine with the resolution catalyst. In this

Chapter, an approach by applying a nanoparticle preparation of palladium as the racemization catalyst is investigated.

References

1. Sheldon, R.A., *Chirotechnology **1993***, Marcel Dekker Inc., New York, 50-61.
2. Hutt. A.J., Valentova, *J., Acta Facult. Pharm. Univ. Comenianae 50* **2003**, 7-23.
3. Breuer, M., Ditrich, K., Habicher, T., Hauer, B., Keβeler, M., Stürmer, Zelinski, T., *Angew. Chem. Int. Ed*. **2004**, *43*, 788-824.
4. Graff. G., from *Reed Busines Information*, **2002**, Reed Elsevier, Inc.
5. Blaser, H. U., Splinder, F., Studer, *M., Appl. Catal. A: General* **2001**, *221*, 119-143.
6. Li, W., Zhang, X., *J. Org. Chem*. **2000**, *65*, 5871-5875.
7. Xiao, D., Zhang, X., *Angew. Chem. Int. Ed*. **2001**, *40*, 3425-3428.
8. Uematsu, N., Fujii, A.; Hashiguchi, S., Ikariya, T., Noyori, R., *J. Am. Chem. Soc.* **1996**, *118*, 4916-4917.
9. Tang, W., Zhang, X., *Org. Lett.* **2002**, *4*, 4159-4161
10. Blaser, H.U., Buser, H-P., Coers, K., Hanreich, R., Jalett, H-P., Jelsch, E., Pugin, B., Schneider, H-D., Spindler, F., Wegmann, A., *Chimia* **1999**, *53*, 275.
11. Blaser, H. U., Malan, C., Pugin, B., Spindler, F., Steiner, H., Studer, M., *Adv. Synth. Catal* **2003**, *345*, 103-151
12. Williams, G. D., Pike, R., Wade, C. E, Wills. M., *Org. Lett.* **2003**, *5*, 4227-4230.
13. Ohmura, T., Hartwig, J. F., *J. Am. Chem. Soc*. **2002**, *124*, 15164-15165
14. Kiener, C. A., Shu, C., Incarvito, C., Hartwig, J. F., *J. Am. Chem. Soc.*, **2003**, *125*, 14272-14273
15. Ellman, J. A., *Pure Appl. Chem*. **2003**, *75*, 39-46
16. Prakash, G. K. S., Mandal, M., Olah, G. A., *Angew. Chem. Int. Ed*. **2001**, *40*, 589-590.
17. Futugawa, T., Canvat, J. P., Cavoy, E., Deleers, M., Hamende, M., Zimmerman, V. (UCB, S.A., Daicel Chemical Industries), *US006107492A* (**2000**).
18. Cavoy, E., Hamende, M., Deleers, M., Canvat, J. P. , Zimmerman, V.(UCB, S. A.), *US006124473A* (**2000**).
19. Powell, K. A., Ramer, S. W., Del Cardayré, S. B., Stemmer, W. P. C., Tobin, M. B., Longchamp, P. F., Huisman, G., *Angew. Chem. Int. Ed*. **2001**, *40*, 3948-3959.
20. Panke, S., Held, M., Wubbolts, M., *Curr. Op. Biotechnol*. **2004**, *15*, 272-279
21. Van Damme, E. J., Cerdobbel, A., Soetaert, W., *Chim. Oggi* **2005**, *23*, 47-51.
22. Hieber, G., Ditrich, K., *Chimi. Oggi* **2001**, *19*, 16-20
23. Chen, C. S., Fujimoto, Y., Girdaukas, G., Sih, C. J., *J. Am. Chem. Soc.*, **1982**, *104*, 7294-7299.

24. Straathof, A. J. J., Jongejan, J. A., *Enz. Microb. Technol*. **1997**, *21*, 559-571
25. Dakin, H.D., *J. Physiol*. **1905**, *32*, 199-206.
26. Martinelle, M., Hult, K., *Biochim. Biophys. Acta*. **1995**, *1251*, 191-197
27. Ema, T., *Curr.Org. Chem*. **2004**, *8*, 1009–1025.
28. Van Rantwijk, F., Sheldon, R. A., *Tetrahedron* **2004**, *60*, 501-519
29. Bornscheuer, U.T, Kazlauskas, R. J., *Hydrolases in Organic Synthesis*, **1999,** Willey-VCH Verlag GmbH, Weinhei, Germany, p. 17-18.
30. Pleiss, J., Fischer, M., Schmid, R. D., *Chem. Phys. Lipids*. **1998**, *93*, 67-80
31. Hoff, B. H., Anthonsen, H. W., Anthonsen, T., *Tetrahedron: Asymmetry* **1996**, *7*, 3187-3192.
32. Ottoson, J., Hult, K., *J. Mol. Catal. B: Enzymatic* **2001**, *11*, 1025-1028.
33. Kazlauskas, R. J., Weissfloch, A. N. E., *J. Mol. Catal. B: Enzymatic* **1997**, *3*, 65-72
34. Kitaguchi, H., Fitzpatrick, P.A., Huber, J. E., Klibanov, A. M., *J. Am Chem. Soc*. **1989**, *111*, 3094-3095
35. Nakamura, K., Takebe, Y., Kitayama, T., Ohno, A., *Tetrahedron Lett*. **1991**, *32*, 4941-4944
36. Tawaki, S., Klibanov, A. M., *J. Am. Chem. Soc*. **1992,** *114*, 1882-1884
37. Gutman, A. L., Meyer, E., Kalerin, E., Polyak, F., Sterling, *J., Biotechnol. Bioeng.* **1992**, *40*, 760-767.
38. Alkema, W. B., Dijkhuis, A. J., De Vries, E., Janssen, D. B., Eur. *J. Biochem*. **2002**, *269*, 2093-2100
39. Duggleby, H. J., Tolle, s. P., Hill, C.P., Dodson, E. J., Dodoson, G., Moody, P. C. E., *Nature* **1995**, *373*, 264-268.
40. Huang, H. T., Seto, T. A., Shull, G. M., *Appl. Microbiol*. **1963**, *11*, 1-6.
41. Cole, M., *Biochem, J*. **1969**, *115*, 733-739
42. Vanderhaeghe, H., Claesen, M., Vlietnick, A., Parmentier, G., *Appl. Microbiol*. **1968**, *16*, 1557-1563.
43. Kaufmann, W., Bauer, K., *Nature* **1964**, *203*, 520
44. Lafuente, R. F., Rosell, C. M., Guisan, J. M., *Enzyme Microb. Technol*. **1998**, *22*, 538-587
45. Van Langen, L. M., Oosthoek, N. H. P., Van Rantwijk, F., Sheldon, R.A., *Adv. Synth. Catal*. **2003**, *345*, 797-801
46. Kasche, V., *Enzyme Microb. Technol*. **1986**, *8*, 4-16.
47. Guranda, D. T., Van Langen, L. M., Van Rantwijk, F., Sheldon, R. A., Svedas, V. K., *Tetrahedron Asymm*. **2001**, *12*, 1645-1650.
48. Carr, R., Alexeeva, M.., Enright, A., Eve, T. M. S. C., Dawson, M.J., Turner, N. J., *Angew. Chem. Int. Ed.* **2003**, *42*, 4807-4810.

49. Dunsmore, C. J., Carr, R., Fleming, T., Turner, N. J., *J. Am. Chem. Soc.* **2006**, *128*, 2224-2225.
50. Alexeeva, M., Enright, A., Dawson, M. J., Mahmoudian, M., Turner, N. J., *Angew. Chem. Int. Ed*. **2002**, *41*, 3177-3180
51. Carr, R., Alexeeva, M., Dawson, M. J., Gotor-Fernández, V., Humphrey, C. E., Turner, N. J., *ChemBioChem*. **2005**, *5*, 637-639
52 Shin, J. S., Kim, B. G., *Biotechnol. Bioeng*. **1998**, *60*, 534-540
53. Shin, J. S., Kim, B. G., *Biotechnol. Bioeng*. **1999**, *65*, 206-211
54. Pannuri, S., Kamat, S. V., Garcia, A. R. M. (Banner & Witcoff), *US 2006/0228 2778 A1* (**2006**).
55. Shik, S. J., Gee, K. B., Andreas, L., Christian, W., *Biotech. Bioeng*. **2001**, *73*, 179-187.
56. Wu, W., Bhatia, M. B., Lewis, C. M., Lang, W., Wang, A.L., Matcham, G. W. (Celgro), *WO 99/46398* (**1999**)
57. Matcham, G., Bhatia, M., Lang, W., Lewis, C., Nelson, R., Wang, A., Wu, W., *Chimia* **1999**, *53*, 584-589.
58. Zaks, A., *Curr. Opin. Biotechnol*. **2001**, *5*, 130-136.
59. Ebbers, E. J., Ariaans, G. J. A., Houbiers, J. P. M., Bruggink, A., Zwanenburg, B., *Tetrahedron* **1997**, *53*, 9417-9476.
60. Samec, J. S. M., Bäckvall, J. E., *Chem. Eur.J*. **2002**, *8* (13), 2955-2961
61. Ell, A. H, Samec, J. S. M., Brasse, C., Bäckvall, J. E., *Chem. Commun*, **2002**, 1144-1145.
62. Riechers, H., Simon, J., Höhn, A., Funke, F., Siegel, W., Nübling, C. (BASF), *USP 006049007A* (**2000**).
63. Reetz, M., Schimossek, K., *Chimia* **1996**, *50*, 668-669
64. Choi Y. K, Kim, M. J., Ahn Y., *Org. Lett.* **2001**, *3*(25), 4099-4101.
65. Parvulescu, A., De Vos, D., Jacobs, P., *Chem. Commun*. **2005**, 5307-5309
66. Paetzold, J., Backval, J. E., *J. Am. Chem. Soc*. **2005**, *127*, 17620-17621
67. Stirling, M., Blacker, J., Page, M. I., *Tetrahedron Lett.* **2007**, *48*, 1247-1250.
68. Pohl, T., Waldman, H., *Tetrahedron Lett*.**1995**, *36*, 2963-2966.
69. Huerta, F. F., Minidis, A. B. E., Bäckvall, J. E, *Chem. Soc. Rev*. **2001**, *30*, 321-331

2

(*R*)-Phenylglycine Ester as Acyl Donor for Fully Enzymatic Amine Resolution*

ABSTRACT

Fully enzymatic resolution of chiral amines was accomplished by acylation with (*R*)-phenylglycine ester in the presence of *C. antarctica* lipase (CaLB), followed by amide deacylation catalyzed by penicillin acylase from *A. faecalis.* The CaLB-catalyzed acylation of aliphatic amines gave moderate-to-poor enantioselectivity (E<12), while the amine containing an aromatic group was resolved better (E=81). The enzymatic hydrolysis was fast at room temperature (90% conversion in 3.5 h), affording liberated amines with high enantiopurity (*ee*>98%). In conclusion, the proof of principle of the fully enzymatic amine resolution under mild reaction conditions was confirmed.

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*Some part of the works in this chapter was performed in collaboration with R. M. Lau.

INTRODUCTION

As mentioned in Chapter 1, the resolution of chiral alkyl- and arylamines *via* the enantioselective lipase-catalyzed acylation is widely applied[1,2]. The common chemical hydrolysis step, however, requires harsh reaction conditions, which are incompatible with sensitive amines. Accordingly, an enzymatic deacylation at close to neutral pH and ambient temperature would be much preferred. An exclusively enzymatic method for both acylation and deacylation steps would allow milder reaction condition for the resolution of chiral amines. Furthermore, an additional advantage of enzymatic hydrolysis is the possibility to polish the resolution by making use of the enantiopreference of both enzymes, which would increase the enantiopurity of the obtained amine ('*polishing effect*').

In this Chapter, the principle of fully enzymatic strategy is investigated by applying a CaLB-catalyzed acylation in organic solvent, followed by penicillin acylase-catalyzed deacylation in aqueous medium. The scheme is presented in Figure 1.

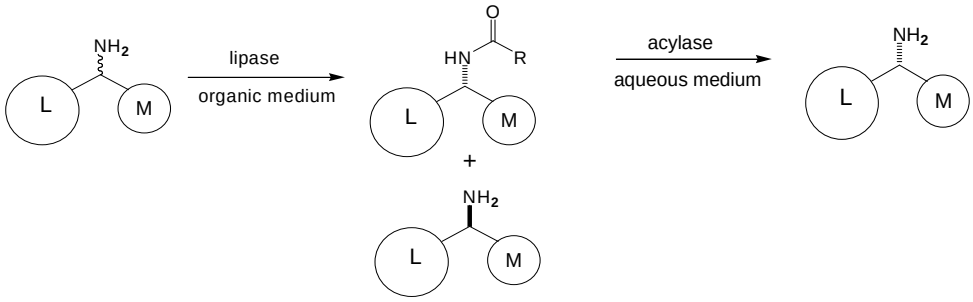


Figure 1. Fully enzymatic resolution with CaLB and penicillin acylase as the catalysts, L = large substituent ; M = medium substituent

Penicillin acylase used in the deacylation step is an amide hydrolase, which is well known for its industrial application in the hydrolysis of the side chain amide bond in penicillin G. Characterization of penicillin acylase from *E. coli* established that the acyl donor-binding site of penicillin acylase has a very high affinity for phenylacetic acid. However, several small α-substituents such as –OH, -NH_2, -F, -Cl, -Br, -OCH_3, -CH3, -CN, and -OCHO are accepted[3,4,5].

Hence, penicillin acylase is known to accept phenylglycine (α-amino phenylacetic acid) derivatives in its active site[5]. The fact that penicillin acylase is able to accept an α-amino acid derivative in its binding site was later found to be an advantage in applying this enzyme in the proposed fully enzymatic strategy.

Making use of an α-aminoacid ester as the acylating agent would lead to an α-aminoacid amide as the product. The enzymatic hydrolysis of this α-aminoacid amide would be quite facile, due to the high stability of the zwitterionic hydrolysis product that renders the hydrolysis reaction energetically favourable. Hence, applying an α-aminoacid ester as acyl donor would make the fully enzymatic methodology feasible. Here we investigated the use of (*R*)-phenylglycine ester as the acyl donor in the resolution of chiral alkyl- and arylamines, as presented in Figure 2.

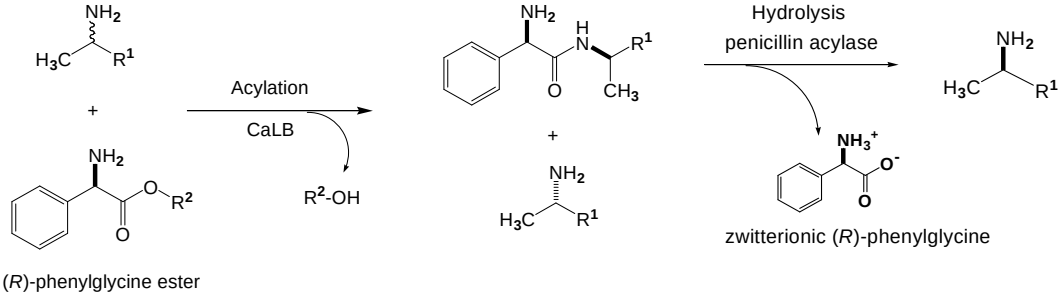


Figure 2. The fully enzymatic resolution of a chiral amine with (*R*)-phenylglycine ester as the acyl donor.

In the acylation step, lipase from *C. antarctica* (CaLB) was used as the catalyst. The ability of CaLB to accept the phenylglycine derivatives has been shown, and enantioselective ammonolysis of racemic phenylglycine methyl ester in *tert*-butyl alcohol showed a modest enantiopreference of CaLB for the (*R*)-phenylglycine methyl ester[6, 7].

In the deacylation step, two penicillin acylases from different sources (from *Escheria coli* and *Alcaligenes faecalis*) were tested as the catalyst. Penicillin acylase from *Alcaligenes faecalis,* a relatively unknown enzyme, was reported

to efficiently mediate the kinetic resolution of chiral amines in aqueous medium[8], and has a similar catalytic mechanism to that of penicillin acylase from *E. coli*[9]. Since this enzyme is known to accept (*R*)-phenylglycine amide as its substrate[10], and its enantiopreference is similar to lipases, combining this enzyme with lipase in the fully enzymatic strategy would appear to be an interesting possibility.

RESULTS AND DISCUSSION

1. Acylation with (R)-phenylglycine esters as acyl donor

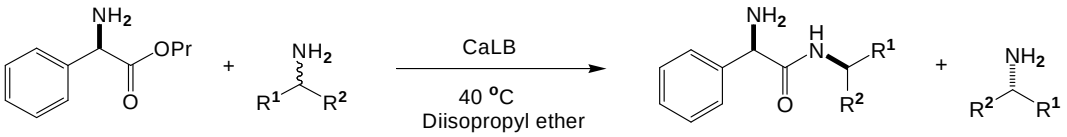
In kinetic resolutions, the leaving group in the acyl donor may affect the rate and enantioselectivity of CaLB[11]. Hence, the rate of the acylation with different esters of (*R*)-phenylglycine in the resolution of 2-heptylamine (**2b**) was compared under similar conditions (see Figure 3). The results are presented in Table 1.

Table 1. Comparison of some (*R*)-phenylglycine esters as acyl donor in the resolution 2-heptylamine[a]

Ester	Time (h)	Conversion (%)
Methyl	144	36
Ethyl	120	32
Propyl	72	41
Butyl	48	20
Isobutyl	48	21

[a]Conditions: Amine (5 mmol), (*R*)-phenylglycine propyl ester (5 mmol), diisopropyl ether (5 mL), catalyst Novozym 435 (100 mg), internal standard 1,3-dimethoxybenzene, molecular sieves 4 A and 5 A (150 mg each). Incubation temperature 40 ℃

The propyl ester was selected for further study on account of its activity. Accordingly, resolution of some other chiral amines (**2a** – **2d**) was carried out using (*R*)-phenylglycine propyl ester as the acyl donor as illustrated in Figure 3, and the results are shown in Table 2.



(1) **(2)** **(3)**

2a) phenylethylamine $R^1 = C_6H_5$ $R^2 = CH_3$
2b) 2-heptylamine $R^1 = C_5H_{11}$ $R^2 = CH_3$
2c) 2-pentylamine $R^1 = C_3H_7$ $R^2 = CH_3$
2d) 2-butylamine $R^1 = C_2H_5$ $R^2 = CH_3$

Figure 3. Acylation of chiral amines with (*R*)-phenylglycine propyl ester in the presence of CaLB as the catalyst.

Table 2. Acylation of chiral amines with (*R*)-phenylglycine propyl ester[a]

Amine	**Time (h)**	**Conv (%)**	***ee*$_{substrate}$ (%)**	***de*$_{product}$ (%)[b]**	***E***
1-phenylethylamine (**2a**)	24	51	96 (*S*)	97(*R,R*)	81
2-heptylamine (**2b**)	5.5	51	73 (*S*)	96(*R,R*)	12
2-pentylamine (**2c**)	3	53	55 (*S*)	94(*R,R*)	5
	3[c]	40	46 (*S*)	n.d.[d]	8.5
2-butylamine (**2d**)	3	50	20 (*S*)	76(*R*)	1.7
	3[c]	38	33 (*S*)	n.d.[d]	4

[a]Conditions: Amine (0.5 mmol), (*R*)-phenylglycine propyl ester (1 mmol), reaction medium diisopropyl ether (5 mL), catalyst novozym 435 (100 mg), internal standard 1,3-dimethoxybenzene, molecular sieves of 4 A and 5 A (150 mg each). Incubation temperature 40 ℃ or room temperature ([c])
[b]after recrystallization in hexane.
[d]n.d. = not determined

Previous experiments with 1 M amine in the presence of the same amount of enzyme, gave very slow acylation (>100 h to reach 50% conversion; data not shown). Hence, a lower concentration of amine (0.1 M) was used and a fast acylation was observed, resulting in 50% conversion of the aliphatic amines in less than 6 h. 1-Phenylethylamine (**2a**) was converted slower, however, and 51% conversion was reached in 24 h. It was clearly observed that longer

aliphatic chains in the amine slow down the reaction. The time course of the acylation of the amines **2a**-**2d** with **1** is shown in Figure 4.

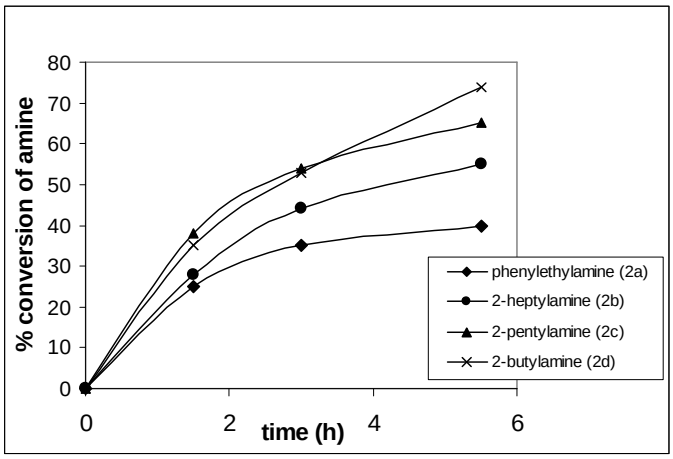


Figure 4. Profile of the acylation of **2a**-**2d** with (*R*)-phenylglycine propyl ester

In general, all amines were resolved with moderate-to-poor enantioselectivity with enantiopreference for the (*R*)-amines as is commonly observed with lipases[12], affording the (*R,R*)-diastereoisomer of the substituted phenylglycin-amide as the major product. 1-Phenylethylamine (**2a**), which was acylated the slowest, showed the best enantiomeric ratio (*E* = 81). It would seem that the enantiorecognition of CaLB is better with amines containing aromatic groups or long aliphatic chains, such as **2b**. The enantioselectivity decreased with shorter aliphatic chains, therefore 2-butylamine (**2d**) was the poorest resolved with *E* = 1.7. Decreasing the reaction temperature (40 ^{o}C) to room temperature slightly increased the enantioselectivity; the enantiomeric ratio (*E*) increased from 5 to 8.5 (**2c**) and from 1.7 to 4 (**2d**). Although the enantioselectivities were low, the diastereomeric purity of the amides was high (de = 97%, 96%, 94% and 76% for **2a**, **2b**, **2c** and **2d,** respectively), suggesting that diastereomeric enrichment takes place during product work-up and purification.

Furthermore, the ability of CaLB to discriminate the enantiomers is known to relate with the structure of the acyl donor[13]. Hence, we also investigated the effect of the α-amino configuration in the acyl donor on the resolution, by

using (*S*)-phenylglycine methyl ester as the acyl donor. The results are presented in Table 3.

Table 3. Acylation of **2a**- **2d** with (S)-phenylglycine methyl ester in the presence of CaLB[a]

Amine	**Time (h)**	**Conversion of amine (%)**	**ee_{amine} (%)**	***E***
1-Phenylethylamine (**2a**)	24	55	~100	100
2-heptylamine (**2b**)	24	50.3	80	21
2-pentylamine (**2c**)	24	58	66	5.3
2-butylamine (**2d**)	24	90	19	2

[a]Conditions: amine (0.5 mmol), (*S*)-phenylglycine methyl ester (1 mmol), reaction medium diisopropyl ether (5 mL), catalyst Novozym 435 (100 mg), 1,3-dimethoxybenzene as internal standard, molecular sieves of 4 A and 5 A (150 mg each), reaction temperature 40 ℃.

Compared to the resolution with (*R*)-**1** (see Table 2), the acylations with the (*S*)-phenylglycine methyl ester were very slow, which is not surprising since (*S*)-phenylglycine derivatives are known as the slow reacting enantiomer with CaLB[7]. Almost all amines need 24 h to reach 50% conversion; while with (*R*)-**1** the same conversions were reached in less than 6 h. The resolution of **2a** and **2b**, however, showed slightly better enantioselectivity than that of (*R*)-**1** (*E*=100 *vs* 81 for **2a**, and 21 *vs* 11 for **2b**), but no improvement was detected in the resolution of **2c** and **2d**. In conclusion, making use of the (*S*)-enantiomer of phenyglycine derivative as the acyl donor does not improve the resolution, and we conclude that (*R*)-phenylglycine propyl ester is the better acyl donor in the CaLB-catalyzed resolution.

2.Deacylation of the (R,R)-phenylglycinamides.

The second step in the fully enzymatic method is the hydrolysis of the obtained amides in the presence of penicillin acylase. Since the relatively unknown penicillin acylase from *A. faecalis* was reported to show better performance than the penicillin acylase from *E.coli*[10], we first compared the activity of these two penicillin acylases in the hydrolysis of *N*-(2-heptylamine)-(*R*)-phenyglycinamide (**3b,** see Figure 5). The reactions were carried out in aqueous phosphate buffer pH 7.0 at room temperature. The results are presented in Table 4.

Table 4. Comparison of penicillin acylase from *E.coli* and *A. faecalis* in the hydrolysis of *N*-(2-heptylamine)-(*R*)-phenyglycinamide[a]

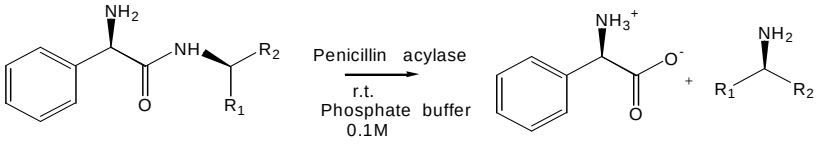
Enzyme	Time (h)	Conv (%)
Penicillin G acylase from *E. coli*	17	10
Penicillin G acylase from *E. coli*, with co-solvent EtOH (20 %).	24	<2
Penicillin G acylase from *A. faecalis*	6	98
Penicillin V acylase from *F. oxysporum*	17	<2

[a]Conditions: amide (5 mg, 0.02 mmol) in phosphate buffer ph 7.0 (3 mL), enzyme: *A. faecalis* penicillin acylase (immobilized, 30 mg; 8.2 BPU), *E.coli* penicillin acylase (30 mg; 9.6 BPU), incubation at room temperature.

We found that *A. faecalis* penicillin acylase was much more active than the *E. coli.* An attempt to increase the hydrolysis rate of the latter enzyme, by addition of 20% ethanol to improve the amide solubility, resulted in deactivation of the enzyme. Consequently, *A. faecalis* penicillin acylase was chosen for further studies of the deacylation step.

The deacylation of the amides **3a**–**3d** was investigated at synthetically relevant concentrations (0.1 M) in phosphate buffer pH 7 (Figure 5). The cross-linked preparation (CLEA) of *A. faecalis* penicillin acylase was used, on account of the high activity of the CLEA preparation in aqueous medium[14]. The amides used had been recrystalized to improve the diastereomeric

excess (*de*) of the (*R,R*)-amides. The crude amide (**3c**[b] in Table 5), which has a lower diastereomeric excess (74% *de*), was also hydrolyzed to study the expected enantio-enrichment of the amine (‘*the polishing effect’*). The results are presented in Table 5.



(3) **(4)**

3a) *N*-(phenylethylamine)-(*R*)-phenyglycinamide ($R^1 = CH_3$ $R^2 = C_6H_5$).
3b) *N*-(2-heptylamine)-(*R*)-phenyglycinamide ($R^1 = CH_3$ $R^2 = C_5H_{11}$).
3c) *N*-(2-pentylamine)-(*R*)-phenyglycinamide ($R^1 = CH_3$ $R^2 = C_3H_7$).
3d) *N*-(2-butylamine)-(*R*)-phenyglycinamide ($R^1 = CH_3$ $R^2 = C_2H_5$).

Figure 5. Deacylation of (*R,R*)-phenylglycinamides catalyzed by *A*. *faecalis* penicillin acylase in aqueous solution.

Table 5. Hydrolysis of the amides catalyzed by *A. faecalis* penicillin acylase[a].

Amide	de_{amide} (%)	Time (h)	Hydrolysis (%)	$ee_{liberated\ amine}$ (%)
3a	97 (*R,R*)	3.5	89	> 99 (*R*)
3b	96 (*R,R*)	3.5	90	99 (*R*)
3c	94 (*R,R*)	7	99	99 (*R*)
3c[b]	74 (*R,R*)	3.5	91	91 (*R*)
3d	76 (*R,R*)	1	84	98 (*R*)

[a]Conditions: amide (0.02 mol) in phosphate buffer pH 7.00 (3 ml), CLEA of *A. faecalis* penicillin acylase (30 mg), incubation at room temperature. Benzamide was used as external standard.
[b]Isolated amide without recrystalization.

We observed that, in general, all enantiopure amides (**3a**, **3b**, **3c**, and **3d**) were hydrolyzed fast; around 90% conversions were reached within 3.5 hours. This fast hydrolysis rate is ascribed to the stability of the liberated *zwitterionic* (*R*)-phenylglycine, which precipitated under the reaction conditions and accordingly does not inhibit penicillin acylase. No significant difference was observed in the hydrolysis rates of **3a**-**3c**; but **3d** was hydrolyzed faster (84%

conversion in 1 h). The hydrolysis of the crude amide (**3c**^{b}) was slower than the recrystalized one. We tentatively ascribe this to the lower puritiy of the amide, which the excess of the unconverted acyl donor (**2**) was hydrolyzed first.

The enantiopurity of the liberated amine was high in all cases, with *ee* >98 %. Furthermore, we observed the expected *'polishing effect'*: (*R*)-2-pentylamine with *ee* = 91% was obtained from the hydrolysis crude amide **3c** having a *de* of 74%.

CONCLUSION

A fully enzymatic resolution of chiral amines using (*R*)-phenylglycine propyl ester as the acyl donor was achieved. The resolution method involves a CaLB-catalyzed acylation in organic solvent, followed by amide deacylation in aqueous medium catalyzed by *A. faecalis* penicillin acylase. In the acylation step, CaLB catalyzed the reaction with moderate-to-poor enantioselectivity. The reaction rate and enantioselectivity was related to the length of the aliphatic chain in the amine. The longer chain aliphatic amines gave lower reaction rates with better enantioselectivity, and the amine containing an aromatic group was acylated the slowest (51% conversion after 24 h) with the best enantiomeric ratio (*E* = 81). The enzymatic hydrolysis of the obtained amide was fast; almost 90% conversions were reached within 3.5 h, liberating highly enantiopure amines (*ee* > 98%) as the hydrolysis products.

EXPERIMENTAL PART

1. Materials

Enzymes: Novozym 435 (immobilized *Candida antarctica* lipase B) was kindly donated by Novozymes; penicillin acylase from *Alcaligenes faecalis* was obtained from the fermentation of recombinant *E. coli*[15]. *A. faecalis* penicillin acylase CLEA was self-prepared using glutaraldehyde (25% w/v) as the cross linker. Other chemicals were purchased from Sigma and Acros.

Preparation of CLEA of A. faecalis penicillin acylase

An aqueous solution of *A. faecalis* penicillin acylase (in 30% propylene glycol, 0.8 kU/ml; 2.5 mL) was diluted 20 times with cold water; ammonium hydroxide solution (1 M) was used to adjust the pH to 7.0. Aggregation of the enzyme was induced by addition of ammonium sulfate (51.6 g) under gentle stirring at 4 ^{o}C. The enzyme aggregates were crosslinked by addition of glutaraldehyde (25% w/v; 4 mL) then stirred at 4 ^{o}C for 60 min. The crosslinked enzyme was separated from the supernatant by centrifugation, and washed 3 times with phosphate buffer pH 7.0 (0.1 M). After addition of glycine (380 mg), the mixture was left over night under stirring. The excess of glycine was removed and the enzyme was washed 2 times, followed by reduction of the free aldehyde groups with sodium borohydride (25 mg). The crosslinked enzyme was then washed 2 times with phosphate buffer pH 7.0. The CLEA preparation was kept as a suspension in phosphate buffer pH 7.0 in a refrigerator (4 ^{o}C).

Activity test of A. faecalis penicillin acylase

The activity of penicillin acylase was assayed in the hydrolysis of penicillin G potassium salt in sodium phosphate buffer pH 8.0 at 37 ^{o}C. One unit (U) of penicillin acylase liberated 1 μmol of product phenylacetic acid per minnute. One g of immobilized penicillin acylase from *A. faecalis* (*Separase*) consists of 267 BPU; One g CLEA preparation consists of 10.600 BPU.

Activity test of lipase from Candida antractica (CaLB)

The activity of CaLB was assayed in the hydrolysis triacetin (0.1 M) in sodium phosphate buffer pH 7.5 at room temperature. The activity was determined by titration of the formed acid with potassium hydroxide 0.1 M. One g of CaLB (Novozym 435) consists of 297 U.

2. Methods

2.1 Enzymatic acylation of chiral amines

(*R*)-phenylglycine propyl ester (1.0 mmol) and the amine (0.5 mmol) were dissolved in 5 mL of diisopropyl ether. Novozym 435 (50 mg), and internal standard 1,3-Dimethoxybenzene (100 µL) and molecular sieves 4A and 5A (150 mg each) were added to the reaction mixture. The reaction was incubated at 40 ℃. A sample (100 µL) was taken every 1.5 h to follow the reaction over time. The conversion was monitored by gas chromatography; chiral HPLC and chiral GC analysis were used to analyze the enantiomeric excess of the unreacted amine.

The reaction was stopped at around 50% conversion by removal of the enzyme and molecular sieves. The unreacted amine was removed by extraction with sodium hydroxide solution pH 8.5. The solvent was evaporated *in vacuo* and pure amide was obtained by recrystallization in hexane (yields: *N*-(2-butylamine)-(*R*)-phenylglycinamide 56%, *N*-(2-pentylamine)-(*R*)-phenylglycinamide 30%, *N*-(2-heptylamine)-(*R*)-phenylglycinamide 40%, *N*-(1-phenylethyl-amine-(*R*)-phenylglycinamide 48%).

2.2 Amide deacylation with penicillin acylases from E. coli and A. faecalis

The amide (5 mg) was added to 5 mL phosphate buffer 0.1 M pH 7.0. Enzyme was added: *A. faecalis* penicillin acylase (immobilized, 30 mg=8.2 BPU), *E. coli* penicillin G acylase (immobilized, 30 mg; 9.6 BPU). The reaction was carried out at room temperature. A solution of (*R*)-phenylglycinamide was used as external standard.

2.3 Enzymatic deacylation catalysed by CLEA A. faecalis penicillin acylase

The amide (0.3 mmol) was added to 3 mL phosphate buffer 0.1M pH 7, then 3 mL of CLEA of *faecalis* Penicillin acylase from *A.* (30 mg; 320 BPU) was added. The reaction was carried out at room temperature.

2.4 Chemical synthesis of racemic amides (reference compounds)

(*R*)-phenylglycine chloride hydrochloride (11 g; 1.2 eq.) was added to a stirred solution of amine (1 eq.) in dichloromethane (50 mL) at –10 ^{o}C. The reaction mixture was allowed to stand for 2 h at –5 ^{o}C. Cold water (50 mL) was then

added, the pH adjusted to 1.5 with hydrochloride acid and the organic layer discarded. The amide was isolated from the water phase (pH 8.5) by extraction with dichloromethane. The solvent was then removed *in vacuo,* and white crystals of amide were obtained by recrysitallization in *n*-hexane.

N-(R)-phenylglycyl-2-butylamide: ^{1}H-NMR (300 MHz), ($CDCl_3$): δ 7.27-7.39 (5H, aromatic protons, m), δ 7.25 (*s,* 1H, CONH,), δ 6.85 (*s*,1H, CONHCH), δ 4.48 (*s*, 1H, COCHNH$_2$), δ 3.84-3.91 (*m*, 1H, NHCHCH$_3$), δ 1.75 (*s*, 2H, COCNH$_2$), δ 1.31-1.41 (*m*, 2H, -(CH$_2$)-), δ 1.10 (*d*, 3H, -CHCH$_3$), δ 0.84 (*t*, 3H, -CH$_2$CH$_3$).

N-(R)-phenylglycyl-2-pentylamide: ^{1}H-NMR (300 MHz), ($CDCl_3$): δ 7.27-7.39 (*m*, 5H, aromatic protons), δ 7.25 (*s*, 1H, CONH), δ 6.85 (*s*, 1H, CONHCH), δ 4.5 (*s*, 1H, COCHNH$_2$), δ 3.92-3.97 (*m*, 1H, NHCHCH$_3$), δ 1.75 (*s*, 2H, COCNH$_2$), δ 1.31-1.41 (*m*, 4H, -(CH$_2$)-), δ 1.10 (*d*, 3H, -CHCH$_3$), δ 0.87 (*t*, 3H, -CH$_2$CH$_3$).

N-(R)-phenylglycyl-2-heptylamide: ^{1}H-NMR (300 MHz), ($CDCl_3$): δ 7.27-7.39 (5H, aromatic protons, m), δ 7.25 (s, 1H, CONH), δ 6.85 (*s*, 1H, CONHCH,), δ 4.5 (*s*, 1H, COCHNH$_2$), δ 3.92-3.97 (*m*, 1H, NHCHCH$_3$), δ 1.74 (*s*, 2H, COCNH$_2$), δ 0.97-1.42 (*m*, 8H, -(CH$_2$) -), δ 1.10 (*d*, 3H, -CHCH$_3$), δ 0.9 (*t*, 3H, -CH$_2$CH$_3$).

N-(R)-phenylglycyl-1-phenylethylamide: ^{1}H-NMR (300 MHz), ($CDCl_3$): δ 7.23-7.41 (*m*, 10H, aromatic protons), δ 7.25 (*s*, 1H, CONH), δ 5.25 (*m*, 1H, C_6H_5CHCH$_3$), δ 4.91 (*s*, 1H, COCHNH$_2$), δ 1.74 (*s*, 2H, COCNH$_2$), δ 1.31-1.41 (*m*, 4H,-(CH$_2$)-), δ 1.49 (*d*, 3H,-CHCH$_3$).

3. Analysis

3.1 Acylation of amines

Analysis was performed by gas chromatography using a CP Sil 5 CB column (50 m x 0.53 mm). Chiral analysis of the enantiomeric excess of the amine

was executed in a β-PH gas chromatography column (Chiraldex), after derivatization with trifluoroacetic anhydride, at 80 ℃ (2-heptylamine) or 160 ℃ (phenylethylamine). The enantiomeric excess of benzoylated 2-butylamine and 2-pentylamine was determined by HPLC using a Chiracel OD column (Daicel Chemical Industries) at 250 nm using eluent hexane/isopropanol 95:5 (v/v) with a flow rate of 0.6 mL/min.

Analysis of enantiomeric purity of the diastereomers *N*-substituted-(*R*)-phenylglycinamides was performed by HPLC on a C_{18} column, using as eluent MeOH-H_2O 50:50 (v/v), 1g/L SDS, pH 3.5 at 215 nm and flow of 0.8 mL/min.

3.2. Amide deacylation

Analysis was performed by HPLC on a C_{18} Reverse Phase Symmetry column, using as eluent MeOH-H_2O 65:35 (v/v), 1 g/L SDS, 1 g/L KH_2PO_4 pH 3.5 at 250 nm at 1 mL/min. Analysis of aliphatic amines was performed after allowing a precise amount of reaction mixture and solution of 2-pentylamine to react for one minute with a commercial solution of *o*-phthaldialdehyde in the presence of 2-mercaptoethanol (1 mL). Formation of free amine was monitored using as eluent MeOH-H_2O 80:20 (v/v) with at 1 mL/min, UV detection at 340 nm.

For the analysis of the enantiomeric excess of the aliphatic amines, the pH of the reaction mixture was adjusted to 11 and the amine was extracted into hexane and analyzed by chiral GC (see above). Enantiomeric purity of phenylethylamine was analyzed by chiral HPLC on a Crownpack CR^+ column, with aqueous 0.1 M $HClO_4$ pH 1.5 at 0.6 mL/min, UV detection 250 nm.

References

1. Hieber, G., Ditrich, K., *Chim. Oggi* **2001**, *16*, 16-20.
2. Van Rantwijk, F., Sheldon, R. A., *Tetrahedron* **2004**, *60*, 501-519.
3. Huang, H. T., Seto, T. A., Shull, G. M., *Appl. Microbiol*. **1963**, *11*, 1-6.
4. Fuganti, C., Rosell, C. M., Servi, S., Rigoni, R., Servi, S., Tagliani, A., Terreni, M., *Biotechnol. Lett*. **1992**, *14*, 543-546.

5. Fuganti, C., Rosell, C. M., Servi, S., Tagliani, A., Terreni, M., *Tetrahedron: Asymmetry* **1992**, *3*, 383-386.
6. Wegman, M.A., Hacking, M. A. P., Rops, J., Pereira, P., Van Rantwijk, F., Sheldon, R. A. *Tetrahedron: Asymmetry* **1999**, *10*, 1739-1750.
7. De Zoete, M.C., Kock-Van Dalen, A. C., Van Rantwijk, F., Sheldon, R.A., *Recl. Trav. Chim. Pays-Bas* **1995**, *114*, 171-174.
8. Van Langen, L. M., Oosthoek, N. H. P., Guranda, D. T., Van Rantwijk, F., Svedas, V. K., Sheldon, R.A.,*Tetrahedron: Asymmetry*, **2000**, *11*, 4593-4600.
9. Svedas, V., Guranda, D., Van Langen, L. M., Van Rantwijk, F., Sheldon, R.A., *FEBS Lett*. **1997**, *417*, 414-418.
10. Guranda, D.T., Khimiuk, A.I., Van Langen, L. M., Van Rantwijk, F., Sheldon, R. A., Svedas, V. K., *Tetrahedron: Asymmetry* **2004**, *15*, 2901-2906.
11. Gonzales-Sabin, J., Gotor, V., Rebolledo, F., *Tetrahedron: Asymmetry* **2004**, *15*, 481-448.
12. Cygler, M., Grochulski, P., Kazlauskas, R. J., Schrag, J. D., Bouthiller, F., Rubin, B., Serreqi, A.N., Gupta, A. K., *J. Am. Chem. Soc*. **1994**, *116*, 3180-3186.
13. Hoff, B. H., Anthonsen, H. W., Anthonsen, T., *Tetrahedron: Asymmetry* **1996**, *7*, 3187-3192.
14. Lopez-Serrano, P., Cao, L., Van Rantwijk, F., Sheldon, R.A., *Biotechnol. Lett*. **2002**, *24*, 1379-1383.
15. Lau, R. M., PhD Thesis, Delft University of Technology, Delft, The Netherlands, **2003**.

3

Effects of a ß-Heteroatom in the Acyl Donor*

ABSTRACT

Glycine derivative-analogues substituted with a heteroatom X (X = O, NH, or S) were investigated as the acyl donor in the fully enzymatic resolution of 2-heptylamine and 1-phenylethylamine. The fully enzymatic resolution method involves a CaLB-catalyzed acylation step followed by amide deacylation step, which was catalyzed by penicillin acylase or CaLB. Interstingly, CaLB was found to be able to hydrolyse amides derived from CH_3-X-CH_2CO_2H. The presence of the β-heteroatom (O, N, and S) in the acyl donor was found to influence the enzymatic acylation and deacylation results.

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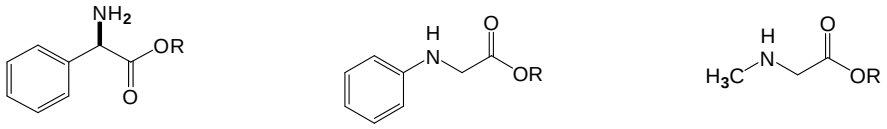
*Some part of the works in this chapter was performed in collaboration with R. M. Lau

INTRODUCTION

A fully enzymatic methodology for chiral amine resolutions has been presented in Chapter 2. Proof of principle was obtained and acylation and deacylation were accomplished under mild reaction conditions. Further improvement is still needed, however. The second step, amide hydrolysis in the presence of penicillin acylase worked very well, but the low enantioselectivity and reaction rate of the CaLB-catalyzed acylation indicated that the acyl donor used was not the optimum one for this latter enzyme. Hence, the following issue is now to find an appropriate acyl donor, which is accepted well by both enzymes in the acylation and deacylation steps, and affords a better enantioselectivity in the former.

As presented in Chapter 2, an ester of an amino acid, (*R*)-phenylglycine, was used as the acyl donor. The advantage of using an amino acid as the acyl donor is the easy deacylation step, due to the liberation of the thermodynamically highly stable *zwitterion*. We found that besides being less suitable in combination with CaLB, the latter ester also has the disadvantage that recycling the acid after the hydrolysis step, is laborious and generates an equivalent of salt. Accordingly, here we extended our investigation to achiral glycine derivatives, which have the added advantage that they do not generate a mixture of diastereomeric amides.

The enzyme used for the acylation step was CaLB, a widely used catalyst in the resolution of chiral alcohols, amines, esters, and carboxylic acids[1,2]. Studies on the substrate specificity of lipases have shown that the size and structure of the acyl donor influence the course of the enzymatic reaction. Since CaLB has a funnel-like binding site, the chain length of the acyl donor affects its ability to reach its active site[3]. Furthermore, the enantioselectivity is strongly influenced by the chain length of the alkyl part of the acyl donor[4, 5]. Consequently, we wished to investigate such effects in amine acylation, and adopted esters of *N*-methyl and *N*-phenylglycine ester (Figure 1b and 1c).



(a) (b) (c)

Figure 1. Esters of glycine derivatives: (*R*)-phenylglycine ester (a), *N*-phenylglycine ester(b) and *N*-methylglycine ester (c).

Beside the size, the electronic properties of the acyl donor are expected to affect the enzymatic performance. The electron density and projection area of a carboxylic carbon was reported to influence the rate and K_m of the CaLB-catalyzed esterification of alcohols[6]. The presence of a heteroatom with different electronegativity is accordingly expected to affect the results of CaLB-catalyzed acylation. Hence, here we also investigated the effect of the β-heteroatom in the acyl donor, by substituting the nitrogen atom in *N*-phenylglycine and *N*-methylglycine esters with an oxygen, sulfur, or carbon atom.

The second enzyme used in the fully enzymatic resolution is penicillin acylase, a serine hydrolase with a mechanism that is very similar to that of the lipases and serine proteases. Penicillin acylase is highly selective for the phenylacetyl moiety (penicillin G acylase) or phenoxyacetyl moiety (penicillin V acylase)[7]. Small α-substituents such as -O-, -NH-, and -CH_2- in the bridging structure of the phenylacetyl moiety were reported to be tolerated by penicillin G acylase from *E. coli,* although at the cost of a decrease in the reaction rate[8,9,10]. Further studies on other sources of penicillin acylases showed that the substrate specificity of the acyl donor-binding site might vary with the type of reaction that is catalyzed. Here we used penicillin acylase from *Acaligenes faecalis*, which was reported to show a high enantioselectivity in the hydrolysis of *N*-phenylacetamides[11].

Accordingly, in this Chapter several acyl donors are investigated with regard to the acceptability to both enzymes. A number of glycine derivative-analogues, which have a heteroatom X (X=O, NH, S, or CH_2) substituted at the β-position were used. Figure 2 presents the scheme of the fully enzymatic resolution of a chiral amine investigated in this Chapter.

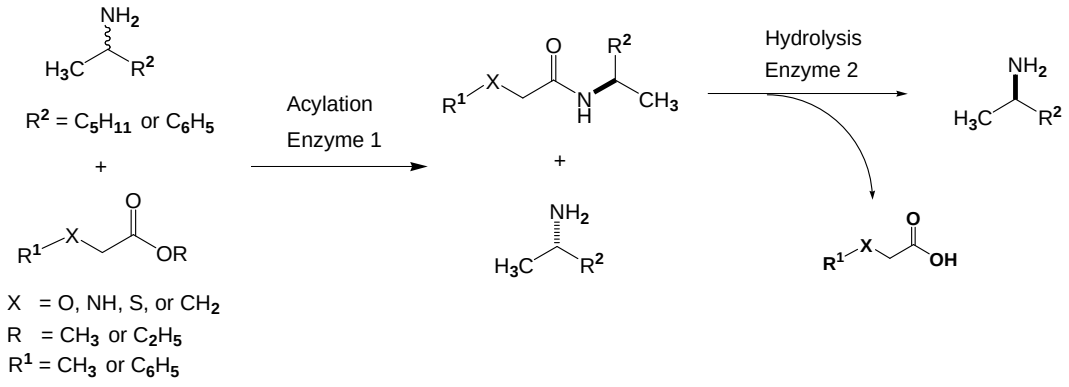


Figure 2. The fully enzymatic resolution of chiral amines

RESULT AND DISCUSSION

1. Acylation of 1-phenylethylamine and 2-heptylamine

Esters with the general formula CH_3-X-CH_2CO_2R (R = methyl or ethyl; X = CH_2, NH, O or S) were applied as acyl donors in the enzymatic resolution of 2-heptylamine (**2a**) and 1-phenylethylamine (**2b,** see Figure 1). Considering that a β-heteroatom may activate the acyl donors, the reaction conditions should be adjusted to avoid the uncatalyzed background reaction. Here, 1,2-dimethoxyethane was chosen as the reaction medium, as this solvent dissolves the substrates and products very well. Moreover, the high polarity of 1,2-dimetyhoxyethane is expected to suppress any uncatalyzed background reaction, since such reaction rate was found to decrease with increasing solvent polarity[12].

Novozym 435 (immobilized *C. antarctica* lipase B) was used as the catalyst, on account of its broad specificity towards acyl donors and high degree of selectivity towards the nucleophile[13]. The reaction scheme is presented in Figure 3.

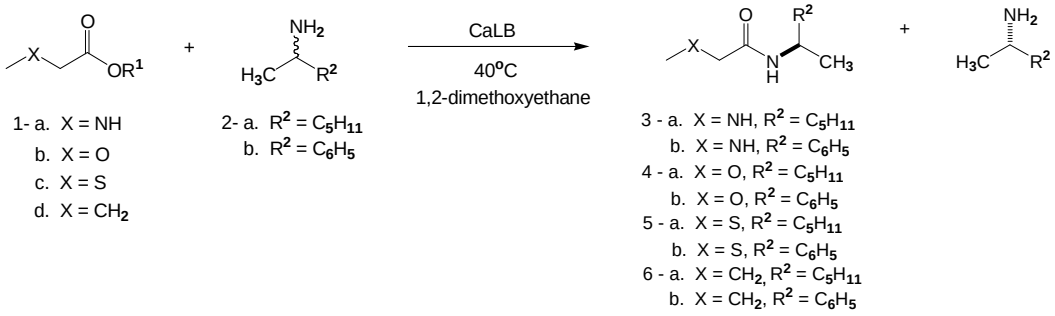


Figure 3. Acylation of 1-phenylethylamine and 2-heptylamine with CH_3-X-CH_2CO_2R as acyl donor catalyzed by CaLB (Novozym 435)

Table 1. Resolution of 2-heptylamine (**2a**) and 1-phenylethylamine (**2b**) with the acyl donor CH_3-X-CH_2CO_2R[a]

Ester	**X**	*R*	**Amine**	**Time (h)**	**Conv. (%)**	**ee_{amine} (%)**	**E**
1a	NH	ethyl	**2a**	63	51	97	94
			2b	128	45	80	>100
1b	O	methyl	**2a**	5	50	91	51
			2b	19	50	99	>100
1c	S	methyl	**2a**	24	50	91	80
			2b	48	48	91	>100
1d	CH_2	ethyl	**2a**	216	42	76	>100
			2b	216	40	73	>100

[a]Conditions: Amine (5 mmol) and acyl donor (3 mmol) dissolved in 1,2-dimethoxyethane (5 mL), catalyst Novozym 435 (100 mg), molecular sieves of 4 A and 5 A powder (each 150 mg), internal standard 1,3-dimethoxybenzene (150 µL).

We found that a heteroatom (X) at the β-position profoundly affected the amine acylation. As regards the reaction time, the acylation rate increased in the order X = CH_2 < NH < S < O (Table 1). The trend of the heteroatom-effect on the initial rate is clearly observed in the resolution of **2b** (Figure 4), revealing that a β-oxygen in the acyl donor significantly increases the reaction rate. Accordingly, methyl methoxyacetate (CH_3-O-$CH_2CO_2CH_3$) is the acyl donor of choice, as it converts 50% of **2a** or **2b** in 5 h and 19 h respectively. The blank reaction tests showed that non–enzymatic background reaction was not detected. The conversion curve is shown in Figure 4.

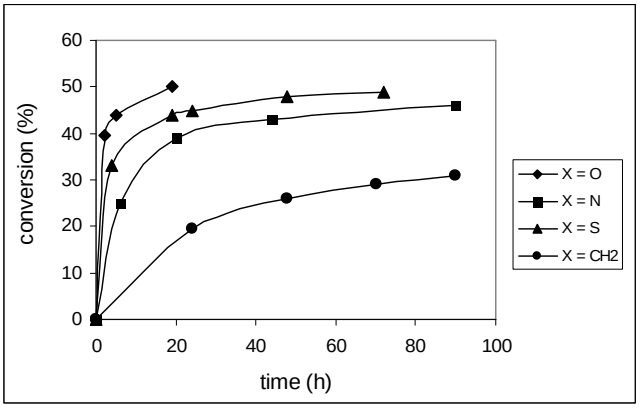


Figure 4. The profile of the acylation of 1-phenylethylamine (**2b**) with various acyl donors

Good to excellent enantioselectivities were obtained with all the acyl donors **1a**–**1d**. The enantiomeric ratio (*E*) in the resolution of **2b** was higher than 100 with all acyl donors. With **2a,** *E* ranged form 51 (X = O) to >100 (X = CH_2). The enantiomeric ratio decreased in the order of X = CH_2 **>** NH > S > O.

We further investigated the effect of acyl donor's size, by substituting the terminal methyl group in **1a-1d** with a phenyl group (**7a**-**7d**; C_6H_5-X-CH_2CO_2R, see Figure 5).

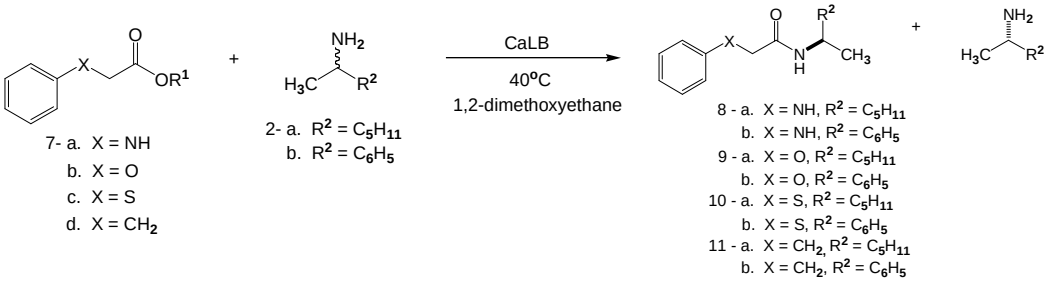


Figure 5. Enzymatic acylation of 1-phenylethylamine and 2-heptylamine with C_6H_5-X-CH_2CO_2R as the acyl donor

Table 2. Resolution of 2-heptylamine (**2a**) and 1-phenylethylamine (**2b**) with C_6H_5–X-CH_2CO_2R as acyl donors[a]

Ester	X	R	Amine	Time (h)	Conv. (%)	ee_{amine} (%)	E
7a	NH	ethyl	**2a**	216	48	63	11
			2b	118	51	54	12
7b	O	methyl	**2a**	7	50	99	>100
			2b	24	50	98	>100
7c	S	methyl	**2a**	200	48	68	14
			2b	200	43	66	23
7d	CH_2	ethyl	**2a**	13 days	8	-	-
			2b	13 days	7	-	-

[a]Conditions: amine (5 mmol) and acyl donor (3 mmol) in 1,2-dimethoxyethane (5 mL), catalyst Novozym 435 (100 mg), molecular sieves of 4 A and 5 A powder (each 30 mg/mL), internal standard 1,3-dimethoxybenzene (150 µL)

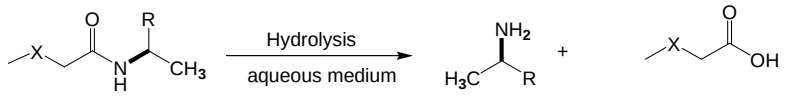
With the exception of **7b**, the phenyl-substituted acyl donors are much less active than **1a**–**d**. However, order remained the same: X = CH_2 < NH < S < O, which correlates with the electronegativity of the heteroatom X. Accordingly, a significant difference in the reaction rate was observed between acyl donors with and without oxygen (less than 24h *versus* 13 days to reach 50% conversion of **2a** and **2b**).

As regards the enantioselectivity, the enantiomeric ratio of the unreacted amine was found to be much lower than that of **1a**-**1d,** except in the case of **7b**. Therefore, we conclude from the results in Table 2 that only the phenoxy-substituted acyl donor (**7b**) has potency as acyl donor.

2. Hydrolysis of the acylated amines

2. 1. Hydrolysis of amides derived from CH_3-X-CH_2CO_2H

Here, two enzymes have been tested to hydrolyse the amides obtained from the acylation step. *A. faecalis* pencillin acylase was the first choice, as we found that this enzyme showed high activity in hydrolysing the amide bond (Chapter 2). The second enzyme was CaLB, which is known to be a modestly active amidase[14,15,16]. Since we observed in the acylation step that the esters of CH_3-X-CH_2CO_2H were well accepted by CaLB, we expected that in aqueous medium CaLB is also able to hydrolyse those amides. The hydrolytic activity of CaLB (Novozym 435) was tested in the hydrolysis of **3a** – **5b** in phosphate buffer pH 7 at 70 ^oC. The scheme of the reaction is illustrated in Figure 6 and Table 3 presents the results of the hydrolysis of **3a** – **5b** with Novozym 435 and *A. faecalis* penicillin acylase.



3 - a. X = NH, $R^2 = C_5H_{11}$
b. X = NH, $R^2 = C_6H_5$
4 - a. X = O, $R^2 = C_5H_{11}$
b. X = O, $R^2 = C_6H_5$
5 - a. X = S, $R^2 = C_5H_{11}$
b. X = S, $R^2 = C_6H_5$

11- a. $R^2 = C_5H_{11}$
b. $R^2 = C_6H_5$

12

Figure 6. Hydrolysis of amides derived from CH_3-X-CH_2CO_2H catalyzed by *A. faecalis* penicillin acylase and Novozym 435.

Table 3. Hydrolysis of the amides catalyzed by Novozym 435 and penicillin acylase[a].

X	***Amide***	***A. faecalis* penicillin acylase***		***C. antarctica* lipase B****	
		Time (h)	*Hydrolysis (%)*	*Time (h)*	*Hydrolysis (%)*
N	**3a**	80	3	120	22
	3b	144	7	120	15
O	**4a**	120	5	120	74
	4b	120	< 2	120	100
S	**5a**	120	6	120	36
	5b	144	7	120	85

[a]Conditions: amide (5 mg, 0.03 mmol) in phosphate buffer 0.1 M pH 7.0 (3.5 ml), enzyme *A. faecalis* penicillin acylase (immobilized, 30 mg; 8 BPU) or Novozym 435 (30 mg; 9 U), diphenylacetic acid as internal standard. Incubation at room temperature (*) or 70 ^{o}C (**).

The hydrolysis of **3a**-**5b** with *A. faecalis* penicillin acylase was indeed quite slow; in all cases less than 10% conversion was detected after 4 days. This result confirms that the structures of the amides derived from CH_3-X-CH_2CO_2H are not accepted well by *A. faecalis* penicillin acylase. This is not surprising in view of the enzyme's known specificity for phenylacetic acid derivatives.

Interestingly, we found that Novozym 435 was able to hydrolyze the amides. The hydrolysis of **3a** – **5b** with Novozym 435 in phosphate buffer pH 7 at 70 ^{o}C was observed to be faster than with penicillin acylase. The reaction rate was also observed to increase in the order of X = NH < S < O, similar to the trend in the acylation step, indicating that the ß-heteroatom X in the amides affects the hydrolysis as well. Amides bearing a β-oxygen (**4a** and **4b**) hydrolyzed fastest and 100% conversion was reached within 120 h.

We further investigated the hydrolysis of **3a** and **4a** with a number of hydrolases. None of these enzymes showed better activity than CaLB, however. With all enzymes tested, less than 10% conversion was detected

after 24 h (Table 4), showing that Novozym 435 was the best catalyst for the hydrolysis of the amides derived from CH_3-X-CH_2CO_2H.

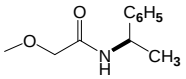
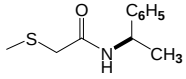
Table 4. Results of the hydrolysis of amide **3a** and **4a** with some acylases[a]

Amide	X	Enzyme	Time (h)	Conversion (%)
3a	NH	Acylase I from *Aspergillus melleus*	24	2
		Thermolysin CLEC	40	2
4a	O	Acylase I from *Aspergillus melleus*	27	7
		Acylase I from Porcine kidney	27	8

[a]Conditions: amide (5 mg, 0.03 mmol) in phosphate buffer 0.1 M pH 7.0 (3.5 mL), enzyme (30 mg), diphenylacetic acid as internal standard, incubation at room temperature.

Encouraged by the performance of Novozym 435 in amide hydrolysis, we extended our work to a cross-linked enzyme aggregate (CLEA) of CaLB, as CLEA are known to show high activity in water[17]. Thus, a CaLB CLEA was applied to the hydrolysis of more concentrated solutions of **4b** and **5b** (see Table 5).

Table 5. The hydrolysis of 4b and 5b in the presence of CaLB CLEA at 40°C and 60°C

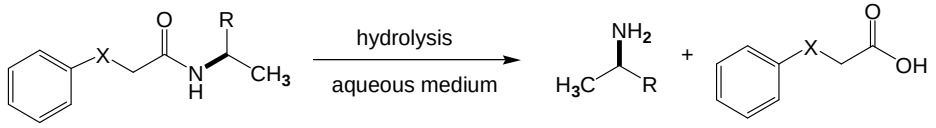
Amide	Time (h)	Conversion at 40°C (%)	Conversion at 60°C (%)
 **4b**	48	100	87
	72	100	90
 **5b**	48	94	48
	72	100	67

Conditions: amide (0,3 mmol) in phosphate buffer 0.1 M pH 7.0 (3 mL), CaLB CLEA (90 mg, 600 U), diphenylacetic acid as internal standard, incubation at 40 °C and 60 °C

In the presence of the CaLB CLEA, hydrolysis was much faster than with Novozym 435. Interestingly, an increase in reaction temperature reduced the hydrolysis rate; only 87% (**4b**) and 48% (**5b**) conversion was reached in 48 h, whereas almost 100% conversion was reached within 48 h at 40 ^{o}C. It would seem that thermal deactivation is significant at 60 ^{o}C. This result demonstrated the principle of facile enzymatic deacylation, in which removal of the acyl donor was achieved under mild reaction conditions.

2.2. Hydrolysis of the phenyl-sustituted amides C_6H_5-X-CH_2CONR_2

The hydrolysis of the amides derived from C_6H_5-X-CH_2CO_2H (X= CH_2, O, NH, S) was investigated using *A. faecalis* penicillin acylase and Novozym 435 as the catalyst (Figure 7). The hydrolysis of these amides with penicillin acylase was expected to be easier, due to the closer similarity of their structures to the substrate specificity of penicillin acylase. The results are presented in Table 6.



8 - a. X = NH, R = C_5H_{11}
b. X = NH, R = C_6H_5
9 - a. X = O, R = C_5H_{11}
b. X = O, R = C_6H_5
10 - a. X = S, R = C_5H_{11}
b. X = S, R = C_6H_5

11- a. R = C_5H_{11}
b. R = C_6H_5

12

Figure 7. Hydrolysis of amides derived from C_6H_5-X-CH_2CO_2H catalyzed by *A. faecalis* penicillin acylase and CaLB

Table 6. Hydrolysis of **8a**-**10b** catalyzed by *A. faecalis* penicillin acylase and CaLB (Novozym 435)[a].

X	Amide	*A. faecalis p*enicillin acylase [b]		*C. antarctica* lipase B[c]	
		Time (h)	Hydrolysis (%)	Time (h)	Hydrolysis (%)
N	**8a**	48	17	90	30
		48[d]	22		
		48[e]	15		
	8b	71	41	120	3
O	**9a**	120	66	120	21
	9b	101	78	120	42
S	**10a**	120	3.5	120	50
	10b	120	12	144	39

[a])Conditions: amide (5 mg, 0.015 mmol) in phosphate buffer 0.1 M pH 7.0 (3.5 mL), *A. faecalis* pencillin acylase (30 mg; 8 BPU) or Novozyme 435 (30 mg; 9 U), diphenylacetic acid as internal standard. Incubation was done at room temperature ([b]) or 70 °C ([c]).
[d])With addition of co-solvent *t*-butyl alcohol (10%).
[e])With addition of co-solvent acetonitrile (10%).

Indeed, the hydrolysis of **8a**-**10b** with penicillin acylase was found to be faster than that of **3a** – **5b** (see also Table 3). Apparently, the presence of a phenyl group in the acyl pocket favoured the reaction rates with this enzyme. The substitution of β-oxygen atom was also found to increase the hydrolysis rate, with **9a** and **9b** (X = O) as the fastest hydrolyzed. However, the hydrolysis rate is still considered as very low (>100 h to reach 78 % conversion), which is ascribed to the poor solubility of the amides in aqueous medium at room temperature. Attempts to increase the solubility of **8a** by adding co-solvents, e.g. *t*-BuOH (10%), resulted in a slight increase in the hydrolysis rate, whereas no improvement was observed upon the addition of acetonitrile (10%).

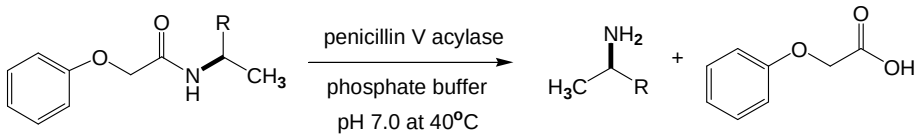
In the hydrolysis catalyzed by CaLB, a considerable effect of the molecule size on the reaction rate was observed. Compared to the hydrolysis of the

amides from CH_3-X-CH_2CO_2H (**3a**-**5b**, see Table 3 and Table 6), the phenyl-substituted amides (**8a-10b**) were hydrolyzed much slower. This low hydrolysis rate is not ascribed to the poor solubility of the amides, since the reactions were carried out at 70°C and the amides were dissolved better in the reaction medium. The difficulty to reach the active site of CaLB due to the structure bulkiness is more likely to be the reason. Summarizing, CaLB is not a good catalyst for the hydrolysis of amides derived from C_6H_5-X-CH_2CO_2H.

2.3. Penicillin V acylase in the hydrolysis of amides derived from phenoxyacetate.

Results from Table 6 revealed that *A. faecalis* penicillin acylase did not tolerate the heteroatom X in the acyl moiety. The fastest hydrolysed amides (**9a** and **9b**) were still hydrolysed very slowly (more than 100 hours to reach <80% conversion).

Here, we attempted to hydrolyse **9a** and **9b** using other penicillin acylases as the catalyst**.** It is known that in some cases, different sources of penicillin acylase can accept the phenoxyacetyl moiety. In particular penicillin V acylase, which naturally hydrolyze phenoxyacetic acid derivatives, is expected to show a better activity in the hydrolysis of **9a** and **9b.** Hence, a number of penicillin V acylase from different sources and different formulations were tested to hydrolyze **9a** and **9b** (Figure 8).



9 - a. R = C_5H_{11}
b. R = C_6H_5

Figure 8. Hydrolysis of amides derived from phenoxyacetic acid catalyzed by penicillin V acylase

We found that semacylase (a commercial preparation from Novozymes) showed the best activity in the hydrolysis of **9a** and **9b**, with almost 100% amide hydrolyzed within 120 h.

Table 7. Hydrolysis of **9a** and **9b** catalyzed by several penicillin V acylases[a].

Amide	Acylated amine	Penicillin V acylase	Time (h)	Conversion (%)
9a	2-heptylamine	Semacylase	120	98
		Cryptococcus sp	120	17
		Biocat V	120	14
		F. oxysporum	120	46
9b	1-phenylethylamine	Semacylase	75	95
		Cryptococcus sp	120	21
		Biocat V	120	52
		F. oxysporum	120	16

[a]Conditions: amide (0.015 mmol) in phosphate buffer 0.1 M pH 7.0 (3.5 mL), enzyme pencillin V acylase (30 mg), diphenylacetic acid as internal standard, incubation at 40 °C.

Further investigations with other hydrolases (thermolysin CLEC, chymotrypsin, trypsin, maxatase, maxacal, papain, acylase I from porcine kidney, and penicillin V acylase) in the hydrolysis of **8a** (X = N) showed that none of these enzymes was capable to accept the amides. In general, all of the tested enzymes showed conversions of approximately 5% within 48 h (Table 8).

Table 8. Hydrolysis of **8a** catalyzed by different hydrolases at pH 7 at 40 °C.

Enzyme	Time (h)	Conversion (%)
Protease *Rhizopus* sp	24	4
Subtilisin A	24	3
Trypsin	24	4
Chymotrypsin	24	5
Maxatase	24	4
Maxacal	24	2
Papain	24	3
Acylase I porcine kidney	48	4
Acylase I *Aspergillus melleus*	48	4

In conclusion, we found that hydrolysis of amides of CH_3-X-CH_2CO_2H was easily carried out in the presence of CaLB as the catalyst. On the other hand, amides derived from C_6H_5-X-CH_2CO_2H were hydrolyzed easier with penicillin acylase from *A. faecalis*. Particularly in the hydrolysis of phenoxyacetamides (X = O), penicillin V acylase (semacylase) emerged as the best catalyst. In all cases, the oxygen atom in the amide molecule increased the hydrolysis rate, both with CaLB as well as with penicillin acylase.

CONCLUSION

The principle of a fully enzymatic resolution using substituted β-heteroatom acyl donors has been investigated. The presence of a β-heteroatom (O, N, and S) in the acyl donor was found to influence the enzymatic acylation and deacylation results. Compounds with substituted β-oxygen have performed best in the acylation well as deacylation steps. In the deacylation step, CaLB has interestingly proven its ability to hydrolyse the amides derived from CH_3-X-CH_2CO_2H, with better activity than penicillin acylase. Penicillin acylase was the better catalyst for the hydrolysis of amides derived from C_6H_5-X-CH_2CO_2H. Hence, two possible approaches in the fully enzymatic resolution have been achieved: (1) by using methyl methoxyacetate (CH_3-O-$CH_2CO_2CH_3$) as the acyl donor, and CaLB was used as the catalyst in both acylation and deacylation steps; (2) by using methyl phenoxyacetate (C_6H_5-O-$CH_2CO_2CH_3$) as the acyl donor, which is started with CaLB-catalyzed acylation followed by penicillin V acylase-catalyzed deacylation.

EXPERIMENTAL PART

1. Materials

Novozym 435 (immobilized *Candida antarctica* lipase B*)* was kindly donated by Novozymes. CLEA CaLB was kindly donated by CLEA Technology BV. Penicillin acylase from *A. faecalis* was obtained from fermentation of

recombinant *E. coli*[18]. Penicillin acylase (PGA-300) was purchased from Roche Diagnostics. The penicillin V acylase preparations were kind gifts from Novozymes (Semacylase), Biochemie (Biocat V), The Academy of Sciences of the Czech Republic (*Cryptococcus* sp), Gist-brocades Sweden (*Fusarium oxysporum*). All other compounds were purchased from ACROS and SIGMA or synthesized as described below.

2. Methods

The progress of the acylation reactions was monitored by gas chromatography using a CP Sil 5 CB column (50 m x 0.53 mm). Chiral analysis of the unconverted amine was performed on a β-PH gas chromatography column, after derivatization with trifluoroacetic anhydride, at 80 ℃ (2-heptylamine) or 100 ℃ (1-phenylethylamine).

The hydrolysis reactions were monitored by HPLC on a C_{18} Symmetry column, eluent MeOH-H_2O 65:35 (v/v), 1g/L SDS, 1g/L KH_2PO_4 pH 3.5 at 1 mL/min, UV detection at 250 nm. Analysis of aliphatic amines was performed after allowing a precise amount of reaction mixture and solution of 2-pentylamine to react for 1 min with a commercial solution of *o*-phthaldialdehyde in the presence of 2-mercaptoethanol (1 mL). Formation of free amine was monitored using HPLC with C_{18} Symmetry column and MeOH-H_2O 80:20 (v/v) as eluent at 1 mL/min, UV detection at 340 nm.

The enantiomeric excess of 2-heptylamine was measured after extracting the amine into hexane and analyzed by chiral GC (see above). The enantiomeric purity of 1-phenylethylamine was determined by chiral HPLC on a Crownpack CR^+ column, with aqueous 0.1 M $HClO_4$ pH 1.5 at a flow of 0.6 mL/min, with UV detection at 250 nm.

3. Enzymatic reactions

3.1. Acylation of amines

Acyl donor (3 mmol) and amine (5 mmol) were dissolved in 5 mL 1,2-dimethoxyethane. Enzyme Novozym 435 (100 mg; 29.7 U) and molecular sieves of 4 A and 5 A (150 mg each) were added to the reaction mixture. 1,3-Dimethoxybenzene (150 µL) was used as internal standard. The reaction mixture was shaken at 40 ℃; samples (100 µL, diluted in 0.5 mL of 1,2-dimethoxyethane) were taken in order to follow the reaction over time.

The reaction was stopped at 50% conversion of amine, by removal of the enzyme and the molecular sieves. The unreacted amine was removed by extraction with sodium hydroxide solution at pH 8.5, then the solvent was evaporated under vacuum and pure amide was obtained by recrystallization from n-hexane.

3.2. Hydrolysis of amides

Amide (5mg, 0.015 or 0.03 mmol) was added to 3.5 mL 0.1M phosphate buffer pH 7. Enzyme was added: Novozym 435 (30 mg; 9 U), Penicillin acylase V (30 mg; 5 BPU), or Penicllin G acylase (30 mg; 8 BPU). The reaction was carried out at the temperature indicated in the table title.

4. Synthesis of non-commercially available acyl donors

4.1. Synthesis of ethyl 3-phenylpropionate

Solution of 3-phenylpropionyl chloride (10 g, 60 mmol) in 50 mL of absolute ethanol and 4.6 g (60 mmol) of pyridine was stirred overnight at room temperature. The excess of ethanol was removed under vacuum. Ethyl acetate (20 mL) was added and the resulting solution was washed with cold water. The organic layer was dried over Na_2SO_4. Ethyl 3-phenylpropionate was obtained as a colourless oil (yield 6.136 g, 57.4%). GC-MS: (MW)=178.

^{1}H NMR: δ 1.21 (*t*, 3H, $OCH_2C\underline{H_3}$), δ 2.57-2.62 (*m*, 2H, $CH_2C\underline{H_2}CO$), δ 2.91-2.96 (*t*, 2H, $CC\underline{H_2}CH_2$), δ 4.07-4.14 (*q*, 2H, $OC\underline{H_2}CH_3$), δ 7.15-7.29 (*m*, 5H, aromatic protons).

4.2. Synthesis of methyl phenylthioacetate

Thiophenol (10 g, 0.09 mol) was dissolved in a mixture of 50 mL dry methanol and 10 mL solution of sodium methoxide in methanol (30%, (wt)). Ethyl chloroacetate (16.6 g, 0.15 mol) was added slowly and the resulting mixture was stirred overnight at room temperature. The excess of methanol was removed under vacuum and dichloromethane (50 mL) was added. The resulting solution was washed with cold water and dried over Na_2SO_4. Kugelrohr distillation afforded 7.5 g of unpleasantly smelling oil. GC-MS: (MW)=182. ^{1}H NMR: δ 3.64 (*s*, 3H, $COOC\underline{H_3}$), δ 3.70 (*s*, 2H, $SC\underline{H_2}CO$), δ 7.21-7.41 (*m*, 5H, aromatic protons).

References

1. Van Rantwijk, F., Hacking, M. A. P.; Sheldon, R. A. *Monatsh . Chem.* **2000**, *131*, 549-569.
2. De Castro, M.S., Gago, J.V.S., *Tetrahedron* **1998**, *54*, 2877-2892.
3. Pleiss, J., Fischer, M., Schmid, R.D., *Chem.Phys. Lipids.* **1998**, *93* 67-80.
4. Hoff, B. H., Anthonsen, H. W., Anthonsen, T., *Tetrahedron: Asymmetry* **1996**, *7*, 3187-3192.
5. Ottoson, J., Hult, K., *J. Mol. Catal. B: Enzymatic* **2001**, *11*, 1025-1028.
6. Kobayashi, T., Adachi, S., Matsuno, R., *Biotechnol. Lett.* **2003**, *25*, 3-7.
7. Huang, H. T., Seto, T. A., Shull, G. M., *Appl. Microbiol*. **1963**, *11*, 1-6.
8. Zmijevski, M. J., Briggs, B. S., Thompson, A. R., Wright, I. G., *Tetrahedron Lett.* **1991**, *32*, 1621-1622.
9. Yin, G.F., Hoo, C. T., *Biosci. Biotechnol. Biochem*. **1993**, *57*, 121-122.
10. Briggs, B. S., Wright, I. G., Zmijewski, M. J., *New J. Chem*. **1994**, *18*, 425-434.
11. Guranda, D.T., Van Langen, L. M., Van Rantwijk, F., Sheldon, R. A., Svedas, V.K. *Tetrahedron: Asymmetry* **2001**, *12*, 1645-1650.
12 Van Rantwijk, F., Sheldon, R.A., *Tetrahedron* **2004**, *60*, 501-519.
13. Arroyo, M., Sinisterra, J. V., *J. Org. Chem*. **1994,** *59*, 4410-4417.

14. Smidt, H., Fischer, A., Schmid, R. D., *Biotechnol. Techn.* **1996**, *10*, 335-338.
15. Reyes Duarte, D., Castillo, E., Barzana, E., Lopez-Munguia, A., *Biotechnol. Lett*. **2000**, *22*, 1811-1814.
16. Buciarelli, Forni, A., Moretti, I., Pratti, F., Torre, G., *Tetrahedron: Asymmetry* **1993**, *4*, 903-906.
17. Lopez-Serrano, P., Cao, L., Van Rantwijk, F., Sheldon, R.A., *Biotechnol. Lett*. **2002**, *24*, 1379-1383.
18. Lau, R. M., PhD Thesis, Delft University of Technology, Delft, The Netherlands, **2003**.

4

Pyridylacetate Ester as Acyl Donor for the Fully Enzymatic Resolution

ABSTRACT

Ethyl 4-pyridylacetate was used as acyl donor in the fully enzymatic resolution of amines. The resolution method involved a lipase-catalyzed acylation, followed by penicillin acylase-catalyzed deacylation. The results showed that ethyl 4-pyridylacetate is well accepted by CaLB and acts as good acyl donor in the acylation of chiral amines. Introduction of the pyridyl ring was found to increase the enantioselectivity and the acylation rate. Furthermore, the pyridyl amides were also hydrolyzed easily by penicillin acylase from *A. faecalis* with enantiopreference for the (*R*)-amides.

INTRODUCTION

In Chapter 2, an exclusively enzymatic method for the acylation and deacylation steps was demonstrated. This fully enzymatic method allows milder reaction conditions, which was achieved by combining a lipase-catalyzed amine acylation and penicillin acylase-catalyzed deacylation, as illustrated in Figure 1.

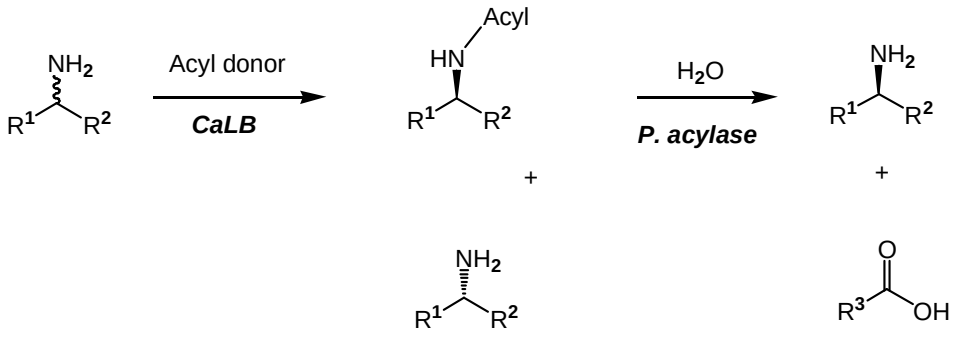


Figure 1. The fully enzymatic strategy for amine resolution.

Penicillin acylase used in the hydrolysis step is a well-known enzyme; it is the industrial catalyst for the hydrolysis of the side chain amide bond in penicillin G and was also involved in various academic studies of amide and ester hydrolysis.

However, penicillin acylase has an exceptionally high affinity for the phenylacetyl moiety and only tolerates minor changes in the phenylacetyl structure in the substrate. Hence, the use of penicillin acylase has been largely restricted to the hydrolysis of phenylacetamides[1,2,3]. These hydrolysis reactions are generally slow, which has greatly hampered their practical application. The low reaction rate is presumably caused by the poor solubility of phenylacetamides in the aqueous reaction medium. Moreover, the phenylacetic acid liberated in the course of the reaction inhibits the activity of penicillin acylase at mM concentrations.[4,5].

Waldman *et al*., have addressed these solubility issues by replacing the phenylacetyl moiety with a pyridylacetyl one. Indeed, pyridylacetic esters were efficiently hydrolyzed by penicillin acylase with better enantioselectivity than the corresponding phenylacetic esters under identical conditions[6]. We surmised, on the basis of these results, that hydrolysis of pyridylacetamides by penicillin acylase should be fast and enantioselective.

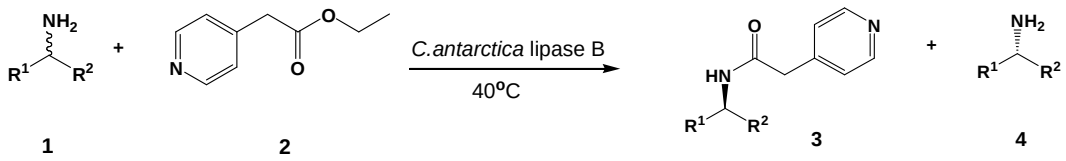
In the hydrolysis experiments, we used a relatively unknown penicillin acylase from *Alcaligenes faecalis,* which has been found to show high activity and enantioselectivity towards chiral amines[7]. Investigations on *Alcaligenes faecalis* penicillin acylase revealed that this enzyme has a catalytic mechanism similar to that of pencillin acylase from E coli[4].

As regards the acylation step, we have found that amine acylation with phenylacetic acid esters in the presence of CaLB is quite slow. However, insertion of a β-nitrogen atom in the acyl group exerts an accelerating effect (Chapter 3). In this Chapter, the results of the enantioselective acylation of chiral amines with ethyl 4-pyridylacetate as acyl donor, followed by deacylation in the presence of penicillin acylase are reported.

RESULTS AND DISCUSSION

1. Enzymatic acylation of chiral amines

The performance of ethyl 4-pyridylacetate as acyl donor was investigated in the resolution of 1a-1d, in the presence of *Candida antarctica* lipase B as the catalyst (Figure 2).



1 2 3 4

1a = 1-phenylethylamine ($R^1 = CH_3$; $R^2 = C_6H_5$)
1b = 2-heptylamine .($R^1 = CH_3$; $R^2 = C_5H_{11}$)
1c = 2-pentylamine .($R^1 = CH_3$; $R^2 = C_3H_7$)
1d = 2-butylamine .($R^1 = CH_3$; $R^2 = C_2H_5$)

Figure2. Enzymatic acylation of chiral amines catalysed by *C. antarctica* lipase B.

It is known that the reaction medium influences the outcome of an enzymatic acylation[8]. The conformational mobility of enzymes in organic media is suspected to have a relationship with the enzyme activity and selectivity[9,10]. Moreover, the unwanted non-enzymatic background reaction, which could erode the enantiopurity of the product, can also be influenced by the polarity of the solvent[2].

In order to find the best reaction medium for the enzymatic resolution, the acylations of **1a, 1b, 1c** and **1d** with ethyl 4-pyridylacetate were carried out in several organic solvents: diisopropyl ether (DIPE), 1,2-dimethoxyethane (DME), 1,4-dioxane, tetrahydrofuran (THF), and hexane. Blank experiments confirmed that no detectable product was formed in the absence of enzyme after 24h. The results are shown in Table 1.

Table 1. Enzymatic resolution of chiral amines[a]

Amine	Solvent	Time (h)	Conversion (%)	ee_{amine} (%)	*E*
1-Phenylethylamine	DME	27	43.6	74	101
(**1a**)	DIPE	5	51	89	35
2-Heptylamine (**1b**)	DME	27	52.6	99.7	105
	DIPE	5	50	83	26
2-Pentylamine (**1c**)	DME	5.5	48	76	24
	DME[b]	24	51	92	46
	DIPE	5.5	59	89	13
	Dioxane	5.5	63	88	8
	THF	5.5	44	52	7
	Hexane	5.5	47	48	7
2-Butylamine (**1d**)	DME	5	40	63	4
	DME[b]	21	50	21	6

[a]Conditions: amine in organic solvent (0.2M, 5 mL), Novozym 435 (20 mg/mL), mol. sieves 4 A and 5 A (each 30 mg/mL), internal standard 1,3-dimethoxybenzene, incubation at 40 ^{o}C.
[b]Room temperature.

The best enantiomeric ratio in these resolutions was obtained when the acylation was carried out in DME or DIPE. Although reactions in DME were slower than those in DIPE, DME is much preferred on account of its comparatively high enantiomeric ratio. Accordingly, further acylations were carried out in DME.

The resolution of **1a** and **1b** with ethyl 4-pyridylacetate gave satisfactory results as regards the reaction rate and enantioselectivity. In general, amines with a longer side chain reacted slower (24-30 h to reach 50% conversion of **1a** and **1b**, compared to 5.5 h in the case of **1c** and **1d**). The enantiomer discrimination for amines with larger R groups (**1a** and **1b**) was excellent (*E* > 100). As expected, amines with shorter R groups showed lower *E* (28 and 4

for **1c** and **1d** respectively). Presumably the small size difference between the methyl and the ethyl or propyl groups is the reason that CaLB has difficulties in discriminating these two groups. Fortunately, the enantioselectivity increased from 28 to 46 (**1c**) and from 4 to 6 (**1d**) upon a decrease of the reaction temperature from 40^{o}C to room temperature.

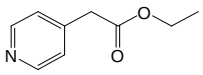
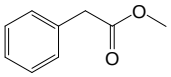
Acyl donor: effect of the nitrogen atom in the aromatic ring

Beside the reaction medium that plays an important role in the enzymatic reaction, the right choice of acyl donor is also essential for optimization of the reaction[11,12, 13]. The electronic property of an acyl donor is known to influence the success of the enzymatic resolution.

The results presented in Table 1 show that CaLB accepts ethyl 4-pyridylacetate as its substrate to perform a fast and enantioselective amine acylation. The nitrogen atom in the pyridine ring is expected to have important role in the reaction; since it is known that a heteroatom in the acyl donor influences the reaction rate and the enantioselectivity of CaLB catalysed amine acylation (Chapter 3).

Here the effect of the nitrogen substituent in the acyl donor was assessed by comparing the acylation of **1a** and **1b** with methyl phenylacetate and ethyl 4-pyridylacetate, as regards enantiomeric bias and enantioselectivity. The results are shown in Table 2.

Table 2.Effect of nitrogen in the phenyl ring: phenylacetic acid ester and pyridylacetic acid ester as acyl donor[a].

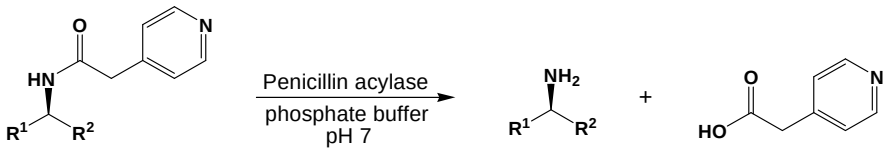
Acyl donor	Amine	Results		
		Time (h)	Conversion (%)	*E*
	2-heptylamine (**1b**)	5	42	>100
	1-phenylethylamine (**1a**)	5	29	> 100
	2-heptylamine (**1b**)	5	38	30
	1-phenylethylamine (**1a**)	5	18	23

[a]Conditions: amine (1 mmol) in 1,2-dimethoxyethane (5 mL), acyl donor (1 mmol), Novozym 435 (100 mg), molecular sieves 4 A and 5 A (each 150 mg), internal standard 1,3-dimethoxybenzene, incubation at 40 ^{o}C.

The results in Table 2 demonstrate that the pyridyl nitrogen in the acyl donor indeed influences the results of the amine acylation. *E* increased from 30 (**1b**) and 23 (**1a**) to more than 100, and the acylation of **1a** becomes faster as well. In conclusion, the pyridyl ring in the acyl donor improved both the rate and the enantiodiscrimination.

2. Deacylation of pyridylacetyl amides by penicillin acylase A. faecalis

As discussed above, penicillin acylase is the preferred biocatalyst for the hydrolysis of *N*-pyridylacetyl-(*R*)-2-aminoalkanes (Figure 3). The acylase from *A. faecalis* was chosen on account of its high enantioselectivity towards chiral amines[14]. The results of the enzymatic hydrolyses are shown in Table 3.



(3)

3a. $R^1 = CH_3$; $R^2 = C_6H_5$
3b. $R^1 = CH_3$; $R^2 = C_5H_{11}$
3c. $R^1 = CH_3$; $R^2 = C_3H_7$
3d. $R^1 = CH_3$; $R^2 = C_2H_5$

Figure 3. Hydrolysis of pyridylacetamides catalyzed by *A. faecalis.*penicillin acylase

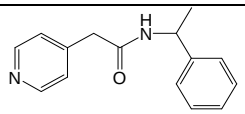
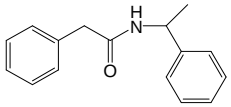
Table 3. Hydrolysis of pyridylacetamides catalyzed by *A. faecalis* penicillin acylase[a]

Amide		**Reaction time (h)**	**Amine**	
Compound	***ee*[b] (%)**		**Conversion (%)**	***ee* (%)**
3a	97	4	93	96
3b	*97*	4	96	97
3c	*88*	4	91	90
3d	*57*	4	89	n.d.

[a] Conditions: amide (0.02 mmol) in phosphate buffer 0.1 M pH 7.0 (3.5 mL), *A. faecalis* penicillin acylase (30 mg; 8 BPU), diphenylacetic acid as internal standard, incubation at room temperature.
[b] Calculated from the *ee* of unconverted amine and the conversion in the acylation step.

The enantio-enriched pyridylacetamides were hydrolyzed fast. Almost 100 % conversion of the amides was reached within 4 h, liberating the corresponding amines with high enantiopurity (ee > 90 %). This fast reaction rate is ascribed to the good solubility of the pyridyl amides, as it was visually observed that all amides were dissolved well in the aqueous reaction medium. The effect of the pyridyl ring in the hydrolysis was further investigated by comparing the enzymatic hydrolysis of **3a** and *N*-phenylacetyl phenylethylamide, as regards the hydrolysis rate and the enantioselectivity. The results are presented in Table 4 and Figure 4.

Table 4. Effect of the nitrogen atom: the hydrolysis of racemic phenylacetylamides and pyridylacetamide catalyzed by *A. faecalis* penicillin acylase[a].

Racemic amide	Results after 4 hours		
	Conv. (%)	***ee*** **amine (%)**	***E***
	51	70 *(R)*	13
	35	33 *(R)*	6

[a]Conditions: amide (0.02 mmol) in phosphate buffer 0.1 M pH 7.0 (3.5 mL), *A. faecalis* penicillin acylase *(*immobilized, 30 mg, 8 BPU), diphenylacetic acid as internal standard, incubation at room temperature.

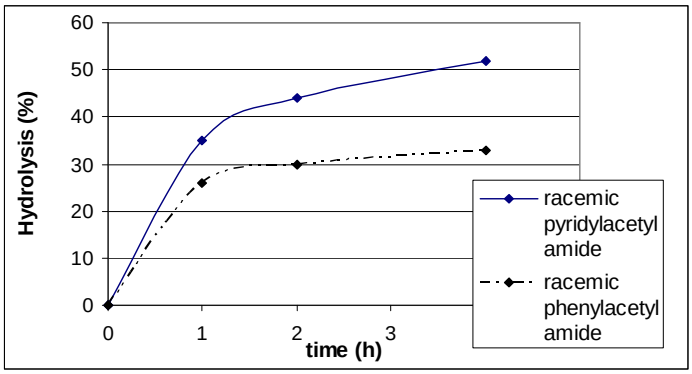
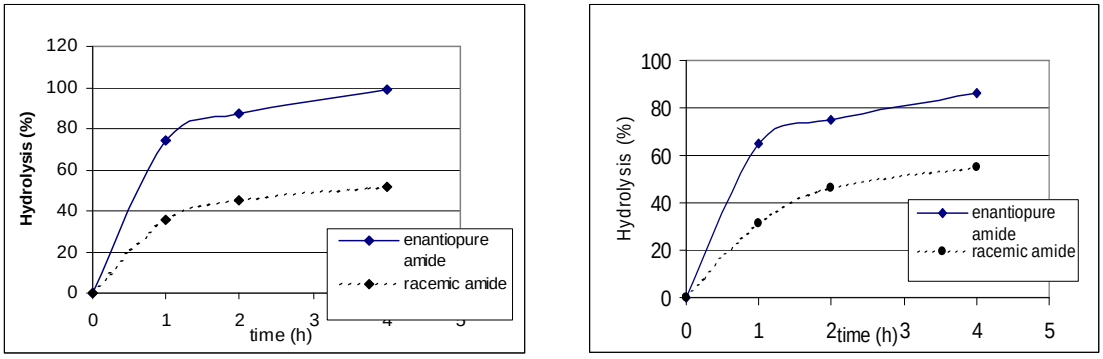


Figure 4. The progress curve of the hydrolysis of racemic amides catalyzed by *A. faecalis* penicillin acylase

Results in Table 4 and Figure 4 show that **3a** is hydrolyzed faster with better enantioselectivity, compared to the corresponding phenylacetyl amide (*E* = 13 *vs* 6). The enantioselectivity of the hydrolysis reaction could be observed, when the hydrolysis of the enantiopure of (*R*)-**3a** and (*R*)-**3c** was compared with the hydrolysis of the racemates. The results are shown in Figure 5.



(3a) **(3c)**

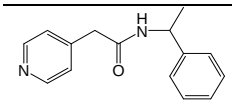
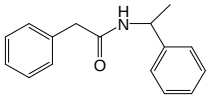
Figure 5. The hydrolysis of **3a** and **3c** in the presence of penicillin acylase from *A. faecalis*

In both cases, approximately 50% of racemic amides were hydrolyzed after 4 hours, while almost 100% of enantiopure (*R*)-amides was hydrolyzed within the same time. These results demonstrate the preference of *A. faecalis* penicillin acylase for the (*R*)-amide. However, almost all of the racemic amide eventually hydrolyzed after 24 hours (data not shown).

We also observed that the hydrolysis rates of the enantiopure amides decreased after ~80% conversion was reached (Figure 5), presumably due to inhibition by the liberated pyridylacetic acid. Inhibition of pencillin acylase by phenylacetic acid is one common issue with regard to the slow rate in the hydrolysis of phenylacetamide.

Accordingly, we investigated the inhibition by pyridylacetic acid. One equivalent of pyridylacetic acid was added to the hydrolysis of **3a**. Similarly, the hydrolysis of corresponding phenylacetylamide was performed in the presence of one equivalent of phenylacetic acid (Table 5 and Figure 6).

Table 5. Effect of the presence of acid on the hydrolysis of racemic amides[a]

Amide	Time (h)	Hydrolysis of amide (%)	
		No acid	With 1 eq. acid
	4	51	23
	24	81	40
	4	35	12
	24	48	12

[a] Reaction conditions: amide (0.02 mmol) in phosphate buffer 0.1 M pH 7.0 (3.5 mL), *A. faecalis* penicillin acylase (immobilized, 30 mg; 8 BPU), diphenylacetic acid as internal standard, incubation at room temperature. Addition of phenylacetic acid or pyridylacetic acid is 1 equivalent to the amine.

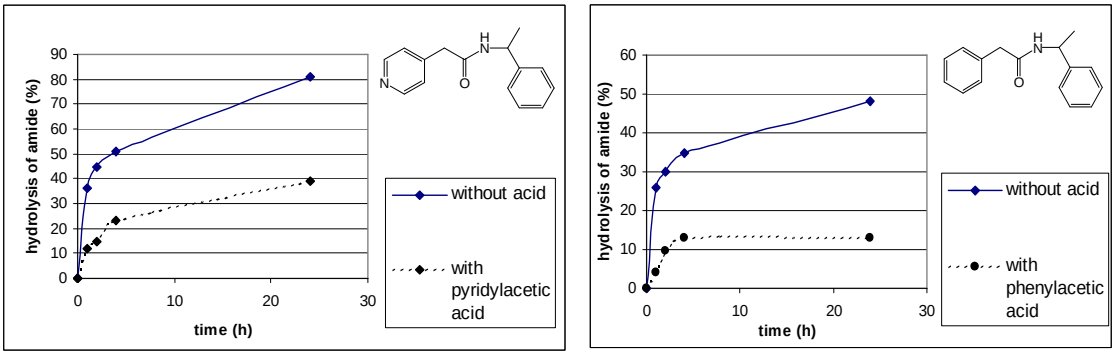


Figure 6.Curve of the hydrolysis of amide in presence of the liberated acid.

Both acids suppressed the hydrolysis; indicating the inhibition of the enzyme's activity by pyridylacetic acid as well as phenylacetic acid. Inhibition by phenylacetic acid was much more severe, however, and brought the reaction to a standstill (see Figures 6a and b).

In conclusion, *A faecalis* penicillin acylase catalyzed the hydrolysis of pyridylacetamides with enantiopreference for the (*R*)-amide. The enzyme inhibition effect caused by the liberated pyridylacetic acid was less than that of observed with phenylacetic acid, which allowed the hydrolysis to reach almost 100% conversion after 4 hours.

CONCLUSION

A fully enzymatic method for the resolution of chiral amine using ethyl 4-pyridylacetate as acyl donor has been demonstrated. It is achieved by a lipase-catalyzed acylation and a penicillin acylase-catalyzed deacylation. Making use of ethyl 4-pyridylacetate as acyl donor resulted in an enantioselective acylation catalyzed by CaLB, followed by a fast deacylation step catalyzed by *A. faecalis* penicillin acylase.

EXPERIMENTAL PART

1. Materials

Enzymes: Novozym 435 (immobilized *Candida antarctica* lipase B) was kindly donated by Novozymes; Penicillin acylase from *Alcaligenes faecalis* was obtained from fermentation of recombinant *E. Coli*[15]. Chemicals: ethyl 4-pyridylacetate was chemically synthesized by esterification of 4-pyridylacetic acid (Acros) with ethanol. Other chemicals were purchased from Sigma and Acros.

2. Synthesis of non-commercial ethyl pyridylacetate

A mixture of 4-pyridylacetic acid hydrochloride (4 g), absolute ethanol (16 mL) and concentrated sulfuric acid (6.8 g) was heated under stirring at 95 ^{o}C for 4 hours. After cooling to room temperature, the mixture was poured into 100 g of crushed ice then a sufficient amount of ammonium hydroxide was added to adjust the pH to alkaline (pH 9). The product was extracted with 4 x 50 mL diethyl ether and the combined ether layers were washed with water and saturated sodium bicarbonate. After drying over anhydrous sodium sulfate, the solvent was removed under vacuum, affording ethyl pyridylacetate as a yellowish oil (yield 2.9 g; 87 %), Mw of 165, ^{1}H NMR: δ 1.24 (*t*, 3H, CH_3), 3.62 (*s*, 2H, $COC\underline{H}_2CH$), 4.19 (*q*, 2H, $OC\underline{H}_2CH_3$), 7.24 (*d*, 2H, aromatic protons), 8.5 (*d*, 2H, aromatic protons). ^{13}C NMR (300 MHz): 170.35, 150.20, 143.28, 124.84, 61.60, 41.01, 14.43.

3. Enzymatic acylation of chiral amines

Chiral amine (**1a**, **1b**, **1c** or **1d**, 0.5 mmol) and ethyl 4-pyridylacetate (82.5 mg, 0.5 mmol) were dissolved in 2.5 mL of solvent. Molecular sieves 4 A and 5 A (30 mg/mL each), 25 mg of enzyme (Novozym 435), and 1,3-dimethoxy benzene (internal standard) were added. The reaction mixture was then incubated at 40 ^{o}C, samples (100 μL, diluted in 400 mL of 1,2-dimethoxyethane) were taken every 2 h to follow the reaction over time. Analysis to follow the conversion was performed by Gas Chromatography, and chiral HPLC was used to analyze the enantiomeric excess of the un-reacted amine.

4. Enzymatic synthesis of enantiopure amides

Chiral amine (**1a**, **1b**, **1c** or **1d,** 10 mmol) and ethyl 4-pyridylacetate (6 mmol) were dissolved in 10 ml of 1,2-dimethoxyethane. Molecular sieves of 4 A and 5 A (300 mg each) and 200 mg of enzyme (Novozym 435) were added, and then the reaction was incubated at 40^{o}C (for **1a** and **1b**) or room temperature (for **1c** and **1d**). The reaction was stopped at 50 % conversion of the amine. After removing the enzyme and molecular sieves by filtration, the organic solvent was evaporated under vacuum then the residue was dissolved in 20 ml of dichloromethane. The excess of un-reacted amine was extracted with aqueous hydrochloric acid (pH 6). Crude products of **3a**, **3b**, or **3c** were obtained as residue after dichloromethane was evaporated under vacuum.

Removal of the excess of amine in **3d** was done by evaporation, followed by extraction of the amide with a solution of hydrochloric acid (pH 4.0) and vacuum evaporation of the solvent. Recrystalizations of all amides were done using chloroform and petroleum ether, to give the pure amide that confirmed by GC-MS, ^{1}H NMR and ^{13}C NMR analysis.

a. *N*-(1-phenylethyl)-4-pyridylacetamide (3a)

Product obtained as white-yellowish crystals (yield 41 %), Mw of 240, ^{1}H NMR: δ 1.43 (*d*, 3H, NHCHC$\underline{H}_3$), 3.50 (*s*, 2H, COC$\underline{H}_2$C), 5.10 (*q*, 1H,

NHCHC), 6.15 (*d*, 1H, CONHCH), 7.18-7.34 (*m*, 7H, aromatic protons), 8.51 (*d*, 2H, aromatic protons). ^{13}C NMR (75 MHz): 168.33, 150.12, 144.27, 142.95, 128.91, 127.70, 126.26, 124.64, 49.31, 43.02, 21.78.

b. *N*-(1-pentylethyl)-4-pyridylacetamide (3b)

Product obtained as white-yellowish crystals (yield 45 %), Mw of 231, ^{1}H NMR: δ 0.86 (*t*, 3H, $CH_2CH_2CH_3$), δ 1.10 (*t*, 3H, NHCHCH_3), 1.24-1.38 (*m*, 8H, CH(CH_2)$_4CH_3$), 3.50 (*s*, 2H, COCH_2C), 3.95-3.98 (*m*, 1H, CH_3CHCH_2), 5.45 (*d*, 1H, CONHCH), 7.21-7.28 (*d*, 2H, aromatic protons), 8.55 (*d*, 2H, aromatic protons). ^{13}C NMR (100 MHz): 168.23, 150.10, 144.26, 124.42, 45.66, 43.15, 36.72, 31.57, 25.64, 22.53, 20.86, 13.98.

c. *N*-(1-propylethyl)-4-pyridylacamide (3c)

Product obtained as white crystals (yield 38 %), Mw of 206, ^{1}H NMR: δ 0.89 (*t*, 3H, $CH_2CH_2CH_3$), δ 1.10 (*t*, 3H, NHCHCH_3), 1.31-1.36 (*m*, 4H, CH(CH_2)$_2CH_3$), 3.51 (*s*, 2H, COCH_2C), 3.95-4.05 (*m*, 1H, CH_3CHCH_2), 5.38 (*d*, 1H, CONHCH), 7.21-7.28 (*d*, 2H, aromatic protons), 8.56 (*d*, 2H, aromatic protons).

d. *N*-(1-methylethyl)-4-pyridylacetamide (3d)

Product obtained as white-yellowish crystals (HCl crystal, yield 35 %), ^{1}H NMR: δ 0.90 (*t*, 3H, $CHCH_2CH_3$), δ 1.20 (*t*, 3H, NHCHCH_3), 1.55-1.60 (*m*, 2H, CH(CH_2)CH_3), 3.50 (*s*, 2H, COCH_2C), 3.95-3.99 (*m*, 1H, CH_3CHCH_2), 5.40 (*d*, 1H, CONHCH), 7.28-7.35 (*d*, 2H, aromatic protons), 8.60 (*d*, 2H, aromatic protons).

5. Chemical synthesis of racemic amides

4-Pyridylacetic acid (500 mg, 3 mmol) and pyridine (2 mL) were dissolved in 5 mL of dichloromethane, then a solution of 450 mg dicyclohexylcarbodiimide

(DCC) in dichloromethane was added, followed by the addition of 4 mmol amine. The reaction mixture was stirred at room temperature for 24 h.
After removing the precipitating dicyclohexylurea (side product), the excess of amine was removed by extraction using a solution of hydrochloric acid (pH 6). The solvent was evaporated after been washed several times with water and dried over magnesium sulfate. Recrystalization of the residu from chloroform and petroleum ether afforded pure amide that was confirmed by GC-MS and ^{1}H- NMR.

6. Enzymatic hydrolysis of amides

The amide (0.02 mmol) dissolved in 3.5 mL of phosphate buffer (100 mM pH = 7.0). *A. faecalis* penicillin acylase (immobilized, 30 mg) and diphenylacetic acid (internal standard) was added. The reaction mixture was shaken at room temperature, and 200 μL of samples were taken every hours. After removing the enzyme, the solution was subjected to HPLC analysis to monitor the hydrolysis of the amide and the enantiomeric excess of the liberated amine.

7. Analysis

7.1. Gas Chromatography:

Analysis was performed using a CP SIL 5 CB column with programmed temperature from 50 ^{o}C–250 ^{o}C. The retention times were (minutes): 4-pyridine ethyl ester (18), 2-heptylamine (8), 1-phenylethylamine (13), *N*-(1-pentylethyl)-4-pyridylacetamide (29 min), and *N*-(1-phenylethyl)-4-pyridylacetamide (32). *N*-(1-propylethyl)-4-pyridylacetamide (22 min), *N*-(1-methylethyl)-4-pyridylacetamide (17 min).

7.2. HPLC:

Samples were analyzed by HPLC using a Waters M6000 pump, a nucleosil C-18 column and a shimadzu SPD-6A UV detector at 250 nm. The eluent was a

mixture of methanol/water (68/32) and 0.1 % sodium dodecylsulfate with pH adjusted to 3.5, and the flow rate was 1.0 mL/min. The retention times (minutes) were as follows: 1-phenylethylamine (13.5), diphenylacetic acid (9.6), 4-pyridylacetic acid (5.1), *N*-(1-phenylethyl)-4-pyridylacetamide (14), *N*-(1-pentylethyl)-4-pyridylacetamide (14 min), *N*-(1-propylethyl)-4-pyridylacetamide (12 min), *N*-(1-methylethyl)-4-pyridylacetamide (11 min).

Chiral analysis of the liberated 1-phenylethylamine (**1a**) was carried out using a crown pack Cr^{+} column and a Shimadzu SPD-6A UV detector at 250 nm. The eluent was a solution of perchloric acid pH 1.5 and 5 % methanol, with a flow rate of 0.6 mL/min. The retention times (minutes) were as follows: *(R)*-1-phenylethylamine (21.7 min) and *(S)*-1-phenylethylamine (16.3 min).

Chiral analysis for the liberated 2-heptylamine (**1b**) and 2-pentylamine (**1c**) was carried out using an OD column after derivatization with benzoyl chloride. A mixture of hexane/isopropanol (95/5) was used as the eluent with a flow rate of 0.6 mL/min. The retention times (minutes) were as follows: (*R*)-2-heptylamine (19.9), (*S*)-2-heptylamine (17.7), (*R*)-2-pentylamine (18.4), (*S*)-2-pentylamine (14.9).

REFERENCES

1. Cole, M., *Biochem*. *J*. **1969**, *115*, 741
2. Van Rantwijk, F., Sheldon, R. A, *Tetrahedron* **2001**, *60*, 501-509
3. Margolin, A. L., *Tetrahedron Lett.* **1993**, *34*, 239-1242
4. Svedas, V., Guranda, D., Van Langen, L., Van Rantwijk, F., Sheldon, R. A., *FEBS Lett.* **1997**, *417*, 414-418.
5. Fuganti, C., Grasselli, P., Casati, P., *Tetrahedron Lett.* **1986**, *27*, 3191
6. Pohl, T., Waldman, H., *Tetrahedron Lett*. **1995**, *36*, 2963-2966.
7. Guranda, D. T., Van Langen, L. M., Van Rantwijk, F., Sheldon, R. A., *Tetrahedron: Asymmetry* **2001**, *12*, 1645-1650.
8. Santoz, A. M., Vidal, M., Pachecho, Y., Frontera, J., Baez, C., Ornellas, O., Barletta, G., Griebenow, K., *Biotechnol. Bioeng.* **2001**, *74*, 295-308

9. Griebenow, K., Vidal, M., Baez, C., Santoz, A. M., Barletta, G., *J. Am. Chem. Soc.* **2001,** *123*, 5380-5381
10. Schmitke, J. L., Wescout, C. R., Klibanov, A. M., *J. Am. Chem. Soc.* **1996**, *118*, 3360.
11. Gotor, V., *Bioorg. Med. Chem.* **1999**, *7*, 2189-2197.
12. Takayama, S., Lee, S. T., Hung, S. C., Wong, C. H., *Chem. Commun.* **1999**, 127-128
13. Ottoson, J., Hult, K., *J. Mol. Catal. B: Enzym.* **2001**, *11*, 1025-1028
14. Guranda, D. T., Khimiuk, A. I., Van Langen, L. M., Van Rantwijk, F., Sheldon, R. A., Svedas, V. K., *Tetrahedron Asymmetry* **2004**, *15*, 2901-2906.
15. Lau, R. M., PhD Thesis, Delft University of Technology, Delft, The Netherlands, 2003.

5

Improved Acyl Donor:

the Pyridyloxyacetate Ester

ABSTRACT

An ester of (3-pyridyloxy)acetate was applied as acyl donor in the fully enzymatic resolution of 1-phenylethylamine and 2-heptylamine. Oxygen substitution at the β-position in the acyl donor significantly increased the reactivity in the CaLB-catalyzed acylation. The enzymatic resolution at low reaction temperature (–10 ^{o}C) was quite fast with good enantioselectivity (*E*=51 for 2-heptylamine and *E*>100 for 1-phenylethylamine). The hydrolysis of the amides was carried out in the presence of CaLB or penicillin V acylase as the catalyst; approximately 90% of the amides were hydrolyzed in 24 h, liberating enantiopure amines (*ee* > 95%).

INTRODUCTION

In Chapter 4, a fully enzymatic method for the resolution of chiral amines with ethyl 4-pyridylacetate as the acyl donor has been demonstrated. We found that the pyridyl ring in the acyl donor was beneficial in both the enzymatic acylation and deacylation steps. In the acylation step, which was performed in the presence of CaLB, the pyridyl ring in the acyl donor increased the reaction rate and enantioselectivity. Furthermore, the corresponding pyridylacetyl amides were more soluble in aqueous medium than the corresponding carbocyclic compounds, resulting in a faster enzymatic hydrolysis[1]. However, the acylation step was still undesirably slow (>20 h to reach 50% conversion) and might be improved. Therefore, in this Chapter we attempted to improve this method through a structural modification of the acyl donor.

As presented in Chapter 3, a heteroatom at the β-position in the acyl donor increases the reaction rate in the order O > S > NH > CH_2. A comparison of the enzymatic acylation of 1-phenyethylamine with methyl phenoxyacetate and with methyl 3-phenylpropionate illustrates the trend (Figure 1).

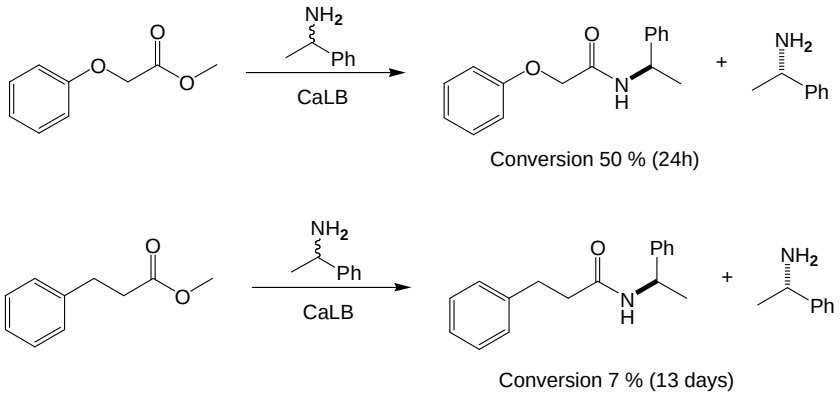


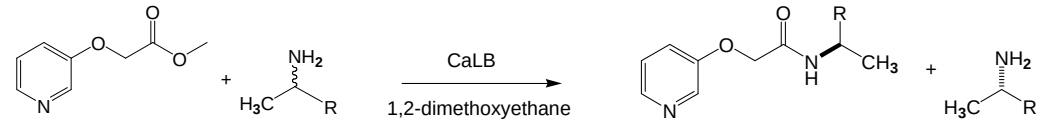
Figure 1. Effect of oxygen at the β-position in the acyl donor

Accordingly, an oxygen atom at the β-position in a pyridyl-substituted acyl donor is expected to improve the reaction rate, too. Here a new acyl donor, methyl 3-pyridyloxyacetate, has been synthesized and investigated as regards reaction rate and enantioselectivity.

RESULTS AND DISCUSSION

1. Enzymatic acylation

Methyl 3-pyridyloxyacetate was employed as the acyl donor in the acylation of 1-phenylethylamine (**1a**) and 2-heptylamine (**1b**), see Figure 2.



methyl pyridoxy acetate (1) (2)

1a. 1-phenylethylamine (R = C_6H_5)
1b. 2-heptylamine (R = C_5H_{11})
2a. *N*-(1-phenylethyl)pyrid-3-yloxyacetamide (R = C_6H_5)
2b. *N*-(2-heptyl)pyrid-3-yloxyacetamide (R = C_5H_{11})

Figure 2. Enzymatic acylation of amines with methyl 3-pyridyloxyacetate

The acyl donor was synthesized chemically from 3-hydroxypyridine and methyl chloro-acetate through the *Williamson ether synthesis* method[2,3]. A low yield was obtained (18% yield) due to the formation of by-product and difficulties in the product isolation. This problem presumably can be solved by optimization of the synthesis procedure.

We found methyl 3-pyridyloxyacetate to be a highly reactive acyl donor in the CaLB-catalyzed acylation, which is ascribed to the oxygen atom at the β-position of the acyl donor. However, the enantioselectivity was much lower than expected on the basis of our previous experience. In the acylation of **1a** and **1b** at 40 ^{o}C, 65% of amine was converted in 45 min. but with enantiomeric ratio (*E*) of only 10 and 7, respectively (data not shown). Blank tests on both amines showed that no non-enzymatic reaction was involved.

Since reducing the reaction temperature tends to improve the enantioselectivity in a lipase-catalyzed resolution[4,5], we conducted the resolution of **1a** and **1b** at a temperature range from room temperature to –10 ^{o}C. The results are shown in Table 1 and Figure 3.

It became apparent that lowering the reaction temperature successfully increased the enantioselectivity. In the resolution of **1a**, reducing the temperature to –10 °C increased *E* from 46 to >100. However, resolution of **1b** under similar condition only gave a moderate enantiomeric ratio (*E* = 51). A high reactivity of the ester was still observed at –10 °C, giving 50% conversion of **1a** and **1b** in less than 5 h.

Table 1. Enzymatic acylation with methyl 2-(3-pyridyloxy)acetate as acyl donor[a].

Amine	Temperature (°C)	Time (min)	Conversion (%)	ee_{amine} (%)	*E*
1a	r.t.	45	48	72	46
	5	45	45	78	86
	-10	45	26	34	130
		300	48	89	121
1b	r.t.	45	59	79	19
	5	45	44	66	27
	-10	45	30	42	48
		120	50	93	51

[a]Conditions: amine in 1,2-dimethoxyethane (0.1 M), acyl donor (0.1 M), Novozym 435 (10 mg/mL), mol. sieves 4 A and 5 A (60 mg/mL), internal standard 1,3-dimethoxybenzene.

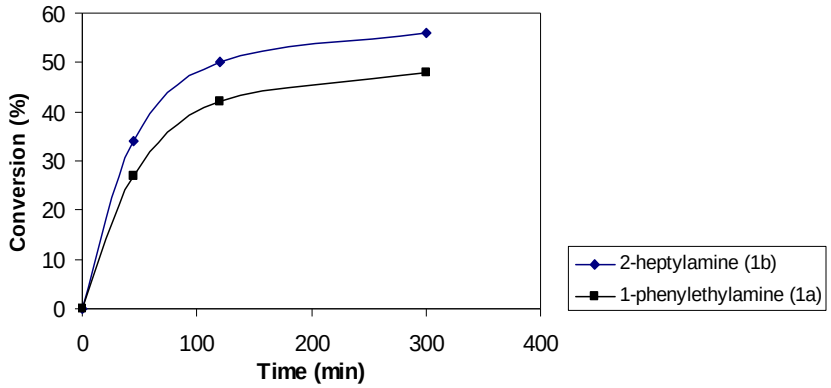
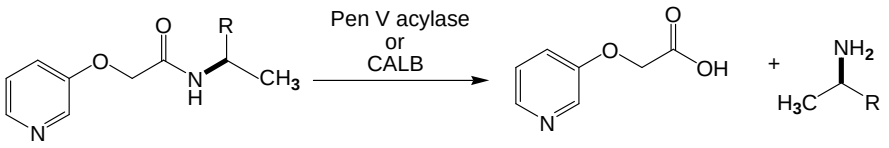


Figure 3. Enzymatic acylation of **1a** and **1b** in the presence of CaLB at –10 °C

2. Enzymatic deacylation

The enzymatic hydrolysis of the amide in the presence of penicillin V acylase and CaLB was investigated. Penicillin V acylase (Semacylase) was chosen on account of its substrate specificity for the phenoxyacetyl moiety[6,7], and accordingly is expected to be able to hydrolyse the pyridyloxy-substituted amides. CaLB (Novozym 435) was also employed as the catalyst, because of its high affinity for the pyridyloxyl moiety and the ability to hydrolyse amides (Chapter 3)[8,9,10]. The hydrolysis reaction is shown in Figure 4.



(**2**) (**3**) (**1**)

2a. *N*-(1-phenylethyl)pyrid-3-yloxyacetamide ($R = C_6H_5$)

2b. *N*-(2-heptyl)pyrid-3-yloxyacetamide ($R = C_5H_{11}$)

1a = 1-phenylethylamine ($R = C_6H_5$)

1b = 2-heptylamine ($R = C_5H_{11}$)

Figure 4. Hydrolysis reaction of the amides catalyzed by penicillin V acylase or CaLB.

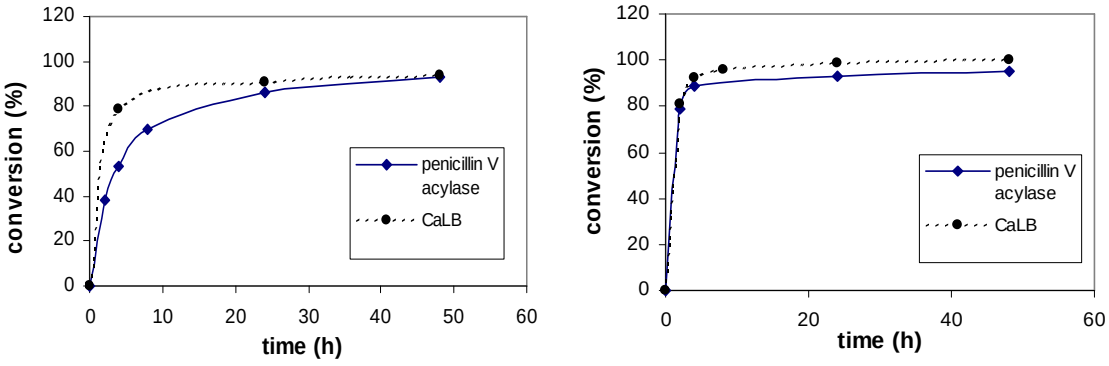
The results from the enzymatic hydrolysis with both enzymes show that deacylation of the amides was easy, affording amines with high enantioselectivity (ee_{amine} >95%) as the hydrolysis product. In general, about 90% of the amides were hydrolyzed within 24 h (**2a**) or 4h (**2b**). The results are shown in Table 2.

Table 2. Hydrolysis of amides catalyzed by pencillin V acylase and CaLB[a].

Amide	Penicillin V acylase (semacylase)		*C. antarctica* lipase (CaLB)	
	Conversion (%)	ee_{amine} (%)	Conversion (%)	ee_{amine} (%)
2a	95 (48h)	98.5	90 (24 h)	99
2b	89 (4h)	92	92 (4 h)	93

[a]Reaction conditions: amide (0.015 mmol) in phosphate buffer pH 7.0 (1.5 mL), penicillin V acylase (30 mg, 5.5 U) or Novozym 435 (30 mg; 9 U), internal standard diphenylacetic acid, incubation at 70 °C (in CaLB treatment) or 40 °C (in penicillin V acylase treatment).

We found that the hydrolytic activity of CaLB for these amides was comparable to that of penicillin V acylase. No significant difference was observed in the hydrolysis rates of both enzymes, even higher initial rate was observed in the hydrolysis of **2a** with CaLB, though in both case almost complete hydrolysis was reached after 48h (Figure 5).

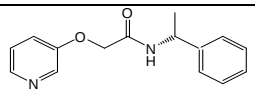
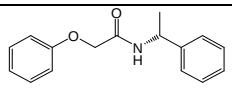
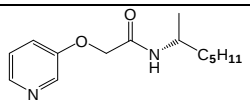
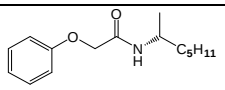


(**2a**) (**2b**)

Figure 5. Hydrolysis of *N*-(1-phenylethyl)pyirid-3-yloxyacetamide (**2a**) and *N*-(2-heptyl)pyirid-3-yloxyacetamide (**2b**) catalyzed by penicillin V acylase and CaLB .

Compared to the hydrolysis of phenoxacetamides (see Chapter 3), it is clear that the pyridyl group significantly increased the hydrolysis rate, which is ascribed to the better solubility of the pyridyloxyacetamides in aqueous medium. Table 3 shows the comparison of both amides performance in the enzymatic hydrolysis reaction.

Table 3. Hydrolysis of 3-pyridyloxylacetamides and phenoxylacetamides catalyzed by penicillin V acylase[a]

	Conversion after 24 h(%)	
Amide	**Penicillin V acylase**	**CaLB Novozym 435**
	86	90
	38	42 (in 120 h)*
	93	99
	66	21 (in 120 h)*

** Data also presented in Chapter 3*

[a]Reaction conditions: amide (0.015 mmol) in phosphate buffer pH 7.0 (1.5 mL), penicillin V acylase (30 mg; 5.5 U) or Novozym 435 (30 mg; 9 U), internal standard diphenylacetic acid, incubation at 70 ^{o}C (in the CaLB-catalyzed hydrolysis) or 40 ^{o}C (in the penicillin V acylase-catalyzed hydrolysis).

Summarizing, introducing a pyridyl group and a β-oxygen in the amide improved the performance of the enzymatic hydrolysis catalyzed by CaLB as well as by penicillin V acylase.

CONCLUSION

Methyl 3-pyridyloxyacetate was shown to be an effective donor for the fully enzymatic resolution of 1-phenylethylamine and 2-heptylamine. The enzymatic resolution with CaLB at –10 ^{o}C was smooth with good

enantioselectivity (*E*=51 for 2-heptylamine and *E*>100 for 1-phenyl-ethylamine). The following amide hydrolysis with penicillin V acylase and CaLB as catalyst in aqueous medium was faccile, liberating enantiopure amines as the hydrolysis product.

EXPERIMENTAL PART

1. Materials

Enzymes: Novozym 435 (immobilized *Candida antarctica* lipase B) and pencillin V acylase (*Semacylase*) were kindly donated by Novozymes.

Chemicals: methyl 3-pyridyloxyacetate was chemically synthesized from 3-hydroxypyridine and methyl chloroacetate through the 'Williamson ether synthesis' procedure. Other chemicals were purchased from Sigma and Across.

2. Synthesis of methyl 2-(3-pyridyloxy)acetate[11,12]

A mixture of 3-hydroxypyridine (0.1 mol), methyl chloroacetate (0.12 mol), and anhydrous potassium carbonate (0.15 mol) in dry acetone (170 mL) was refluxed (70 ^{o}C) for 21 hours. The reaction progress was monitored by thin layer chromatography, until sufficient amount of product was detected.

The reaction mixture was cooled to room temperature and the precipitated by product was removed by filtration. Crude product (dark-coloured residue) was obtained after evaporating the solvent in *vacuo*, and the main product was isolated by column chromatography using ethyl acetate as the eluent. Pure methyl 3-pyridyloxyacetate was obtained after recrystalization from petroleum ether 40/60 (yield 18%), mw of 168, ^{1}H-NMR (300 MHz): δ 3.81 (*s*, 3H, C$\underline{H}_3$), 4,69 (*s*, 2H, COC$\underline{H}_2$O), 7.21-8.35 (*m*, 4H, aromatic protons). ^{13}C NMR (75 MHz): 52.39, 65.43, 121.54, 123.87, 138.10, 143.27, 154.09, 168.72.

3. Enzymatic acylation of chiral amines

Chiral amine (**1a** or **1b** 0.25 mmol) and methyl 3-pyridyloxyacetate (0.25 mmol) were dissolved in 2.5 mL of 1,2-dimethoxyethane. Molecular sieves of 4 A and 5 A (75 mg each), 25 mg of enzyme (Novozym 435), and 1,3-dimethoxybenzene (internal standard) were added. The reaction mixture was incubated at 40 ^{o}C, room temperature, 5 ^{o}C, or –10 ^{o}C. Samples (50 μL, diluted in 200 μL of 1,2-dimethoxyethane) were taken regularly to follow the reaction over time. Analysis was performed by gas chromatography and chiral HPLC.

4. Enzymatic synthesis of enantiopure amides

Chiral amine (**1a** or **1b,** 1 mmol) and methyl pyridyloxyacetate (1 mmol) were dissolved in 10 mL of 1,2-dimethoxyethane. Molecular sieves of 4 A and 5 A (300 mg each) and 100 mg of enzyme (Novozym 435) were added; the reaction mixture was incubated at 5 ^{o}C, and the reaction was stopped at 50% conversion of the amine. After removing the enzyme and molecular sieves by filtration, the organic solvent was evaporated under vacuum. The residue was dissolved in a solution of sodium hydroxide pH 12. The excess of unreacted amine was extracted with hexane, then the aqueous phase was neutralized with hydrochloric acid solution. After evaporation of the aqueous phase, residu was dissolved in dichloromthane. Products were obtained after removing the precipitated sodium chloride and evaporation of the organic solvent.

4.1. *N*-(1-phenylethyl)pyrid-3-yloxyacetamide (2a)

Product obtained as brownish crystals (yield 10 %), Mw of 256, ^{1}H NMR: δ 1.53-1.55 (*d*, 3H, NHCHC$\underline{H}_3$), 4.51 (*s*, 2H, COC$\underline{H}_2$C), 5.21-5.26 (*m*, 1H, NHC$\underline{H}$C), 6.84 (*d*, 1H, CON$\underline{H}$CH), 7.17-7.32 (*m*, 7H, aromatic protons), 8.27-8.34 (*d*, 2H, aromatic protons). ^{13}C NMR (75 MHz): 166.41, 153.51, 143.45, 142.54, 138.39, 128.77, 127.59, 126.15, 124.05, 121.22, 67.55, 48.47, 21.68.

4.2. *N*-(2-heptyl)pyrid-3-yloxyacetamide (2b)

Product obtained as yellowish crystals (yield 15 %), Mw of 250, ^{1}H NMR: δ 0.85-0.89 (*t*, 3H, $CH_2CH_2C\underline{H}_3$), δ 1.16-1.19 (*d*, 3H, $NHCHC\underline{H}_3$), 1.26-1.48 (*m*, 8H, $CH(C\underline{H}_2)_4CH_3$), 4.05-4.10 (*m*, 1H, NHC<u>H</u>), 4.51 (*s*, 2H, $COC\underline{H}_2C$), 6.84 (*d*, 1H, CON<u>H</u>CH), 7.23-7.27 (*m*, 7H, aromatic protons), 8.30-8.39 (*d*, 2H, aromatic protons). ^{13}C NMR (100 MHz): 166.49, 153.57, 143.52, 138.52, 124.03, 121.06, 67, 60, 45.20, 36.78, 31.60, 25.65, 22.54, 20.92, 13.98.

5. Enzymatic hydrolysis of amides

Amide (0.015 mmol) was dissolved in 1.5 mL of phosphate buffer (100 mM pH = 7.0). Penicillin V acylase (30 mg) or Novozym 435 (30 mg) was added. Diphenylacetic acid was used as the internal standard. The reaction mixture was shaken at 70 ^{o}C (with CaLB) or 40 ^{o}C (with pencillin V acylase). Samples (50 μL) were taken every 2 h to monitor the hydrolysis over time. After removing the enzyme, the samples were subjected to a C-18 HPLC column to monitor the hydrolysis of the amide, the enantiomeric excess of the liberated amine was further analyzed by chiral HPLC.

6. Analysis

6.1. Thin Layer Chromatography

Thin layer chromatography was performed on silicagel 60 F_{254} plates, with eluent of ethyl acetate: petroleum ether 40/60 (9:1), detection was done by UV 254 nm

6.2. Gas Chromatography:

The progress of the acylation reactions was monitored by gas chromatography using a CP Sil 5 CB column (50 m x 0.53 mm) on a Varian STAR 3400 CX instrument. Chiral analysis of the unconverted amine was

performed on a β-PH gas chromatography column, after derivatization with trifluoroacetic anhydride, at fixed temperature of 80 ℃ (2-heptylamine) or 100 ^{0}C (1-phenylethylamine).

6.3. HPLC

Samples were analyzed by HPLC using a Waters M-6000 pump, a nucleosil C-18 column and a shimadzu SPD-6A UV detector at 250 nm. The eluent was a mixture of methanol/water (70/30) and sodium dodecylsulfate (0.1%) with pH adjusted to 3.5, at the flow rate of 1.0 mL/min. The retention times (minutes) were as follows: diphenylacetic acid (13), pyridyloxyacetic acid (5), *N*-(2-heptyl)pyrid-3-yloxyacetamide (45), *N*-(1-phenylethyl)pyrid-3-yloxyacetamide (21).

Chiral analysis of the liberated 1-phenylethylamine (**1a**) was carried out using a crown pack Cr^+ column and a Shimadzu SPD-6A UV detector at 250 nm. The eluent was solution of perchloric acid pH 1.3 with 2% methanol, flow rate of 0.6 mL/min. The retention times (minutes) were as follows: *(R)*-phenylethylamine (24.8 min) and *(S)*-phenylethylamine (19.6 min).
Chiral analysis for the liberated 2-heptylamine (**1b**) was carried out using an OD column after derivatization with benzoyl chloride. A mixture of hexane/isopropanol (90/10) was used as the eluent with flow rate of 0.6 ml/min. The retention times (minutes) were as follows: (*R*)-2-heptylamine (19.9), (*S*)-2-heptylamine (17.7), (*R*)-2-pentylamine (18.4), (*S*)-2-pentylamine (14.9).

References

1. Pohl, T., Waldman, H., *Tetrahedron Lett.* **1995**, *36*, 2963-2966.
2. Mertes, M.P., Borne, Hare, L.E., *J. Heterocyclic. Chem*. **1968**, *5*, 281-283
3. Hertog, D.H. J., Maas, J., *J. Chem. Pharm. Bull*. **1975**, *23*, 3008-3010.
4. Sakai, T., *Tetrahedron Assymetry* **2004**, *15*, 2749-2756.

5. Van Rantwijk, F., Hacking, M. A. P. J., Sheldon, R.A., *Monatsh. Chem.* **2000**, *131*, 549-569
6. Shewale, J. G.; Sudhakaran, V., *Enzyme Microb. Technol.*, **1997**, *20*, 402-410.
7. Huang, H. T.; Seto, T. A.; Shull, *G. M. Appl. Microbiol*. **1963**, *11*, 1-6.
8. Reyes Duarte, D.; Castillo, E.; Barzana, E.; Lopez-Munguia, A. *Biotechnol. Lett*. **2000**, *22*, 1811-1814.
9. Smidt, H.; Fischer, A.; Schmid, R. D. *Biotechnol. Techn*. **1996**, *10*, 335-338.
10. Buciarelli, Forni, A.; Moretti, I.; Pratti, F.; Torre, G. *Tetrahedron: Asymmetry* **1993**, *4*, 903-906.
11. Mertes, M.P.; Borne, Hare, L.E.; *J. Heterocyclic. Chem*. **1968**, *5*, 281-283
12. Hertog, D.H. J.; Maas, J.; *J. Chem. Pharm. Bull*., **1975**, *23*, 3008-3010

6

Dynamic Kinetic Resolution of Amines

ABSTRACT

A palladium nanoparticle catalyst was used as racemization catalyst in the dynamic kinetic resolution (DKR) of 1-phenylethylamine. Instead of introducing hydrogen gas, ammonium formate was used as a hydrogen source to maintain the reducing environment. The palladium-formate system has proven that *in-situ* racemization of 1-phenylethylamine by palladium nanoparticles is feasible, and 90% yield of enantiopure amide (ee_{amide} 98%) was obtained after 96 h. From a scaled up reaction, after 88% conversion according to GC analysis, 76% isolated yield was obtained with an ee_{amide} of 98%.

INTRODUCTION

The enzymatic resolution of amines remains one of the easiest and practical methods to produce chiral amines. However, the 50% yield limit in the kinetic resolution is a major drawback of this method. Racemization *in situ* of the slow-reacting enantiomer would permit a 100% yield of the enantiomerically pure amide. Such a combination of kinetic resolution with *in situ* racemization as illustrated in Figure 1 is called dynamic kinetic resolution (DKR),

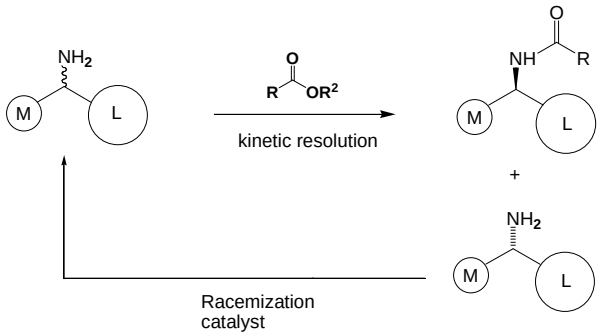


Figure 1. Scheme of the dynamic kinetic resolution of amines
L = large substituent; M = medium susbtituent

The DKR of alcohols in the presence of lipase combined with a metal catalyst is now well developed[1,2,3]. Reports on the DKR of amines are scarce, in contrast, presumably because amine racemization is more problematic, requires harsh conditions[4,5], and is difficult to combine with enzymatic resolution.

The reported DKR methodologies for amines employed a palladium catalyst in combination with CaLB[6,7,8]. These reports revealed the difficulties of amine racemization, such as high temperature, long reaction time, or the need for an excessive amount of catalyst. Summarizing, there is ample room for improving amine racemization, as regards the catalyst reactivity.

Recently, metal nanoparticle catalysts have attracted much attention, and studies in the field have grown explosively over the past decade. The nanocatalysts are known to have a high surface-to-volume ratio and a high surface energy, which make their surface atoms very active[9]. Applications of

transition metal nanoparticles as catalysts in several organic reactions have been reported[10,11,12]. Palladium nanoparticle catalysts have been applied in the Heck and Suzuki reactions, and proved to be efficient and recyclable[13,14]. Encouraged by these reports, we have set out to investigate the application of palladium nanoparticles as the racemization catalyst in the DKR of amines.

RESULTS AND DISCUSSION

In this Chapter, two preparations of palladium nanoparticles were employed as the catalyst in the racemization of 1-phenylethylamine. The first preparation consisted of palladium nanoparticles prepared in poly(ethylene glycol) 400. This catalyst was chosen for its 'green' and simple preparation process, which involved stirring palladium acetate in PEG 400 at room temperature. In this procedure, PEG 400 appeared to act as both reducing agent and stabilizer[11]. The stability of the nanoparticles becomes an important issue, since the very active surface atoms could provoke the aggregation and inactivation of the nanoparticles[5].

The second preparation was a suspension of palladium nanoparticles in toluene. The nanoparticle preparation was derived from the methods described by Shen and Brust *et al.* [15,16], which involved borohydride reduction of a palladium (II) salt in the presence of 1-dodecanethiol as the stabilizer. Since the nanoparticles form a stable dispersion in non-polar solvents such as toluene, this latter catalyst seems more readily compatible with lipase catalyzed resolution.

1. Racemization and DKR of amine with palladium nanoparticles in PEG 400

The first preparation of palladium nanoparticles was made and kept in PEG 400. Since PEG 400 also acts as the nanoparticle stabilizer, this solvent was further used as reaction medium in the racemisation and DKR experiments.

Considering that the PEG 400 has terminal alcohol groups that might react enzymaticaly with the acyl donor[6], an excessive amount of acyl donor was used in the DKR experiments. Here, ethyl acetate was used as the acyl donor (Figure 2). The results are presented in Table 1.

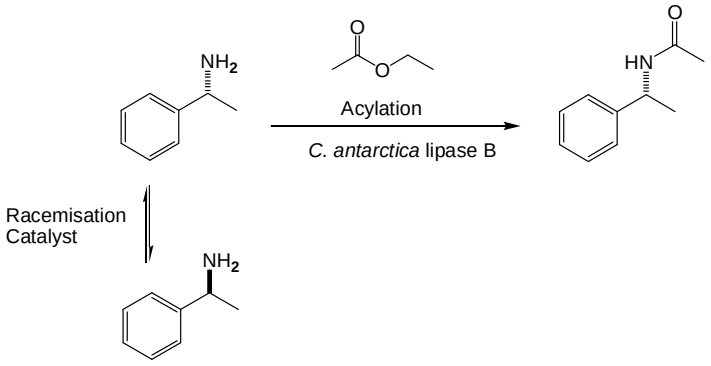


Figure 2. The dynamic kinetic resolution of 1-phenylethylamine

Table 1. Racemization, DKR, and kinetic resolution of 1-phenylethylamine[a]

Entry	Treatment	Time (h)	Conversion of amine (%)	ee_{amine} (%)	E
1	Racemization of (*S*)-1-phenylethylamine	20 h	n.d	56	
		4 d	n.d	50	
2	Dynamic kinetic resolution (DKR)	20 h	43		
		4 d	77	n.d.	
3	Kinetic resolution in PEG 400 as solvent	20 h	45		
		4 d	59	99.6	38
4	Kinetic resolution in ethyl acetate as solvent	3 h	44	74	63
		18 h	58	~100	62

[a]Conditions:
Racemization: (*S*)-1-phenylethylamine (0.5 mmol), palladium nanoparticles dispersed in PEG (0.05 mmol/mL; 2 mL). Incubation at 70 ^{o}C under vigorous stirring after hydrogenation (pH_2 = 1 atm).
DKR: (*R/S*)-1-phenylethylamine (0.5 mmol), PEG 400 (2 mL), palladium nanoparticles dissolved in PEG (0.05 mmol/mL; 2 mL), Novozyme (100 mg), isopropyl acetate (3 mL), 1,3-dimethoxybenzene as internal standard. Incubation at 50 ^{o}C under vigorous stirring, after hydrogenation (pH_2 = 1 atm).
Kinetic resolution: similar to DKR, without palladium catalyst and hydrogen.

We found that the palladium nanoparticle catalyst was able to racemize (*S*)-1-phenylethylamine, as the ee_{amine} decreased from 100% to 56%, but almost no further racemisation was observed after 20 hours. The catalyst thus loses its activity, and precipitation of palladium black and aggregation of nanoparticles were observed by visual inspection. The tendency of the nanoparticles to aggregate in the course of the reaction has been reported previously, and is ascribed to the very high activity of the surface atoms, especially at high temperature[14]. Apparently, the short chain of PEG 400 was not sufficient to stabilize the nanoparticles. The stabilizing effect of PEG is known to increase with the polymer chain length[9]. In this experiment, we chose PEG 400 because of its compatibility with the kinetic resolution. The precipitation of palladium black was presumably caused by over-reduction of the palladium nanoparticles by hydrogen. It would seem that the oxidation state of the palladium catalyst should be carefully controlled.

In the DKR (Table 1), 77% yield of (*R*)-amide was obtained within 4 days, which is higher than the yield of the kinetic resolution (58%) under otherwise identical conditions. The reaction was very slow, however, which we tentatively ascribed to PEG 400 as the reaction medium. We indeed observed that the kinetic resolution was much slower in PEG 400 than in ethyl acetate (18 h vs 4 d to reach 58% yield). The enzymatic side-reaction between the terminal hydroxyl groups in PEG 400 with the acyl donor was suspected to be one reason. Besides, since PEG 400 acted as the reducing agent in the catalyst preparation, some hydroxyl groups were oxidized to form aldehydes[13]. Accordingly, another side reaction between the aldehyde groups and 1-phenylethylamine could take place.

In conclusion, despite the slow reaction, the principle of in situ dynamic kinetic resolution using a palladium nanoparticle catalyst and CaLB was confirmed. However, further improvement is needed, because the PEG 400 system is not very effective for amine DKR. Accordingly, we extended the experiments by applying a palladium nanoparticle catalyst prepared in toluene as solvent with dodecanethiol as stabilizer.

2. Palladium nanoparticles in toluene as the racemization catalyst.

The racemization activity of the palladium nanoparticle catalyst in toluene towards 1-phenylethylamine was investigated in the racemization of (*S*)-1-phenylethylamine. As we have observed that the presence of hydrogen could cause over-reduction of the palladium catalyst, we also investigated the effect of alternative reductants.

Thus, H_2 gas (pH_2 = 1 atm) as reductant was compared with formate salts and isopropyl alcohol (Table 2). These latter compounds are known to act as the hydrogen sources in some metal-catalyzed reactions. We note that combinations of formate or isopropyl alcohol with palladium and ruthenium catalysts have been applied in some hydrogen transfer reactions[17,18,19,20]. In the racemization in the presence of H_2 gas, precipitation of the palladium black was noticed quite early in the reaction and almost no racemization was observed. Ammonium formate and isopropyl alcohol induced amine racemization, as ee_{amine} decreased to 65% and 87% respectively. The small amount of formate and isopropyl alcohol proved sufficient to maintain the reducing environment.

Table 2. Racemization of (*S*)-1-phenylethylamine with palladium nanoparticles in toluene[a].

Hydrogen source	**Time (h)**	**ee_{amine} (%)**
H_2 gas (pH_2 = 1 atm)	24	98
Ammonium formate	24	65
Isopropyl alcohol	24	87

[a]Conditions: (*S*)-1-phenylethylamine (0.5 mmol) in a mixture of toluene and dimethoxyethane (3:2; 5 mL), palladium nanoparticles (0.024 mmol), ammonium formate (4 mg), isopropanol (10 µL). Incubation under vigorous stirring at 70 ^{o}C.

Considering that the DKR requires a rather long reaction time, an appropriate acyl donor that maintains a high enantioselectivity in such long reactions is needed. Hence, we investigated several acyl donors in the kinetic resolution of 1-phenylethylamine, during 96 hours at 50 ^{o}C and 70 ^{o}C. The results are presented in Table 3.

Table 3. Kinetic resolution of 1-phenylethylamine within 96 hours at 50 ^{o}C.

Acyl donor	Time to reach ~50% conv. (h)	Initial rate (mmol/h)	Yield (after 96 h) (%)	ee_{amide} (after 96 h) (%)
Ethyl acetate	40	0.038	64	
Isopropyl acetate	48	0.040	51	98
Methyl methoxyacetate	4	0.093	74	62
Isopropyl methoxyacetate	4	0.092	76	67

[a]Conditions: (R/S)-1-phenylethylamine (0.5 mmol), a mixture of dimethoxyethane-toluene (3:2) as the solvent (5 mL), Novozym 435 (100 mg), acyl donor (3 mmol), internal standard 1,3-dimethoxybenzene. Incubation at 50 ^{o}C.

Although isopropyl acetate reacted slower, it appeared as the best choice since amide with high enantiopurity was still obtained after 96 h (ee_{amide} = 98%) indicating that the reaction came to a standstill at 50% conversion. The more reactive acyl donors (methyl- and isopropyl methoxyacetate) reacted faster, but afterwards the unwanted *S*-enantiomer was acylated quite fast and causes drop of the ee_{amide} (after 96 h= 62% and 67%). Further investigation showed that in the presence of CaLB, the acylation of (*S*)-1-phenylethylamine by methyl methoxyacetate occurred quite fast; 30% amine was converted in 24 h (data not shown).

Hence, here the DKR of 1-phenylethylamine was carried out using isopropyl acetate as the acyl donor. The reduction activity was also investigated by comparing H_2 gas and the addition of ammonium formate. The results are presented in Table 4 and Figure 3.

Table 4. DKR and kinetic resolution of 1-phenylethylamine[a]

Treatment	Hydrogen source	Results	
		Yield of amide 96 h (%)	*ee* product (%)
Dynamic kinetic resolution	H_2 gas (pH_2 = 1 atm)	54	n.d
	Ammonium formate (2 eq. to amine)	90	98
	No hydrogen source addition	68	98
Kinetic resolution	--	50 (48h)	99

[a]Conditions: (R/S)-1-phenylethylamine (0.5 mmol), a mixture of dimethoxyethane-toluene (3:2) as solvent (5 mL), palladium nanoparticle (13 mg; 5 mol%), Novozym 435 (100 mg), isopropyl acetate (2 mmol), ammonium formate (6 mg), internal standard 1,3-dimethoxybenzene. Incubation at 50 ^{o}C under vigorous stirring.

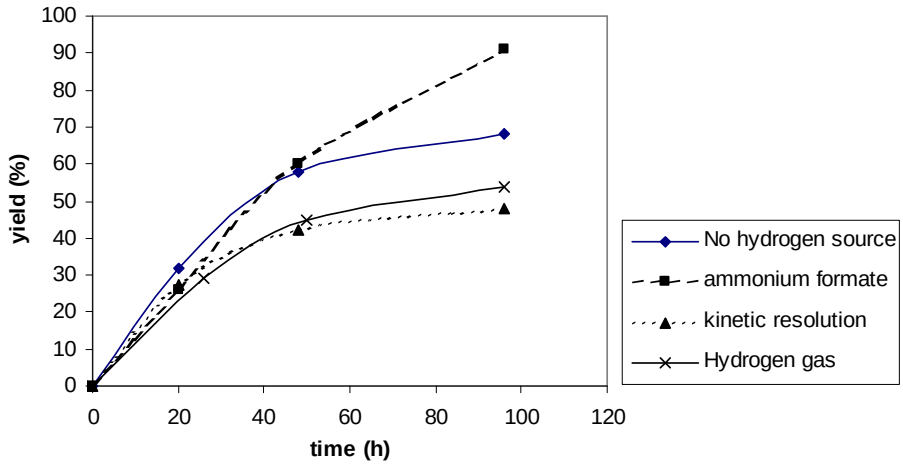


Figure 3. Profile of the kinetic resolution and DKR of 1-phenylethylamine

As before, palladium black precipitated early in the reaction with H_2 and the role of racemization was negligible. Ammonium formate as the hydrogen source gave much better results, and 90% yield (ee_{amide} of 98%) was reached after 96 h. Presumably, the poor solubility of the formate salt in the solvent has maintained a sufficient amount of the dissolved reducing agent. This may prevent over-reduction of the palladium nanoparticle catalyst during the reaction.

Interestingly, the DKR without any addition of hydrogen source also gave higher yield than the kinetic resolution (68% *vs* 50% yield, ee_{amide} = 98%), indicating that some racemization occurred. It would seem that the liberated isopropyl alcohol from the acyl donor functioned as hydrogen source. Furthermore, acetone, the oxidation product of isopropanol presumably evaporated under the reaction conditions. The yield obtained is however lower than that with ammonium formate.

We conclude that the palladium-formate system was superior for the dynamic kinetic resolution of 1-phenylethylamine. From a scaled up reaction (25 mL), after 88% conversion according to GC analysis, 76 % crude crystalline amide was isolated with ee 98 %.

An attempt to improve the results by increasing the reaction temperature to 70 ^{o}C was not successful, as higher yields were not achieved, and moreover a decrease of the ee_{amide} was noticed. Presumably the higher temperature caused deactivation of the palladium nanoparticle catalyst. We attempted, furthermore, to increase the racemization rate by increasing the amount of the palladium catalyst. The results are presented in Table 5.

Table 5. DKR of 1-phenylethylamine with various palladium nanoparticles concentration[a]

Palladium (mol%)	time (h)	Yield (%)
50	24	54
	72	70
5	24	62
	72	83
0.5	24	61
	72	77

[a]Conditions: (*R/S*)-1-phenylethylamine (0.5 mmol), a mixture of dimethoxyethane-toluene (3:2) as the solvent (5 mL), Novozyme (100 mg), isopropyl acetate (3 mmol), ammonium formate (4 mg), internal standard dimethoxybenzene. Incubation at 50 ^{o}C under vigorous stirring.

We found that increasing the amount of palladium catalyst from 5 to 50 mol% slowed down the reaction and decreased the yield to 70% after 72 h. Furthermore, reducing the amount of catalyst by a factor of 10 hardly affected the yield (77% after 72 h). It would seem that at high palladium concentrations the tendency of the nanoparticles to aggregate increases, and less palladium is accordingly preferred. Summarizing, a palladium nanoparticle racemization catalyst combined with CaLB effectively mediated the DKR of 1-phenylethylamine.

Nevertheless, further improvement is still needed, especially with regard to enhancing the activity and stability of the palladium nanoparticle catalyst. Such improvement will be sought by optimizing the catalyst synthesis, as the synthesis procedure is known to affect the nanoparticle size and shape[9], which in turn, strongly influences the activity and stability[20.] An established method for the preparation of the nanoparticles is needed, since we found that it is difficult to reproduce the palladium nanoparticles with similar activity. Hence, the best results from the DKR of 1-phenylethylamine were not successfully reproduced using other palladium nanoparticles from different preparations.

CONCLUSIONS

A nanoparticulate preparation of palladium was shown to catalyze the racemization of 1-phenylethylamine. The dynamic kinetic resolution of 1-phenylethylamines has accordingly been achieved, by applying palladium nanoparticle as the racemisation catalyst combined with CaLB.

EXPERIMENTAL PARTS

1. Materials

Enzymes: Novozym 435 (immobilized *Candida antarctica* lipase B) was kindly donated by Novozymes. Chemicals were purchased from Sigma and Across.

2. Synthesis of acyl donor isopropyl methoxyacetate

A mixture of methoxyacetylchloride (0.8 mmol), isopropyl alcohol (7 mL) and pyridine (7 mL) in 30 mL dichloromethane was stirred at 30 ^{o}C for 5 h. After cooling to room temperature, the reaction mixture was washed successively with cold hydrochloric acid solution (0.25 M), cold water, a saturated solution of sodium bicarbonate, and cold water. The organic layer was dried over Na_2SO_4, the ester was obtained after evaporation of the solvent in vacuum. Isopropyl methoxyacetate was obtained as colourless oil (yield 70%), GC-MS: (MW)=132, ^{1}H- NMR = δ 1.27 (*m*, 6H, CH(C*H*$_3$)$_2$), 3.44 (*s*, 3H, OC*H*$_3$), 3.99 (*s*, 2H, OC*H*$_2$CO), 5.06-5.10 (*m*, 1H, OC*H*(CH$_3$)$_2$).

3. Preparation of a palladium nanoparticle catalyst in PEG 400

Palladium acetate (0.052 mmol) and 1,10-phenanthroline (0.0199 mmol) were dissolved in 10 mL of poly(ethylene glycol) 400. The mixture was stirred at room temperature, until spontaneous reduction of palladium (II) occurred and the color turned from light yellow to dark brown-black. The nanoparticles were kept as solution in PEG 400.

4. Preparation of a palladium nanoparticle catalyst in toluene[15]

Palladium (II) chloride (85 mg; 0.48 mmol) was dissolved in 20 mL of 0.5 M hydrochloric acid. A solution of tetra-*n*-octylammonium bromide (1.1 g; 2 mmol) in 40 mL toluene was added under vigorous stirring, whereupon the Pd (II) was transferred from the aqueous phase to the toluene phase, and then dodecanethiol (230 µL; 0.96 mmol) was added to the solution. The mixture was stirred for 15 min, and 10 mL of a sodium borohydride solution (20 mg/mL) was added slowly. The mixture was kept under vigorous stirring for 2 h. After separating the water layer, the organic solvent was removed by vacuum evaporation. Methanol (200 mL) was later added to precipitate the palladium nanoparticles. The black particles were collected by centrifugation, and washed three times with methanol. The black precipitate then was re-dispersed in 10 mL toluene.

5 Kinetic resolution of 1-phenylethylamine

1-Phenylethylamine (0.5 mmol) and acyl donor (1.0 mmol) were dissolved in 5 mL of 1,2-dimethoxyethane. Molecular sieves 4 A and 5 A (150 mg each), 100 mg enzyme (Novozym 435), and 1,3-dimethoxybenzene (internal standard) were added. The reaction mixture was incubated at 40 ^{o}C, 50 ^{o}C or 70 ^{o}C. Samples (100 µL, diluted in 500 µL of 1,2-dimethoxyethane) were taken regularly to follow the reaction over time. Analysis was performed by Gas Chromatography and Chiral GC/HPLC.

6. Racemization of 1-phenylethylamine with palladium catalyst in PEG 400

1-Phenylethylamine (0.5 mmol) was added to the solution of palladium nanoparticle in PEG (0.05 mmol/ml; 4 mL). After introduction of H_2 gas (pH_2 =1 atm, 5 min) the reaction mixture was incubated under stirring at 50 ^{o}C. Samples were taken every 24 h. After adjusting the pH to 12 with NaOH solution (0.1N), the amine was extracted with diethyl ether. After removing diethyl ether by vacuum evaporation, the residue was dissolved in the HPLC eluent (solution of perchloric acid pH 1.3 with 2% methanol) and injected on a chiral HPLC column (Crownpak CR^{+})

7. Dynamic kinetic resolution of 1-phenylethylamine with palladium nanoparticle in PEG 400 as the racemization catalyst.

1-Phenylethylamine (0.5 mmol) was added to a palladium nanoparticles solution in PEG (0.05 mmol/mL; 2 mL) and was mixed well. The acyl donor (3 mmol), Novozym 435 (100 mg) and internal standard 1,3-dimethoxybenzene (50 µL) were added to the mixture. After introduction of H_2 gas (pH_2 =1 atm) or addition of ammonium formate (4 mg), the reaction mixtures were incubated at 50 ^{o}C or 70 ^{o}C.

Samples (200 µL) were taken every 24 h to follow the reaction progress. After diluting with water and removal of the palladium catalyst by microfiltration, the

samples were analyzed by HPLC. The chiral analysis for the enantiomeric excess (*ee*) of amine was performd by chiral HPLC using Crownpak CR^+ column.

8. Racemization of 1-phenylethylamine with palladium catalyst in toluene as catalyst

1-Phenylethylamine (0.5 mmol) was dissolved in 3 mL of 1,2-dimethoxyethane, then a palladium nanoparticle solution in toluene (0.0125 mmol/ml; 2 ml) was added. After introducing hydrogen gas (pH_2 =1 atm), ammonium formate (4 mg) or isopropyl alcohol (10 µL), the reaction mixtures were incubated at 70 ^oC.

Samples were taken every 24 h. After removing the excess of dimethoxyethane by vacuum evaporation, the palladium catalyst was precipitated with 1 mL methanol and removed by centrifugation. After evaporating methanol in vacuum, the residue was dissolved in the chiral HPLC solvent (solution of perchloric acid pH 1.3 with 2% methanol) and subjected to a chiral HPLC column (Crownpak CR^+).

9. Dynamic kinetic resolution of 1-phenylethylamine with palladium in toluene as the racemization catalyst.

1-Phenylethylamine (0.5 mmol) was dissolved in 3 mL of 1,2-dimethoxyethane. The acyl donor (3.0 mmol), a palladium nanoparticles solution in toluene (0.0125 mmol/mL; 2 mL), internal standard 1,3-dimethoxybenzene and Novozym 435 (100 mg) were added to the reaction mixture. After introduction of H_2 gas (pH_2 =1 atm) or addition of ammonium formate (4 mg), the reaction mixtures were incubated at 50 ^oC or 70 ^oC.

Samples (200 µL) were taken every 24 h to follow the reaction progress. After evaporating the excess of dimethoxyethane, the palladium catalyst was precipitated with 1 mL methanol and separated by centrifugation. The filtrate was subjected to GC column to monitor the conversion over time. The enantiomeric excss of the amide was analyzed with chiral GC.

10. Analysis

a. Gas Chromatography:

The progress of the acylation reactions was monitored by gas chromatography using a CP Sil 5 CB column (50 m x 0.53 mm) on a Varian STAR 3400 CX instrument. Chiral analysis of the amide was performed on a β-PH gas chromatography column, at programmed temperature from 110 ℃ to 230℃.

b. HPLC:

Samples were analyzed by HPLC using a Waters M6000 pump, a Nucleosil C-18 column and a Shimadzu SPD-6A UV detector at 250 nm. The eluent was a mixture of methanol/water (50/50) and trifluoroacetic acid (0.1%) with flow rate of 1.0 ml/min. The retention times (min) were as follows: 1-phenylethylamine (3.4), 1,2-dimethoxybenzene (20), N-(1-phenylethyl)-acetamide (7.4).

The chiral analysis for 1-phenylethylamine was carried out using a crown pack Cr^{+} column and a shimadzu SPD-6A UV detector at 250 nm. The eluent was solution of perchloric acid pH 1.3 with 2 % methanol, flow rate of 0.5. The retention times (minutes) were as follows: *(S)*- phenylethylamine (20 min) and *(R)*-phenylethylamine (18 min).

References

1. Kim, M. J., Han, Y., Park, J., *Curr. Opin.Biotechnol.* **2002**, *13*, 578-587
2. Huerta, F. F., Minidis, A. B. E., Bäckvall, J. E, *Chem. Soc. Rev*. **2001**, *30*, 321-331
3. Dijksman, A., Elzinga, J. M., Li, Y. X., Arends, I.W.C.E., Sheldon, R.A., *Tetrahedron: Asymmetry* **2002**, *13*, 879-884.
4. Ebbers, E. J., Ariaans, G. J. A., Houbiers, J. P. M., Brugink, A., Zwanenburg, B., *Tetrahedron* **1997**, *53*, 9417-9476.
5. Pamies, O., Ell A. H., Samec, J. S. M., Hermanns, N., Bäckvall, J.E., *Tetrahedron Letters*, **2002**, *43*, 4699-4702.

6. Reetz, M., Schimossek, K., *Chimia* **1996**, *50*, 668-669
7. Choi Y. K., Kim, M. J., Ahn Y., *Organic Lett.* **2001,** *3(25)*, 4099-4101.
8. Parvulescu, A., De Vos, D., Jacobs, P., *Chem. Commun.* **2005**, 5307-5309
9. Narayanan R., El-Sayed, M.A., *J. Phys. Chem. B.* **2005**, *109*, 12663-12676
10. Chen, C.W., Serizawa, T., M.; Akashi, M., *Chem. Mater.* **1999**, *11*, 1381.
11. Toshima, N., Wang, L., *Adv. Mater*. **1994**, *6*, 245-247
12. Umpierre, A. P., Machado, G., Fecher, G.H., Morais, J., Dupont, *J. Adv. Synth. Catal.* **2005**, *347*, 1404-1412.
13. Reetz, M. T., Breinbauer, R., Wanninger, K., *Tetrahedron Lett*. **1996**, *37*, 4499-4502.,
14. Luo, C., Zhang, Y., Wang, Y., *J. Mol. Catal. A: Chem*. **2005**, *229*, 7-12
15. Shen, C.M., Su, Y.K., Yang, H.T., Yang, T.Z., Gao, H.J., *Chem. Phys. Lett.*, **2003**, *373*, 39-45.
16. Brust, M.; Walker, M., Bethell, D., Schiffrin, D. J., Whyman, R. *J. Chem. Soc. Chem . Commun.* **1994**, *7*, 801-802.
17. Prasad, K., Slade, J.S., Clemens, J., Repic, O., Blacklock, T., *Adv. Synth. Catal*. **2005**, *347*, 1769-1773.
18. Samec, J. S. M., Bäckvall, J. E., *Chem. Eur. J*. **2002**, *8*, 2955-2961.
19. Kopinke, F. D., Mackenzie, K., Koehler, R., Georgi, A., *Appl. Catal. A: General*, **2004**, *271*, 119-128.
20. Ella, A. H; Samec, J. S. M, Brasse, C., Bäckvall, J. E., *Chem. Commun.* **2002***, 1144-1145.*

SUMMARY

Enzymatic enantioselective acylation is one of the fastest growing methodologies for the resolution of chiral amines. However, presently, the subsequent chemical deacylation of the amide involves harsh reaction conditions, which are incompatible with sensitive functional groups. Accordingly, a strategy based on enzymatic hydrolysis at mild reaction conditions would be much preferred, and a fully enzymatic procedure employing enzymatic reactions in both acylation and deacylation is proposed. The resolution strategy involves an enantioselective lipase-catalyzed amine acylation followed by penicillin acylase-catalyzed deacylation. Considering that most enzymes need a specific substrate, the choice of acyl donor that is compatible with both enzymes becomes important. This Thesis concerns investigations on the use of non-conventional acyl donors, with the objective of complementing the enzymatic acylation with the enzymatic deacylation.

In Chapter 2, the utilization of (*R*)-phenylglycine ester as acyl donor was investigated. Applying an amino acid as acyl donor was expected to facilitate the deacylation step, because the amide hydrolysis will be easier due to the liberation of thermodynamically stable *zwitterions*. We found that the CaLB-catalyzed amine acylation was slow with a moderate-to-poor enantioselectivity. However, the enzymatic hydrolysis was quite facile at room temperature, affording amines with high enantiopurity (*ee*>98%). Continuing these results, the use of several glycine derivatives/analogues substituted with a ß-heteroatom X (X = O, NH, or S) was investigated in Chapter 3. The results reveal that the presence of a β-heteroatom X in the acyl donor influences both enzymatic acylation and deacylation steps, and compounds having a β-oxygen performed the best. Interestingly, CaLB catalyzed the hydrolysis of amides derived from CH_3-X-CH_2CO_2H with better activity than penicillin acylase. Hence, here another approach in the fully enzymatic resolution of amine is proposed, which CaLB is applied in both acylation and deacylation steps.

Since amide solubility in aqueous medium is often considered as the cause of the low hydrolysis rate, utilization of a more water-soluble acyl donor: ethyl 4-pyridylacetate, was investigated in Chapter 4. In the acylation step, a reasonable rate with good enantioselectivity was obtained, revealing that this acyl donor is well accepted by CaLB. Furthermore, the obtained acylated amines were better soluble in aqueous medium, resulting a fast enzymatic hydrolysis in the presence of *A. faecalis* penicillin acylase.

Considering that a β-oxygen in the acyl donor increases the reactivity (Chapter 3), we attempted to improve the acyl donor discussed in Chapter 4 by inserting a β-oxygen in the molecule. As presented in Chapter 5, it is shown that oxygen substitution at the β-position has significantly increased the rate of the CaLB-catalyzed acylation. A fast enzymatic amine acylation was observed even at low reaction temperatures (–10 ^{o}C), affording a good enantioselectivity (E = 51 for 2-heptylamine and *E* >100 for 1-phenylethylamine). The amide hydrolysis in the presence of CaLB or penicillin V acylase in aqueous medium was facile, liberating amines with high enantiopurity.

In Chapter 6, a dynamic kinetic resolution (DKR) method that allows *in-situ* recycling of the unwanted enantiomer in parallel with the kinetic resolution was investigated. Here, a palladium nanoparticle catalyst in combination with ammonium formate was used as racemization catalyst in DKR of 1-phenylethylamine. The palladium-formate system has proven that *in-situ* racemization of 1-phenylethylamine by palladium nanoparticles is feasible, and 90% yield of enantiopure amide (ee_{amide} 98%) was obtained after 96 h.

Samenvatting

Een van de snelst groeiende methoden voor de resolutie van chirale amines is enantioselectieve enzymatische acylering. Voor de daaropvolgende deacylering van het amide zijn echter ruwe reactie condities nodig. Deze condities zijn slecht te combineren met verbindingen die gevoelige functionele groepen dragen. Vandaar dat de voorkeur wordt gegeven aan een strategie die gebaseerd is op enzymatische hydrolyse onder milde condities. In dit onderzoek wordt een volledige enzymatische procedure gepresenteerd die gebruik maakt van enzymatische reacties voor zowel de acylering en deacylering. Deze resolutie strategie maakt gebruik van amine acylering gekatalyseerd door een enantioselectieve lipase gevolgd door een deacylering gekatalyseerd door penicilline acylase. Aangezien de meeste enzymen een specifiek substraat nodig hebben is de keuze voor een acyl donor die te combineren is met beide enzymen belangrijk. Dit Proefschrift beschrijft het onderzoek naar het gebruik van onconventionele acyl donoren, met als doel het combineren van de enzymatische acylering met de enzymatische deacylering.

In hoofdstuk 2 werd onderzoek gedaan naar het gebruik van (R)-fenylglycine ester als acyl donor. Verwacht werd dat het gebruik van een aminozuur als acyl donor de deacylerings stap zou vereenvoudigen, omdat de hydrolyse van het amide makkelijker zou zijn door het vrijkomen van thermodynamisch stabiele *zwitterionen.* We vonden dat de amine acylering gekatalyseerd door CaLB langzaam was met een redelijk tot slechte enantioselectiviteit. De enzymatische hydrolyse was echter vrij gemakkelijk bij kamertemperatuur en resulteerde in amines met hoge enantiozuiverheid (ee>98%). Voortbordurend op deze resultaten werd in Hoofdstuk 3 onderzoek gedaan naar verschillende analogen/derivaten van glycine die gesubstitueerd zijn met een β-heteroatoom X (X = O, NH of S). De resultaten laten zien dat de aanwezigheid van een β-heteroatoom X in de acyl donor invloed heeft op zowel de enzymatische acylerings als deacylerings stap. Verbindingen met een β-zuurstof leverden de beste resultaten op. Het was opmerkelijk om te zien dat CaLB een betere activiteit heeft dan penicilline acylase voor de

hydrolyse van amides afgeleid van CH_3-X-CH_2CO_2H. Vandaar dat hier een andere benadering wordt voorgesteld voor de volledig enzymatische resolutie van amines, waar CaLB wordt toegepast in zowel de acylerings als deacylerings stap.

Aangezien de oplosbaarheid van amides in een waterig milieu vaak wordt aangedragen als de oorzaak van de lage hydrolyse snelheid werd in Hoofdstuk 4 het gebruik onderzocht van een beter water oplosbare acyl donor: ethyl 4-pyridylacetaat. Een redelijke snelheid en goede enantio-selectiviteit werden behaald in de acylerings stap. Dit toont aan dat deze acyl donor goed wordt geaccepteerd door CaLB. De verkregen geacyleerde amines waren beter oplosbaar in waterig milieu, hetgeen leidde tot een snelle enzymatische hydrolyse.

Aangezien een β-zuurstof in de acyl donor de reactiviteit vergroot (Hoofdstuk 3), hebben we geprobeerd om de acyl donor, besproken in Hoofdstuk 4, te verbeteren door een β-zuurstof in het molecuul te plaatsen. In Hoofdstuk 5 wordt aangetoond dat de zuurstof substitutie op de β-positie de snelheid van de CaLB gekatalyseerde resolutie significant verhoogt. Een snelle enzymatische amine acylering werd zelfs waargenomen bij lage reactie temperaturen (-10 ^{o}C), hetgeen leidde tot een goede enantioselectiviteit. De hydrolyse van het amide in de aanwezigheid van CaLB of penicilline V acylase in waterig milieu was makkelijk, wat amines met een hoge enantiozuiverheid opleverde.

In Hoofdstuk 6 werd onderzoek gedaan naar een dynamische kinetische resolutie (DKR) methode. Deze methode combineert *in-situ* recyclen van het ongewilde enantiomeer met de kinetische resolutie. Een palladium nanodeeltje werd gebruikt in combinatie met ammonium formaat als racemisatie katalysator in de DKR van 1-fenylethylamine. Met dit palladium formaat systeem is de *in-situ* racemisatie van 1-fenylethylamine met palladium nanodeeltjes haalbaar. Een 90% yield van enantiozuiver amide (ee_{amide} 98%) werd bereikt na 96 uur.

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Delft, May 2007
Hilda Ismail

Curriculum vitae

Hilda Ismail was born on October 22, 1967 in Yogyakarta, Indonesia. After finishing her secondary education at *SMA Negeri I* Yogyakarta in 1986, she studied pharmacy in Gadjah Mada University, Yogyakarta, Indonesia with specialization in pharmacochemistry. She finished her bachelor degree on August 1991 (*cum laude*) and achieved the title of 'Apotheker' on August 1992.

In 1993 she was accepted as a junior lecturer at the Faculty of Pharmacy, Gadjah Mada University, where she has been working untill now. She achieved her Master degree in Pharmacy from the same university in Januari 1999.

In September 2001 she started her PhD research at the Biocatalysis and Organic Chemistry group, TU Delft, under the supervision of Prof. Roger A. Sheldon and Dr. Fred van Rantwijk. The results of this research are described in this Thesis.