Lipase catalyzed acylation of (hetero-substituted) nitrogen nucleophiles
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Chapter 1

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INTRODUCTION

Enzymatic catalysis has developed into a mature science over the past decades with numerous industrial applications in food and personal care products, detergents and in the production of fine chemicals, pharmaceuticals and agrochemicals. Because of the obvious advantages of enzymes, high chemo-, stereo- and enantioselectivity, mild reaction conditions and very low environmental contamination, biocatalysis is of increasing significance in the fine chemical industry. For instance, the traditional chemical multi-step process for the production of several important β-lactam antibiotics (a laborious chemical route that uses chlorinated hydrocarbons, acid chlorides, pyridine and chlorotrimethylsilane at -40 °C) is being superseded by an enzymatic reaction near ambient temperature in water\(^1\).

Lipases

Due to their stability combined with the promiscuous acceptance of non-natural reactants lipases have evolved into nearly universal catalysts for transformations that involve the carboxyl group. Studies of lipases date back to the beginning of this century\(^2\). Industrial applications of lipases include improving characteristics of oils and fats for food applications\(^3\) and the production of chiral synthons\(^4,5\) through kinetic resolution of chiral esters\(^6\), alcohols and acids.

Lipases have a broad substrate specificity, are relatively stable and are cofactor-independent. They normally function at the surface of emulgated microscopic fat droplets; some of them are activated by lipid water interfaces and a large number of lipases can function in the presence of water miscible cosolvents or even in dry organic media. The natural substrate of the lipase, a triacyl glycerol such as triolein, is a rather large molecule, therefore most lipases have a relatively large active site compared to some other enzymes.

2
Figure 1. The natural reaction of lipases: hydrolysis of oils and fats.

In vivo, lipases catalyze the hydrolysis of triacyl glycerides. *In vitro*, they can be used to catalyze the reverse reaction—esterification—by performing the reaction in an organic medium and removing the water which is formed. Because of the large variety in size and polarity in natural substrates and nucleophiles that a lipase should be able to convert (water, glycerol, alcohols, monoacyl, diacyl and triacyl glycerols with varying alkyl chain length) they are also able to convert a diversity of non-natural substrates and nucleophiles. Although the natural substrates of lipases are achiral, the enzymes themselves are chiral molecules and they are also, as one would expect, able to catalyze the enantioselective conversion (kinetic resolution) of racemic substrates.
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Calculation of the enantiomeric ratio \((E)\) of a kinetic resolution

The enantiomeric ratio (abbreviation: \(E\)) is a convenient measure of the enantioselectivity of the kinetic resolution of a racemate. It can in principle be calculated at any point in time in the course of the reaction using a set of logarithmic formulas\(^7\). If two of the three parameters -conversion \((c)\), enantiomeric excess of the substrate \((ee_s)\) and enantiomeric excess of the product \((ee_p)\)- are known, the enantiomeric ratio and the missing parameter can be calculated\(^a\). All parameters are numbers between 0 and 1. Most often used are the formulae with conversion and substrate \(ee\) or product \(ee\). The latter tends to be more accurate at low conversions.

\[
E = \frac{\ln[(1-c)(1-ee_s)]}{\ln[(1-c)(1+ee_p)]} \quad E = \frac{\ln[(1-c)(1+ee_p)]}{\ln[(1-c)(1-ee_p)]}
\]

When determination of the conversion is difficult it is also possible to calculate the enantiomeric ratio from the substrate \(ee\) and the product \(ee\)\(^8\) according to:

\(^a\) The enantiomeric ratio represents the ratio of the second order rate constants of the two enantiomers of a chiral substrate \((S)\). In practice this means that the formula is only valid for substrate concentrations much lower than \(K_M\).

\[
E + S \xrightleftharpoons[k_2]{k_1} ES \xrightarrow{k_3} E + P \quad K_M = \frac{k_2 + k_3}{k_1}
\]

The Michaelis constant \((K_M)\) is composed of the rate constants for the formation \((k_1)\) and dissociation \((k_2)\) of the enzyme-substrate complex \((ES)\) and the rate of formation \((k_3)\) of product \((E + P)\), also referred to as \(k_{cat}\), and represents the substrate concentration that will give half of the maximum rate. The formation of product from the enzyme-substrate complex is often the rate determining step in hydrolase catalysis.
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\[
E = \frac{\ln[(1 - ee_s)/(1 + \frac{1}{ee_p})]}{\ln[(1 + ee_s)/(1 + \frac{ee_s}{ee_p})]}
\]

The unknown parameter can be calculated with one of the two following simple formulae:

\[
ee_p = (\frac{1}{c} - 1)ee_s
\]
\[
ee_s = (\frac{c}{1-c})ee_p
\]

The above formulae are valid in the case of an irreversible reaction. In the case of a reversible reaction the equilibrium constant enters into the equations. One of the advantages of reaction is that, in general, it can be regarded as irreversible, which simplifies analysis and data interpretation.

The implicit assumption made in these formulas, that an achiral nucleophile reacts with an equal rate with a R- and a S-acyl enzyme intermediate is not always valid because these intermediates are diastereomers and not enantiomers and therefore not equal. Nevertheless, the enantiomeric ratio or apparent enantiomeric ratio has developed into a standard parameter for comparing enantioselectivity of enzymatic transformations.

Catalytic mechanism of a lipase

The mechanism of action of a lipase is the same as that of serine proteases (Figure 2). It involves the so-called catalytic triad of serine, histidine and aspartate in the active site. The negative charge of the oxygen atom in the so-called tetrahedral intermediate is stabilized by hydrogen bonding in a subsite of the lipase that is referred to as the oxanion hole (Figure 2).
In the third step of the catalytic cycle, the acyl enzyme intermediate is formed by elimination of an alcohol or water (R' = H, alkyl) molecule from the tetrahedral intermediate. The proton on the histidine is donated to the alkoxy moiety (OR') during this process and the histidine reverts to its original state (Figure 2).

Figure 2. The catalytic cycle of a lipase and several other hydrolases.

Unnatural nucleophiles

The acyl enzyme intermediate can in principle be cleaved by any nucleophile\textsuperscript{10}. In vivo this is water, but alcohols\textsuperscript{11} phenols\textsuperscript{12}, hydrogen peroxide\textsuperscript{13,14}, alkyl hydroperoxides\textsuperscript{15}, sugar derivatives\textsuperscript{16}, oximes\textsuperscript{17} and even nitrogen nucleophiles have been used with success. Since the seminal publication regarding enzymatic activity in organic solvents\textsuperscript{18}, numerous articles that describe lipase catalyzed acylation of nitrogen nucleophiles have been published.
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Lipase catalyzed aminolysis has definite advantages over reaction with oxygen nucleophiles. Although all enzymatic processes are reversible, aminolysis can effectively be considered as irreversible, which is a big advantage in a kinetic resolution. Evidence has been presented that lipases are able to hydrolyze amide bonds but only under drastic conditions\(^ 19 \), or using exotic substrates\(^ 20 \). Lipases are even able to catalyze transamidation reactions under special circumstances e.g. when a highly activated trifluoroethylamide\(^ 21 \) is used as a substrate. Aminolysis was found to be more enantioselective than hydrolysis or alcoholysis in a number of cases and obviously a whole range of (chiral) amides and related compounds can be synthesized using this method.

In this Chapter an overview will be given of the advantages and limitations of lipase catalyzed aminolysis with a variety of (hetero-substituted) nitrogen nucleophiles.

Ammonolysis

Enzymatic ammonolysis of esters (Figure 3) was discovered in our group\(^ 22, 23, 24, 25 \). A saturated solution of dry ammonia in tert-butyl alcohol gives good results with Novozym 435 (a commercially available immobilized form of *Candida antarctica* lipase B) as well as with many other lipases. Quantitative conversion to the amide was reached within 24 hours. The product precipitates from the reaction mixture and the catalyst suffers no significant loss of activity. tert-Butyl alcohol is a suitable solvent as it is not a nucleophile for the enzyme; presumably because it cannot access the acyl enzyme intermediate in the active site due to its bulky nature. Enantioselective ammonolysis of chiral esters constitutes a potentially interesting source of optically active amides. Moreover, the latter can be readily converted, e.g. via the Hofmann degradation, to the corresponding chiral amines containing one carbon atom less, with retention of optical activity.
Figure 3. The ammonolysis of ethyl octanoate using ammonia saturated tert-butyl alcohol.

Ammonolysis is not possible in, or in the presence of large amounts of water; nor can the free acid be used. In the presence of water the hydrolytic reaction becomes dominant and the free acid forms an ion pair with ammonia that cannot be converted by the lipase. The rigorous exclusion of water is well tolerated by Candida antarctica lipase B; it is fully active under extremely dry conditions and can even be dried over P₂O₅ or activated zeolites without losing activity¹⁶.

Figure 4. Ammonolysis of ibuprofen chloroethyl ester compared to other kinetic resolutions (all reactions 10% substrate in tert-butyl alcohol at 40 °C).
We note that some lipases, such as *Thermomyces lanuginosus* lipase, that require some water to catalyze esterification still act as ammoniolysis catalyst in dry medium. Presumably, ammonia acts as a water replacement.

The ammonolysis reaction was found to be more enantioselective than hydrolysis or alcoholysis in some cases e.g. in the ammonolysis of ibuprofen chloroethyl ester (Figure 4). Amino acid esters can be resolved by lipase catalyzed ammonolysis. Surprisingly the ammonolysis is compatible with racemisation catalysts such as pyridoxal and salicylaldehyde. Aromatic aldehydes are known to form stable imines with primary amines and the racemization is believed to proceed via such an imine. In a dynamic kinetic resolution D-phenylglycine amide (D-FGA), the side chain donor for the enzymatic synthesis of the antibiotics ampicillin and cefalexin, is produced in high yield.

\[
\begin{align*}
\text{NH}_2 & \quad \text{OCH}_3 \\
\text{O} & \quad \text{NH}_2 \\
\text{Pyridoxal} & \quad \text{NH}_3/\text{t-BuOH} \\
& \quad \text{Novozym 435} \\
\text{NH}_2 & \quad \text{NH}_2 \\
\text{OCH}_3 & \quad \text{NH}_2 \\
& \quad \text{D-FGA, 61% ee at 87% Conversion}
\end{align*}
\]

*Figure 5.* *The dynamic kinetic resolution of phenylglycine methyl ester.*

Another application of enzymatic ammonolysis is the conversion of (achiral) substrates that are not compatible with conventional ammonolysis which often requires high pressure and temperature for an acceptable rate. Under these
conditions esters containing double and triple bonds can give side reactions, especially if they are in conjugation with a carbonyl function. Lipase catalyzed ammonolysis\textsuperscript{29} and aminolysis\textsuperscript{30} of α,β-unsaturated esters takes place without side reactions. In fact the reaction works so well that they are frequently used as activated esters in the enantioselective acylation of alcohols and amines\textsuperscript{31}. In a similar way amides of β-keto esters can be prepared\textsuperscript{32}. 

\begin{figure}
\centering
\includegraphics[width=0.8\textwidth]{figure6.png}
\caption{Ammonolysis of α,β-unsaturated esters.}
\end{figure}

**Aminolysis**

The aminolysis of esters with aliphatic amines has been known for quite some time. The first publications on aminolysis appeared in the mid 80's when Klibanov\textsuperscript{18} and Japanese scientists\textsuperscript{23,34} found enzymatic activity in organic solvents with freeze-dried lipases. The aminolysis reactions that are described in literature almost exclusively take place in organic media and not in water or aqueous-organic mixtures. This is not only because of the competing hydrolysis that would take place when large amounts of water are present, but also because ionization in general is suppressed in organic media and therefore the lipases are not deactivated by bases that would harm them in water. For example, an unbuffered solution in water of a compound such as butylamine would have a pH between 12 and 13; not at all the pH optimum of most
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enzymes. Buffering would spare the enzyme but renders the nucleophile inactive by protonation.

The first enantioselective aminolysis reactions were described at the end of the 80's. Gotor et al. reported the enantioselective aminolysis of ethyl 2-chloropropionate\textsuperscript{35} catalyzed by Candida cylindracea lipase (now renamed Candida rugosa lipase) forming the (S)-product. Similarly, β-hydroxy esters were effectively resolved via Candida antarctica lipase catalyzed aminolysis with benzylamine (Figure 7)\textsuperscript{36}. In this reaction the stereogenic center is more removed from the ester function. In general, compounds in which the stereogenic carbon atom is not directly connected to the reacting functional group are converted with lower enantioselectivity because of the decreased influence of the chiral group.

\[
\begin{align*}
\text{CAL-B} & \quad \text{dioxane} \\
\text{R} & \quad \text{E} = > 100 \quad \text{(S)} \\
\text{R} = \text{H} & \quad \text{E} = 52 \quad \text{(S)} \\
\text{R} = \text{CH}_3 & \quad \text{E} = > 100 \quad \text{(R)} \\
\end{align*}
\]

\[\text{OH} \quad \text{H} \quad \text{N} \quad \text{NH}_2 \quad \text{OH} \quad \text{O} \]

\[\begin{align*}
\text{R} & \quad \text{OH} \quad \text{O} \\
\text{R} = \text{H} & \quad \text{E} = > 100 \quad \text{(S)} \\
\text{R} = \text{CH}_3 & \quad \text{E} = 52 \quad \text{(S)} \\
\text{R} = \text{Cl} & \quad \text{E} = > 100 \quad \text{(R)} \\
\end{align*}
\]

\[\begin{align*}
\text{OH} & \quad \text{O} \\
\text{R} & \quad \text{OH} \quad \text{O} \\
\end{align*}\]

\textbf{Figure 7.} Enantioselective aminolysis of β-hydroxy esters.

The absolute configurations of the amides formed in this reaction can be \(R\) or \(S\), depending on the priority of the substituent on \(C_5\) but for all products the orientation of the hydroxyl group in space is equal.
As already mentioned, aminolysis can be more selective than hydrolysis or transesterification. In some cases the change in rate for one of the enantiomers is so large that there is an actual inversion of the selectivity. For example, Candida rugosa lipase preferentially converts the \((R)\)-enantiomer of octyl 2-chloropropanoate in a transesterification with butanol and the \((R)\)-enantiomer of 2-chloropropanoic acid in an esterification with butanol, but in the aminolysis of ethyl 2-chloropropanoate and ethyl 2-bromopropanoate the \((S)\)-enantiomers are selectively converted\textsuperscript{37}.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure8.png}
\caption{Enantioselective aminolysis of \(\alpha\)-halo esters with Candida rugosa lipase.}
\end{figure}

\textbf{Aminolysis of diesters}

Alkylamines have been used for the regioselective aminolysis of diesters. For example, protected diesters of L-glutamic acid can be aminolyzed selectively at \(C_1\) with Candida antarctica lipase B (CAL-B) to yield the protected amino acid amides (Figure 9)\textsuperscript{38}. Protecting groups such as acetyl, benzylxoy carbonyl, \textit{tert}-butoxy carbonyl and, surprisingly, even diphenylacetyl (although less active) were tolerated by the enzyme. It was found that substrates with alkoxy carbonyl protecting groups in general reacted faster than those with acyl protecting groups.
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Figure 9. Regioselective aminolysis of a N-protected diethyl glutamate.

When the N-protected D-diester of glutamic acid was used as the substrate (Figure 10) the regioselectivity was lower and significant amounts of the 5-ester were aminolysed\textsuperscript{39}. Several amines were used (R = isopropyl, pentyl, benzyl, cyclohexyl and phenyl) and only aniline did not react. There are only few examples of lipase catalyzed aminolysis with an aromatic amine as nucleophile, although many authors have tested aromatic amines. The fact that cyclohexylamine can be used in this reaction as a nucleophile in contrast to aniline indicates that the inactivity of aromatic amines is caused by electronic factors and not by steric factors.

Figure 10. Non-selective aminolysis of N-protected diethyl glutamate.

When the ε-amino group is not protected, a non-enzymatic intramolecular aminolysis takes place (Figure 11). However, the reaction product can also be aminolyzed with pentylamine in the presence of Candida antarctica lipase B\textsuperscript{40}. Starting from the racemate there is a preference for the D-enantiomer.
Figure 11. Enantioselective aminolysis of ethyl pyroglutamate.

When dimethyl succinate or dimethyl adipate was used as acyl donor with amino alcohols (Figure 12) a number of products was formed\textsuperscript{41}. Besides the major products, mono amide and $\alpha,\omega$-amido ester, evidence was found for the formation of macrocycles and $N$-alkylsuccinimides. The yield of macrocycles could be increased by diluting the reaction mixture or raising the temperature.

Figure 12. The lipase catalyzed formation of an $N$-amino succinimide derivative.
Unfortunately no isolated yield or conversion is given for this reaction although a number of \(\omega\)-pyrrolo alkanols were prepared by LiAlH\(_4\) reduction of the succinimide derivatives. The synthesis of the macrocyclic compounds (Figure 13) was better described.

![Chemical Reaction Diagram]

Figure 13. *Lipase catalyzed transesterification and aminolysis forming a macrocyclic compound.*

Using porcine pancreas lipase it was possible to synthesize macrocyclic bislactams from diesters and diamines\(^{42}\).

**Transformations with prochiral compounds**

Prochiral diesters constitute an important group of diester substrates. A lipase will convert, in the ideal case, one of the two enantiotropic ester moieties to generate an optically active molecule. Conversion of a prochiral diester has the advantage over a kinetic resolution of a racemate that theoretically 100% yield of the desired enantiomer can be obtained (a so-called desymmetrization). For example, in the
Chapter 1

*Candida antarctica* lipase B catalyzed aminolysis of dimethyl 3-hydroxyglutarate\(^{43}\) with ammonia, allylamine, butylamine or benzylamine (Figure 14), yields up to 98% and enantiomeric excess of 80% were achieved. In all cases the \((S)\)-amide was formed independent of the amine which was used.

![Chemical reaction](image)

**Figure 14.** Enantioselective aminolysis of a prochiral diester.

Similarly, prochiral diamines can be converted, by reaction with an achiral ester, to give a theoretical yield of 100% of one enantiomer of the monoacylated diamine\(^{44}\).

**Aminolysis of dialkyl carbonates**

Dialkyl carbonates form a special group of diesters. Although they contain two alkoxy moieties, lipase catalyzed aminolysis of carbonates always leads to the carbamate\(^{45}\); no formation of urea derivatives has ever been reported, even when activated carbonates were used. Reaction rates tend to be lower than with carboxylic acid esters. Lipase catalyzed acylations with dibenzyl carbonate (Figure 15)\(^{45}\) or dialllyl carbonate can be used for the conversion of racemic amines to the benzylxycarbonyl or allyloxycarbonyl derivatives of the optically active amines. These are both easily removable and often used protecting groups in organic chemistry that are generally prepared by phosgene-based chemistry.
**Figure 15.** Lipase catalyzed kinetic resolution and protection.

The rates of these reactions are generally low but this problem can be circumvented by using activated vinyl carbonates\(^{46,47}\). In this case the choice of enzyme was also *Candida antarctica* lipase B; one of the few lipases that catalyzes this type of reaction effectively\(^{45}\). The reaction is enantioselective with respect to both the amine and the vinyl carbonate (Figure 16). This procedure not only works well with chiral amines but also with vinyl carbonates derived from chiral alcohols because the acylation reaction yields vinyl alcohol, which immediately tautomerizes to acetaldehyde rendering the reaction irreversible.

Vinyl carbonates derived from chiral alcohols can be resolved with chiral amines in a doubly enantioselective reaction (Figure 17)\(^{48}\). One stereogenic center of the product has the S configuration and the other has the R configuration. Moderate to high diastereomeric excess and enantiomeric excess were obtained in a one step procedure.
Figure 16. Kinetic resolution of a chiral carbonate and a chiral amine.

Figure 17. Double kinetic resolution of a chiral vinyl carbonate and a chiral amine.

Hetero-substituted nitrogen nucleophiles

In practice the group of hetero-substituted nitrogen nucleophiles comprises only the hydrazine and hydroxylamine derivatives. Compounds that contain other elements than nitrogen or oxygen directly bonded to the nucleophilic nitrogen atom are not stable enough to be used in enzymatic transformations.
The chemical behavior of hydroxylamine and hydrazine is different from normal amines in a number of respects. The heteroatom lowers the basicity of the nitrogen atom but increases its nucleophilicity because of the so-called α-effect\textsuperscript{49}. Normal amines are so basic that they form an ion pair with carboxylic acids. Lipases cannot convert carboxylic acids in the anionic form, therefore there are very few examples of amide synthesis directly from a carboxylic acid and an amine\textsuperscript{50}. In contrast, hydrazine and hydroxylamine undergo reaction with carboxylic acids as well as with their corresponding esters (Figure 18)\textsuperscript{51}.

\[
\begin{align*}
\text{O} & \quad \text{R}^1-X\text{NH}_2 \quad \text{CAL} \\
& \quad \text{O} \quad \text{N} \quad X \\
& \quad \text{H} \quad \text{R}^1
\end{align*}
\]

\(R = H, \text{Et} \quad X = \text{NH, O} \quad R^1 = \text{H, Me, Ph, Bz}\)

**Figure 18.** Acylation of hetero-substituted nitrogen nucleophiles.

In general, the ester reacts much faster than the acid, although this depends on the nature of the nucleophile. Relatively basic nucleophiles such as hydrazine react much faster with the ester whereas with weakly basic phenylhydrazine there is hardly any difference in initial rates. Hydroxylamine can be acylated with fatty acids\textsuperscript{52}, their methyl esters and triglycerides\textsuperscript{53}, all with a high rate and in good yields, even in the presence of water. Water does not present a problem because hydroxamic acids and acyl hydrazines are thermodynamically more stable than normal amides. Amidases are also able to convert short chain amides into hydroxamic acids\textsuperscript{54} and acyl hydrazines\textsuperscript{55} in aqueous solution with hardly any hydrolysis.

A remarkable difference in enantioselectivity is observed in the reactions of hydrazine and hydroxylamine, respectively, with ibuprofen or ibuprofen methyl ester (Figure 19), in the presence of *Candida antarctica* lipase B. Hydrazine is acylated with a
much higher enantioselectivity by both the acid and the ester. In contrast, when benzylhydrazine and benzylhydroxylamine are used, both reactions are highly selective\textsuperscript{51}. When crosslinked enzyme crystals of *Candida rugosa* lipase (CLEC-CR) were used in water with hydroxylamine the biologically active hydroxamic acid derivative of (S)-ibuprofen, (S)-ibuproxima\textsuperscript{56} was formed in good yield and with high enantioselectivity.

![Chemical structure](image)

**Figure 19.** *Aminolysis of ibuprofen derivatives.*

Hydrazinolysis and hydroxylaminolysis are also enantioselective with respect to the alkoxy moiety of an ester\textsuperscript{51}. For example, the aminolysis of 1-phenylethyl butanoate with hydrazine and hydroxylamine and their benzyl derivatives in the presence of *Candida antarctica* lipase B was highly selective in all cases (Figure 20).

Acyl hydrazines theoretically still have a nucleophilic nitrogen that can be acylated. When hydrazine is acylated the pK\textsubscript{a} of the nitrogen atom drops from 8.0 to 3.4 (below the pK\textsubscript{a} of aniline and pyridine). However, because of the \(\alpha\)-effect the nucleophilicity of this nitrogen atom is still high enough to serve as an effective nucleophile in lipase catalyzed transformations. Hence, it is possible to use acyl hydrazines as nucleophiles for regioselective\textsuperscript{57} and enantioselective\textsuperscript{58} aminolysis of carboxylic esters (Figure 21).
Figure 20. Aminolysis of 1-phenylethyl butanoate.

Figure 21. Regio and enantioselective reactions with acyl hydrazines.

Gotor et al. even reported the formation of triacylated hydrazine derivatives. At elevated temperatures dimethyl succinate formed \( N \)-amino succinimides\(^{59} \) in the presence of \textit{Pseudomonas cepacia} lipase (Figure 22).
Figure 22. Lipase catalyzed formation of a N-amino succinimide.

Diacyl hydrazines can be formed directly in a rapid reaction from hydrazine hydrate and carboxylic acids in an organic solvent in the presence of a lipase. Water slows down the reaction because it acts as a competing nucleophile but it does not affect the endpoint, which is very close to 100% conversion. Different lipases have very different selectivities towards hydrazine and acyl hydrazines. In some cases hydrazine is the preferred nucleophile but, for example, Pseudomonas lipoprotein lipase converts the intermediate monoacyl hydrazine so fast that its equilibrium concentration is lower than 1% in the course of the reaction (Figure 23).

Figure 23. Lipase catalyzed formation of a diacyl hydrazine.
Chiral acyl hydrazines can be acylated enantioselectively with carboxylic acids\textsuperscript{60}. This method can be used to resolve carboxylic acids that are normally not a substrate for a particular lipase (Figure 24). By esterification and chemical hydrazinolysis the racemic hydrazine can be prepared and used as an acyl acceptor instead of an acyl donor.

\begin{center}
\textbf{Figure 24.} Kinetic resolution using a chiral acyl hydrazine as nucleophile. The lipase that is used does not accept ibuprofen as a substrate.
\end{center}

Lipases generally have more tolerance for large substrates in the alcohol pocket than in the acyl donor pocket. In the natural reaction the nucleophile (diacyl glyceride) is much larger than the acyl donor (carboxylic acid). In the case of ibuprofen, the acylation of its hydrazine is much faster than the aminolysis of its ester. This may be a promising method for the resolution of very bulky substrates that are not accepted as acyl donor by lipases.
In summary, lipase catalyzed acylations of (hetero-substituted) nitrogen nucleophiles is a fascinating part of biocatalysis where things that seem impossible are possible and things that seem obvious do not work. The aim of this thesis is to investigate the scope, advantages and limitations of the use of (hetero-substituted) nitrogen nucleophiles in lipase catalyzed transformations and to apply this technique to the synthesis of potentially interesting fine chemicals and pharmaceutical intermediates.

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Chapter 2

Lipase catalyzed reactions of aliphatic and arylaliphatic carbonic acid esters

Summary

Symmetrical dialkyl carbonates and dibenzyl carbonate reacted with various nucleophiles in the presence of Candida antarctica lipase B in organic solvents. For example, reaction of dibutyl and dibenzyl carbonate with an alcohol gave a mixture of the mono- and disubstituted products. Aminolysis, however, afforded only the carbamates, without subsequent reaction to the ureum derivatives. The reaction rates were rather low compared with carboxylic esters; the reactivity increased in the order dimethyl < diethyl < dibutyl = dibenzyl carbonate. Aminolysis of dibenzyl carbonate by chiral benzylamines gave the corresponding enantiomerically pure benzylxycarbonyl amines. This route has the advantage that the use of phosgene is circumvented.
Chapter 2

INTRODUCTION

During the last decade it has become clear that lipases (E.C. 3.1.1.3) efficiently catalyze a variety of reactions with non-natural acyl acceptors\(^1\) such as alcohols, hydrogen peroxide, ammonia, amines and oximes. The choice of the acyl donor, on the other hand, has generally been limited to simple carboxylic acid esters. Lipase-catalyzed transformation of the structurally closely related carbonic acid derivatives\(^2\) has almost exclusively involved activated compounds such as vinyl\(^3\), phenyl\(^4\) or oxime\(^5\) esters, pyrocarbonates\(^6\) or mixed anhydrides\(^6\), and only a few examples of lipase-catalyzed alcoholysis of dialkyl carbonates have been published.\(^7\)\(^8\)\(^9\)\(^10\)

We have found that *Candida antarctica* lipase B catalyzes the alcoholysis and aminolysis of dibutyl and dibenzyl carbonate. These reactions are of potential interest because they provide a route to carbonic acid derivatives which does not involve the use of phosgene. Because the benzylloxycarbonyl (Z) group in particular is a frequently used protecting group that can be cleaved by hydrogenolysis under very mild conditions, we have also explored the synthesis of enantiomerically pure Z-amines from racemic precursors.

RESULTS AND DISCUSSION

A number of immobilized lipases and esterases were screened for activity in the alcoholysis of dibutyl carbonate by 1-propanol in *tert*-butyl alcohol. Only Novozym 435 (*Candida antarctica* lipase B) showed a useful rate of reaction; lipoprotein lipase from *Pseudomonas* reacted approx. 10 times slower. Other lipases and esterases showed no significant activity. Hence, Novozym 435 was used as catalyst in all further experiments.
Table 1. Effect of the alkyl chain length on the hydrolysis of dialkyl carbonates\textsuperscript{a}.

<table>
<thead>
<tr>
<th>Carbonate</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dimethyl carbonate</td>
<td>13</td>
</tr>
<tr>
<td>Diethyl carbonate</td>
<td>48</td>
</tr>
<tr>
<td>Dibutyl carbonate</td>
<td>66</td>
</tr>
<tr>
<td>Dibenzyl carbonate\textsuperscript{b}</td>
<td>56</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Reaction conditions: 5.0 mmol dialkyl carbonate with 5 mmol water was shaken at 40 °C with 50 mg Novozym 435 in 5 ml tert-butyl alcohol for 48 h.

\textsuperscript{b} Concentration 0.5M (121gr/L).

The effect of the chain length on the reactivity was assessed by subjecting dimethyl, diethyl, and dibutyl carbonate to Novozym 435 catalyzed hydrolysis. From the results (Table 1) it becomes clear that the reaction rate increases in the order methyl < ethyl < butyl; we ascribe this effect to the increasing hydrophobicity of the reactant. Moreover, inhibition of the lipase by the liberated alcohol, which would be more substantial with ethanol and particularly methanol, might play a role. Dibenzyl carbonate (which eluded previous attempts at enzymatic reaction\textsuperscript{8}) reacted slower than dibutyl carbonate. On the basis of these results, we selected dibutyl and dibenzyl carbonate for further study. The latter compound is of particular interest because its aminolysis would afford the highly interesting class of Z-protected amines as reaction product.

Dibutyl carbonate was subjected to Novozym 435 mediated hydrolysis, alcoholysis and aminolysis. The general course of these reactions is depicted in Figure 1. Alcoholysis of dibutyl carbonate with 1-propanol gave a statistical mixture of the starting compound and its two products (Table 2). In contrast, isopropyl alcohol gave exclusively butyl isopropyl carbonate; it would seem that the alcoholysis of the second butyl group is prevented by steric hindrance in this case.
Figure 1. Reactions of alkyl carbonates. $R, R^1 = \text{alkyl, arylalkyl}, R^2 = H, \text{alkyl, arylalkyl}$.

Aminolysis, which is known to be irreversible due to the stability of the amide bond\textsuperscript{11}, did not proceed beyond the carbamate stage. In control experiments, dibutyl carbamate showed no reaction at all in hydrolysis, alcoholysis and aminolysis under the same conditions as used with dibutyl carbonate, even after very long reaction times. Carbamates are known to be slow reacting substrates or even reversible inhibitors of lipases\textsuperscript{12}.

Dibutyl carbonate seems to react slower than carboxylic acid esters under comparable conditions. The Novozym 435 catalyzed ammonolysis of ethyl octanoate was approx. four times faster than that of dibutyl carbonate\textsuperscript{13}.

Dibenzyl carbonate (Table 3) reacted in much the same manner. The aminolysis of dibenzyl carbonate by simple amines such as 1- or 2-aminopropane gave high conversions at a moderate rate.
**Table 2. Transformations of dibutyl carbonate catalyzed by C. Antarctica lipase.**

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>Equivalents</th>
<th>Reaction time (h)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>5</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>2</td>
<td>96</td>
<td>74(^b)</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>2</td>
<td>96</td>
<td>60(^c)</td>
</tr>
<tr>
<td>Ammonia</td>
<td>2.5</td>
<td>96</td>
<td>99</td>
</tr>
<tr>
<td>1-Aminopropane</td>
<td>1.2</td>
<td>168</td>
<td>100</td>
</tr>
<tr>
<td>2-Aminopropane</td>
<td>1.2</td>
<td>168</td>
<td>72</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>1.2</td>
<td>96</td>
<td>98</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 5.0 mmol dibutyl carbonate, nucleophile (25 mmol water, 10 mmol alcohol or 6 mmol amine) and 100 mg Novozym 435 in 5 ml tert-butyl alcohol at 40 °C.

b. The total conversion was 74%. Two products were formed: dipropyl carbonate (22%) and butyl propyl carbonate (52%).

c. Only one product was formed: butyl isopropyl carbonate.

Because enzyme catalyzed reactions are inherently enantioselective, the aminolysis of dibenzyll carbonate can be used as a one step synthesis of optically active Z-amines from the racemic amine. In order to develop a practical synthesis, we studied the effect of the solvent on the reaction of 1-phenylethylamine and dibenzyl carbonate; initial rates are listed in Table 4.

The high initial rate in tert-butyl alcohol made it look like a promising solvent but the reaction rate dropped dramatically in the course of the reaction. Higher conversions (Table 4) were reached in apolar solvents such as hexane and isooctane. These solvents had the extra advantage that they dissolved the product only sparingly which allowed its isolation by simple filtration in a number of cases. We note that the rates of lipase catalyzed reactions are generally higher in hydrophobic solvents than in water miscible ones.\(^{14,15}\)
Chapter 2

Table 3. Transformations with dibenzyl carbonate. a

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>Equivalents</th>
<th>Reaction time (h)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>5</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td>1-Propanol</td>
<td>2</td>
<td>48</td>
<td>90b</td>
</tr>
<tr>
<td>2-Propanol</td>
<td>2</td>
<td>48</td>
<td>70c</td>
</tr>
<tr>
<td>Ammonia</td>
<td>5</td>
<td>48</td>
<td>100</td>
</tr>
<tr>
<td>1-Aminopropane</td>
<td>1.2</td>
<td>96</td>
<td>100</td>
</tr>
<tr>
<td>2-Aminopropane</td>
<td>1.2</td>
<td>96</td>
<td>66</td>
</tr>
<tr>
<td>Benzylamine</td>
<td>1.2</td>
<td>96</td>
<td>100</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 2.5 mmol dibenzyl carbonate, nucleophile (12.5 mmol water, 5 mmol alcohol or 3 mmol amine) and 100 mg Novozym 435 in 5 ml tert-butyl alcohol at 40 °C

b. The total conversion was 90%. Two products were formed: dipropyl carbonate (28%) and benzyl propyl carbonate (62%).

c. Only one product was formed: benzyl isopropyl carbonate.

The high initial rate that was measured in tert-butyl alcohol did not translate into a correspondingly high yield (Table 4). To gain more insight, the progress of the reactions in hexane and tert-butyl alcohol were monitored over time (Figure 2). It became apparent that in tert-butyl alcohol the reaction soon abated and became stagnant at approx. 8% conversion, for which, beside deactivation of the enzyme, product inhibition would seem the most likely cause. In contrast, when the reaction was carried out in hexane medium it continued at a steady rate. The rates of lipase catalyzed reactions are generally higher in hydrophobic solvents than in water miscible solvents. Product inhibition may have been lower in the beginning of the reaction in tert-butyl alcohol because of lower product activity. The product is very soluble in tert-butyl alcohol but precipitated from hexane before 2% conversion is reached.

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### Table 4: Initial rate of benzylxycarbonylation of 1-phenylethylamine in various solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial rate (μmol·h⁻¹·g⁻¹)</th>
<th>Conversion (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tert-Butyl alcohol</td>
<td>716</td>
<td>7.6</td>
</tr>
<tr>
<td>Hexane</td>
<td>402</td>
<td>15.6</td>
</tr>
<tr>
<td>Isooctane</td>
<td>396</td>
<td>16.1</td>
</tr>
<tr>
<td>tert-Butyl methyl ether</td>
<td>359</td>
<td>8.9</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>356</td>
<td>14.9</td>
</tr>
<tr>
<td>Diisopropyl ether</td>
<td>331</td>
<td>12.9</td>
</tr>
<tr>
<td>tert-Amyl alcohol</td>
<td>265</td>
<td>7.0</td>
</tr>
<tr>
<td>No solvent</td>
<td>244</td>
<td>16.9</td>
</tr>
<tr>
<td>Dioxane</td>
<td>198</td>
<td>3.7</td>
</tr>
<tr>
<td>Diethyl ether</td>
<td>158</td>
<td>6.3</td>
</tr>
<tr>
<td>Dimethoxyethane</td>
<td>147</td>
<td>3.4</td>
</tr>
<tr>
<td>Benzene</td>
<td>135</td>
<td>8.2</td>
</tr>
<tr>
<td>Toluene</td>
<td>133</td>
<td>7.9</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>72</td>
<td>2.7</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>65</td>
<td>2.8</td>
</tr>
<tr>
<td>Pyridine</td>
<td>22</td>
<td>0.4</td>
</tr>
<tr>
<td>Dichloromethane</td>
<td>16</td>
<td>1.0</td>
</tr>
<tr>
<td>N-methylpyrrolidone</td>
<td>8</td>
<td>0.4</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 2.5 mmol dibenzyl carbonate, 200 mg 1,3-dimethoxybenzene (internal standard), 4 mmol 1-phenylethylamine and 50 mg Novozym 435 in 5 ml solvent at 40 °C

b. Percentage of amine acylated
Figure 2. The formation of Z-1-phenylethylamine in hexane (◊) and tert-butyl alcohol (▲). Reaction conditions: 605 mg dibenzyl carbonate (2.5 mmol), 485 mg 1-phenylethylamine (4.0 mmol), 5 ml solvent and 50 mg Novozym 435 at 40 °C.

We attempted the resolution of several chiral aliphatic and aryl aliphatic amines via lipase catalyzed benzyloxy carbonyl transfer. 2-Aminopentane and 2-amino octane reacted rather slowly in comparison with 1-phenylethylamine and the enantiomeric ratio was only 2 at 40 °C (data not shown). We tentatively conclude that the apolar 2-alkyl carbamoyl moiety is an even more efficient lipase inhibitor. At 20 °C the reaction rate was still lower but a modest enantiomeric selectivity was achieved (Table 5). The enantiomers of the benzyllic amines were efficiently discriminated by Novozym 435 with $E$ factors ranging from 19 to 72. Because the reactions were carried out in hexane the products precipitated from the reaction mixture which allowed for their isolation by filtration. The precipitated product was enantiopure in all three cases but the recovery was low.

At 60 °C, 1-phenylethylamine reacted faster (27% conversion was reached in 96 hours) but $E$ decreased from 50 to 34.
**Table 5. Benzylxycarbonylation of amines in hexane.**

<table>
<thead>
<tr>
<th>Amine</th>
<th>Conversion (%)</th>
<th>ee of product</th>
<th>$E^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Amine" /></td>
<td>21</td>
<td>55</td>
<td>4 (-)</td>
</tr>
<tr>
<td><img src="image" alt="Amine" /></td>
<td>22</td>
<td>60</td>
<td>5 (-)</td>
</tr>
<tr>
<td><img src="image" alt="Amine" /></td>
<td>27</td>
<td>95</td>
<td>50 ($R, +$)</td>
</tr>
<tr>
<td><img src="image" alt="Amine" /></td>
<td>39</td>
<td>95</td>
<td>72 (+)</td>
</tr>
<tr>
<td><img src="image" alt="Amine" /></td>
<td>22</td>
<td>87</td>
<td>19 (+)</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 5 mmol dibenzyl carbonate (1210 mg), 360 mg 1,3-dimethoxybenzene (internal standard), 8 mmol amine and 250 mg Novozym 435 in 10 ml hexane for 7 days at 40°C; aliphatic amines 12 days at room temperature.

b. $E$ value with the absolute configuration or sign of rotation of the product.

In the case of benzylxycarbonyl-1-phenylethylamine the absolute configuration was determined by coelution with the chemically produced ($R$)-product on chiral HPLC. It is known that *Candida Antarctica* lipase B has a strong tendency to catalyze acylation of ($R$)-enantiomers of chiral alcohols and amines$^{15,17,18}$ containing one hydrogen atom and a methyl group attached to the chiral center. Hence, we expect that all products have the ($R$)-configuration although the aliphatic products have the opposite optical rotation.
CONCLUSIONS

C. antarctica lipase catalyzes the hydrolysis, alcoholysis and aminolysis of aliphatic and arylaliphatic carbonic acid esters. Aminolysis and ammonolysis do not proceed beyond the carbamate stage.

Resolution and protection of chiral benzylamine derivatives was accomplished in one step by a C. antarctica lipase catalyzed reaction with dibenzyl carbonate, which provides a route that avoids the use of phosgene.

EXPERIMENTAL

Materials

Immobilized Candida antarctica lipase B (Novozym 435) was a gift from Novo Nordisk A/S, Denmark and was used as received. Pseudomonas lipoprotein lipase was a gift of Boehringer Mannheim. It was immobilized on Accurel EP 100 according to a published procedure\textsuperscript{19}. Solvents and reagents except amines were dried on Zeolite CaA (Uetikon, activated at 400 °C for 24 h before use). Amines were purchased from Aldrich and used as received.

Dibutyl carbonate: metallic sodium (230 mg, 10 mmol) was dissolved in n-butanol (74.0 g, 1.0 mol) and dimethyl carbonate (88.5 g, 0.75 mol) was added. The mixture was slowly distilled and the fraction with a boiling point of 201-204 °C was collected, yield 76.6 g (88%). Characterization by \textsuperscript{1}H NMR (CDCl\textsubscript{3}): δ 0.94 (t, 6H, CH\textsubscript{3}), 1.41 (m, 4H, CH\textsubscript{2}), 1.66 (m, 4H, CH\textsubscript{2}), 4.13 (t, 4H, CH\textsubscript{2}).

Dibenzyl carbonate: metallic sodium (230 mg 10 mmol) was dissolved in benzyl alcohol (108.0 g, 1.0 mol) and dimethyl carbonate (88.5 g, 0.75 mol) was added. The methanol and excess of dimethyl carbonate were removed by slow distillation at
normal pressure. Subsequent distillation at reduced pressure afforded dibenzyl carbonate as an oily liquid that slowly crystallized at room temperature, yield 102.9 g, 85%, mp 29 °C, bp 194 °C at 13 mbar. Characterization by $^1$H NMR (CDCl$_3$): δ 5.16 (s, 4H, CH$_2$), 7.4-7.3 (m, 10H, CH).

**Analysis and Equipment**

GC analysis of the achiral products was performed on a Varian Star 3400 chromatograph, equipped with a 50m x 0.53 mm CP-Sil 5CB column and a FID detector. The carrier gas was nitrogen at a flow of 3.0 ml/min. Temperature program: 50 °C (5 min) to 280 °C (15 °C/min), the max. temp was held for 10 min. Analysis was also performed with reversed phase HPLC, using a Waters 8x100 mm 4µ Novapak C18 Radial Pak cartridge contained in a Waters 8x10 compression unit, a Waters 510 pump, a Shodex RI SE-61 refractometer and a SP 4270 integrator. The eluent was methanol/aqueous 0.1 M NaOAc/HOAc buffer pH 4.3 (65:35, v/v) at a flow of 1.0 ml/min. Conversion was measured against an internal standard (dibutyl ether, for HPLC as well as GC analysis).

The progress of the kinetic resolution experiments was monitored with reversed phase HPLC as described above. The enantiomeric excess was measured by chiral HPLC on a 4.6x25 mm 10µ Chiralcel OD column with a Waters 510 pump, a Shimadzu SPD-6A UV detector at 254 nm and a SP 4270 Spectra-Physics integrator. In this case 1,3-dimethoxybenzene was used as internal standard. Hexane/isopropyl alcohol (90/10, v/v) was used as eluent for reactions with 1-phenylethylamine, 1-amino indane and 1-aminotetrahydronaphtalene. For 2-aminopentane and 2-aminoctane, hexane/isopropyl alcohol (99/1, v/v) was used as eluent. Samples of 50 µL were taken, dissolved in 1.0 ml of tert-butyl methyl ether and filtered over MgSO$_4$ to adsorb the unreacted amine. $^1$H and $^{13}$C NMR spectra were recorded using a 400 MHZ Varian-VXR 400S spectrometer. Attempts to measure MS or GC-MS spectra
from reaction mixtures using a variety of ionization techniques were not successful. Specific rotations were measured with a Perkin Elmer 241 polarimeter at 589 nm (Na-lamp).

Reactions

General procedure. The reactions of dibutyl carbonate were carried out on a 5.0 mmol scale. To 5.0 ml of a stock solution of 1.0 M dibutyl carbonate (174 g/L) and 100 g/L dibutyl ether (internal standard) in tert-butyl alcohol, 25 mmol of water, 10 mmol of an alcohol or 6.0 mmol of an amine was added. In the ammonolysis reaction, the same amount of dibutyl carbonate and internal standard were weighed in and 5.0 ml of a saturated solution of ammonia in tert-butyl alcohol (2.5 M, 12.5 mmol) was added. The solutions were shaken for 10 days with 100 mg of Novozym 435 at 40°C. At regular intervals samples (50 µL) were taken and analyzed by GC. The products coeluted with chemically prepared samples on reversed phase HPLC as well as on GC. The procedure for reactions of dibenzyl carbonate with achiral nucleophiles was similar. The stock solution consisted of 0.5M dibenzyl carbonate (121 g/L) and 100 g/L dibutyl ether (internal standard). To 5.0 ml of this solution 12.5 mmol of water, 5.0 mmol of an alcohol or 3.0 mmol of an amine was added. The ammonolysis reaction was done with the same amount of dibenzyl carbonate and internal standard and 5.0 ml of a saturated solution of ammonia in tert-butyl alcohol (12.5 mmol). Novozym 435 was added (100 mg) and the mixtures were shaken for 10 days at 40 °C and samples (50 µL) were taken at regular intervals for GC analysis. The products coeluted with chemically prepared samples on reversed phase HPLC as well as on GC.
Initial rate experiments

The initial rate experiments were performed only with dibenzyl carbonate as acyl donor. A solution of 605 mg (2.5 mmol) dibenzyl carbonate, 200 mg 1,3-dimethoxybenzene and 485 mg (4.0 mmol) 1-phenylethylamine in 5.0 ml of organic solvent was shaken with 50 mg of Novozym 435 at 40 °C. At regular intervals, samples (50 μL) were taken and analyzed by HPLC. The products coeluted with chemically prepared samples on reversed phase as well as on chiral HPLC. Response factors were determined with the chemically produced racemic compound.

Acylation of chiral amines

A solution of 1210 mg dibenzyl carbonate (5 mmol) and 360 mg 1,3-dimethoxybenzene with 8.0 mmol of a chiral amine in 10 ml hexane was shaken with 250 mg Novozym 435 at 40 °C. Reactions with aliphatic amines were performed at room temperature. Conversions were measured by reversed phase HPLC and ee values by chiral straight phase HPLC. The reactions were performed in duplo. Precipitated product, if present, was filtered off and analyzed by chiral HPLC and with $^1$H and $^{13}$C NMR. To the duplo reaction mixture, isopropanol was added until it was homogeneous and samples for HPLC were taken. Products were isolated by column chromatography over silica with 1:15 (v/v) ethyl acetate/petroleum ether for Z-2-aminopentane and Z-2-aminoctane and 1:10 (v/v) ethyl acetate/petroleum ether for the other products. The products coeluted with chemically prepared samples in all cases on straight-phase and reversed-phase HPLC.
Characterization

(-)-2-benzylxycarbonylaminopentane, $^1$H NMR (400 MHz) (CDCl$_3$): $\delta$ 0.91 (t, 3H, CH$_3$), 1.13 (d, 3H, CH$_3$), 1.37 (m, 4H, CH$_2$), 3.72 (m, 1H, CH-NH), 4.52 (s, 1H, NH), 5.10 (s, 2H, O-CH$_2$), 7.3 (m, 5H, ring H). $^{13}$C NMR (400 MHz) (CDCl$_3$): Pentyl moiety, $\delta$ 13.922 (C5), 19.150 (C1), 21.229 (C4), 39.377 (C3), 46.926 (C2). $\delta$ 66.471 (O-CH$_2$), ring: $\delta$ 128.047, 128.085, 128.518, 136.742. $\delta$ 155.786 (C=O). Specific rotation: $[\alpha]_D^{23} = -11.9^\circ$ Ethanol abs. c = 1.0.

(-)-2-benzylxycarbonylaminooctane, $^1$H NMR (400 MHz) (CDCl$_3$): $\delta$ 0.87 (t, 3H, CH$_3$), 1.13 (d, 3H, CH$_3$), 1.26 (m, 4H, CH$_2$), 1.40 (m, 2H, CH$_2$), 3.70 (m, 1H, CH-NH), 4.52 (s, 1H, NH), 5.09 (s, 2H, O-CH$_2$), 7.3 (m, 5H, ring H). $^{13}$C NMR (400 MHz) (CDCl$_3$): Octyl moiety, $\delta$ 14.066 (C8), 21.236 (C1), 22.587 (C7), 25.902 (C6), 29.157 (C5), 31.767 (C4), 37.207 (C3), 47.192 (C2). $\delta$ 66.455 (O-CH$_2$), ring: $\delta$ 128.047, 128.085, 128.510, 136.750. $\delta$ 155.778 (C=O). Specific rotation: $[\alpha]_D^{23} = -8.1^\circ$ Ethanol abs. c = 1.0.

(R)-N-benzylxycarbonyl-1-phenylethylamine, $^1$H NMR (400 MHz) (CDCl$_3$): $\delta$ 1.47 (d, 3H, CH$_3$), 4.66 (q, 1H, CH-NH), 5.07 (m, 3H, NH, O-CH$_2$), 7.3 (m, 10H, ring H). $^{13}$C NMR (400 MHz) (CDCl$_3$): $\delta$ 22.458 (CH$_3$), 50.735 (NH-CH), 66.721 (O-CH$_2$), ring: $\delta$ 125.391, 127.327, 128.100, 128.495, 128.639, 136.492 (C-CH-NH), 143.495 (C-CH$_2$-O). $\delta$ 155.543 (C=O). Specific rotation: $[\alpha]_D^{23} = +45.0^\circ$ Ethanol abs. c = 1.0.

(+)-N-benzylxycarbonyl-1-aminindane, $^1$H NMR (400 MHz) (CDCl$_3$): $\delta$ 1.78 (m, 1H, CH$_2$-2), 2.56 (m, 1H, CH$_2$-2), 2.8 (br. m, 2H, CH$_2$-3), 5.0-5.3 (NH, CH-NH, CH$_2$-O), 7.0-7.3 (m, 9H, rings). $^{13}$C NMR (400 MHz) (CDCl$_3$): $\delta$ 30.025 (C2), 34.240 (C3), 56.419 (C1), 66.711 (CH$_2$-O), aromatic rings: $\delta$ 123.981, 124.760, 126.704, 127.984, 128.094, 128.502. $\delta$ 136.523, 143.132 (C-CH-NH, C-CH$_2$-O). $\delta$ 156.176 (C=O). Specific rotation: $[\alpha]_D^{23} = +68.5^\circ$ Ethanol abs. c = 1.0.

(+)-N-benzylxycarbonyl-1-amintetrahydroanaphthalene, $^1$H NMR (400 MHz) (CDCl$_3$): $\delta$ 1.81 (m, 3H, CH$_2$-CH$_2$), 2.05 (1H, NH-CH-CH$_2$), 2.78 (m, 2H, Ph-CH$_2$), 4.8-5.2 (m, 4H, O-CH$_2$, NH, NH-CH), 7.0-7.4 (m, 9H, rings). $^{13}$C NMR (400 MHz) (CDCl$_3$):
\( \delta 19.848 \) (C2), 29.180 (C3), 30.447 (C4), 49.316 (C1), 66.721 (O-CH\(_2\)), aromatic rings: \\
\( \delta 126.226, 127.053, 127.327, 128.100, 128.533, 128.662, 129.132, \delta 136.590, 136.697, \\
137.425 \) (C-CH-NH,C-CH\(_2\)-O and C-CH\(_2\)-CH\(_2\)). \( \delta 155.907 \) (C=O). Specific rotation: \\
\([\alpha]_D^{23} = 46.0^\circ\) Ethanol abs. c= 1.0.

REFERENCES


Chapter 3

Dynamic kinetic resolution of phenylglycine esters via lipase-catalyzed ammonolysis*

Summary

Ammonolysis of D,L-phenylglycine methyl ester catalyzed by Candida antarctica lipase B (Novozym 435) gave D-phenylglycine amide with 78% ee at 47% conversion. Combination of this reaction with in situ racemisation of the unconverted ester, catalyzed by pyridoxal or salicylaldehyde, gave D-phenylglycine amide with 61% ee at 87% conversion in a dynamic kinetic resolution. The racemisation rate of the amide was much lower than that of the ester under the reaction conditions.

* In cooperation with M. A. Wegman.
INTRODUCTION

The use of enzymes as catalysts in reactions which they do not perform in nature is destined to play a prominent role in the current trend towards the use of catalytic procedures to minimize waste and to reduce the number of reaction steps. In this context lipases (E.C. 3.1.1.3) are known to be very stable and versatile catalysts that readily effect acyl-transfer reactions to a broad spectrum of non-natural nucleophiles\(^1\) such as hydrogen peroxide\(^2,3,4\) and amines\(^5\). The lipase catalyzed ammonolysis of carboxylic esters\(^6, 7, 8, 9, 10, 11, 12, 13\) which affords the corresponding amides in high yields under mild reaction conditions, is more a recent addition to the synthetic repertoire. We also employed the inherent enantioselectivity of lipase catalysis in the ammonolysis of racemic phenylglycine methyl ester 1\(\text{a}\) into D- phenylglycine amide 2\(^{14}\). The best results were obtained with Candida antarctica lipase (B type) in ammonia saturated tert-butyl alcohol.

![Figure 1. Enantioselective ammonolysis of phenylglycine esters](image)

As a result of recent developments in the manufacture of penicillin and cephalosporin antibiotics\(^{15}\), whereby multi-step chemical procedures for the coupling of the D-phenylglycine and D-(4-hydroxyphenyl)glycine side chains with the β-lactam nuclei are being replaced by enzymatic alternatives (Figure 2), 2 may become an important synthetic intermediate.
Figure 2. Enzyme catalyzed coupling of D-phenylglycine amide and 6-aminopenicilllic acid.

Ammonolysis would become much more attractive if the slow racemisation of the ester, that is observed\(^\text{16}\) (5% over 24 h), in the course of the reaction could be accelerated to the rate of the ammonolysis reaction, which would make a 100% yield - theoretically - possible. For such a scheme to be effective, the product should racemise much slower than the reactant.

The racemisation of amino acid esters is known to be efficiently catalyzed by aromatic aldehydes such as 2-hydroxybenzaldehyde (salicylaldehyde) and 3-hydroxy-2-methyl-5-hydroxymethyl-pyridine-4-carboxaldehyde (pyridoxal) under basic conditions\(^\text{17,18}\). The use of aldehyde-based racemisation catalysts under ammonolytic conditions would seem questionable, however, because a reaction of the aldehyde group with ammonia\(^\text{17,18}\) would be expected to be facile.

The problem of undesired racemisation of D-phenylglycine amide 2 would seem more manageable, because the amide 2 is racemised slower than ester 1 and at high
concentrations it precipitates and is thus effectively removed from the reaction mixture.

We surprisingly found that pyridoxal and salicylaldehyde readily racemise phenylglycine methyl ester 1a in ammonia saturated tert-butyl alcohol. On this basis a dynamic kinetic resolution process has been developed.

RESULTS AND DISCUSSION

Ammonolysis of phenylglycine esters

Because Candida antarctica lipase B (Novozym 435) has emerged from our initial study\textsuperscript{14} as the catalyst of choice, it was used throughout the present work. The influence of the chain length of the ester group on the course of the reaction was briefly investigated. The data in Table 1 show that the butyl ester 1c reacted at one third of the rate of the methyl ester 1a. The effect on the enantiomeric ratio\textsuperscript{19} $E$, which varied between 16 and 19 was negligible\textsuperscript{a,b}. In view of these results all further experiments were performed with 1a.

\textit{a.} Enantiomeric ratios for the ester ($E_e$) and the amide ($E_a$) were calculated from the conversion $c$ and the optical purities of the ester ($ee_e$) and the amide ($ee_a$) as follows:

For the ester: $E_e = \frac{\ln[(1-c)(1-ee_e)]}{\ln[(1-c)(1+ee_e)]}$

For the amide: $E_a = \frac{\ln[1-c(1+ee_a)]}{\ln[1-c(1-ee_a)]}$

\textit{b.} Experimental values for $E$ were lower than those found previously by De Zoete et al. because the enantiomeric analysis has been improved.
Table 1. Effect of the chain length of the ester in the ammonolysis of phenylglycine esters

<table>
<thead>
<tr>
<th>Ester</th>
<th>Conversion (%)</th>
<th>ee\textsubscript{L-ester} (%)</th>
<th>ee\textsubscript{D-amide} (%)</th>
<th>E\textsubscript{ester}</th>
<th>E\textsubscript{amide}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1a</td>
<td>47</td>
<td>69</td>
<td>78</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>1b</td>
<td>36</td>
<td>47</td>
<td>84</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>1c</td>
<td>16</td>
<td>17</td>
<td>89</td>
<td>19</td>
<td>20</td>
</tr>
</tbody>
</table>

a. Reaction conditions: D,L-phenylglycine ester HCl 1 (1 mmol) was shaken with Novozym 435 (50 mg) in ammonia-saturated tert-butyl alcohol (5 ml, 12.5 mmol NH\textsubscript{3}) at 40 °C for 4 h.

The concentration of D,L-methyl ester 1a was varied between 50 and 800 mM. The initial reaction rate of L-1a was linearly proportional to the concentration over the whole range measured, whereas D-1a followed Michaelis-Menten kinetics (Figure 3). The apparent Michaelis constant K\textsubscript{m}, which is approximately 830 mM for D-1a, was deduced by fitting the experimental data to the Michaelis-Menten rate equation.

![Figure 3](image)

**Figure 3.** Initial rate (Vi) as a function of the concentration of D-1a (▲) and L-1a (●)
Chapter 3

Reaction times increased with the concentration of 1a, and a slight decrease in E (16 to 14) was observed over this concentration range (data not shown). Due to the deviation of D-1a from first order kinetics, E decreased at increased substrate concentrations. The concentration of ammonia had no influence on E or the reaction rate in the range 1.7 to 2.5 M (data not shown). The E value of 16 that we consistently observed in the ammonolysis of 1a is rather low for an efficient kinetic resolution. We would like to note, however, that in an ideal dynamic kinetic resolution this would result in a satisfactory product ee of 88%.

Racemisation via Schiff base intermediates

D-methyl ester 1a and D-amide 2 were subjected to pyridoxal or salicylaldehyde catalyzed racemisation under ammonolysis conditions (tert-butyl alcohol, 2.5 M NH₃). In all cases the ee decreased to 0% according to first order kinetics. The initial racemisation rate obeyed Michaelis-Menten kinetics according to:

\[
\nu = \frac{k_{rac} [D-PGX]}{K_{ds} + [D-PGX]} [rac \text{ cat}]
\]  

We conclude that the reaction takes place via a substrate binding step with dissociation constant \( K_{ds} \) and catalytic rate constant \( k_{cat} \) (Figure 4). The kinetic parameters were determined from Lineweaver-Burk plots (Table 2). It should be noted that the measured racemisation rate constant is half the kinetic rate constant \( k_{cat} \).

c. In an ideal dynamic kinetic resolution the racemisation of the reactant is fast compared with its transformation into product, whereas the product racemisation is negligible.
**Figure 4.** Racemisation via Schiff base intermediates.

**Ammonolysis with in situ racemisation**

We next combined ammonolysis and racemisation of 1a in one reaction. From the time-course and ee of reactant and product (Figure 1) we conclude that L-1a is efficiently racemised by pyridoxal. Its ee at 50% conversion is just over 40%, compared with 70% in the absence of racemisation catalyst.
Chapter 3

The racemisation catalyst also accelerated the reaction from 50% to 60% conversion in 4h, because it counteracts the depletion of fast-reacting D-1a. On the other hand, the downward trend of the ee of 2 in the course of the reaction shows that pyridoxal-catalyzed racemisation of 2 is quite significant.

**Table 2. Kinetic data of the racemisation of 1a and 2**

<table>
<thead>
<tr>
<th>Racemisation catalyst</th>
<th>X</th>
<th>$K_{\text{dis}}$ (mM)</th>
<th>$k_{\text{cat}}$ (10^{-3} s^{-1})</th>
<th>$V_{\text{max}}$ (μM·s^{-1})</th>
<th>$k_{\text{cat}}/K_{\text{dis}}$ (M^{-1}·s^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pyridoxal</td>
<td>OCH$_3$</td>
<td>180</td>
<td>20.8</td>
<td>13.5</td>
<td>0.116</td>
</tr>
<tr>
<td></td>
<td>NH$_2$</td>
<td>45</td>
<td>0.74</td>
<td>0.48</td>
<td>0.016</td>
</tr>
<tr>
<td>Salicylaldehyde</td>
<td>OCH$_3$</td>
<td>44</td>
<td>7.56</td>
<td>5.36</td>
<td>0.172</td>
</tr>
<tr>
<td></td>
<td>NH$_2$</td>
<td>28</td>
<td>0.25</td>
<td>0.18</td>
<td>0.009</td>
</tr>
</tbody>
</table>

a. Conditions: variable concentrations of 1a or 2 in ammonia saturated tert-butyl alcohol (approx. 2.5 M), containing 1.3 mM pyridoxal or salicylaldehyde at 40 °C.

**Figure 5.** Composition and ee of ester 1a and amide 2 as function of time (□) ee ester 1a, (■), amount of ester 1a, (○) ee amide 2, (●) amount of amide 2. Reaction conditions: D,L-phenylglycine methyl ester 1a (200 mM) was shaken with Novozym 435 (50 mg) and pyridoxal (2 mM) in ammonia-saturated tert-butyl alcohol (5 ml, 12.5 mmol) at 40 °C.

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In order to establish optimal conditions for ammonolysis with *in situ* racemisation, we varied the concentration of pyridoxal between 40 μM and 2 mM. Similar experiments were performed with salicylaldehyde (1 to 4 mM) as racemisation catalyst. The results have been compiled in Table 3, but no clear optimum as regards concentration and ee is apparent. Racemisation catalyst concentrations over 1 mM mainly affect the conversion rate. Pyridoxal and salicylaldehyde at 1 and 2 mM perform similarly as regards ee, but in the case of pyridoxal a higher conversion is reached.

**Table 3. Ammonolysis with in situ racemisation**

<table>
<thead>
<tr>
<th>Racemisation catalyst (mM)</th>
<th>Pyridoxal Conversion (%)</th>
<th>Pyridoxal ee&lt;sub&gt;p&lt;/sub&gt; (%)</th>
<th>Salicylaldehyde Conversion (%)</th>
<th>Salicylaldehyde ee&lt;sub&gt;p&lt;/sub&gt; (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.0</td>
<td>91.2</td>
<td>53.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>90.0</td>
<td>55.6</td>
<td>86.3</td>
<td>55.9</td>
</tr>
<tr>
<td>1.0</td>
<td>86.8</td>
<td>60.7</td>
<td>78.5</td>
<td>60.9</td>
</tr>
<tr>
<td>0.4</td>
<td>81.5</td>
<td>57.1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2</td>
<td>75.1</td>
<td>50.5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.04</td>
<td>62.1</td>
<td>54.7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.0&lt;sup&gt;b&lt;/sup&gt;</td>
<td>83.1</td>
<td>60.6</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

a. Reaction conditions: D,L-phenylglycine methyl ester-HCl 1a (1 mmol, 0.2 M) was shaken with Novozym 435 (50 mg) and pyridoxal or salicylaldehyde in ammonia saturated *tert*-butyl alcohol (5 ml, 12.5 mmol) at 40 °C for 17 hours.

b. D,L-phenylglycine methyl ester-HCl 1a (4 mmol, 0.8 M) was shaken with Novozym 435 (200 mg) and pyridoxal (0.02 mmol, 4 mM) in ammonia saturated *tert*-butyl alcohol (5 ml, 12.5 mmol) at 40 °C for 17 hours.

55
In order to gain more insight into the effect of the racemisation catalysts, reactions were followed in time and the ee values were plotted against the conversion (Figures 6a and 6b). Up to 50% conversion, the results appeared to be independent of the concentration of the racemisation catalyst. Even when no racemisation catalyst was added, the same result was obtained. For conversions higher than 50% we observed that at low pyridoxal concentrations (40 to 200 μM, see Fig. 2) the racemisation of 1a was too slow to be effective.

![Graph A](image)

![Graph B](image)

**Figure 6.** Enantiomeric excess of amide 2 as a function of the pyridoxal (6A) and salicylaldehyde (6B) concentration. 6a: (Δ) 2, (O) 1, (□) 0.4 (●) 0.2 (■) 0.04 mM pyridoxal; 6b: (O) 4, (□) 2, (Δ) 1 mM salicylaldehyde.

However, at increased pyridoxal concentrations (400 μM and above) it seems that the concentration of pyridoxal has only a small effect on ee vs conversion. For salicylaldehyde we observed a very similar pattern (Figure 6b). It would seem that, once the racemisation catalyst concentration exceeds a critical limit, higher concentrations effect 1a and 2 equally. Consequently, the reaction proceeds faster due to faster racemisation of L-1a, but the racemisation of D-2 is also accelerated. Because 2 would be expected to precipitate and hence to be effectively withdrawn from the reaction mixture, we would expect that high concentrations of 1a would
accelerate its racemisation relative to 2. However, when we increased the concentration of 1a and pyridoxal four-fold (to 0.8 M and 4 mM, respectively) no effect on ee vs concentration became apparent (Table 3). This is probably due to the lower E value at high concentrations of 1a (Figure 3). Rapid exchange of precipitated D-2 with product in solution may also contribute.

**Reactions with free phenylglycine methyl ester**

To get better insights in the effects of precipitation and high substrate concentrations, a number of experiments were performed with the free ester of 1a that was prepared by careful distillation of a basic mixture. In this way the formation of large amounts of ammonium chloride in more concentrated reaction mixtures could be avoided and the precipitation in the now clear reaction mixture would be visible. The use of the free methyl ester gave very similar results in terms of reaction rate and enantiomeric ratio to the reaction with the hydrochloric salt. No significant new information about the reaction was obtained but the results were somewhat more accurate because the reaction mixture was homogeneous, which made sampling easier.

**Experiments with other solvents**

A number of other solvents were briefly investigated. Most solvents are either not compatible with Novozym 435 (e.g. methanol) or do not dissolve ammonia very well. For a smooth reaction, constant bubbling of ammonia through the reaction mixture was necessary in all cases. In most solvents it proved to be rather difficult to suppress hydrolysis as a side reaction, probably because the wateractivity was higher in these solvents than in tert-butyl alcohol. The reaction rate in tert-amy1 alcohol was slightly higher than in tert-butyl alcohol. In dimethoxyethane, acetonitrile, dioxane and tert-
butyl methylether rates were lower but in the case of dioxane and tert-butyl methyl ether there was an increase in selectivity. The influence of different solvents on this reaction is still under investigation.

**EXPERIMENTAL**

**Materials**

Immobilized *Candida antarctica* lipase B (Novozym 435) was a gift from Novo Nordisk A/S, ( Bagsværd, Denmark). Salicylaldehyde was purchased from Janssen Chimica, pyridoxal hydrochloride from Aldrich. Racemic phenylglycine was obtained from Acros. Enantiomerically pure phenylglycine, phenylglycine methyl ester and phenylglycine amide were kindly donated by DSM (Geleen, The Netherlands). Solvents were dried on Zeolite CaA (Uetikon, activated at 400 °C for 24 h before use). The methyl, ethyl and butyl esters of phenylglycine were synthesized from the amino acid according to the literature 20.

**Isolation of free phenylglycine methyl ester**

D,L-Phenylglycine methyl ester hydrochloride (4.0 g, 20 mmol) was dissolved in 15 ml dry methanol in the distillation flask of a kugl-rohr. Sodium methoxide (19.9 mmol; 30% solution in methanol) was added and the mixture was carefully distilled under vacuum. The free ester distilled at 100 °C (1 mbar) as a colorless liquid that solidified in the cooling flask. 2.78 g of a low melting white solid was obtained (85%). The free ester was rather stable when kept under cool and dry conditions. Attempts to isolate the free D-ester were unsuccessful, as it racemised significantly, even at lower pressure and lower distillation temperature.
Analysis and equipment

The reaction mixtures of phenylglycine methyl ester were analyzed by chiral HPLC on a Daicel Chemical Industries Ltd. 4.6 × 150 mm 5 μm Crownpak CR (+) column using a Waters 625 LC pump and a Waters 486 UV detector. The eluent was aqueous HClO₄, pH 1.5 at a flow of 0.6 ml/min, the column temperature was 18 °C. The reaction mixtures of the ethyl and butyl esters of phenylglycine were analyzed by chiral HPLC on a 4.6 × 250 mm 10 μm Chiralcel OD column with a Waters 510 pump, and a Shimazu SPD-6a UV detector. Hexane/isopropyl alcohol/diethylamine (90/10/0.1, v/v) at 0.5 ml/min was used as eluent. ¹H and ¹³C NMR spectra were recorded using a 400 MHz Varian-VXR 400S spectrometer.

Ammonolysis of phenylglycine esters

A mixture of 1.0 mmol phenylglycine ester hydrochloride (201 mg methyl ester 1a, 216 mg ethyl ester 1b or 232 mg butyl ester 1c) and 50 mg Novozym 435 in 5.0 ml ammonia saturated tert-butyl alcohol (2.5 M) were shaken in 40 ml reaction vessels at 40 °C. Reactions were monitored in time. The reactions were stopped by adding 0.6 ml concentrated formic acid and the reaction mixture was dissolved by adding water. A sample was taken for HPLC analysis.

Racemisation of phenylglycine methyl ester and phenylglycine amide

All kinetic measurements were made in 40 ml reaction vessels at 40 °C; ammonia saturated tert-butyl alcohol was used as solvent. When pyridoxal was used as racemisation catalyst the concentration of 1a was varied between 50 and 400 mM, with salicylaldehyde between 30 and 300 mM. The concentration of phenylglycine
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amide 2 was in both cases varied between 40 and 160 mM. The concentration of pyridoxal as well as salicylaldehyde was kept constant at 1.3 mM. Samples were periodically withdrawn and analyzed by HPLC. The kinetic constants were determined by fitting the experimental data in a Lineweaver-Burk plot\textsuperscript{21}.

**Ammonolysis with *in situ* racemisation**

1.0 mmol 1a, racemisation catalyst and 50 mg Novozym 435 in 5.0 ml tert-butyl alcohol saturated with ammonia (2.5 M) were shaken in 40 ml reaction vessels at 40 °C. The reactions were carried out under 1 atmosphere ammonia by bubbling dry ammonia gas through the reaction mixtures. The concentration of the racemisation catalyst was varied between 0.04 and 4 mM for pyridoxal and between 1 and 4 mM for salicylaldehyde. The reactions were followed in time. The reactions were stopped by adding 0.6 ml formic acid and the reaction mixture was dissolved by adding water. A sample was taken for HPLC analysis.

**CONCLUSIONS**

Pyridoxal or salicylaldehyde catalyzed racemisation of D-phenylglycine methyl ester 1a via a Schiff base intermediate is compatible with lipase catalyzed enantioselective ammonolysis. D-Phenylglycine amide 2 was obtained with 61\% ee at 87\% conversion.

**REFERENCES**

Dynamic kinetic resolution of phenylglycine methylester


Chapter 4

Lipase and esterase catalyzed acylation of hydroxylamine

Summary

A variety of lipases and esterases were shown to catalyze the acylation of hydroxylamine with octanoic acid or ethyl octanoate. *Candida antarctica* lipase B gave superior results and the influence of solvent on the initial reaction rate was investigated for this enzyme. Initial rates were highest in water but the optimum overall productivity was observed in dioxane. Octanoic acid (250 g/l) was converted for 93% into the hydroxamic acid in 36 hours with only 1% (w/w) *Candida antarctica* lipase B (Novozym 435) in dioxane at 40 °C. This translates to a catalyst productivity of 68.5 g·g⁻¹·d⁻¹ and a space time yield of 149 g·l⁻¹·d⁻¹, unprecedented figures for the enzyme catalyzed reaction of an acid with a nitrogen nucleophile in an organic solvent.

Enantioselectivity was observed when either the acyl group or the alkoxy moiety of the ester were chiral. For example, hydroxylaminolysis of 1-phenylethyl butanoate gave an *E* value > 100. Racemic ibuprofen afforded the (*R*) or the (*S*) enantiomer of the corresponding hydroxamic acid depending on the enzyme used. Cross-linked enzyme crystals of *Candida rugosa* lipase (CLEC-CR) in aqueous medium preferentially converted the biologically active *S*-enantiomer with an *E* value of 40; the hydroxamic acid product precipitated from the reaction mixture.
INTRODUCTION

It is now well established that in organic media lipases (EC 3.1.1.3) readily catalyze a variety of acylation reactions which they do not perform in nature\(^1\). For example, carbohydrates\(^2\), alkylamines\(^3,4\), ammonia\(^5,6,7,8,9,10\) and hydrogen peroxide\(^11,12\) have been successfully used as acyl acceptors.

In contrast, hydroxylamine has scarcely been investigated as a nucleophile in enzymatic reactions. The synthesis of hydroxamic acids (N-acyl hydroxylamines) by lipase-catalyzed hydroxylaminolysis of triglycerides and fatty acids in water was recently reported\(^13,14\), but the scope of the reaction was not further examined\(^15\).

An enzymatic route for the synthesis of hydroxamic acids with the inherent advantages of selectivity, mild reaction conditions and enantioselectivity, is of synthetic interest. First, the hydroxamic acid moiety has a low toxicity and is widely under investigation for use in new pharmaceuticals\(^16,17\) and highly selective complexing agents for metal ions\(^18\). Ibuproxam, the hydroxamic acid derived from ibuprofen, for example, is a pro-drug\(^19\) which made (S)-ibuproxam an interesting target compound. Furthermore, hydroxamic acids can be chemically converted to amines and derivatives thereof via the Lossen\(^20\) rearrangement. This rearrangement is closely related to the Hofmann reaction. Because the key step in these reactions - formation and rearrangement of an acylminate intermediate - takes place with retention of the configuration at the α-carbon atom, an enantioselective route to hydroxamic acids would also give access to the corresponding enantiomerically pure amines.

\[
\begin{align*}
\text{Ac}_2\text{O} & \quad \text{pH 9} & \quad \text{H}_2\text{O} \\
\text{R}^1\text{N=C=O} & \quad \text{R}^1\text{N=C=O} & \quad \text{R}^1\text{N=C=O}
\end{align*}
\]

**Figure 1.** The Lossen rearrangement. \(\text{R}^1, \text{R}^2, \text{R}^3 = \text{H}, \text{alkyl}, \text{aryl}\).
In this Chapter we report the scope of the lipase and esterase catalyzed acylation of hydroxylamine using ethyl octanoate and octanoic acid as model compounds, as well as the lipase-catalyzed enantioselective synthesis of (S)-ibuprofam and enantioselective hydroxylaminolysis of 1-phenylethyl butyrate.

RESULTS AND DISCUSSION

Hydroxylaminolysis of ethyl octanoate

The initial experiments were performed with ethyl octanoate and hydroxylamine in tert-butyl alcohol, which was a good solvent for ester ammonolysis. The lipases that had previously shown activity in the ammonolysis reaction were also active in the hydroxylaminolysis reaction provided that a small amount of water was present. In nearly anhydrous medium (using NH₂OH·HCl and triethylamine, data not shown) there was no product formation at all with any of the enzymes, only a small amount of hydrolysis. In ammonolysis, the corresponding mixture of NH₄Cl and triethylamine works well for a number of enzymes. The inability to acylate hydroxylamine in the absence of water is rather surprising because Novozym 435 is known to be active at very low water activity, also in the presence of nitrogen nucleophiles. The initial reaction rate, using Novozym 435 and 0.5 M hydroxylamine, was highest at 1.75 M water (data not shown). In the case of the reactions with NH₂OH·HCl and triethylamine the optimum water concentration was even higher, about 2.5 M. We attribute the water requirement to the instability of hydroxylamine in the absence of water or acid. Disintegration to a variety of products - N₂, NH₃, H₂O, HNO₂ and N₂O - is known to take place under such conditions. We compared the activity and yield with a number of immobilized lipases in the hydroxylaminolysis of ethyl octanoate (Table 1). Especially Candida antarctica lipase
B, lipoprotein lipase from *Pseudomonas* and Novo SP 523 from *Thermomyces lanuginosus* rapidly transformed the starting compound into octanohydroxamic acid.

Table 1. Lipase catalyzed hydroxylaminolysis of ethyl octanoate.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Initial rate(^b) (µmol·g(^{-1})·min(^{-1}))</th>
<th>Yield(^d) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.92 M H(_2)O(^c)</td>
<td>1.75 M H(_2)O</td>
</tr>
<tr>
<td><em>C. antarctica</em> B</td>
<td>1110</td>
<td>1270</td>
</tr>
<tr>
<td><em>C. antarctica</em> B (Novozym 435)(^e)</td>
<td>910</td>
<td>980</td>
</tr>
<tr>
<td><em>Pseudomonas alcaligenes</em></td>
<td>120</td>
<td>170</td>
</tr>
<tr>
<td><em>Thermomyces lanuginosus</em> (SP 523)</td>
<td>420</td>
<td>630</td>
</tr>
<tr>
<td><em>Pseudomonas</em> lipoprotein</td>
<td>180</td>
<td>320</td>
</tr>
<tr>
<td><em>Rhizomucor miehei</em> (Lipozym IM 20)(^f)</td>
<td>n.d.</td>
<td>50</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 688 mg ethyl octanoate, 0.5 M NH\(_2\)OH, 10 mg lipase (on Accurel EP 100 unless indicated otherwise) in 10 ml tert-butyl alcohol at 40 °C.

b. Initial rates were actually maximum rates because there was a small lag phase. This was caused by the total absence of acid in the beginning of the reaction which causes some disintegration of the hydroxylamine. In a control experiment, to exclude warming and wetting effects, we added 5% of octanoic acid at the start of the reaction. The lag phase disappeared almost completely. This problem did not occur in the other experiments. The amount of catalyst includes the carrier.

c. This water concentration results from the use of a commercial 50% (17.0 M) hydroxylamine solution in water.

d. Reaction conditions: 5 ml 0.5 M NH\(_2\)OH with 1.75 M of water in tert-butyl alcohol, 344 mg ethyl octanoate and 50 mg immobilized lipase, 24 h at 40 °C.

e. Commercial immobilized enzyme (Novo Nordisk) on Lewatit E.

f. Commercial immobilized enzyme (Novo Nordisk) on anion exchange resin (Duolite A568).
Due to the presence of water some competing hydrolysis took place. 10 to 15% octanoic acid accumulated in the course of the reaction but this was subsequently transformed into the hydroxamic acid. Apparently, there are two pathways leading to the hydroxamic acid (Figure 2) as would be expected on the basis of the report by Servat\textsuperscript{13}.

\textbf{Figure 2.} Different pathways leading to the formation of hydroxamic acid.

\textbf{Acylation of hydroxylamine with octanoic acid}

The further conversion of initially formed octanoic acid in the reaction of ethyl octanoate with hydroxylamine suggested that a direct lipase catalyzed condensation of carboxylic acid and hydroxylamine could be a more attractive option. Accordingly, octanoic acid was completely converted into its hydroxamic acid, even in the presence of water. This was quite unexpected. For example, the acylation of ammonia with a fatty acid in water is not feasible because the carboxylic acid and ammonia form an ion pair and lipases cannot convert a deprotonated acid nor a protonated nucleophile.
Table 2. Basicity of some nitrogen nucleophiles.

<table>
<thead>
<tr>
<th>nucleophile</th>
<th>pKₐ of protonated form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia</td>
<td>9.3</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>6.0</td>
</tr>
<tr>
<td>Pyridine</td>
<td>5.2</td>
</tr>
</tbody>
</table>

An interesting property of hydroxylamines is that due to the α-effect, they are much stronger nucleophiles than one would expect from their basicity (Table 2).

Figure 3. Destabilisation of the ground state of hydroxylamine.

The ground state of the nucleophile is destabilized by repulsion between the adjacent pairs of electrons. The highest molecular orbital resulting from this destabilisation is the HOMO that will donate an electron for bonding. Another factor is the stabilization of the transition state by the extra pair of electrons. Due to the electron withdrawing effect of the oxygen, the electron density of the lone pair (and therefore the basicity) at the nitrogen atom in hydroxylamine is lower than in ammonia. The pKₐ of hydroxylamine of 6.0 is so close to that of octanoic acid (pKₐ=4.9) that
significant amounts of unprotonated hydroxylamine and octanoic acid are expected to be present at e.g. pH 5.5. So reaction can take place, even between the free acid and the free base in water.

The initial rates were reduced by almost an order of magnitude compared to the ethyl ester (Table 3). This is not surprising, considering that carboxylic acids are less reactive acyl donors than esters; moreover, the acid is partially inactivated by deprotonation. Some water is required for the reaction to proceed, but its concentration is not as critical as with ethyl octanoate.

Table 3. Lipase catalyzed acylation of hydroxylamine with octanoic acid.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Initial rate (^{a}) (µmol·g(^{-1})·min(^{-1}))</th>
<th>Yield (^{b}) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida antarctica</em> B (SP 525)</td>
<td>140</td>
<td>95</td>
</tr>
<tr>
<td><em>Candida antarctica</em> B (Novozym 435)(^{c})</td>
<td>160</td>
<td>89</td>
</tr>
<tr>
<td><em>Pseudomonas alcaligenes</em></td>
<td>16</td>
<td>67</td>
</tr>
<tr>
<td><em>Thermomyces lanuginosus</em> (SP523)</td>
<td>130</td>
<td>95</td>
</tr>
<tr>
<td><em>Pseudomonas lipoprotein</em></td>
<td>39</td>
<td>79</td>
</tr>
<tr>
<td><em>Rhizomucor miehei</em> (Lipozym IM 20)(^{d})</td>
<td>39</td>
<td>61</td>
</tr>
</tbody>
</table>

---

\(^{a}\) Reaction conditions: 576 mg octanoic acid (4 mmol), 0.5 M NH\(_2\)OH, 0.92 M water, 10 mg catalyst (enzyme on Accurel EP 100 unless indicated otherwise) in 10 ml tert-butyl alcohol at 40 °C.

\(^{b}\) Reaction conditions: 288 mg octanoic acid (2 mmol), 0.5 M NH\(_2\)OH, 0.92 M water, 50 mg catalyst (enzyme on Accurel EP 100 unless indicated otherwise) in 5 ml tert-butyl alcohol at 40 °C for 24 h.

\(^{c}\) Commercial immobilized enzyme (Novo Nordisk) on Lewatit E.

\(^{d}\) Commercial immobilized enzyme (Novo Nordisk) on anion exchange resin (Duolite A568).
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Although tert-butyl alcohol performs well as a solvent in a number of lipase catalyzed reactions, it suffers from the disadvantage of partially deactivating the catalyst\textsuperscript{23,24,25}. Indeed the use of aprotic solvents accelerated the reaction of octanoic acid and hydroxylamine (Table 4). The highest initial rates were observed with the high log P solvents hexane and toluene. However, reaction rates decreased rapidly due to deactivation of the catalyst, although high conversions were eventually achieved. Dioxane proved to be the best solvent for this reaction: in a preparative experiment a catalyst productivity of 68.5 g·g\textsuperscript{-1}·d\textsuperscript{-1} and a space time yield of 149 g·l\textsuperscript{-1}·d\textsuperscript{-1} were achieved with only 1% of catalyst, which are unprecedented figures in the direct coupling of an acid with a nitrogen nucleophile in an organic solvent.

Table 4. Initial reaction rate for the acylation of hydroxylamine with octanoic acid in different solvents.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial rate \textsuperscript{a} (μmol·g\textsuperscript{-1}·min\textsuperscript{-1})</th>
<th>Yield \textsuperscript{b} (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexane</td>
<td>1400</td>
<td>83</td>
</tr>
<tr>
<td>Toluene</td>
<td>1680</td>
<td>84</td>
</tr>
<tr>
<td>tert-Butyl methyl ether</td>
<td>470</td>
<td>87</td>
</tr>
<tr>
<td>Tetrahydrofuran</td>
<td>110</td>
<td>80</td>
</tr>
<tr>
<td>1,2-Dimethoxyethane</td>
<td>260</td>
<td>89</td>
</tr>
<tr>
<td>Dioxane</td>
<td>450</td>
<td>91</td>
</tr>
<tr>
<td>tert-Amyl alcohol</td>
<td>140</td>
<td>95</td>
</tr>
<tr>
<td>tert-Butyl alcohol</td>
<td>160</td>
<td>95</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Reaction conditions: 576 mg octanoic acid (4 mmol), 0.5 M NH\textsubscript{2}OH, 0.92 M water and 10 mg Novozym 435 in 10 ml solvent at 40 °C.

\textsuperscript{b} Reaction conditions: 288 mg octanoic acid (2 mmol), 0.5 M NH\textsubscript{2}OH, 0.92 M water and 50 mg Novozym 435 in 5 ml solvent at 40 °C for 24 h.
The deactivation of the catalyst mentioned above seems to be caused by the low solubility of water and hydroxylamine in high log P media. In consequence, hydroxylamine is mainly present as a concentrated aqueous phase, as is the case with hexane and toluene. In the case of tert-butyl methyl ether the reaction mixture was homogeneous in the beginning but separated into two phases after some time. The concentration of hydroxylamine in the water layer is very high, probably over 10 M. This combined with the fact that water is a very polar solvent that promotes the undesired hydroxylaminolysis of peptide linkages in the enzyme, leads to rapid enzyme deactivation.

As mentioned above, high yields of octanohydroxamic acid could be achieved in high log P media with a high thermodynamic activity of water. From this observation, combined with the report by Servat\(^\text{13}\) we tentatively conclude that hydroxamic acids are hydrolytically stable under the reaction conditions. Hydroxamic acids are thermodynamically more stable than normal amides; in fact hydroxylamine liberates ammonia from amides\(^\text{26}\). Hence, it should be possible to perform the hydroxamidation of octanoic acid in aqueous solution which would also open the opportunity to use enzymes that generally do not perform well in systems with low water activity and obviate the necessity for immobilization. Accordingly, octanoic acid was smoothly converted into octanohydroxamic acid in the presence of a number of lipases and esterases. The best enzyme in terms of productivity per unit of hydrolytic activity is SP 525 (\textit{C. antarctica} lipase B), which is also very stable under the reaction conditions. Most active were SP 523 and SP 524 (from \textit{Th. lanuginosus} and \textit{Rh. miehei}, respectively); CLEC-CR and -PC also gave good results (see Table 5 for further data). For SP 525 and \textit{P. alcaligenes} lipase the amounts of enzyme adsorbed on the carrier are known, which makes it possible to compare the initial rates in tert-butyl alcohol and water. Per g of lyophilisate, SP 525 is 4.4 times more active in water than in tert-butyl alcohol, for \textit{P. alcaligenes} lipase this factor amounts to 2.6. These ratios are much lower than those usually found for enzyme activity in water \textit{vs.} organic media.
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This relatively low activity is caused by the abundance of water in the system. Thus, the rate of hydroxamic acid production is decreased due to the competing non-productive attack of water (more than 100 equivalents per equivalent of hydroxylamine) on the acyl enzyme intermediate.

**Table 5. Acylation of hydroxylamine with octanoic acid in water catalyzed by lipases and esterases.**

<table>
<thead>
<tr>
<th>Lipase source</th>
<th>Initial rate (µmol·g⁻¹·min⁻¹)</th>
<th>Enzyme (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Th. lanuginosus</em> (SP 523)</td>
<td>10.4·10³</td>
<td>1.8</td>
<td>79</td>
</tr>
<tr>
<td><em>Rh. miehei</em> (SP 524)</td>
<td>6350</td>
<td>1.4</td>
<td>70</td>
</tr>
<tr>
<td><em>C. antarctica</em> B (SP 525)</td>
<td>4780</td>
<td>1.7</td>
<td>66</td>
</tr>
<tr>
<td><em>C. antarctica</em> A (SP 526)</td>
<td>15</td>
<td>50.0</td>
<td>18</td>
</tr>
<tr>
<td><em>Ps. lipoprotein</em></td>
<td>550</td>
<td>3.0</td>
<td>15</td>
</tr>
<tr>
<td><em>P. alcaligenes</em></td>
<td>350</td>
<td>11.6</td>
<td>23</td>
</tr>
<tr>
<td><em>P. cepacia</em> (CLEC-PC)</td>
<td>530</td>
<td>10</td>
<td>58</td>
</tr>
<tr>
<td><em>C. rugosa</em> (CLEC-CR)</td>
<td>1200</td>
<td>10</td>
<td>54</td>
</tr>
</tbody>
</table>

**Esterase source**

<table>
<thead>
<tr>
<th>Esterase source</th>
<th>Initial rate (µmol·g⁻¹·min⁻¹)</th>
<th>Enzyme (mg)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. rugosa</em> cholesterol esterase</td>
<td>270</td>
<td>20</td>
<td>38</td>
</tr>
<tr>
<td><em>M. miehei</em> esterase</td>
<td>100</td>
<td>20</td>
<td>18</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 576 mg (4 mmol) octanoic acid and 0.5 M NH₂OH, in 10 ml water at 40 °C.
b. Reaction time 19 h.
c. Addition of 10 mM CaCl₂ gave 37% conversion in 19 hrs. For the other reactions there was no significant difference.
Some enzymes, particularly *Pseudomonas alcaligenes* lipase and *Pseudomonas* lipoprotein lipase, which performed well in organic media, did not maintain their initial reaction rate in aqueous medium and seem to be deactivated. In the case of *Pseudomonas alcaligenes* lipase this could be remedied by addition of 10 mM Ca$^{2+}$ which stabilized the enzyme and allowed the reaction to proceed.

Several enzymes were tested but showed no activity in this reaction; they are listed in Table 9 in the experimental part. In a somewhat similar manner, the *Candida rugosa* cholesterol esterase catalyzed reaction started rather well but soon came to a standstill. In this context we note that, analogous to proteases$^{27}$, lipases probably suffer from competitive inhibition by hydroxamic acids, which effect would be less in an organic medium because of the better solubility and therefore lower activity of hydroxamic acids.

A number of other lipases and esterases were inactive in the acylation in water (Table 9). In the case of *C. rugosa* lipase this is due to instability because, in contrast, its cross-linked crystals (CLEC-CR) showed activity.

The low solubility of the product in water causes practical problems. At about 50% conversion the reaction mixture formed gels and the rates decreased. Above 70% conversion the reaction mixtures solidified and the reactions came to a standstill.

It is noteworthy that the condensation of octanoic acid with hydroxylamine takes place so readily. Only a single example of a lipase catalyzed condensation of a carboxylic acid and an alkylamine has been reported previously$^{28}$. As noted earlier, this can be rationalized on the basis of the fact that the pK$_a$ of hydroxylamine (6.0) is, compared to that of ammonia (pK$_a$ 9.3), much closer to the pK$_a$ of octanoic acid (4.9). Hence, reaction can take place, even between the free acid and the free base in water.

The question arises whether, analogous to ammonolysis$^1$ a protease would perform hydroxylaminolysis. An attempt was made to acylate hydroxylamine with $N$-acetyl-L-phenylalanine in the presence of SP 539 (an alkaline endoprotease from Novo Nordisk) in water, but the conversion remained very low (4% in 48 h). This
disappointing result is ascribed to the well documented inhibition of proteases by hydroxamic acids\textsuperscript{27}.

We also investigated the direct acylation of hydroxylamine with amino acids but without success. For example, no reaction was observed between phenyl glycine and hydroxylamine in the presence of \textit{Candida antarctica} lipase B in water for prolonged time. From other experiments it was known (Chapter 3 of this thesis) that phenyl glycine derivatives are substrates for this enzyme. It is possible that the thermodynamic equilibrium of this reaction is too far towards the hydrolysis side. Experiments with analogs of octanoic acid with a lower pK\textsubscript{a} value, such as 3-azaoctanoic acid (pK\textsubscript{a} 2.2), 3-oxaoctanoic acid (pK\textsubscript{a} 3.8) and 3-thiaoctanoic acid (pK\textsubscript{a} 3.3) and \textit{Candida antarctica} lipase B showed no formation of hydroxamic acid. It is possible that the low pK\textsubscript{a} value of these acids resulted in a low concentration of unprotonated acid in the reaction mixture or an unfavorable thermodynamic equilibrium.

\textbf{Enantioselective transformations}

Ibuprofen is an interesting reactant for enantioselective hydroxylamidation because its (S)-hydroxamic acid is a prodrug. Moreover, ibuprofen esters have developed into a standard substrate for enantioselective hydrolysis and we already had experience with the ammonolysis of ibuprofen esters\textsuperscript{10} and could compare the results of the two methods. By starting from the acid we saved an esterification step compared with other methods.

Only \textit{Candida antarctica} lipase B and \textit{Candida rugosa} lipase cross-linked crystals (CLEC-CR; freely dissolved enzyme was inactive) catalyzed the condensation of ibuprofen and hydroxylamine. \textit{Candida antarctica} lipase B converted the (R)-enantiomer, similar to hydrolysis, transesterification and ammonolysis of ibuprofen derivatives. The enantioselective ratio $E$ was somewhat lower than the value observed
in the ammonolysis of the 2-chloroethyl ester\textsuperscript{10}. The 2-chloroethyl ester was tested as a substrate but it gave a significant blank reaction with hydroxylamine in \textit{tert}-butyl alcohol, even at room temperature.

\begin{center}
\begin{displaymath}
\begin{array}{c}
\text{OH} \\
\downarrow \text{NH}_2\text{OH} \\
\text{CLEC-CR} \\
\end{array}
\end{displaymath}
\end{center}

\begin{center}
\begin{array}{c}
\text{(S)-ibuproxam} \\
\text{(R)-ibuprofen}
\end{array}
\end{center}

\textbf{Figure 2.} Enzymatic synthesis of (S)-ibuproxam from racemic ibuprofen.

\textit{Candida rugosa} lipase CLEC is known\textsuperscript{29} to convert the physiologically active (S)-enantiomer selectively in hydrolysis of ibuprofen esters. This was also the case in hydroxylaminolysis, although the enantiomeric ratio was lower than in the hydrolysis of ibuprofen esters with the same catalyst\textsuperscript{29}. However, during the workup the enantiomeric purity of ibuproxam was increased, probably by crystallization, and a yield of 33\% enantiomerically pure (S)-ibuproxam could be obtained.

Most of the enzymes that tolerate the conditions of this reaction do not accept ibuprofen derivatives as a substrate. \textit{Candida rugosa} cholesterol esterase gave traces of product with a small preference for the \textit{R}-enantiomer. Because the wrong enantiomer was formed, this reaction was not further optimized. In water both enantiomers of ibuproxam could be obtained depending on the enzyme (Table 6).

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Table 6. Enantioselective conversion of ibuprofen into ibuproxam.

<table>
<thead>
<tr>
<th>Lipase preparation</th>
<th>Conversion (%)</th>
<th>ee&lt;sub&gt;s&lt;/sub&gt; (%)</th>
<th>ee&lt;sub&gt;p&lt;/sub&gt; (%)</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. antarctica B (SP 525)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>39</td>
<td>49</td>
<td>78&lt;sup&gt;d&lt;/sup&gt;</td>
<td>13 (R)</td>
</tr>
<tr>
<td>C. rugosa CLEC-CR&lt;sup&gt;c&lt;/sup&gt;</td>
<td>41</td>
<td>64</td>
<td>92&lt;sup&gt;d&lt;/sup&gt;</td>
<td>40 (S)</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 103 mg ibuprofen (0.5 mmol), 2.5 mmol NH<sub>2</sub>OH and 25 mg enzyme or CLEC in 5 ml water.

b. 96 hours at 40 °C.

c. 96 hours at room temperature.

d. Calculated.

The commercial immobilized form of *Candida antarctica* lipase B, Novozym 435 was used for enantioselective transformations in organic solvents. Three different substrates were tested in tert-butyl alcohol at room temperature and at 40 °C. Ibuprofen and ibuprofen methyl ester reacted with similar selectivity but there was an order of a magnitude difference in initial reaction rate. At 40 °C the methyl ester gave 0.5% blank reaction but because of the modest enantioselectivity this could be neglected. The competing hydrolysis in this reaction was less selective than the hydroxylaminolysis. Lowering the reaction temperature from 40 °C to room temperature gave a four-fold decrease in reaction rate but no increase of selectivity. To prove that the reaction is also selective towards the alcohol part of an ester we performed the hydroxylaminolysis of 1-phenylethyl butyrate (Figure 4). The reaction was (R)-specific and turned out to be three times as fast as the ammonolysis of the same substrate with Novozym 435, with only one fifth of the nucleophile concentration, which attests to the disproportionally high nucleophilicity of the weakly basic hydroxylamine.
Figure 4. Hydroxylaminolysis of 1-phenylethyl butyrate

The hydroxylaminolysis of 1-phenylethyl butyrate was completely selective at room temperature and 40 °C, just like the ammonolysis and hydrolysis of this substrate with the same enzyme. The initial rate of the reaction was an order of a magnitude higher than the hydroxylaminolysis of ibuprofen methyl ester. The difference in initial rate between room temperature and 40 °C was a factor two in this case. Results are compiled in Table 7.

The hydroxylaminolysis of the ibuprofen methyl ester appears to be more selective at 40 °C than at room temperature. Alternatively, a similar mechanism as described in Figure 2 may be the reason for this apparent increase of selectivity. Ibuprofen methyl ester is hydrolyzed and the resulting (R)-ibuprofen will also react with hydroxylamine to form (R)-ibuproxam with a high optical purity, even though the rate of the second reaction is lower. In theory the enantiomeric ratios of the two consecutive reactions are multiplied for the part of the ibuproxam that is formed by this route. The initial synthesis hydrolysis ratio at 40 °C is 1.7 and at room temperature this is 3.0. Hence, at 40 °C a larger percentage of the (R)-ibuproxam will be formed via initial hydrolysis.

Table 7. Enantioselective transformations in tert-butyl alcohol.
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<table>
<thead>
<tr>
<th>Substrate</th>
<th>Temp.</th>
<th>Initial rate ( (\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}) )</th>
<th>( E )</th>
<th>Conv. ( (%) ), h</th>
<th>( ee_s )</th>
<th>( ee_p )</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image1.png" alt="Image" /></td>
<td>rt</td>
<td>0.11</td>
<td>8</td>
<td>17,240</td>
<td>15</td>
<td>75</td>
</tr>
<tr>
<td><img src="image2.png" alt="Image" /></td>
<td>40 °C</td>
<td>0.43</td>
<td>7</td>
<td>33,168</td>
<td>33</td>
<td>69</td>
</tr>
<tr>
<td><img src="image3.png" alt="Image" /></td>
<td>rt</td>
<td>1.1</td>
<td>8^b</td>
<td>42,72</td>
<td>47</td>
<td>71^c</td>
</tr>
<tr>
<td><img src="image4.png" alt="Image" /></td>
<td>40 °C</td>
<td>3.7</td>
<td>11^b</td>
<td>58,41</td>
<td>86</td>
<td>72^d</td>
</tr>
<tr>
<td><img src="image5.png" alt="Image" /></td>
<td>rt</td>
<td>8.2</td>
<td>&gt; 100</td>
<td>50,40</td>
<td>99^e</td>
<td>99</td>
</tr>
<tr>
<td><img src="image6.png" alt="Image" /></td>
<td>40 °C</td>
<td>16</td>
<td>&gt; 100</td>
<td>50,15</td>
<td>99^e</td>
<td>99</td>
</tr>
</tbody>
</table>

a. Conditions: 2 mmol substrate, 2.5 mmol hydroxylamine and 200 mg Novozym 435 in 5 ml tert-butyl alcohol. The configuration of all products in this table is \((R)\) and of all remaining substrates (S).

b. Calculated from ee of the ester.

c. Products: 31.4% \((R)\)-ibupropan (ee 71%), 10.2% \((R)\)-ibuprofen (ee 50%).

d. Products: 40.2% \((R)\)-ibupropan (ee 72%), 17.9% \((R)\)-ibuprofen (ee 38%).

The rate of coupling of hydroxylamine and ibupropan catalyzed by Novozym 435 can be enhanced by changing the solvent. Dioxane was slightly more selective than tert-butyl alcohol but gave no increase in reaction rate. In all the other solvents studied, enantiomeric ratios were lower than in tert-butyl alcohol (Table 8). The reaction is particularly fast in apolar solvents, with initial rates up to 15 times higher than in tert-butyl alcohol. There is a significant drawback however. Reactions in tert-butyl methyl ether, benzene and toluene start out as a homogeneous system but in the course of the reaction a biphasic system is formed. Isooctane is biphasic from the beginning.

When these reaction mixtures are diluted with isopropyl alcohol, to take a
homogeneous sample, precipitation occurs, indicating that the enzyme is leached from the carrier in contact with the aqueous phase.

In Table 8, Novozym 435 in water was used under the same conditions as in the organic media for comparison. The rate is somewhat higher but the selectivity is significantly lower than in the reaction with the free enzyme in Table 6. We did not investigate whether this was a pH or a carrier effect.

A number of the reactions in Table 8 were also performed at room temperature and at 40 °C with the double amount of solvent. In neither case an significant increase of selectivity was found but only a decrease of reaction rate.

**Table 8. Solvent effect on formation of (R)-ibuproxam**

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial rate (μmol·g⁻¹·min⁻¹)</th>
<th>Conversion (%)</th>
<th>eeₛ</th>
<th>eeₚ</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1.40</td>
<td>57, 96</td>
<td>55</td>
<td>41</td>
<td>4</td>
</tr>
<tr>
<td>Dioxane</td>
<td>0.42</td>
<td>32, 168</td>
<td>39</td>
<td>80</td>
<td>13</td>
</tr>
<tr>
<td>Dimethoxyethane</td>
<td>0.26</td>
<td>16, 96</td>
<td>11</td>
<td>59</td>
<td>4</td>
</tr>
<tr>
<td>tert-Amyl alcohol</td>
<td>0.68</td>
<td>35, 168</td>
<td>35</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>tert-Butyl methyl ether</td>
<td>1.98</td>
<td>22, 96</td>
<td>16</td>
<td>56</td>
<td>4</td>
</tr>
<tr>
<td>Benzene</td>
<td>2.50</td>
<td>47, 48</td>
<td>53</td>
<td>61</td>
<td>7</td>
</tr>
<tr>
<td>Toluene</td>
<td>2.85</td>
<td>48, 48</td>
<td>59</td>
<td>65</td>
<td>8</td>
</tr>
<tr>
<td>Isooctane</td>
<td>6.25</td>
<td>59, 48</td>
<td>76</td>
<td>52</td>
<td>7</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 412 mg ibuprofen (2 mmol), 2.5 mmol hydroxylamine and 200 mg 1,3-dimethoxybenzene in 5 ml solvent at 40 °C.

b. Novozym 435 was also used in this case but the catalyst is not designed for use in water. The enzyme is not covalently bonded and will bleed off the carrier. This leads to relatively high activity but the catalyst is destroyed.
It would seem that the absence of a larger alkyl group in the ester moiety exerts a negative effect on the enantioselectivity. The effect of decreasing enantioselectivity with decreasing ester chain length has also been found by others\textsuperscript{29}, and an acid can be viewed in steric terms as an ester with chain length zero. Margolin \textit{et al.} found a similar selectivity in the hydrolysis of the ibuprofen methyl ester with CLEC-CR as we found in the hydroxylamidation of ibuprofen but better results were obtained with the pentyl ester.

\textbf{CONCLUSIONS}

The acylation of hydroxylamine with acids or esters is catalyzed by a variety of lipases and esterases even in the presence of large amounts of water. The best results in terms of performance and stability were obtained with \textit{Candida antarctica} lipase B (Novozym 435).

The reaction is enantioselective with respect to both the acyl and the alkoxy moiety of an ester. Ibuprofen is enantioselectively converted into the biologically active (S)-enantiomer of ibuprofen by cross-linked crystals of \textit{Candida rugosa} lipase (CLEC-CR). The method is applicable to the synthesis of optically active hydroxamic acids as pharmaceuticals or chiral intermediates in organic synthesis. In combination with a subsequent Lossen rearrangement it constitutes a convenient route to optically active amines from chiral carboxylic acids.
MATERIALS AND METHODS

Materials

Hydroxylamine was purchased as a 50% aqueous solution (17 M) from Merck (Note: higher concentrations of the free base are unstable or explosive). All solvents were of analytical purity and were dried over activated Uetikon CaA zeolite prior to use. All other reagents were purchased from Aldrich or Acros and used as received.

Enzymes

The enzymes were used as delivered for the screening reactions in water. The suppliers and activities are given in Table 9. Enzymes were immobilized on Accurel EP 100 according to a published procedure\textsuperscript{31}. A slightly different procedure was followed in the immobilization of the lipases from \textit{C. antarctica} B and \textit{P. alcaligenes}. Accurel EP 100 (1 g) was pretreated as described by Petersen and Eigtved\textsuperscript{31} and added to a solution of \textit{C. antarctica} lipase B (Novo SP 525, 0.2 g 35 kLU) in 25 ml 0.01 M phosphate buffer pH 9 (1 LU will liberate 1 μmol of butyric acid from tributyrin per minute). After 20 h shaking at room temperature 8.3 kLU of lipase activity was still found in the supernatant. Hence, an amount of lipase equivalent to 0.15 g of SP 525 had been deposited on the catalyst, corresponding to a loading of 13% (w/w). The solid material was removed by filtration, washed with 30 ml buffer and dried \textit{in vacuo} for 16 h at 40 °C.

\textit{P. alcaligenes} lipase (0.3 g, 425 kLU) was dissolved in 25 ml water (pH 9 due to buffer salts present in the preparation). After removal of the solid material by centrifugation, 380 kLU of lipase activity was recovered in the supernatant. After addition of Accurel EP 100 (2 g) no residual lipase activity could be detected in the supernatant. Hence the preparation contains 12% (w/w) of lipase.
Attempts to assess the hydrolytic activity of the adsorbed lipases were unsuccessful, because hydrolysis of tributyrin was in part due to lipase that leached into the solution and not to adsorbed lipase\textsuperscript{32}. The PLU test\textsuperscript{33} (propyl laurate units) for immobilized enzymes in organic solvents was also tried but gave low activity for some enzymes that were active in the hydrolysis test, in particular \textit{Pseudomonas} lipoprotein lipase and \textit{Pseudomonas alcaligenes} lipase. We conclude that there is no suitable standard essay for measuring enzyme activity in organic solvent. Conditions that are suitable for one enzyme in terms of temperature, water activity and solvent are disastrous for another. The amount of adsorbed activity (units added minus units left in the supernatant after immobilization) or even the number of active sites on the carrier gives no information about the activity of the enzyme in its immobilized form, in some cases this can be zero. An enzyme essay in an organic solvent gives meaningful information only for different preparations of the same enzyme but is not suitable for comparing activities of different enzymes.

\textbf{Analysis and equipment}

The derivatives of octanoic acid and ibuprofen were analyzed by HPLC on a Waters 8 x 100 mm 4 μ Novapak C\textsubscript{18} reversed phase RCM column with a Waters 510 pump, a Shimadzu SPD-6A UV detector, a Shodex RI SE-61 RI detector and a Spectra-Physics SP 4270 integrator. The eluent was 65/35 methanol/water (v/v), with 0.05 M acetate buffer pH 4.3 at a flow of 1.0 ml/min.
Table 9. Used enzymes and their suppliers.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Activity^a</th>
<th>Supplier</th>
</tr>
</thead>
<tbody>
<tr>
<td>SP 523 (<em>Thermomyces lanug.</em> lipase)</td>
<td>4000</td>
<td>Novo Nordisk</td>
</tr>
<tr>
<td>SP 524 (<em>Rhizomucor miehei</em> lipase)</td>
<td>2590</td>
<td>Novo Nordisk</td>
</tr>
<tr>
<td>SP 525 (<em>Candida antarctica</em> lipase B)</td>
<td>147</td>
<td>Novo Nordisk</td>
</tr>
<tr>
<td>SP 526 (<em>Candida antarctica</em> lipase A)</td>
<td>28</td>
<td>Novo Nordisk</td>
</tr>
<tr>
<td>Porcine pancreas lipase^b</td>
<td>300</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td><em>Pseudomonas alcaligenes</em> lipase</td>
<td>unknown</td>
<td>Gist-brocades</td>
</tr>
<tr>
<td><em>Rhizopus arrhenius</em> lipase^b</td>
<td>3000</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td><em>Candida rugosa</em> lipase^b</td>
<td>690</td>
<td>Sigma</td>
</tr>
<tr>
<td><em>Candida lipolytica</em> lipase^b</td>
<td>0.91</td>
<td>Fluka</td>
</tr>
<tr>
<td>CLEC-PC (<em>Pseudomonas cepacia</em> lipase)</td>
<td>unknown</td>
<td>Altus</td>
</tr>
<tr>
<td>CLEC-CR (<em>Candida rugosa</em> lipase)</td>
<td>unknown</td>
<td>Altus</td>
</tr>
<tr>
<td><em>Pseudomonas</em> lipoprotein lipase</td>
<td>100</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td><em>Thermoanaerobium brockii</em> esterase^b</td>
<td>1.7</td>
<td>Fluka</td>
</tr>
<tr>
<td><em>Candida rugosa</em> cholesterol esterase</td>
<td>10.5</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td><em>Mucor miehei</em> esterase</td>
<td>2.2</td>
<td>Fluka</td>
</tr>
<tr>
<td><em>Candida rugosa</em> esterase^b</td>
<td>unknown</td>
<td>Altus</td>
</tr>
<tr>
<td><em>Bacillus stearothermophilus</em> esterase^b</td>
<td>unknown</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Pig liver esterase^b</td>
<td>260</td>
<td>Boehringer Mannheim</td>
</tr>
<tr>
<td>Lumafast (<em>Pseudomonas mendoza</em>)^b</td>
<td>unknown</td>
<td>Genencor</td>
</tr>
<tr>
<td>E-002 (experimental esterase)^b</td>
<td>1.0</td>
<td>Thermogen</td>
</tr>
<tr>
<td>E-005 (experimental esterase)^b</td>
<td>6.7</td>
<td>Thermogen</td>
</tr>
<tr>
<td>E-019 (experimental esterase)^b</td>
<td>0.9</td>
<td>Thermogen</td>
</tr>
</tbody>
</table>

^a. Activity: 1U will hydrolyze 1 μmol of substrate in 1 minute. The substrates and test conditions are not all equal.

^b. No activity as free enzyme in hydroxylaminolysis in water (Table 5).
Chapter 4

Chiral HPLC was performed on a Baker 4.6 x 250 mm 5 μ Chiracel OD column with a Waters 510 pump and a Waters 486 UV detector operating at 254 nm. As eluent for ibuprofen and 1-phenylethanol 98/2/0.1 hexane/isopropyl alcohol/formic acid was used at a flow rate of 0.5 ml/min. Ibuprofen methyl ester was analyzed on the same column with 100% hexane as eluent at a flow rate of 0.5 ml/min. Samples were filtrated over MgSO₄ to adsorb slow eluting compounds. Derivatized ibuproxam (the O-benzyl derivative, procedure later described) was also analyzed on the chiralcel OD column using 90/10 hexane/isopropyl alcohol as eluent at a flow rate of 0.5 ml per minute.

¹H and ¹³C NMR spectra were recorded on a Varian VXR-400S spectrometer.

Mass spectra were recorded on a VG 70 SE spectrometer with the EI method. Reactions were performed in 30 ml glass vessels with a teflon coated cap, in a stirred thermostated oil bath at 40°C. All reactions with octanoic acid and ethyl octanoate were performed with 0.4 M of substrate and 0.5 M of nucleophile in 10 ml solvent. Higher concentrations of hydroxylamine gave rise to a blank reaction with ethyl octanoate.

The enzyme and the solvent were stirred for one hour for thermal stabilization and wetting of the enzyme. Samples of 50 μL were taken at regular intervals and analyzed. The initial rate was calculated by fitting a line through the data points at < 5 % conversion.

Protease catalysis

N-acetyl-L-phenylalanine (0.5 mmol) was dissolved in 1 M aqueous NH₂OH (5 ml) and

---

a. Analysis of 1-phenylethanol with 90/10 hexane/isopropyl alcohol would have been less time consuming and gave good separation of (R)- and (S)-1-phenyl ethanol. However, the starting compound 1-phenethyl butyrate contained about 0.2% styrene, with very strong UV absorption, that could not be baseline separated from the starting material at 90/10.
SP 539 (10 mg) was added. According to HPLC 4% product had been formed after 48 h. Some byproduct that could be detected was probably due to aminolysis of the N-acetyl group.

Isolation of the products

For analytical purposes the octanohydroxamic acid was isolated from different reaction mixtures. After prolonged shaking of the reaction mixtures at 40 °C to reach full conversion, the solvent was evaporated in vacuo and the residue was recrystallized from petroleum ether (bp 40-60 °C).

Preparative-scale synthesis of octanohydroxamic acid

Octanoic acid (25 g, 174 mmol) was dissolved in dioxane (75 ml) together with 50% aqueous hydroxylamine (12.75 ml, 216 mmol). Novozym 435 (250 mg) was added and the mixture was shaken at 40 °C. The reaction mixture remained homogeneous throughout the reaction. After 36 h 93% octanohydroxamic acid had been formed according to HPLC. This translates to a catalyst productivity of 62.3 g·g⁻¹·d⁻¹ and a space time yield of 155 g·l⁻¹·d⁻¹. After addition of 10 ml of acetone to destroy excess hydroxylamine, the product was isolated by evaporation of the solvent, water and acetone oxime in vacuo. Recrystallisation from hexane afforded 22.6 g (82%) of pure octanohydroxamic acid.

Ibuproxam

(R,S)-Ibuprofen (103 mg, 0.5 mmol) and hydroxylamine (2.5 mmol) were dissolved in water (5 ml). Lipase (25 mg) was added and the reaction was monitored by reversed-phase HPLC. The enantiomeric purity of the unreacted ibuprofen was monitored by
chiral HPLC as described above. The hydroxamic acid precipitated from the reaction mixture as platelets. The pH of the suspension was adjusted to pH 7 with 0.1 M NaOH in a pH stat. The crystals were isolated by filtration, washed with a small amount of water and dissolved in acetone. After removal of the CLEC by filtration, the solvent was removed in vacuo to yield the 36.8 mg (33%) of the enantiomerically pure product. The enantiomeric ratio \( E \) was calculated from the conversion and the \( ee \) of the reactant, because separation of the product enantiomers was not observed on the Chiralcel OD and Chiralcel OB column. A rotation of \([\alpha]_D^{22} = +44.8^\circ \text{ c 0.30; Ethanol abs.}\) was found for the product. (lit.\(^{34}\) \([\alpha]_D^{23} = +44.4^\circ \text{ c 0.30; Ethanol abs.}\)) From this result we concluded that during the workup enrichment of (S)-ibuproxam occurred and only one enantiomer was obtained. An attempt to verify this by \(^1H\) NMR with several different chiral shift reagentia failed. Derivatisation of the isolated ibuproxam to the O-benzylated product showed a single peak on chiral HPLC.

The reactions in organic media were performed with 412 mg (2 mmol) ibuprofen or 440 mg ibuprofen methyl ester, 1,3-dimethoxybenzene (100 mg, internal standard), hydroxylamine (147 \(\mu l\) commercial 50 % solution, 2.5 mmol) and 200 mg Novozym 435 in 5 ml solvent. Reference compounds for NMR and HPLC were prepared chemically. The chiral ester aminolysis reactions were performed with 384 mg (2 mmol) 1-phenyl ethyl butyrate, 2.5 mmol nucleophile and 200 mg Novozym 435 in 5 ml tert-buty alcohol room temperature.

Reference compounds for NMR and HPLC were prepared chemically from racemic ibuprofen methyl ester and enantiopure (S)-ibuprofen methyl ester.

**Derivatisation of ibuproxam for analysis on chiral HPLC**

A sample of 50 \(\mu l\) was taken from a reaction mixture containing ibuproxam. The sample was diluted with 250 \(\mu l\) isopropyl alcohol, 100 \(\mu l\) 10% benzyl bromide in isopropyl alcohol was added and the mixture was stirred in a GLC bottle with 125 mg
anhydrous $K_2CO_3$ for three hours. Longer reaction time gave side products that interfere with the analysis. The sample was diluted with 1 ml eluent, filtered over a little $MgSO_4$ in a pipet with cotton and immediately injected.

**Characterization by $^1H$- and $^{13}C$ NMR**

Structural assignments of the isolated products were made on the basis of $^1H$ and $^{13}C$ NMR. Both the $^1H$ and $^{13}C$ NMR spectra were recorded in CDCl$_3$ at room temperature.

Octanohydroxamic acid, $^1H$ NMR: $\delta$ 7.23 (2H, NH, OH, s), $\delta$ 2.15 (2H, CH$_2$-2, t), $\delta$ 1.64 (2H, CH$_2$-3, q), $\delta$ 1.29 (8H, CH$_2$A-7, m), $\delta$ 0.88 (3H, CH$_3$, t). $^{13}C$ NMR: $\delta$ 171.81 (C=O), $\delta$ 33.53 (C2), $\delta$ 31.65 (C3), $\delta$ 29.09, 28.93 (C4,5), $\delta$ 25.38 (C6), $\delta$ 22.59 (C7), $\delta$ 14.04 (C8). MS: m/z = 160 (3%, m+1), 159 (2%), 127 (86%), 109 (17%), 84 (8%), 75 (31%), 67 (10%), 57 (100%). mp 77 °C.

Ibuprofen, $^1H$ NMR: (propanohydroxamic acid moiety) $\delta$ 8.5 (2H, NH, OH, s), $\delta$ 3.48 (1H, CH, q), $\delta$ 1.45 (3H, CH$_3$, d), (ring) $\delta$ 7.12 (4H, CH2, 3, 5, 6, q), (isobutyl moiety) $\delta$ 2.47 (2H, CH$_2$, d) $\delta$ 1.84 (1H, CH, m), $\delta$ 0.90 (6H, CH$_3$, d). $^{13}C$ NMR: (proanoyl moiety) $\delta$ 173.14 (C=O), $\delta$ 45.02 (C2), $\delta$ 22.37 (C3), (ring) $\delta$ 141.15 (C1), $\delta$ 136.71 (C4), $\delta$ 129.66 (C2, C6), $\delta$ 127.35 (C3, C5), (isobutyl moiety) $\delta$ 43.57 (C1), $\delta$ 30.17 (C2), $\delta$ 18.00 (C3). MS: m/z =221 (22%), 188 (4%), 145 (5%), 122 (5%), 117 (35%), 105 (13%), 91 (25%), 77 (8%), 65 (6%), 57 (11%), 51 (6%), 43 (17%). Specific rotation: $[\alpha]_D^{22} = +44.8^\circ$ c 0.30; Ethanol abs. Racemate mp 119-121 °C. (S)-ibuprofen 126-127 °C.

**REFERENCES**


Lipase catalyzed acylation of hydroxylamine


33. Assay from Novo Nordisk, personal communication.

Chapter 5

Acylation of hetero-substituted nitrogen nucleophiles catalyzed by Candida antarctica lipase B

Summary

A number of hetero-substituted nitrogen nucleophiles, even weakly basic ones such as phenylhydrazine, were acylated with octanoic acid and ethyl octanoate in the presence of Candida antarctica lipase B. Hydrazine was more enantioselective than hydroxylamine in reactions with chiral carboxylic acids and esters. The aminolysis reaction displayed enantioselectivity both with esters derived from chiral carboxylic acids and chiral alcohols. Substituted hydrazines and hydroxylamines reacted slower with ibuprofen than with ibuprofen methyl ester but in both cases, especially using benzylated derivatives, products of high optical purity could be obtained.
INTRODUCTION

It is now well established that in organic media lipases (EC 3.1.1.3) readily catalyze a variety of acylation reactions which they do not perform in nature\(^1\). For example, carbohydrates\(^2\), alkylamines\(^3,4\), ammonia\(^5,6,7,8,9,10\) and hydrogen peroxide\(^11,12\) have been successfully used as acyl acceptors.

Enzymatic acylation of stable hetero-substituted nitrogen nucleophiles, comprising hydroxylamine and hydrazine derivatives (the sulfur analogue of hydroxylamine is not a stable compound), on the other hand has not been investigated in detail. The synthesis of hydroxamic acids by lipase-catalyzed hydroxylaminolysis of triglycerides\(^13\) and fatty acids in water has recently been reported\(^14\), but the scope of the reaction was not further examined\(^15\).

An enzymatic route for the synthesis of substituted hydroxamic acids and acyl hydrazines with the inherent advantages of selectivity, mild reaction conditions and enantioselectivity, is of synthetic interest (Chapter 4). Furthermore acylated hydroxylamine and hydrazine derivatives can be converted to amines and derivatives thereof via the Lossen\(^16\), Curtius\(^17\) or Hofmann\(^18\) rearrangements. These rearrangements are closely related in mechanism but take place under very different conditions and to a certain extent they are complementary. Some substituents tolerate the conditions of one method while other substituents are more compatible with another method. Because the key step in these reactions - formation and rearrangement of an acylnitrene intermediate - takes place with retention of the configuration at the \(\alpha\)-carbon atom, an enantioselective route to acylated hydroxylamines and hydrazines would also give access to the corresponding enantiomerically pure amines containing one carbon atom less.
Acylation of hydrazine and hydroxylamine derivatives

\[
\begin{align*}
\text{NHOH} & \quad \text{NNNH}_2 & \quad \text{NXR}^4 \\
\text{R}_1^2 & \quad \text{R}_1^3 & \quad \text{R}_1^2 & \quad \text{R}_1^3
\end{align*}
\]

\[
\begin{align*}
\text{pH 9} & \quad \text{Ac}_2\text{O} & \quad \text{HNO}_2 & \quad 1\text{ H}_2/\text{cat.} & \quad 2\text{ Cl}_2\text{ OH}^+ \\
\left[ \text{N} = \text{C} = \text{O} \right] & \quad \text{H}_2\text{O} & \quad \rightarrow & \quad \text{NH}_2
\end{align*}
\]

**Figure 1.** The Lossen, Curtius and Hofmann rearrangement. \( R = \text{aryl, alkyl} \) \( X = \text{O, NH} \)

Due to the \( \alpha \)-effect\(^{19} \) hetero-substituted nitrogen nucleophiles are much stronger nucleophiles than one would expect from their basicity. The ground state of the nucleophile is destabilized by repulsion between the adjacent pairs of electrons (Figure 2). The highest molecular orbital resulting from this destabilisation is the HOMO that will donate an electron pair for bonding. Another factor is the stabilization of the transition state by the extra pair of electrons.

**Figure 2.** Destabilisation of the ground state of hydrazine vs. a normal amine.
The pK\textsubscript{a} of protonated hydroxylamines and hydrazines is in the region 5-8 (Table 1) which is much lower than ammonia and alkylated amines (pK\textsubscript{a} 9-11). It is so close to the pK\textsubscript{a} of octanoic acid (4.9) that significant amounts of both uncharged hetero-substituted nitrogen nucleophile and octanoic acid are expected to be present. Hence, in contrast to ammonolysis a direct reaction between the free acid and the free base is feasible, even in the presence of water.

**Table 1. Basicity of some nitrogen nucleophiles.**

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>pK\textsubscript{a} of protonated form</th>
</tr>
</thead>
<tbody>
<tr>
<td>Triethylamine</td>
<td>11.0</td>
</tr>
<tr>
<td>Ammonia</td>
<td>9.3</td>
</tr>
<tr>
<td>Hydrazine</td>
<td>8.0</td>
</tr>
<tr>
<td>Hydroxylamine</td>
<td>6.0</td>
</tr>
<tr>
<td>Phenylhydrazine</td>
<td>5.3</td>
</tr>
<tr>
<td>Pyridine</td>
<td>5.2</td>
</tr>
</tbody>
</table>

It is known from analogous reactions with alcohols and amines as nucleophile that the type of nucleophile as well as the presence of an alkyl chain can have a positive effect on the reaction rate or the enantioselectivity of the reaction. Therefore we have investigated the effect of alkyl and aryl substituents on the hydroxylaminolysis and hydrazinolysis. O-alkylhydroxylamines have the advantage that they are more stable than hydroxylamine itself. In this Chapter we report the acylation of various hydroxylamine and hydrazine derivatives with ethyl octanoate and octanoic acid as model compounds in the presence of Candida antarctica lipase B, the acylation of hydroxylamine and hydrazine with a chiral acid and enantioselective aminolysis of 1-phenylethyl butyrate.
RESULTS AND DISCUSSION

Aminolysis of ethyl octanoate

The good results in the acylation of hydroxylamine (Chapter 4) prompted us to investigate whether the corresponding reactions of hydroxylamine derivatives, as well as of hydrazine and its derivatives, would take place with equal ease. The acylation of O-methyl- and O-benzylhydroxylamine by ethyl octanoate or octanoic acid proceeded smoothly in the presence of Novozym 435 (*Candida antarctica* lipase B), although the initial rates were ca. 2-5 times lower than the corresponding reactions with hydroxylamine. Hydrazine reacted at approximately half the rate of hydroxylamine, but the reactivity of its methyl and benzyl derivatives was comparable with the corresponding O-alkyl hydroxylamine compounds. Hydrazine was used as the monohydrate but the other reaction mixtures contained no water. Hence, the water activity is apparently less important than in hydroxylaminolysis where there was no reaction at all in the absence of water. It is possible that the addition of water would have had a positive effect on the reaction rate in a number of cases but the water activity was not optimized.

Ethyl octanoate reacts faster than octanoic acid with the various nucleophiles, with the exception of phenylhydrazine, which may be due to the low basicity of this nucleophile. The decrease in reaction rate due to deprotonation of octanoic acid by this nucleophile is probably negligible in organic media. In general, the hetero-substituted nitrogen nucleophiles are acylated readily, in spite of their low basicity. This can be rationalized on the basis of the α-effect which renders the O- and N-substituted amines much stronger nucleophiles than would be expected based on their basicity.

It is surprising that the condensation of octanoic acid with hydroxylamine and hydrazine derivatives is so facile considering that only very few examples of a lipase
catalyzed condensation of a carboxylic acid and an alkyl amine have been reported previously\textsuperscript{20}.

**Table 2. Acylation of various nucleophiles with ethyl octanoate.**

<table>
<thead>
<tr>
<th>Donor</th>
<th>Ethyl octanoate</th>
<th>Octanoic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Initial rate\textsuperscript{a}</td>
<td>Initial rate\textsuperscript{a}</td>
</tr>
<tr>
<td>Nucleophile</td>
<td>(\textmu{}mol·g\textsuperscript{-1}·min\textsuperscript{-1})</td>
<td>(\textmu{}mol·g\textsuperscript{-1}·min\textsuperscript{-1})</td>
</tr>
<tr>
<td>H\textsubscript{2}NOH\textsuperscript{c}</td>
<td>910</td>
<td>160</td>
</tr>
<tr>
<td>H\textsubscript{2}NOMe\textsuperscript{d}</td>
<td>450</td>
<td>77</td>
</tr>
<tr>
<td>H\textsubscript{2}NOPh</td>
<td>n.d.</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>H\textsubscript{2}NOCH\textsubscript{2}Ph</td>
<td>200</td>
<td>87</td>
</tr>
<tr>
<td>H\textsubscript{2}N(CH\textsubscript{2}Ph)OH\textsuperscript{g}</td>
<td>n.d.</td>
<td>&lt; 5</td>
</tr>
<tr>
<td>H\textsubscript{2}NNH\textsubscript{2}\textsuperscript{f}</td>
<td>540</td>
<td>70</td>
</tr>
<tr>
<td>H\textsubscript{2}NNHCH\textsubscript{3}\textsuperscript{g}</td>
<td>530</td>
<td>79</td>
</tr>
<tr>
<td>H\textsubscript{2}NNHPh</td>
<td>52</td>
<td>70</td>
</tr>
<tr>
<td>H\textsubscript{2}NNHBz</td>
<td>200</td>
<td>100</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Reaction conditions: 688 mg (4 mmol) ethyl octanoate or 576 mg (4 mmol) octanoic acid, 0.5 M nucleophile and 10 mg Novozym 435 in 10 ml tert-butyl alcohol at 40 °C.

\textsuperscript{b} Reaction conditions: 57.6 mg (0.4 mmol) octanoic acid, 1.0 mmol nucleophile and 50 mg Novozym 435 in 1.0 ml tert-butyl alcohol, 20 h at 40 °C.

\textsuperscript{c} Reaction mixture contains 0.91 M water.

\textsuperscript{d} Reactions with O-methyl hydroxylamine were performed with a 1:1 mixture of O-methyl hydroxylamine hydrochloride and triethylamine instead of the free base because the latter is very volatile.

\textsuperscript{e} Ratio of acylation of N and O 25:1.

\textsuperscript{f} Reaction mixture contains 0.5 M water.

\textsuperscript{g} Ratio of acylation of the substituted and non-substituted nitrogens 1:15.
Acylation of hydrazine and hydroxylamine derivatives

The rates of the reactions with $N$-benzylhydroxylamine and $O$-phenylhydroxylamine were very low. In the case of $N$-benzylhydroxylamine this is probably because of steric hindrance. Dialkyl amines, even with small substituents, also react very slowly. In the case of $O$-phenylhydroxylamine the sluggish reaction may be due to electronic effects.

**Enantioselective Conversions**

Ibuprofen esters have developed into standard substrates for enantioselective hydrolysis and based on our earlier experience with the ammonolysis of ibuprofen esters we could compare the results of different methods. It was known from those earlier experiments that the type of nucleophile could be of great importance for the reaction rate and enantioselectivity of the reaction. Enantioselective reactions were performed with Novozym 435 which is stable under reaction conditions and is one of the few enzymes that accepts ibuprofen derivatives as a substrate. A number of hydroxylamines and hydrazines were tested with ibuprofen and its methyl ester.

![Chemical structure]

**Figure 2.** Enzymatic synthesis of R-ibuprofen amides from racemic ibuprofen derivatives. ($R = H, CH_3, X = OH, NH_2, OBz, NHBz$)
Unsubstituted hydrazine and hydroxylamine gave the highest reaction rates with ibuprofen methyl ester (Table 3) but the benzylated derivatives, although they reacted at only half the rate, gave a higher enantiomeric ratio. Novozym 435 preferentially converted the (R)-enantiomer, analogous to hydrolysis, transesterification and ammonolysis of ibuprofen esters with this enzyme. The enantiomeric ratio $E$ of the hydrazinolysis was comparable to the value observed in the ammonolysis of the 2-chloroethyl ester ($E=28^8$). Hydroxylaminolysis was less selective but the benzyl derivatives of both hydroxylamine and hydrazine reacted with high selectivity ($E>100$). In the case of hydrazine, which is used as the monohydrate there is almost no hydrolysis. The synthesis hydrolysis ratio is 32 at 46% conversion. Results are depicted in Table 3; some results of the acylation of hydroxylamine from Chapter 4 are given in Tables 3, 4 and 5 for comparison.

**Table 3. Aminolysis of ibuprofen methyl ester**.

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>Initial rate ($\mu$mol·g$^{-1}$·min$^{-1}$)</th>
<th>Conversion (% h)</th>
<th>$ee_s$</th>
<th>$ee_p$</th>
<th>$E^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H$_2$NOH</td>
<td>1.11</td>
<td>42, 72</td>
<td>47</td>
<td>71</td>
<td>8</td>
</tr>
<tr>
<td>H$_2$NNH$_2$</td>
<td>2.36</td>
<td>46, 48</td>
<td>97</td>
<td>86</td>
<td>28</td>
</tr>
<tr>
<td>BzONH$_2$</td>
<td>0.54</td>
<td>18, 96</td>
<td>19$^c$</td>
<td>99$^c$</td>
<td>$&gt;100$ (R)</td>
</tr>
<tr>
<td>BzNHNH$_2$</td>
<td>1.18</td>
<td>42, 96</td>
<td>65$^c$</td>
<td>97$^c$</td>
<td>$&gt;100$ (R)</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 440 mg ibuprofen methyl ester (2 mmol), 2.5 mmol nucleophile, 5 ml tert-butyl alcohol, 200 mg Novozym 435 at r.t.

b. Enantiomeric ratio calculated from product $ee$ except hydroxylamine which is calculated from ester $ee$

c. A small amount of ibuprofen was formed; otherwise $ee$ of ester would be slightly higher
Hydrazinolysis was also performed at 40 °C. There was a blank reaction of about 1% per day but under the same conditions as in Table 3, a conversion of 46% was reached in 22 hours with an enantiomeric ratio of 23. The rate of this reaction is similar to the ammonolysis of ibuprofen chloroethyl ester but the nucleophile concentration is a factor five lower and the ester has no activated alcohol moiety in this case.

Similar selectivities to those in Table 3 were found when ibuprofen was used as acyl donor. The reactions were one to two orders of magnitude slower with the acid than with the methyl ester. The difference was large for the hydrazine derivatives, again because of the stronger basic character of these nucleophiles that led to deprotonation of the free acid and therefore to loss of reaction rate. In fact of all the nucleophiles that were tested, hydrazine gave the highest reaction rate with the ester and the lowest with the acid.

**Table 4. Enantioselective conversion of ibuprofen into ibuprofen amides.**

<table>
<thead>
<tr>
<th>Nucleophile</th>
<th>Initial rate (μmol·g⁻¹·min⁻¹)</th>
<th>Conversion (%)</th>
<th>ee (%)</th>
<th>eeₚ (%)</th>
<th>Eᵣ</th>
</tr>
</thead>
<tbody>
<tr>
<td>NH₂OH</td>
<td>0.11</td>
<td>17</td>
<td>15</td>
<td>75</td>
<td>8</td>
</tr>
<tr>
<td>NH₂NH₂</td>
<td>0.033</td>
<td>5</td>
<td>5</td>
<td>94</td>
<td>34</td>
</tr>
<tr>
<td>BzONH₂</td>
<td>0.079</td>
<td>11</td>
<td>12</td>
<td>98</td>
<td>&gt;100</td>
</tr>
<tr>
<td>BzNHNH₂</td>
<td>0.051</td>
<td>7</td>
<td>7</td>
<td>99</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

---

a. Reaction conditions: 412 mg ibuprofen (2.0 mmol), 100 mg 1, 3-dimethoxybenzene, 2.5 mmol nucleophile and 200 mg Novozym 435 in 5 ml tert-butyl alcohol at room temperature.

b. Conversion after 240 hrs.

c. Calculated from product ee; calculation from substrate ee is unreliable at low conversion.
Chapter 5

The biologically active enantiomer of ibuprofen was left unreacted in the reaction mixtures but because of the low reaction rate this is not a very practical way of producing it. Surprisingly we found that lowering the concentration of hydrazine to 0.5 equivalents dramatically increased the reaction rate and the selectivity. At room temperature the initial reaction rate was three times as high and the enantiomeric ratio was above 100. When the reaction was performed at 40 °C with 0.5 equivalents of hydrazine, the initial rate went up by more than an order of a magnitude and the enantiomeric ratio is still 80. After the same reaction time as in Table 4 a conversion of 32% with 96% ee was reached.

When only 0.5 equivalents of hydrazine are used, a large part of it will be protonated by the ibuprofen in the mixture. Apparently the concentration of free acid is much more rate determining than the concentration of free hydrazine. Presumably hydrazine is such a strong nucleophile that even at low concentration the reaction with the acyl enzyme intermediate is fast compared to the acylation of the native enzyme by the acid. The results from the hydrazinolysis of ibuprofen methyl ester are consistent with this notion. Thus, when equal amounts of water and hydrazine are offered as a nucleophile to the enzyme, hydrazine forms 97% of the product, although water is an excellent nucleophile under normal circumstances.

High enantioselectivities were observed with benzylhydroxylamine and benzylhydrazine using either acid or ester as acyl donor. It would seem that the absence of an alkyl chain on the nucleophile exerts a negative effect on the enantioselectivity in general. The effect of decreasing enantioselectivity with decreasing ester chain length or, in the case of esterification, alcohol chain length has also been found by others. For example, hydrolysis of ibuprofen esters with Candida antarctica lipase B in tert-butyl alcohol is much less enantioselective than transesterification with butanol.
Aminolysis of 1-phenylethyl butanoate

To show that the reaction is also enantioselective with respect to the alcohol part of an ester we also performed the hydrazinolysis and hydroxylaminolysis of 1-phenylethyl butyrate (Figure 4) with both the unsubstituted and the benzylated nucleophiles (Table 5). The reaction was (R)-specific in all cases and proved to be faster than the ammonolysis of the same substrate with Novozym 435 by a factor two, with only one fifth of the nucleophile concentration, which attests to the disproportionally high nucleophilicity of the hydrazines and hydroxylamines. The results were comparable to that found with hydroxylamine.

![Chemical Reaction](image)

**Figure 4.** Aminolysis of 1-phenylethyl butyrate

A problem that occurs in all experiments with hydroxylamine and hydrazine derivatives is the relatively low stability of enzymes in media containing these compounds. Even a very stable enzyme such as *Candida antarctica* lipase B suffers some loss of activity. Amides react chemically with hydroxylamine and hydrazine derivatives to form the hydroxamic acids or acyl hydrazines. Enzymes, being polyamides and therefore liable to aminolysis by hetero-substituted nitrogen nucleophiles are inherently unstable. It is a problem that can be suppressed by
choosing a lower temperature or a solvent with a low polarity or by making sure that there is no layer separation in the course of the reaction but it will always be a disadvantage compared to aminolysis with ammonia or alkylamines where this plays a minor role.

**Table 5. Aminolysis of 1-phenylethyl butanoate**

<table>
<thead>
<tr>
<th>HNu</th>
<th>Conversion (%)</th>
<th>ee&lt;sub&gt;s&lt;/sub&gt; (%)</th>
<th>ee&lt;sub&gt;p&lt;/sub&gt; (%)</th>
<th>E</th>
</tr>
</thead>
<tbody>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;NOH</td>
<td>49</td>
<td>94</td>
<td>99</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;NNH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>50</td>
<td>98</td>
<td>99</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>BzONH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>50</td>
<td>99</td>
<td>99</td>
<td>&gt; 100</td>
</tr>
<tr>
<td>BzNHNH&lt;sub&gt;2&lt;/sub&gt;</td>
<td>50</td>
<td>99</td>
<td>99</td>
<td>&gt; 100</td>
</tr>
</tbody>
</table>

a. Conditions: 384 mg 1-phenylethyl butanoate (2 mmol) with 2.5 mmols of nucleophile in 5 ml tert-butyl alcohol, 40 hours at room temperature with 200 mg of Novozym 435.

b. Substrate ee calculated.

**CONCLUSIONS**

Various hydrazine and hydroxylamine derivatives are readily acylated with acids as well as esters in the presence of *Candida antarctica* lipase B. High conversions can be reached but in a number of cases substantial amounts of enzyme have to be used. The aminolysis is enantioselective with respect to both the alcohol as well as the acid moiety. Acylation of hydrazine was more enantioselective than acylation of hydroxylamine with both ibuprofen and its methyl ester. Benzylation of hydrazine and benzyl hydroxylamine gave products of high optical purity in all cases.
MATERIALS AND METHODS

Materials

Hydroxylamine was purchased as a 50% aqueous solution (17 M) from Merck (Note: higher concentrations of the free base are unstable or explosive). Hydrazine was obtained as the monohydrate from Acros. All solvents were of analytical purity and were dried on activated Uetikon CaA zeolite prior to use. All other reagents were purchased from Aldrich or Acros and used as received. Novozym 435 was kindly donated by Novo Nordisk A/S, Bagsvaerd and used as delivered.

Analysis and Equipment

The derivatives of octanoic acid and ibuprofen were analyzed by HPLC on a Waters 8 x 100 mm 4 μ Novapak C18 reversed phase RCM column with a Waters 510 pump, a Shimadzu SPD-6A UV detector, a Shodex RI SE-61 RI detector and a Spectra-Physics SP 4270 integrator. The eluent was 65/35 methanol/water (v/v), with 0.05 M acetate buffer pH 4.3 at a flow of 1.0 ml/min.

Chiral HPLC was performed on a Baker 4.6 x 250 mm 5 μ Chiralcel OD column with a Waters 510 pump and a Waters 486 UV detector operating at 254 nm. As eluent for ibuprofen and 1-phenylethanol, 98/2/0.1 hexane/isopropyl alcohol/formic acid was used at a flow rate of 0.5 ml/min. For the analysis of ibuprofen methyl ester 100% hexane was used as eluent at a flow rate of 0.5 ml/min. For reaction products of benzylhydrazine and benzylhydroxylamine and derivatized ibuprofenhydrazide (procedure later described) 90/10 hexane/isopropyl alcohol was used at the same flow rate.

1H- and 13C NMR spectra were recorded on a Varian VXR-400S spectrometer. Mass spectra were recorded on a VG 70-SE spectrometer using the EI method.
Reactions were performed in 30 ml glass vessels with a teflon coated cap, in a stirred thermostated oil-bath at 40 °C or at room temperature. All reactions with octanoic acid and ethyl octanoate were performed with 0.4 M of substrate and 0.5 M of nucleophile in 10 ml solvent. Higher concentrations of nucleophile gave rise to a blank reaction with ethyl octanoate. The enzyme and the solvent were stirred for one hour for thermal stabilization and wetting of the enzyme. Samples of 50 µl were taken at regular intervals and analyzed. The initial rate was calculated by fitting a line through the data points at < 5 % conversion.

**Isolation of the Products**

For analytical purposes the octanoyl derivatives of the different nucleophiles were isolated. After prolonged shaking of the reaction mixtures at 40 °C to reach full conversion, the solvent was evaporated *in vacuo* and the residue was recrystallized from petroleum ether (bp 40-60 °C). The liquid reaction products (O-benzyl-N-octanoylhydroxylamine and O-methyl-N-octanoylhydroxylamine were taken up in petroleum ether and washed successively with 0.1 M Na₂CO₃ solution, 0.1 M HCl solution and water and dried over anhydrous Na₂SO₄. Removal of the solvent *in vacuo* yielded the pure product.

**Enantioselective reactions**

The reactions with ibuprofen, ibuprofen methyl ester and 1-phenylethyl butyrate were performed with 2 mmols of substrate, 100 mg 1,3-dimethoxybenzene (internal standard), 2.5 mmols of nucleophile and 200 mg Novozym 435 in 5 ml *tert*-butyl alcohol. The reaction mixtures were worked up to provide an analytical sample for NMR. Reference compounds for NMR and HPLC were prepared chemically.
Acylation of hydrazine and hydroxylamine derivatives

Derivatisation of ibuprofenhydrazide for chiral HPLC

In a GLC bottle a sample of 50 µl, taken from a reaction mixture, in 500 µl tert-butyl methyl ether was extracted with 750 µl saturated KH₂PO₄ solution in water. From the organic layer a sample of 200 µl was taken and mixed in an other GLC bottle with 200 µl 0.5 M benzoic acid anhydride in tert-butyl methyl ether. To this mixture 1.0 ml of a saturated NaHCO₃ solution in water was added and after vigorous shaking 200 µl of the organic layer was taken out and dried over anhydrous Na₂SO₄ in an eppendorf. After centrifugation the sample was immediately injected.

Characterization

Structural assignments of the isolated products were made on the basis of¹H and ¹³C NMR and MS. Both the ¹H and ¹³C NMR spectra were recorded in CDCl₃ at room temperature unless indicated otherwise. Mass spectra were recorded with 70 mV EI. Octanohydroxamic acid, ¹H NMR: δ 7.23 (2H, NH, OH, s), δ 2.15 (2H, CH₂-2, t), δ 1.64 (2H, CH₂-3, qi), δ 1.29 (8H, CH₂4-7, m), δ 0.88 (3H, CH₃, t). ¹³C NMR: δ 171.81 (C=O), δ 33.53 (C2), δ 31.65 (C3), δ 29.09, 28.93 (C4,5), δ 25.38 (C6), δ 22.59 (C7), δ 14.04 (C8). MS: m/z=160 (3%, m+1), 159 (2%), 127 (86%), 109 (17%), 84 (8%), 75 (31%), 67 (10%), 57 (100%). mp 77 °C.

N-octanoyl-O-methylhydroxylamine, ¹H NMR: δ 9.3 (1H, NH, s), δ 3.75 (3H, O-CH₃, s) δ 2.33 (2H, CH₂-2, t), δ 1.62 (2H, CH₂-3, qi), δ 1.27 (8H, CH₂4-7, m), δ 0.87 (3H, CH₃, t). ¹³C NMR: δ 171.31 (C=O), δ 64.26 (O-CH₃), δ 34.20 (C2), δ 33.19 (C3), δ 29.22, 29.10 (C4,5), δ 28.95 (C6), δ 25.46 (C7), δ 14.06 (C8). MS: m/z=173 (2%), 127 (96%), 109 (17%), 102 (16%), 89 (58%), 67 (10%), 57 (100%), 47 (16%), 43 (53%). Product was an oil.

N-octanoyl-O-phenylhydroxylamine, ¹H NMR: δ 8.47, 8.20 (1H, NH, br. d), 7.4-7.0 (5H, ring H, m), 2.30 (2H, CH₂, br. s), 1.67 (2H, CH₂, br. s), 1.3 (8H, CH₂, m) 0.88
(3H, CH$_3$). $^{13}$C NMR: δ 159.503, 129.603, 123.169 113.146 (ring), δ 31.638 (C4), δ 29.188, 28.930 (C5,6), δ 22.587 (C7), δ 14.051 (C8). MS: m/z = 235 (39%), 151 (8%), 142 (9%), 127 (73%), 109 (84%), 94 (95%), 65 (49%), 57 (100%). mp 130 °C.

N-octanoyl-O-benzylhydroxylamine, $^1$H NMR: δ 8.39 (1H, NH, s), δ 7.4-7.2 (5H, ring, m), δ 4.90 (2H, O-CH$_2$, s), δ 2.02 (2H, CH$_2$-2, t), δ 1.60 (2H, CH$_2$-3, q), δ 1.26 (8H, CH$_2$-4-7, m), δ 0.87 (3H, CH$_3$, t). $^{13}$C NMR: δ 171.10 (C=O), δ 129.22, 129.03, 128.61, 128.22 (ring), δ 78.12 (O-CH$_2$), δ 33.31 (CH$_2$-2), δ 31.67 (CH$_2$-3), δ 29.17, 28.96 (CH$_2$-4,5), δ 25.40 (CH$_2$-6), δ 22.60 (CH$_2$-7), δ 14.07 (CH$_2$-8). MS: m/z = 250 (34%, m+1), 214 (32%), 181 (11%), 127 (12%), 91 (100%), 57 (23%). Product was an oil.

Octanohydrazide, $^1$H NMR: δ 6.80 (1H, NH, s), δ 3.60 (2H, NH$_2$, s), δ 2.15 (2H, CH$_2$-2, t), δ 1.64 (2H, CH$_2$-3, q), δ 1.29 (8H, CH$_2$-4-7, m), δ 0.88 (3H, CH$_3$, t). $^{13}$C NMR: δ 174.01 (C=O), δ 34.62 (CH$_2$-2), δ 31.66 (CH$_2$-3), δ 29.24, 28.96 (CH$_2$-4,5), δ 25.51 (CH$_2$-6), δ 22.59 (CH$_2$-7), δ 14.05 (CH$_2$-8). MS: m/z = 158 (14%), 127 (54%), 109 (10%), 83 (6%), 74 (33%), 57 (100%). mp 87 °C.

N-octanoyl-N'-methylhydrazine, $^1$H NMR: δ 7.19 (1H, amide NH, s), δ 4.61 (1H, NH, s), δ 2.61 (3H, NCH$_3$, s), (1H, δ 2.12 (2H, CH$_2$-2, t), δ 1.64 (2H, CH$_2$-3, q), δ 1.29 (8H, CH$_2$-4-7, m), δ 0.88 (3H, CH$_3$, t). $^{13}$C NMR: δ 172.61 (C=O), δ 39.43 (C-N'), δ 34.78 (C2), δ 31.67 (C3), δ 29.18, 28.96 (C4,5), δ 25.51 (C6), δ 22.59 (C7), δ 14.05 (C8). MS: m/z = 172 (36%), 144 (6%), 127 (16%), 101 (6%), 88 (8%), 83 (6%), 73 (13%), 69 (8%), 57 (100%). mp 49-51 °C.

N-octanoyl-N'-phenylhydrazine, $^1$H NMR: δ 9.59 (1H, amide NH, s), δ 7.73 (1H, NH, s), δ 7.2 (2H, m-ringH, m) δ 6.8 (3H, o, p-ringH, m), δ 2.15 (2H, CH$_2$-2, t), δ 1.54 (2H, CH$_2$-3, q), δ 1.27 (8H, CH$_2$-4-7, m), δ 0.88 (3H, CH$_3$, t). $^{13}$C NMR: δ 171.87 (C=O), δ 149.41 (ring C1), δ 128.50 (ring C2, C6), δ 118.22 (ring C4), δ 111.96 (ring C3, C5), δ 33.21 (C2), δ 31.11 (C3), δ 28.49, 28.30 (C4, C5), δ 25.05 (C6), δ 21.95 (C7), δ 13.85 (C8). MS: m/z = 234 (34%), 127 (7%), 108 (100%), 92 (17%), 77 (21%), 65 (14%), 57 (32%). mp 103 °C.
Acylation of hydrazine and hydroxylamine derivatives

N-octanoyl-N'-benzylhydrazine, $^1$H NMR: $\delta$ 6.96 (1H, amide NH, s), $\delta$ 4.93 (1H, NH, s), $\delta$ 3.97 (2H, N-CH$_2$, s), $\delta$ 2.09 (2H, CH$_2$-2, t), $\delta$ 1.60 (2H, CH$_2$-3, qi), $\delta$ 1.27 (8H, CH$_2$-7, m), $\delta$ 0.88 (3H, CH$_3$, t). $^{13}$C NMR: $\delta$ 172.75 (C=O), $\delta$ 137.67 (ring C1), $\delta$ 128.96 (ring C2, C6), $\delta$ 128.50 (ring C3, C5), $\delta$ 127.56 (ring C4), $\delta$ 55.89 (CH$_2$-N), $\delta$ 34.72 (C2), $\delta$ 31.66 (C3), $\delta$ 29.15, 28.95 (C4, C5), $\delta$ 25.52 (C6), $\delta$ 22.59 (C7), $\delta$ 14.06 (C8). MS: m/z = 248 (12%), 144 (46%), 127 (10%), 122 (82%), 106 (87%), 91 (100%), 77 (16%), 65 (21%), 57 (48%). mp 75 °C.

Ibuproxan, $^1$H NMR: (propanohydroxamic acid moiety) $\delta$ 8.5 (2H, NH, OH, s), $\delta$ 3.48 (1H, CH, q), $\delta$ 1.45 (3H, CH$_3$, d), (ring) $\delta$ 7.12 (4H, CH$_2$, 3, 5, 6, q), (isobutyl moiety) $\delta$ 2.47 (2H, CH$_2$, d) $\delta$ 1.84 (1H, CH, m), $\delta$ 0.90 (6H, CH$_3$, d). $^{13}$C NMR: $\delta$ 171.81 (C=O), $\delta$ 33.53 (C2), $\delta$ 31.65 (C3), $\delta$ 29.09, 28.93 (C4, C5), $\delta$ 25.38 (C6), $\delta$ 22.59 (C7), $\delta$ 14.04 (C8). $^{13}$C NMR: (propanoyl moiety) $\delta$ 173.14 (C=O), $\delta$ 45.02 (C2), $\delta$ 22.37 (C3), (ring) $\delta$ 141.15 (C1), $\delta$ 136.71 (C4), $\delta$ 129.66 (C2, C6), $\delta$ 127.35 (C3, C5), (isobutyl moiety) $\delta$ 43.57 (C1), $\delta$ 30.17 (C2), $\delta$ 18.00 (C3, C4). MS: m/z = 221 (22%), 188 (4%), 145 (5%), 122 (5%), 117 (35%), 105 (13%), 91 (25%), 77 (8%), 65 (6%), 57 (11%), 51 (6%), 43 (17%). Racemate mp 119-121 °C. (S)-ibuproxan 126-127 °C.

2-(4-isobutyl-phenyl)propanohydrazide, $^1$H NMR (DMSO-d$_6$): (propanohydrazide moiety) $\delta$ 9.98 (1H, NH, d), 3.60 (1H, CH, q), $\delta$ 3.37 (2H, NH$_2$, br.s), $\delta$ 1.32 (3H, CH$_3$, d), (ring) $\delta$ 7.3-7.0 (4H, CH$_2$, 3, 5, 6, m), (isobutyl moiety) $\delta$ 2.39 (2H, CH$_2$, d) $\delta$ 1.78 (1H, CH, m), $\delta$ 0.93 (6H, CH$_3$, d). $^{13}$C NMR: (propanoyl moiety) $\delta$ 172.067 (C=O), $\delta$ 44.140 (C2), $\delta$ 22.077 (C3), (ring) $\delta$ 139.261 (C1), $\delta$ 138.686 (C4), $\delta$ 128.852, 128.627, 128.960 (C2, C3, C5, C6), (isobutyl moiety) $\delta$ 42.429 (C1), $\delta$ 29.509(C2), $\delta$ 18.205, 18.103 (C3, C4). MS: m/z 220 (27%), 188 (23%), 161 (100%), 145 (9%) 119 (30%), 105 (11%), 91 (21%), 57 (25%). Racemate mp 75 °C, (S)-ibuprofenhydrazide mp 92 °C.

O-Benzyl-2-(4-isobutyl-phenyl)propanohydroxamic acid, $^1$H NMR: (propanohydroxamic acid moiety) $\delta$ 7.9 (1H, NH, br s), 3.4 (1H, CH$_2$, br s), $\delta$ 1.46 (3H, CH$_3$, d), (rings) $\delta$ 7.4-7.0 (9H, m), $\delta$ 4.82 (2H, O-CH$_2$, s), (isobutyl moiety) $\delta$ 2.42 (2H, CH$_2$, d) $\delta$ 1.83 (1H, CH, m), $\delta$ 0.87 (6H, CH$_3$, d). $^{13}$C NMR: (propanoyl moiety) $\delta$
Chapter 5

172.039, 167.228 (C=O, double singlet broadened by exchange), δ 43.958 (C2), δ 22.376 (C3), (rings) δ 140.907 (C1, benzyl), δ 137.428 (C1, phenylpropanoic acid), δ 135.062 (C4, phenylpropanoic acid), 129.617, 129.341, 128.729, 128.554, 127.230 (other ring carbons), δ 78.067 (O-CH$_2$), (isobutyl moiety) δ 45.013 (C1), δ 30.186(C2), δ 18.263 (C3, C4). MS: m/z 311 (6%), 279 (11%), 251 (17%), 203 (13%), 188 (27%), 161 (65%), 144 (35%), 118 (59%), 108 (36%), 91 (100%), 77 (45%), 65 (23%). mp 65 °C (Racemate).

N-Benzyl-2-(4-isobutyl-phenyl)propanohydrazide, $^1$H NMR (DMSO-d$_6$): (propanoyl moiety) δ 9.9 (1H, amide NH, s), δ 3.83 (1H, CH, q), δ 3.4 (1H, NH, br s), δ 1.45 (3H, CH$_3$, d), (rings) δ 7.4-7.0 (9H, m), δ 4.75 (2H, O-CH$_2$, s), (isobutyl moiety) δ 2.42 (2H, CH$_2$, d) δ 1.88 (1H, CH, m), δ 0.87 (6H, CH$_3$, d). $^{13}$C NMR: (propanoyl moiety) δ 176.698, 172.898 (C=O, double singlet broadened by exchange), δ 42.575 (C2), δ 22.390 (C3), (rings) δ 140.587 (C1, benzyl), δ 139.779 (C1, phenylpropanoic acid), δ 135.906 (C4, phenylpropanoic acid), 129.726, 129.159, 128.729, 128.249, 127.499 (other ring carbons), δ 53.268 (N-CH$_2$), (isobutyl moiety) δ 45.042 (C1), δ 30.215(C2), δ 19.406 (C3, C4). MS: m/z 310 (46%), 202 (10%), 188 (23%), 161 (100%), 145 (15%), 122 (83%), 105 (16%), 91 (75%), 77(15%), 65 (15%). mp 51 °C (Racemate).

REFERENCES


Chapter 6

Lipase catalyzed diacylation of hydrazine with alkanoic acids: an indirect method for the kinetic resolution of chiral acids

Summary

The acylation of hydrazine to, eventually, its $N, N'$-diacyl derivative was catalyzed by a number of lipases. The rates of the first and second steps depended on the lipase and the type of solvent used. Water, up to 0.4 M, had no effect on the reaction. Double acylation of hydrazine with ethyl octanoate in the presence of Novozym 435 was as fast as the corresponding reaction with octanoic acid. Although the formation of octanohydrazide is faster with the ester than with the acid, the second acylation step is rate determining in both cases.

Chemical conversion of ibuprofen to its hydrazide makes it a suitable nucleophile for several lipases that do not accept ibuprofen as acyl donor. The rate of the acylation of ibuprofen hydrazide with octanoic acid was unexpectedly high compared to other reactions where ibuprofen is the acyl donor but the chiral recognition of the reaction was low in most cases. The best result was obtained with Pseudomonas lipoprotein lipase on EP 100. The $(R)$-enantiomer of the product ($N$-octanoyl-$N'$-2-(4-isobutylphenyl) propanohydrazide) was formed with an $E$ value of 26.
INTRODUCTION

In the course of our investigation of (hetero) nitrogen nucleophiles we found that the reaction of octanoic acid with hydrazine\(^1\) gave rise to a small amount of an apolar byproduct that was detected by HPLC. This byproduct was subsequently shown to be \(N,N'\)-dioctanoylhydrazine (2), formed \textit{via} further acylation of the initial product octanohydrazide (1).

\[
\text{Octanoic acid + Hydrazine} \rightarrow \text{N,N'-dioctanoylhydrazine}
\]

\[(1)\]

\[
\text{Octanoic acid + Long chain fatty acid} \rightarrow \text{N,N'-long chain diacylhydrazine}
\]

\[(2)\]

\textbf{Figure 1.} The formation of dioctanoylhydrazine.

The formation of \(N,N'\)-diacyl hydrazines from hydrazides is known. Hydrazides of amino acids, for example, are used as carboxyl protecting agents in peptide chemistry\(^2\). They can act as an acyl donor but also as a nucleophile \textit{via} the terminal nitrogen atom of the hydrazide moiety \(^3\). Few authors mention the resulting side products however. Diacyl hydrazines of amino acids can be synthesized from activated
esters such as Z-amino acid esters when they are treated with hydrazine and a protease. Gotor et al. reported the formation of diacyl hydrazines starting from acetohydrazide, formylhydrazine or methyl carbazate. The amount of enzyme in this reaction exceeded the amount of nucleophile by more than a factor of six by weight, even when strongly activated esters such as vinyl crotonate were used. The enzyme catalyzed acylation of alkanohydrazides could be a useful technique for the indirect resolution of carboxylic acids via their hydrazide derivative, synthesized either by enzyme catalysis or chemically. The latter methodology could be used for resolving carboxylic acids that are, e.g. for steric reasons, too unreactive for a conventional enzyme-catalyzed kinetic resolution. Activated carboxylic esters, which have commonly been used as acyl donor in aminolysis reactions, have the disadvantage of a considerable uncatalyzed background reaction with hydrazine which is, due to the α-effect, a very reactive nucleophile. Carboxylic acids have never been used as acyl donor, although they would be attractive reagents because they do not suffer from a background reaction with hydrazine. In this Chapter we report the lipase-catalyzed acylation of hydrazine and alkanohydrazides by alkanoic acids in a one-pot procedure. The influence of the lipase and the reaction medium on the course of the reaction has been explored. We have also investigated the enantioselective acylation of the hydrazide derived from (R,S)-ibuprofen as an example of an indirect kinetic resolution.
RESULTS AND DISCUSSION

Acylation of hydrazine with ethyl octanoate and octanoic acid

In the initial experiments ethyl octanoate and octanoic acid were compared as acyl donors in the lipase-catalyzed acylation of hydrazine. Immobilized *Candida antarctica* lipase B (Novozym 435) was used as catalyst because it had emerged from related work as the catalyst of choice. The reactions were carried out in tert-butyl alcohol because it is inert and dissolved the reactants and products well.

\[ \text{Figure 2. The formation of 1 and 2 from hydrazine and ethyl octanoate (A) or octanoic acid (B). Conditions: 1 mmol hydrazine hydrate, 2 mmol octanoic acid or ethyl octanoate and 25 mg Novozym 435 in 5 ml tert-butyl alcohol at 40 °C. Composition of the mixture on the Y-axis; 100\% of 2 equals 1 mmol. \bullet ethyl octanoate, ◆ octanoic acid, □ 1, ▲ 2.} \]

The course of the reactions is depicted in Figure 2. In the first step, the acylation of hydrazine to octanohydrazide (1), ethyl octanoate reacted 8 times as fast as octanoic acid. Due to the presence of 0.2 M water approx. 20% of the ethyl octanoate was hydrolyzed to octanoic acid. The lower reactivity of the acid (Figure 2B) is to be
expected because carboxylic acids are in general less active acyl donors than their esters. Moreover, part of the octanoic acid is present as its unreactive hydrazinium salt. Surprisingly, the nature of the acyl donor had no effect on the rate of the acylation of 1 to $N,N'$-dioctanoylhydrazine (2). It would seem that in the second step the acylation of the lipase is not rate-limiting.

The acylation of 1 continued unabated for 2 days (Figure 2B) and did not seem to suffer from the accumulating water in the reaction mixture. After two days about 30% conversion was reached but with other enzymes and other solvents quantitative yields of 2 could be obtained. The thermodynamic equilibrium of the reaction seems to be far towards the synthesis side despite the presence of as much as 3 equivalents of water at the end of the reaction. We tentatively conclude that 2 is hydrolytically stable under the reaction conditions. We decided to focus on the unprecedented lipase-catalyzed acylation of 1. Octanoic acid was used as acylating agent because the procedure is simple and is not accompanied by a background reaction.

**Solvent effects**

The initial rates of the acylation of hydrazine and 1 by octanoic acid were measured in a number of solvents, ranging from hydrophilic to non-polar (Table 1). Novozym 435 was again used as catalyst. Typically hydrazine was approx. an order of magnitude more reactive than 1 (Table 1) but the reactions were influenced in different ways by the solvent. The acylation of hydrazine was more than twice as fast in the medium-polarity solvents tert-butyl methyl ether and benzene compared with reaction in a hydrophilic solvent, such as tert-butyl alcohol. It would seem that the inhibition of lipase by tert-butyl alcohol, which has been noted previously, is also apparent in this case. In contrast, the acylation of 1 was only moderately affected by the solvent. The suggested change in rate-limiting step noted above may cause this difference in solvent effect.
\textit{Table 1. Acylation of hydrazine with octanoic acid in different solvents.}

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Initial rate ($\mu$mol $\cdot$ g$^{-1}$ $\cdot$ min$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$1^a$</td>
</tr>
<tr>
<td>Hexane</td>
<td>700</td>
</tr>
<tr>
<td>Isooctane</td>
<td>610</td>
</tr>
<tr>
<td>Benzene</td>
<td>1070</td>
</tr>
<tr>
<td>\textit{t}ert-Butyl methyl ether</td>
<td>1090</td>
</tr>
<tr>
<td>1,2-Dimethoxyethane</td>
<td>400</td>
</tr>
<tr>
<td>Dioxane</td>
<td>320</td>
</tr>
<tr>
<td>\textit{t}ert-Butyl alcohol</td>
<td>430</td>
</tr>
</tbody>
</table>

\begin{itemize}
  \item[a.] Reaction conditions: 576 mg (4 mmol) octanoic acid and 100 $\mu$l (2 mmol) hydrazine monohydrate in 10 ml of solvent are stirred with 10 mg Novozym 435 at 40 °C.
  \item[b.] Reaction conditions: 576 mg (4.0 mmol) octanoic acid and 316 mg (2.0 mmol) 1 in 10 ml of solvent are stirred with 10 mg Novozym 435 at 40 °C.
\end{itemize}

The rate of formation of 2 in \textit{t}ert-butyl alcohol in Table 1 was almost an order of magnitude faster than in the reaction shown in Figure 2B. Apparently water (which was not present in the initial rate experiment) interferes as a competing nucleophile, giving non-productive hydrolysis of the acyl enzyme intermediate, although it does not effect the thermodynamic equilibrium of the reaction.

A number of microbial lipases were compared as catalyst in the consecutive reactions of octanoic acid with hydrazine and 1. Isooctane was chosen as the solvent because it had performed well in the second step (Table 1) and because it dissolves the product only sparingly. Moreover, the relatively high water activity in this solvent is beneficial for lipases that do not perform well at low water activity, such as e.g. \textit{Thermomyces lanuginosus} lipase\textsuperscript{10}.

The course of the reactions are depicted in Figure 3. The activity of the lipases
diverged widely, as becomes apparent from the yields of 2 which ranged from 12% with Pseudomonas alcaligenes lipase to quantitative conversion (Thermomyces lanuginosus lipase) after 450 min. The water that is present in the reaction mixture - 0.2 M at the outset, increasing to 0.4 M at complete conversion - seemingly has no detrimental effect. Apparently the products 1 and 2 are hydrolytically stable under the reaction conditions. It is known already from literature11 that diacyl hydrazines are very stable.

The amounts of the intermediate product 1 that accumulated in the steady state also varied widely, reflecting the preference of the lipases for reaction with either hydrazine or 1. Pseudomonas lipoprotein lipase, Pseudomonas alcaligenes lipase and Candida antarctica lipase A converted 1 faster than hydrazine when both nucleophiles were present in the solution. Consequently it was not possible to calculate initial rates for the formation of 1 from the data points. Compound 1 was also acylated in separate experiments, using the same enzymes. The yields and initial reaction rates are presented in Table 2.

Table 2. Acylation of octanoylhydrazide catalyzed by different lipasesa.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Initial rate (μmol g⁻¹ min⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida antarctica A SP526 (on EP 100)</td>
<td>40</td>
</tr>
<tr>
<td>Candida antarctica B SP525 (on EP 100)</td>
<td>54</td>
</tr>
<tr>
<td>Candida antarctica B (Novozym 435)</td>
<td>54</td>
</tr>
<tr>
<td>Pseudomonas alcaligenes (on EP 100)</td>
<td>64</td>
</tr>
<tr>
<td>Th. lanuginosus. SP523 (on EP 100)</td>
<td>89</td>
</tr>
<tr>
<td>Pseudomonas lipoprotein (on EP 100)</td>
<td>115</td>
</tr>
<tr>
<td>Rhizomucor miehei (Lipozym IM 20)</td>
<td>44</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 576 mg (4.0 mmol) octanoic acid and 316 mg (2.0 mmol) 1 in 10 ml isooctane are stirred with 10 mg immobilized enzyme at 40 °C.
Figure 3. The formation of 1 and 2 in time for different lipases; A. SP525, B. SP523, C. SP526, D. Ps. alcaligenes. E. Ps. lipoprotein, F. Lipozym IM 20. Results for Novozym 435 and SP525 on EP 100 were almost identical. Conditions: 1 mmol hydrazine hydrate, 2 mmol octanoic acid and 50 mg immobilized lipase in 5 ml isooctane at 40 °C. Composition of the mixture on the Y-axis; 100% of 2 equals 1 mmol. ◆ octanoic acid, ■ 1, ▲ 2.
Kinetic resolution of a chiral hydrazide

The hydrazide derived from ibuprofen (2-(4-isobutylyphenyl)-propanohydrazide, 3) was selected as a suitable model reactant for exploring the resolution of chiral hydrazides. Ibuprofen and ibuprofen esters\textsuperscript{12, 13} have been the focus of a considerable effort, aimed at kinetic resolution, and the chiral preferences of the lipases that accept these as acyl donor are known. Candida antarctica lipase B preferentially converts the (R)-enantiomer\textsuperscript{12}, while Candida rugosa lipase is (S)-specific\textsuperscript{13}. None of the other lipases that we tested accepted ibuprofen as a acyl donor.

\begin{equation}
\begin{array}{c}
\text{OH} \\
\text{H}_{3}\text{C}-\text{C}^{\text{---}}\text{NHNH}_{2}
\end{array}
\xrightarrow{1 \text{ H}^+ / \text{MeOH}}
\begin{array}{c}
\text{O} \\
\text{H}_{3}\text{C}-\text{C}^{\text{---}}\text{NHNH}_{2}
\end{array}
\xrightarrow{2 \text{ H}_2\text{NHNH}_2}
\begin{array}{c}
\text{NHNH}_2
\end{array}
\end{equation}

\begin{equation}
\begin{array}{c}
\text{C}_7\text{H}_{15}\text{C}^{\text{---}}\text{NHNH}_{2} \\
\text{Octanoic acid}
\end{array}
\xrightarrow{\text{Lipase}}
\begin{array}{c}
\text{C}_7\text{H}_{15}\text{C}^{\text{---}}\text{NHNH}_{2}
\end{array}
\xrightarrow{\text{Lipase}}
\begin{array}{c}
\text{H}_{3}\text{O}
\end{array}
\end{equation}

**Figure 4.** Indirect kinetic resolution of ibuprofen with lipases that do not accept it as an acyl donor. Chemical conversion to the racemic hydrazide converts it to a nucleophile.
Table 3. Acylation of ibuprofen hydrazide with octanoic acid.

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Initial rate* ( \text{(\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1} \cdot \text{g}^{-1})} )</th>
<th>( E )</th>
<th>Enant.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida antarctica A (SP526 on EP 100)</td>
<td>17</td>
<td>6</td>
<td>( R )</td>
</tr>
<tr>
<td>Candida antarctica B (SP525 on EP 100)</td>
<td>3.0</td>
<td>2</td>
<td>( S )</td>
</tr>
<tr>
<td>Candida antarctica B (Novozym 435)</td>
<td>1.6</td>
<td>2</td>
<td>( S )</td>
</tr>
<tr>
<td>Pseudomonas alcaligenes (on EP 100)</td>
<td>12</td>
<td>2</td>
<td>( R )</td>
</tr>
<tr>
<td>Th. lanuginosus SP523 (on EP 100)</td>
<td>6.0</td>
<td>2</td>
<td>( R )</td>
</tr>
<tr>
<td>Pseudomonas lipoprotein (on EP 100)</td>
<td>22</td>
<td>8</td>
<td>( R )</td>
</tr>
<tr>
<td>Rhizomucor miehei (Lipozym IM 20)</td>
<td>1.9</td>
<td>2</td>
<td>( S )</td>
</tr>
<tr>
<td>Candida rugosa (CLEC-CR)</td>
<td>54</td>
<td>2</td>
<td>( S )</td>
</tr>
<tr>
<td>Pseudomonas cepacia (CLEC-PC)</td>
<td>35</td>
<td>2</td>
<td>( R )</td>
</tr>
</tbody>
</table>

*a. Reaction conditions: 110 mg 3 (0.5 mmol), 72 mg octanoic acid (0.5 mmol) and 50 mg immobilized enzyme or 10 mg CLEC in 5 ml isoctane at 40 °C.

Conversion of ibuprofen to 3 makes it a nucleophile and hence it will bind in a different position in the enzyme. For their natural reaction lipases need more space in the nucleophile binding subsite (diacyl glycerol) than in the acyl binding subsite (fatty acid). Hence, lipases generally accommodate quite bulky nucleophiles in the acceptor subsite, whereas the acyl moiety is subject to much more stringent steric restrictions\(^{14}\).

All lipases tested accepted 3 as nucleophile and, although the reaction rates varied widely (Table 3), the reactions were remarkably fast and several lipases accomplished 100% conversion within one day. This contrasts with the sluggish reactions when the ibuprofen moiety is used in the acyl donor role. Novozym 435 typically converts these donors\(^1\) at rates of 0.1 - 1 \(\mu\text{mol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}\). The specific rates of the two cross-linked enzyme crystal (CLEC) preparations were high, but it should be considered that...
CLECs consist of pure protein, contrary to the adsorbed enzymes. From these, the high activity of *C. antarctica* A, *P. alcaligenes* and *Pseudomonas* lipoprotein lipases is notable. The difference in steric restriction of the acyl and nucleophile subsites is underscored by the extremely slow acylation of 1 by ibuprofen which yields the same product (Figure 4). Novozym 435 catalyzed this reaction at an initial rate of only 0.06 μmol·g⁻¹·min⁻¹.

The enantiomeric differentiation of most lipases was disappointingly low in most cases. *Pseudomonas* lipoprotein lipase and *Candida antarctica* lipase A displayed the highest *E* values (8 and 6 respectively) and both enzymes were (R)-specific. The low *E* values are probably caused by the relatively large distance between the nucleophilic nitrogen and the stereogenic center of 3 interatom distances. Moreover, it should be noted that 3 is rather small compared to cholesterol, which is the natural leaving group for *Pseudomonas* lipoprotein lipase. It is to be expected that the fit of 3 in the nucleophile subsite of this enzyme is insufficient for a good discrimination.

**Table 4. Acylation of ibuprofen hydrazide with octanoic acid at room temperature.**

<table>
<thead>
<tr>
<th>Lipase</th>
<th>Initial rate a (μmol·g⁻¹·min⁻¹·g⁻¹)</th>
<th><em>E</em></th>
<th>Enant.</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Candida antarctica</em> A (SP526 on EP 100)</td>
<td>8.3</td>
<td>11</td>
<td>R</td>
</tr>
<tr>
<td><em>Pseudomonas alcaligenes</em> (on EP 100)</td>
<td>8.5</td>
<td>3</td>
<td>R</td>
</tr>
<tr>
<td><em>Pseudomonas</em> lipoprotein (on EP 100)</td>
<td>9.3</td>
<td>26</td>
<td>R</td>
</tr>
</tbody>
</table>

a. Reaction conditions: 110 mg 3 (0.5 mmol), 72 mg octanoic acid (0.5 mmol) and 50 mg immobilized enzyme in 5 ml isoctane.

Lowering the reaction temperature to room temperature produced an increase in enantioselectivity. The three enzymes with the highest initial rate at 40 °C (except the two CLECs) were tested. With *Pseudomonas* lipoprotein lipase the *E* value increased from 8 to 26. For *Candida antarctica* lipase A and *Pseudomonas alcaligenes* lipases, the increase was even more pronounced. However, the enzyme preparations were not reported to be stable below 40 °C.
lipase the increase was smaller. In all three cases the reaction rate was reduced as would be expected.

CONCLUSIONS

The acylation of hydrazine to, eventually, the $N, N'$-diacyl derivative is catalyzed by a variety of lipases. Octanoic acid is an efficient acyl donor, in particular in the second step. Several lipases convert the intermediately formed $N$-acyl hydrazine faster than hydrazine itself.

The chiral hydrazide derived from ibuprofen ($N$-octanoyl-$N'$-2-(4-isobutyl-phenyl) propanohydrazide) was acylated very rapidly, compared to reactions with ibuprofen as acyl donor, by several lipases but the enantioselectivity was generally. Candida antarctica lipase A and Pseudomonas lipoprotein lipase gave the best results. They were both ($R$) specific and the latter catalyzed the reaction with an enantiomeric ratio of 26 when the reaction was performed at room temperature.

MATERIALS AND METHODS

Chemicals

All solvents were of analytical purity and were dried over activated Uetikon CaA zeolite prior to use. All other reagents were purchased from Aldrich or Acros and used as received unless they had to be synthesized.
Enzymes

Novozym 435, SP 523 (*Thermomyces lanuginosus* lipase), SP 525 (*Candida antarctica* lipase B), SP 526 (*Candida antarctica* lipase A) and Lipozym IM 20 (*Mucor miehei* lipase) were obtained from Novo Nordisk. *Pseudomonas alcaligenes* lipase was obtained from Gist-brocades. *Pseudomonas* lipoprotein lipase was obtained from Boeringer Mannheim. CLEC-CR (crosslinked crystals of *Candida rugosa* lipase) and CLEC-PC (cross linked crystals of *Pseudomonas cepacia* lipase) were obtained from Altus Biologics. Enzymes were used as received (Novozym 435, Lipozym IM 20, CLEC-CR and CLEC-PC) or, in case of freeze dried preparations, immobilized on Accurel EP 100 according to a published procedure\(^5\). A slightly different procedure was followed in the immobilization of the lipases from *Candida antarctica* B and *Pseudomonas alcaligenes*.

Accurel EP 100 (1 g) was pretreated as described by Petersen and Eigtved and added to a solution of *Candida antarctica* lipase B (Novo SP525, 0.2 g 35 kLU) in 25 ml 0.01 M phosphate buffer pH 9 (1 LU will liberate 1 μmol of butyric acid from tributyrin per minute). After 20 h shaking at room temperature 8.3 kLU of lipase activity still was found in the supernatant. Hence, an amount of lipase equivalent to 0.15 g of SP525 had been deposited on the catalyst, corresponding to a loading of 13% (w/w). The solid material was removed by filtration, washed with 30 ml buffer and dried *in vacuo* for 16 h at 40 °C.

*Pseudomonas* alcaligenes lipase (0.3 g, 425 kLU) was dissolved in 25 ml water (pH 9 due to buffer salts present in the preparation). After removal of the solid material by centrifugation, 380 kLU of lipase activity was recovered in the supernatant. After addition of Accurel EP 100 (2 g) no residual lipase activity could be detected in the supernatant. Hence the preparation contains 12% (w/w) of lipase.
Chapter 6

Analysis and Equipment

The progress of the reactions of octanoic acid and ibuprofen derivatives was monitored with HPLC on a Waters 8 x 100 mm 4 μ Novapak C₁₈ reversed phase RCM column with a Waters 510 pump, a Shimadzu SPD-6A UV detector, a Shodex RI SE-61 RI detector and a Spectra-Physics SP 4270 integrator. The eluent was 65/35 methanol/water (v/v), containing 0.05 M acetate buffer pH 4.3 at a flow of 1.0 ml/min. Chiral HPLC was performed on a Baker 4.6 x 250 mm 5 μ Chiralcel OD column with a Waters 510 pump and a Waters 486 UV detector operating at 254 nm. As eluent for N-octanoyl-N'-2-(4-isobutylphenyl)-propanohydrazide, 98/2 isopropyl alcohol/hexane was used at a flow rate of 0.5 ml/min.

¹H and ¹³C NMR spectra were recorded on a Varian VXR-400S spectrometer. Mass spectra were recorded on a VG 70 SE spectrometer with the EI method.

Synthesis of starting and reference compounds

Octanohydrazide (1) was synthesized from 10 ml ethyl octanoate (8.78 g, 51 mmol) and 10 ml hydrazine hydrate (206 mmol) in 25 ml methanol. After 48 hours at 40 °C the solvent was evaporated in vacuo and the residue recrystallized from hexane/isopropyl alcohol (20:1, v/v). Yield 6.53 g (81%).

N,N'-dioctanoylhydrazine (2) was synthesized by slowly adding 1.63 g (10 mmol) octanoyl chloride in 10 ml diethyl ether to a vigorously stirred ice-cold solution of 1.58 g octanohydrazide (1) (10 mmol) and 1ml pyridine in 25 ml diethyl ether. After stirring for one hour 35 ml water was added. The heterogeneous reaction mixture was filtrated, the residue washed with a little ether and recrystallized from isopropyl alcohol. Yield 1.99 g (70%).

Racemic- and (S)-ibuprofen hydrazide (4-isobutylphenyl-2-propanohydrazide) (3) was synthesized from 10 g racemic or (S)-ibuprofen methyl ester (45.5 mmol) and 10 ml
Lipase catalyzed formation of diacyl hydrazines

Hydrazine (206 mmol) hydrate in 25 ml methanol. After 48 hours at 40 °C the solvent was evaporated in vacuo and the residue recrystallized from hexane/isopropyl alcohol (20:1 v/v). Yield 7.31 g (73%).

Racemic- and (S)-N-octanoyl-N'(4-isobutylphenyl)-2-propanohydrazide (4) were synthesized as reference compounds from 1.0 g racemic or S-(4-isobutylphenyl)-2-propanoyl chloride (4.45 mmol), 750 mg octanohydrazide (4.75 mmol) and 1 ml pyridine in 50 ml ice-cold tert-butyl methyl ether. After washing three times with 50 ml 0.1 M HCl, three times with 50 ml 0.1 M NaHCO₃ and three times with 50 ml water, the organic layer was dried on Na₂SO₄ and the solvent was evaporated in vacuo. The residue was recrystallized from hexane. Yield 1.31 g (85%).

Reactions

Reactions were performed in 30 ml glass vessels with a teflon coated cap in a stirred thermostatted oil bath at 40 °C or at room temperature. Initial rate measurements with octanoic acid except for the acylation of 3 were performed with 4 mmol of substrate, 2 mmol of nucleophile and 10 mg immobilized enzyme in 10 ml solvent. The initial rate was calculated by fitting a line through the data points at < 10% conversion. Other reactions with ethyl octanoate or octanoic acid were performed with 2 mmol of acyl donor and 1 mmol hydrazine monohydrate and 50 mg of immobilized lipase at 40 °C. The enantioselective reactions were performed with 110 mg (1 mmol) 3, 72 mg octanoic acid (1 mmol) and 50 mg immobilized lipase or 10 mg CLEC in 5 ml isooctane at 40 °C.

Samples of 50 µl were taken at regular intervals with a cut-off pipet from the stirred reaction mixtures, diluted with 200 µl isopropyl alcohol and analyzed by HPLC. If heavy precipitation occurred separate reactions were performed for each data point.
Isolation of the Products

For analytical purposes the 1 and 2 were isolated. 1 was isolated from the reaction mixture of the reaction with Novozym 435 in isooctane after one hour. The solvent was evaporated in vacuo and the residue taken up in tert-butyl methyl ether and filtered to remove any traces of disubstituted product. The organic layer was washed with 0.1 M NaHCO₃, dried over Na₂SO₄ and concentrated by evaporation in vacuo. Recrystallization of the residue from petroleum ether (bp 40-60 °C) afforded the pure product, which was identical with the product of the chemical synthesis.

Compound 2 could be isolated from the reaction mixture with Pseudomonas lipoprotein lipase by simple filtration. Recrystallization of crude 2 from isopropyl alcohol afforded an analytical sample. The same procedure was used for the isolation of 4 from the reaction mixture of the reaction with Pseudomonas lipoprotein lipase after prolonged reaction time. In this case the product was recrystallized from hexane.

Characterization

Structural assignments of the isolated products were made on the basis of ¹H and ¹³C NMR and MS. Both the ¹H and ¹³C NMR spectra were recorded in CDCl₃ at room temperature unless indicated otherwise. Mass spectra were recorded with 70 mV EI.

Octanoylhydrazide (1): ¹H NMR: δ 6.80 (1H, NH, s), δ 3.60 (2H, NH₂, s), (δ 2.15 (2H, CH₂-2, t), δ 1.64-1.80 (2H, CH₂-3, q), δ 1.29 (8H, CH₂-4-7, m), δ 0.88 (3H, CH₃, t). ¹³C NMR: δ 174.01 (C=O), δ 34.62 (CH₂-2), δ 31.66 (CH₂-3), δ 29.24, 28.96 (CH₂-4,5), δ 25.51 (CH₂-6), δ 22.59 (CH₂-7), δ 14.05 (CH₂-8). MS: m/z = 158 (14%), 127 (54%), 109 (10%), 83 (6%), 74 (33%), 57 (100%). mp. 87 °C.

N,N'-dioctanoylhydrazine (2): ¹H NMR: δ 8.25 (2H, NH, s), δ 2.23 (4H, CH₂-2, t), δ 1.65 (4H, CH₂-3, q), δ 1.28 (16H, CH₂-4-7, m), δ 0.88 (6H, CH₃, t). ¹³C NMR: δ 170.91
Lipase catalyzed formation of diacyl hydrazines

(C=O), δ 33.04 (CH₂-2), δ 31.06 (CH₂-3), δ 28.39, 28.32 (CH₂-4,5), δ 24.94 (CH₂-6), δ 21.94 (CH₂-7), δ 13.84 (CH₂-8). MS: m/z = 284 (3%), 158 (71%), 127 (66%), 74 (29%), 57 (100%). mp. 154 °C.

2-(4-isobutyl-phenyl)propanohydrazone (3), ¹H NMR (DMSO-d₆): (propanohydrazone moiety) δ 9.98 (1H, NH,d), 3.60 (1H, CH, q), δ 3.37 (2H, NH₂, br.s), δ 1.32 (3H, CH₃, d), (ring) δ 7.3-7.0 (4H, CH2, 3, 5, 6, m), (isobutyl moiety) δ 2.39 (2H, CH₂, d) δ 1.78 (1H, CH, m), δ 0.93 (6H, CH₃, d). ¹³C NMR: (propanoyl moiety) δ 172.067 (C=O), δ 44.140 (C2), δ 22.077 (C3), (ring) δ 139.261 (C1), δ 138.686 (C4), δ 128.852, 128.627, 126.960 (C2, C3, C5, C6), (isobutyl moiety) δ 42.429 (C1), δ 29.509(C2), δ 18.205, 18.103 (C3, C4). MS: m/z 220 (27%), 188 (23%), 161 (100%), 145 (9%) 119 (30%), 105 (11%), 91 (21%), 57 (25%). Racemate mp 75 °C, (S)-ibuprofen hydrazide mp 92 °C.

N-octanoyl-N’-(4-isobutylphenyl)-2-propanohydrazone (4): ¹H NMR (CDCl₃): (propanohydrazone moiety) δ 9.02 (2H, NH,d), 3.66 (1H, CH, q), δ 1.57 (3H, CH₃, d), (ring) δ 7.21, 7.08 (4H, CH2,3,5,6, dd), (isobutyl moiety) δ 2.42 (2H, CH₂, d), δ 1.83 (1H, CH, m), δ 0.88 (6H, CH₃, m), (octanoyl moiety) δ 2.21 (2H, CH₂, t), δ 1.60 (2H, CH₂, t), δ 1.13 (8H, CH₂, m), δ 0.88 (3H, CH₃, s). ¹³C NMR: δ 170.765, 169.586 (C=O), (propanoyl moiety), δ 45.042 (C2), δ 22.390 (C3), (ring) δ 140.980 (C1), δ 137.304 (C4), δ 129.603, 127.288 (C2, C3, C5, C6), (isobutyl moiety) δ 44.409 (C1), δ 29.174(C2), δ 18.350 (C3, C4), (octanoyl moiety) δ 34.051, 31.635, 30.150, 28.970, 25.331, 22.594 (C2-C7), δ 14.041 (C8). MS: 328 (6%), 285 (11%), 244 (12%), 220 (13%), 161 (100%), 149 (17%), 119 (26%), 91 (21%), 57 (43%). mp. 115 °C (racemate), 138 °C (S-enantiomer).

REFERENCES

Chapter 7

Lipase catalyzed $O$-Acylation of hydroxamic acids with $in situ$ Lossen rearrangement

Summary

Octanohydroxamic acid can be $O$-acylated with a lipase and vinyl acetate. The reaction product, $O$-acetyl-$N$-octanoyl hydroxylamine can be isolated but easily degrades to form $N,O$-dioctanoyl hydroxylamine, which can be isolated in 64% yield. Attempts to resolve ibuprofam by lipase catalyzed $O$-acylation were unsuccessful. The starting material was consumed in the course of the reaction forming several products but the remaining ibuprofam was racemic.
INTRODUCTION

Lipases are able to catalyze reactions with several unnatural oxygen nucleophiles, in which oxygen is not connected to a sp$^3$ hybridized carbon, e.g. hydrogen peroxide$^{1,2}$, alkyl hydroperoxides$^3$, phenols$^4$ and oximes$^5$. The latter two are electron deficient and when normal esters or acids are used as acyl donor the thermodynamic equilibrium is rather unfavorable. When strongly activated esters or so called irreversible acylating agents are used it is possible to push the thermodynamic equilibrium towards the synthesis side (Figure 1). In the case of irreversible acylating agents isomerization or disintegration of the liberated compound prevents the backward reaction as illustrated for vinyl esters and dialkyl dicarbonates, respectively, in Figure 1.

![Chemical Structures](image)

**Figure 1.** Transesterifications with electron deficient oxygen nucleophiles.
All three esters in Figure 1 are activated but in the case of di-tert-butyl dicarbonate and isopropenyl acetate one reaction product loses CO$_2$ or isomerizes to acetone, respectively, which makes the reaction irreversible.

A variety of substituted hydroxamic acids is currently under investigation as e.g. protease inhibitors$^6$ and anti-bacterial compounds$^7$. Hydroxamic acids have a structure related to oximes but their pK$_a$ of about 9$^8$ puts them closer to carboxylic acids than to alcohols. Lipase catalyzed acylation of a hydroxamic acid would not only constitute a potential route to chiral hydroxamic acids but also to chiral amines via Lossen rearrangement of the O-acylated derivate (Figure 2). The latter is an important method for converting a carboxylic acid to an amine bearing one carbon atom less. The Lossen rearrangement is closely related to the Hofmann reaction and takes place with retention of configuration. Both proceed through an acylnitrone intermediate that is highly unstable and rearranges to an isocyanate that is in most cases hydrolyzed under the reaction conditions (Figure 2). The conversion of the O-acyl hydroxamic acid to the acylnitrone is the rate determining step. Under mild conditions the O-acyl intermediate can be isolated.

![Figure 2. The Lossen rearrangement of the acylated enantiomer of a chiral hydroxamic acid.](image)

As far as we know only one example of an enzyme-catalyzed O-acylation of a hydroxamic acid is known$^9$. The reaction was not stereospecific and the authors did
not mention any degradation of the reaction product. An interesting possibility could be a two step synthesis of an optically active amine by two consecutive enzymatic conversions, starting from a racemic carboxylic acid, e.g. ibuprofen, or its ester. The racemic carboxylic acid or ester is used as acyl donor to acylate hydroxylamine. The resulting optically active hydroxamic acid is then used as the acyl acceptor in the second step which will enrich the enantiomeric purity of the final product, assuming that the stereospecificity of the two enzymes is the same. In the second step of this reaction sequence, an enzyme can be used that does not accept ibuprofen derivatives as a substrate because the hydroxamic acid binds in the, generally much more accessible, nucleophile binding site.

RESULTS AND DISCUSSION

To demonstrate the above concept we isolated the Lossen rearrangement precursor from a reaction mixture. During the acylation of octanohydroxamic acid with vinyl acetate and Novozym 435 in tert-butyl alcohol substantial amounts of the O-acetylated product were observed, especially when a large amount of enzyme was used at room temperature and a low concentration of hydroxamic acid. The conditions of the enzymatic reaction were mild enough to give the O-acylated intermediate an acceptable lifetime for isolation, whereas it would react immediately under chemical Lossen rearrangement conditions (pH 9 and 60 °C).

![Diagram](image_url)

**Figure 3.** The formation of O-acetyl octanohydroxamic acid.
Acylation of hydroxamic acids

Simple evaporation of the solvent in vacuo and dilution with hexane precipitated O-acetyl octanohydroxamic acid (1) (28%). The ratio of 1 to other products decreased gradually after longer reaction times. Several degradation products were formed, some of which had very long retention times on HPLC, which made analysis laborious. At higher reaction temperatures two degradation products were predominantly present, in equal amounts that together made up about 80% of the reaction mixture by refractive index area. Strangely enough, evaporation of the solvent or recrystallization converted the more apolar product into the other compound. The more polar product proved to be N,O-dioctanoyl hydroxylamine, the structure of the other compound could not be determined. N,O-Dioctanoylhydroxylamine seems to be more stable than 1, probably because of extra steric shielding of the N-H by the second octanoyl moiety. It could be isolated in 64% yield by evaporation of solvent followed by recrystallization from hexane. The mechanism by which this compound is formed is uncertain but it is probable that a series of rearrangements took place. For instance the intermediate heptylisocyanate may be able to acylate octanohydroxamic chemically, leading to another type of Lossen rearrangement precursor.

Lipase catalyzed acylation of ibuproxam

From previous work\textsuperscript{10} it was known that \textit{Pseudomonas} lipoprotein lipase catalyzes the acylation of the R-enantiomer of ibuprofen hydrazide with reasonable selectivity. Because of the structural similarity between ibuprofen hydrazide and ibuproxam we reasoned that it could be possible to resolve ibuproxam via acylation in the presence of this enzyme (Figure 2).

Several attempts were made to resolve ibuproxam using different conditions and vinyl esters. In all cases ibuproxam was converted but no single product could be obtained. At approximately 50% conversion, samples were taken and derivatized after
column separation. The unreacted ibuproxam was racemic. It is possible that the reaction was completely non-selective but we cannot rule out the possibility of a chemical transesterification in the reaction mixture or chemical hydrolysis during the work up of the sample.

![Chemical reaction diagram]

**Figure 4.** The lipase catalyzed acylation of R,S-ibuproxam, with a R-specific lipase and a vinyl ester, leaves the biologically active S-enantiomer intact and the R-enantiomer rearranges to R-(1-(4-isobutyl)phenylethylamine).

A two step reaction using *Candida antarctica* lipase B and *Pseudomonas* lipoprotein lipase with ibuprofen methyl ester as acyl donor, gave a complex mixture of compounds. The first step was identical to the hydroxylaminolysis reaction of ibuprofen methyl ester, described in Chapter 4. After removal of hydroxylamine and solvents this mixture was used as starting material for the acylation of optically active ibuproxam (71% ee of the R-enantiomer). It was not possible to determine the enantiomeric excess of the ibuproxam by derivatisation and subsequent chiral HPLC
analysis after the second step. Hence it is not possible at this point to determine whether the acylation reaction is not selective or whether there is a racemization mechanism responsible for the apparent lack of selectivity.

CONCLUSIONS

Acylated hydroxamic acids are very unstable but can be isolated from reaction mixtures. Because of the multitude of possible side reactions and rearrangements it is very difficult to achieve a high yield of a single product. The acylation of ibuproxam with vinyl acetate or vinyl laurate was not enantioselective but this may be caused by subsequent chemical reactions in the reaction mixture.

MATERIALS AND METHODS

Materials

Hydroxylamine was purchased as a 50% aqueous solution (17 M) from Merck (Note: higher concentrations of the free base are unstable or explosive). All solvents were of analytical purity and were dried on activated Uetikon CaA zeolite prior to use. All other reagents were purchased from Aldrich or Acros and used as received.

Analysis and Equipment

The derivatives of octanoic acid and ibuprofen were analyzed by HPLC on a Waters 8 x 100 mm 4 μm Novapak C18 reversed phase RCM column with a Waters 510 pump, a Shimadzu SPD-6A UV detector, a Shodex RI SE-61 RI detector and a Spectra-
Physics SP 4270 integrator. The eluent was 65/35 methanol/water (v/v), with 0.05 M acetate buffer pH 4.3 at a flow of 1.0 ml/min.

Chiral HPLC was performed on a Baker 4.6 x 250 mm 5 μm Chiralcel OD column with a Waters 510 pump and a Waters 486 UV detector operating at 254 nm. As eluent for all chiral substrates 98/2/0.1 hexane/isopropyl alcohol/formic acid was used at a flow rate of 0.5 ml/min.

Derivatisation of ibuprofen for chiral HPLC was performed by the same method as described in Chapter 4.

$^1$H and $^{13}$C NMR spectra were recorded on a Varian VXR-400S spectrometer.

Mass spectra were recorded on a VG 70 SE spectrometer with the EI method.

Reactions

Reactions were performed in 30 ml glass vessels with a teflon coated cap, in a stirred thermostated oil bath at room temperature or 40 °C. The enzyme and the solvent were stirred for one hour for thermal stabilization and wetting of the enzyme.

Synthesis of O-acetyl-N-octanoyl hydroxylamine: 159 mg (1 mmol) octanohydroxamic acid and 50 μl diethylene glycol dibutyl ether (internal standard) were dissolved in 2.5 ml tert-butyl alcohol and 500 μl vinyl acetate was added. After incubation with 50 mg Novozym 435 at room temperature for 48 hours almost all starting material had reacted. The enzyme was filtered off and O-acetyl-N-octanoyl hydroxylamine was isolated in 28% yield by evaporating the solvent and low boiling components of the reaction mixture thoroughly in vacuo and recrystallizing the residue from petroleum ether (bp 40-60 °C).

Synthesis of N,O-dioctanoylhydroxylamine: A suspension of 1.0 g (6.3 mmol) octanohydroxamic acid in 5 ml tert-butyl alcohol, 4 ml hexane and 1 ml vinyl acetate
was incubated for 24 hours at 40 °C with 200 mg Novozym 435. After this period practically all starting material had reacted according to HPLC and the mixture had become homogeneous. The enzyme was filtered off and \( N,O \)-dioctanoylhydroxylamine was isolated in 64% yield by evaporating the solvent and low boiling components of the reaction mixture thoroughly \textit{in vacuo} and recrystallizing the residue from hexane.

Kinetic resolution of ibuproxam: A suspension of 1 mmol ibuproxam, 50 \( \mu \)l 1,3-dimethoxybenzene (internal standard) and 1 mmol vinyl acetate or 1 mmol lauryl acetate in 10 ml isooctane was incubated at room temperature with 50 mg \textit{Pseudomonas} lipoprotein lipase on EP 100. After 48 hours the reaction mixture had become homogeneous but the conversion was almost complete. An other experiment was stopped after 24 hours when the reaction mixture was still a suspension (vinyl laurate, 48% conversion, multiple products). The reaction mixture was homogenized by adding isopropanol and the enzyme was filtered off. The solvent was evaporated \textit{in vacuo} and the residue taken up in \textit{tert}-butyl methyl ether. The residual ibuproxam was isolated by separation from other components over a silica column with hexane/ethyl acetate 90/10 as eluent. Ibuproxam was washed from the column with ethyl acetate. Derivatisation according to the procedure in Chapter 4 showed no enantiomeric excess.

Two enzyme reaction: 2 mmol ibuprofen methyl ester and 2.5 mmol hydroxylamine in 5 ml \textit{tert}-butyl alcohol were incubated with 200 mg Novozym 435 for 70 hours. The conversion was 41% and \( R \)-ibuproxam was formed in 71% ee. Acetone was added to the reaction mixture to destroy the excess hydroxylamine and the solvent was evaporated \textit{in vacuo}. The residue was coevaporated with benzene three times to eliminate residual water. The oily residue was used as starting material for acylation as described above.
Chapter 7

Characterization

Structural assignments of the isolated products were made on the basis of $^1\text{H}$ and $^{13}\text{C}$ NMR. Both the $^1\text{H}$ and $^{13}\text{C}$ NMR spectra were recorded in CDCl$_3$ at room temperature.

$O$-Acetyl-$N$-octanoylhydroxylamine, $^1\text{H}$ NMR: $\delta$ 9.08 (1H, NH, s), $\delta$ 2.25 (2H, CH$_2$-2, t), $\delta$ 2.22 (3H, CH$_3$-acetyl, s), $\delta$ 1.68 (2H, CH$_2$-3, qi), $\delta$ 1.3 (8H, CH$_2$-4-7, m), $\delta$ 0.88 (3H, CH$_3$, t). $^{13}\text{C}$ NMR: $\delta$ 171.66 (C=O), $\delta$ 168.94 (C=O) $\delta$ 32.984 (C2), $\delta$ 31.637 (C3), $\delta$ 29.09, 28.92 (C4,5), $\delta$ 25.06 (C6), $\delta$ 22.58 (C7), $\delta$ 14.05 (C8).

$N,O$-Dioctanoylhydroxylamine, $^1\text{H}$ NMR: $\delta$ 8.92 (1H, NH, s), $\delta$ 2.43 (2H, CH$_2$, t), $\delta$ 2.21 (2H, CH$_2$, s), $\delta$ 1.6 (4H, CH$_2$-3, m), $\delta$ 1.3 (16H, CH$_2$-4-7, m), $\delta$ 0.87 (6H, CH$_3$, t). $^{13}\text{C}$ NMR (DMSO): $\delta$ 171.149 (C=O), $\delta$ 169.684 (C=O) $\delta$ 31.797, $\delta$ 31.031, $\delta$ 30.986, 30.849, $\delta$ 28.277, $\delta$ 28.254, $\delta$ 28.163, $\delta$ 28.110, $\delta$ 24.666, $\delta$ 24.279, $\delta$ 21.927, $\delta$ 21.911, $\delta$ 13.824.

Chemical ionization mass spectrometry: M= 285. Elemental analysis 67.27\% C, 11.13\% H, 5.18\% N, 16.5\% O. Theoretically C$_{16}$H$_{31}$NO$_3$ (285.24) 67.31\% C, 10.95\% H, 4.91\% N, 16.83\% O. mp 66-68 °C (decomposition).

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SUMMARY

The subject of this thesis is the lipase catalyzed acylation of compounds containing a nitrogen atom that acts as the nucleophilic atom. In vivo lipases hydrolyze fats and oils but they are also capable of esterification, transesterification and even aminolysis. They are rather stable enzymes, have a broad substrate specificity, are not dependent on a cofactor and some of them display activity in organic solvents. Chapter 1 is a literature overview of work in the field of nitrogen nucleophiles with lipases. A variety of reactions with amines, hydroxylamines and hydrazines is reviewed. Lipases catalyze the formation of a very different array of compounds and can do this in a stereo- or enantioselective manner.

In Chapter 2 the acylation of amines with dibutyl- and dibenzyl carbonate is described. Carbonic acid esters are accepted as an acyl-donor by some lipases such as Candida antarctica lipase B and acylate amines to form carbamates. The method is used to acylate a number of chiral amines enantioselectively with dibenzylcarbonate to form optically active Z-amines.

A combination of enantioselective ammonolysis and in situ racemization of the substrate is investigated in Chapter 3. This so-called dynamic kinetic resolution of phenylglycine methyl ester to D-phenylglycine amide yields more of the desired enantiomer than a normal kinetic resolution. In this process aromatic aldehydes were used as racemization catalysts; they proved to be compatible with ammonia in the reaction mixture.

In Chapter 4 and 5 the lipase-catalyzed acylation of hydroxylamine and hydrazine derivatives is described with a variety of (chiral) carboxylic acids and esters. Hydroxylamine and hydrazine derivatives are similar in certain aspects of their behavior. They are less basic but more nucleophilic than the corresponding amines. This is why, in contrast to aliphatic amines, they can be acylated with carboxylic acids rapidly in water and organic solvents. The method can be used to prepare S-ibuproxam, the biologically active enantiomer of a prodrug of ibuprofen, and some of its analogues with high optical purity.
Hydrazine has two nucleophilic nitrogen atoms and although it has a very low basicity after monoacylation, it still has nucleophilic properties. As described in Chapter 6, lipases catalyze the double acylation of hydrazine and the enantioselective acylation of chiral hydrazides. *Pseudomonas* lipoprotein lipase is able to rapidly acylate and resolve the hydrazide of ibuprofen though the enzyme does not accept ibuprofen as a substrate.

In chapter 7 the *O*-acylation of hydroxamic acids to form a Lossen rearrangement precursor is described. The rather unstable *O*-acetyl-*N*-octanoyl hydroxylamine could be isolated and characterized but attempts to acylate a chiral hydroxamic acid were unsuccessful.
SAMENVATTING

Dit proefschrift behandelt de lipase gekatalyseerde acylering van verbindingen die een nucleofiel stikstof atoom bevatten. In de natuur hydrolyseren lipasen oliën en vetten maar ze kunnen ook veresterings, transveresterings en zelfs aminolyse katalyseren. Lipasen zijn robuuste enzymen, met brede substraat specificiteit, ze zijn niet afhankelijk van een co-factor en sommige lipasen vertonen activiteit in organisch milieu.

Hoofdstuk 1 geeft een overzicht van de literatuur op het gebied van reacties van lipasen met stikstof nucleofielen. Diverse reacties met amines, hydroxylamines en hydrazines worden belicht. Lipasen zijn in staat verschillende typen stikstofverbindingen stereo- en enantioselectief te acyleren.

In Hoofdstuk 2 wordt de acylering van amines met dibutyl- en dibenzylcarbonaat beschreven. Koolzure esters worden als acyl donor geaccepteerd door sommige lipasen, zoals Candida antarctica lipase B, onder vorming van carbamaten. Deze methode wordt gebruikt om door enantioselectieve aminolyse van dibenzylcarbonaat optisch actieve Z-amines te maken.

Een combinatie van enantioselectieve ammonolyse en racemisatie van het substraat wordt beschreven in Hoofdstuk 3. Door deze zogenaamde dynamische kinetische resolutie wordt D-fenylglycine amide (zijkten voor antibiotica) gemaakt uit de racemische methyl ester. In dit proces werden aromatische aldehyden gebruikt als racemisatie katalysator, dit bleek mogelijk in de aanwezigheid van ammoniak. Door middel van deze methode wordt een hogere opbrengst van het gewenste enantiomeer gevormd dan bij een gewone kinetische resolutie.

In Hoofdstuk 4 and 5 wordt de lipase gekatalyseerde acylering van hydroxylamine- en hydrazine derivaten beschreven met verschillende (chirale) carbonzuren en esters. Hydroxylamine en hydrazine derivaten hebben bepaalde overeenkomsten in hun chemisch gedrag. Ze zijn zwakker basisch maar sterker nucleofiel dan overeenkomstige amines. Ze worden hierdoor gemakkelijker geacyleerd met carbonzuren in de aanwezigheid van lipasen dan amines, door de beperktere ionpaar
vorming. In sommige gevallen kon de reactie worden uitgevoerd in, of in de aanwezigheid van, water. Condensatie van ibuprofen en hydroxylamine in water onder invloed van Candida rugosa lipase CLEC leverde enantiomeer zuiver S-ibuproxam op; het biologisch actieve enantiomeer van een prodrug van ibuprofen. Hydrazine heeft twee nucleofiele stikstofatomen. Monoacylhydrazines hebben praktisch geen basische eigenschappen meer maar zijn nog wel nucleofiel. In Hoofdstuk 6 wordt de lipase gekatalyseerde dubbele acylering van hydrazine beschreven en de enantioselectieve acylering van chirale hydrazides. Pseudomonas lipoprotein lipase is in staat om de acylering van het hydrazide van ibuprofen enantioselectief te katalyseren met een hoge reactiesnelheid hoewel het enzym ibuprofen zelf niet accepteert als substraat.

In Hoofdstuk 7 wordt de vorming van Lossen rearrangement precursors door lipase gekatalyseerde O-acylering van hydroxamzuren beschreven. Het bleek mogelijk het labiele O-acetyl-N-octanoyl hydroxylamine te isoleren maar pogingen om de reactie enantioselectief uit te voeren zijn niet geslaagd.
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