Alternative routes to activated side-chain donors of \( \beta \)-lactam antibiotics

Margreth A. Wegman
Stellingen

behorende bij het proefschrift

'Alternative routes to activated side-chain donors of β-lactam antibiotics'

van Margreth A. Wegman

1. In het onderzoek doe je niets even.

2. Wanneer een bepaalde route nooit eerder is onderzocht, betekent dit niet dat deze (thermodynamisch) onmogelijk is.

3. De lage carboxylase activiteit van B. megaterium pyruvool-2-carboxylaat decarboxylase met CO₂, in plaats van HCO₃⁻, is het gevolg van een te lage pH.


4. Het is een misvatting dat p-hydroxyphenylhydantoïne en phenylhydantoïne dezelfde eigenschappen bezitten en beide moeten dan ook getest zijn voordat ze als substraat voor de recombinante E. coli stam vermeld mogen worden.


5. De lage ee van het niet omgezette substraat (R)-α-n-propylacetamide is geen aanwijzing voor de aanwezigheid van een racemase.


6. Het bepalen van de hoeveelheid geïmmobiliseerd enzym en actief geïmmobiliseerd enzym bij verschillende temperaturen is zinloos.


7. De suggestie dat de ontleding van het product (S)-2-hydroxy-(3-phenoxy)phenylacetonitrile lipase gekatalyseerd is, wordt onvoldoende onderbouwd.


8. Het bepalen van het aantal vrije carboxylgroepen in polyacrylzuur na immobilisatie van alcohol dehydrogenase zegt niets over de immobilisatie opbrengst.

9. Inhalatie-insuline is de neus ophalen voor het echte probleem.


Alternative routes to activated side-chain donors of β-lactam antibiotics
The cover shows a four-leaved clover representing the four alternative routes to activated side-chain donors as described in this thesis.

Designed by Maaike C. Wegman.
Alternative routes to activated side-chain donors

of $\beta$-lactam antibiotics

Proefschrift

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Towards green synthesis of β-lactam antibiotics

Abstract
The production of β-lactam antibiotics is undergoing a remarkable transformation. Traditional chemical processes are gradually being replaced by biological reactions, such as fermentation, bioconversion and enzymatic catalysis. An overview is given of the development of processes for the manufacture of 6-aminopenicillanic acid (6-APA), 7-aminodesacetoxycephalosporanic acid (7-ADCA), side-chain donors and semi-synthetic penicillins and cephalosporins.
Chapter 1

Introduction

The growth of the chemical industry during the 20th century has been substantially higher than that of industry in general.\(^1\) Its general development from 1950 up to the middle of 1970s was characterised by the production of goods and services aimed at satisfying the ever-growing consumer demands. Negative aspects, such as the depletion of non-renewable resources and the effects of waste products on the environment were generally disregarded.\(^2\) International concern for this trend led to the organisation of the Conference on Environment at Stockholm by the United Nations in 1972.\(^3\) Twenty years later international agreements were concluded at the United Nations Conference on Environment and Development\(^4\) about improving economic activity, the rational use of materials and energy, and the quality of life and of the environment. Consequently, the chemical industry had to change from a degradative to a sustainable mode.\(^5\)

De greening of fine chemistry

An urgent problem with respect to sustainability is the depletion of the resources oil, gas and coal, even more since it is expected that the need for raw fossil materials will strongly increase.\(^6\) They are the most important sources of energy, but also of raw materials for the production of chemicals and polymers. However, up through 1990 only 8% of all the fossil fuels was used for industrial purposes.\(^7\) Apart from the raw materials issue, the numerous environmental regulations resulted in increasing costs of waste disposal and the prohibition of certain materials such as phosphates in detergents.

The growing awareness of the environment has brought about a search for an alternative cleaner technology. The past decades enzyme catalysis has become increasingly important in fine chemicals industry.\(^8-11\) The high chemo-, regio-, and enantiospecificity of enzymes under mild reaction conditions - aqueous medium, neutral pH and ambient temperature - avoid the use of organic solvents as well as the reactive group protection/deprotection sequences, resulting in low-waste processes.

The first application of hydrolytic enzymes was their use in the manufacture of washing agents.\(^12\) However, the manufacture of β-lactam antibiotics has spearheaded the introduction of biocatalysis in the fine chemicals industry and is an elegant example of a green development. Subsequent to the successful introduction of the enzymatic hydrolysis of penicillin G in the early 1990s, enzyme catalysed synthesis of β-lactam

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\(^1\) The World Commission on Environment and Development gives the most commonly used definition of sustainable development:\(^5\) Development that meets the needs of the present generations without compromising the ability of future generations to meet their own needs.
antibiotics became highly desirable in order to reduce or eliminate the use of environmentally detrimental chemicals and solvents. The green development of the manufacture of β-lactam antibiotics will be discussed in this chapter.

In conclusion, the proper goal is to develop green processes, which are more efficient in energy, more precise in raw materials and moreover, which afford superior products.

Reduction of waste streams in the fine chemicals industry

Clean and highly selective catalytic procedures have gradually replaced traditional methods in the manufacture of bulk chemicals, such as e.g. vinyl chloride\textsuperscript{13} and acrylonitrile.\textsuperscript{14} In contrast, crude textbook-type, sometimes greatly outdated, procedures are still widely used in the manufacture of fine chemicals. The focus used to be mainly on chemical yield and less on energy efficiency and environmental care.\textsuperscript{15} The generation of large amounts of waste was considered to be acceptable because waste disposal was generally not a problem in the small-scale fine chemicals industry.

Especially the pharmaceutical industry generates an unproportionately large amount of waste in relation to the desired products. The production of pharma products often involves multi-step procedures and in each step reagents are consumed, and waste is generated in stoichiometric amounts. Moreover, the acid/base neutralisation reactions that are widely applied generate large waste streams of inorganic salts, such as sodium chloride or ammonium sulfate, which cannot be reused. Furthermore, the large volumes of organic solvents, in particular halogenated solvents that are used also contribute to waste production. The amount of by-products per kg product, the \textit{E} factor,\textsuperscript{16,17} for the various segments of the chemical industry is shown in Table 1.1.

<table>
<thead>
<tr>
<th>Industry division</th>
<th>Product tonnage/year</th>
<th>kg by-product/kg product</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oil refining</td>
<td>$10^6 - 10^8$</td>
<td>&lt;0.1</td>
</tr>
<tr>
<td>Bulk chemicals</td>
<td>$10^4 - 10^6$</td>
<td>&lt;1 - 5</td>
</tr>
<tr>
<td>Fine chemicals</td>
<td>$10^2 - 10^4$</td>
<td>5 - 50</td>
</tr>
<tr>
<td>Pharmaceuticals</td>
<td>$10 - 10^3$</td>
<td>25 - &gt;100</td>
</tr>
</tbody>
</table>

There is now a marked trend, however, towards higher energy costs and more expensive waste disposal. Moreover, accidental release of hazardous substances is, to an increasing degree, socially unacceptable and limits of allowed employee exposure are progressively tightened. The solution is to be found in the adoption of selective and atom-efficient
catalytic procedures, in imitation of the corresponding revolution in the bulk-chemicals industry alluded to above.\textsuperscript{20}

However, introduction of chemocatalysis as developed in the bulk-chemicals industry could not be easily introduced into the fine-chemicals industry. The fine chemical manufacture is characterised by a large range of different products, often produced in small quantities, which have a relative short economic lifetime. In general, large-scale process technologies from the bulk chemical manufacture were followed and no dedicated fine chemical process engineering was developed.\textsuperscript{19,21} However, fine chemicals are usually complex multi-functional molecules with high boiling points and limited thermal stability. This is often not compatible with the conditions used in the petrochemical catalysis (high pressure and temperature). The introduction of biocatalysis, which is readily compatible with the nature of fine chemicals synthesis, resulted in a markedly improvement of efficiency.\textsuperscript{22}

\textit{The fine chemicals industry in the Netherlands}

The petro- and bulk chemicals clearly contributed most to the huge growth of the chemical industry. However, the fine chemicals and the special products have grown notably during the last 25 years and major, rapid, improvements in efficiency have been made in particular in biocatalysis. Internationally, the Netherlands is one of the largest producers of fine chemicals with a share of 4 to 5\% of the world market. The market share of the Netherlands in the pharmaceutical fine chemicals sector is even 8 to 10\%; hence it is one of the major suppliers to the pharmaceutical industry throughout the world.\textsuperscript{23} To maintain this position, new and environmentally benign processes have gradually been developed, although the number of processes that have actually been commercialised is still relatively small.

\textbf{Semi-synthetic β-lactam antibiotics up to 1985: the high-water mark of chemical synthesis}

The industrial production of semi-synthetic antibiotics has a history of about 40 years. Efficient chemical procedures to semi-synthetic penicillins (derived from 6-aminopenicillanic acid, 6-APA) and semi-synthetic cephalosporins (derived from 7-aminodesacetoxycephalosporanic acid, 7-ADCA) have been developed over this time span. (Figure 1.1).
Towards green synthesis of β-lactam antibiotics

Figure 1.1 The semi-synthetic penicillins and cephalosporins.

Up to 1985, the semi-synthetic penicillins and cephalosporins were obtained by chemical condensation of a protected β-lactam nucleus and an activated protected side-chain. Apart from the fermentation of *Penicillium*, the routes to the protected nuclei and the activated side-chains also involved mainly stoichiometric chemical reaction steps (Scheme 1.1).

Scheme 1.1 Traditional chemical synthesis of β-lactam antibiotics.
Nearly all of these involved organic (often halogenated) solvents, toxic auxiliaries and very low temperatures. The processes involved will be discussed in more detail later in this chapter. As an illustration, the synthesis of cephalixin, with an annual consumption of almost 3000 t, the largest cephalosporin on the world market, generates 30-40 kg waste per kg of end-product. Nowadays, β-lactam antibiotics are still largely produced on an industrial scale using these chemical stoichiometry-based processes with the biocatalytic hydrolysis of penicillin G to 6-APA as a notable exception. It is obvious that environmentally benign production processes for β-lactam antibiotics are highly desirable.

Green routes to 6-APA

In 1928 Alexander Fleming proved that a cell-free extract of a growing mold *Penicillium notatum* culture was active against a variety of bacteria. Flory and Chain finally isolated penicillin G in 1940, followed by its chemical characterisation and clinical use. Unfortunately, an increasing number of bacterial strains became resistant to penicillin G. A breakthrough was the discovery that the nucleus of penicillin G, 6-APA, could be acylated with various side-chains to yield new active semi-synthetic penicillins. In the early 1960s 6-APA was produced by either direct fermentation or enzymatic deacylation of penicillin G. However, both methods were inconvenient, due to low productivity and complex product and enzyme recovery, which necessitated an alternative route.

Efficient deacylation of penicillin G is not trivial, because it contains a tertiary as well as a secondary amide functionality. The secondary one should be cleaved whereas the tertiary one is more susceptible to basic and nucleophilic conditions. An efficient chemical deacylation process of penicillin G was ultimately developed and commercialised by DSM Gist-brocades (at that time Nederlandsche Gist en Spiritusfabriek). The selective cleavage of the secondary amide bond was accomplished by the formation of an imine chloride, using phosphorus pentachloride in dichloromethane, which was subsequently hydrolysed. The key step in this process was the use of the silyl group for the protection of the penicillin carboxyl group. This inexpensive one-pot synthesis of 6-APA is often referred to as ‘Delft Cleavage’ (Scheme 1.2).
Towards green synthesis of β-lactam antibiotics

Scheme 1.2 Chemical deacylation of penicillin G into 6-APA.

Notwithstanding the high energy input due to the low operating temperature and the use of hazardous, highly active chemicals, which are difficult to recover and reuse, the chemical process was universally favoured over the microbial routes mentioned earlier and was used for many years.

However, due to the growing environmental concerns, the enzymatic hydrolysis of penicillin G was again subjected to extensive research. A great step forward was the development of immobilised preparations of purified penicillin acylase, which made recycling of the biocatalyst possible. The enzymatic cleavage proceeded at a slightly elevated temperature in water rather than in halogenated solvent and 6-APA was obtained in nearly quantitative yield (Scheme 1.3). Economic, environmental and operational advantages of the enzymatic process over the chemical process have been realised with the immobilised preparations and the bulk of 6-APA is nowadays produced via enzymatic deacylation of penicillin G.

Scheme 1.3 Enzymatic deacylation of penicillin G to 6-APA.
Green routes to 7-ADCA

In 1953 a new β-lactam antibiotic - cephalosporin C - was isolated from the fermentation broth of *Cephalosporium acremonium*.\(^{35}\) Although its antibacterial activity was too low for therapeutic use, it was argued that the deacylated product 7-aminoccephalosporanic acid (7-ACA) could become as important as 6-APA in the development of highly active, non-toxic antibiotics. However, microbial methods to obtain 7-ACA, such as direct fermentation or enzymatic deacylation of cephalosporin C, which had been successful in the synthesis of 6-APA, failed.\(^{36}\)

Hence, the chemical approach was under investigation again and the first chemical cleavage of cephalosporin C was reported in 1961, although 7-ACA was obtained in an extremely low yield.\(^{37}\) Promptly, Eli Lilly\(^{38}\) and Ciba Geigy\(^{39}\) developed more practical selective deacylation methods (yields from 40 to 75%). However, at Gist-brocades the 'Delft Cleavage' using silyl protection for the synthesis of 6-APA,\(^{30}\) was successfully adapted to the synthesis of 7-ACA starting from cephalosporin C\(^{40}\) (Scheme 1.4).

![Chemical synthesis of 7-ACA from cephalosporin C.](Image)

**Scheme 1.4** Chemical synthesis of 7-ACA from cephalosporin C.

However, the fermentation of *A. chrysogenum* yielded, besides cephalosporin C significant quantities of desacetoxycephalosporin C and desacetylcephalosporin C (Scheme 1.5). Hence, although cephalosporin C could efficiently be deacylated chemically, the fermentation and, moreover, the isolation of cephalosporin C itself resulted in a low yield.
Towards green synthesis of \( \beta \)-lactam antibiotics

\textit{Acremonium chrysogenum}

\[ \text{L-}\alpha\text{ aminoadipic acid } + \text{ L-cysteine } + \text{ L-\textalpha-}\text{valine} \]

\[ \text{ACV tripeptide} \]

\[ \text{Isopenicillin N} \]

\[ \text{Expandase} \]

\[ \text{Penicillin N} \]

\[ \text{Expandase / Hydroxylase} \]

\[ \text{Desacetoxycephalosporin C} \]

\[ \text{Desacetylcephalosporin C} \]

\[ \text{Cephalosporin C} \]

\textbf{Scheme 1.5} Biosynthesis of cephalosporin C.

This obstacle initiated studies into the relationship between cephalosporins and penicillins. It was found that a cephalosporin nucleus could be obtained via chemical, acid-catalysed ring expansion of the penicillin nucleus.\(^{41}\) As a consequence, the 3-substituent was a methyl group rather than an acetoxy group and 7-ADCA was obtained. Again the one-pot procedure, using silyl protection\(^{30}\) was successfully adapted to the synthesis of 7-ADCA\(^{42}\) (Scheme 1.6).

\[ \text{penicillin G} \]

\[ \text{CH}_3\text{COOH} \]

\[ (> 90\% \text{ yield}) \]

\[ \text{H}^+ \]

\[ \text{7-ADCA} \]

\[ (> 90\% \text{ yield}) \]

\[ \text{PCl}_5, \text{ ROH or amidase} \]

\[ (> 80\% \text{ yield}) \]

\textbf{Scheme 1.6} Synthesis of 7-ADCA from penicillin G.
Similar to the 6-APA process, the chemical deacylation was recently replaced by a simple enzymatic conversion, using an immobilised penicillin acylase.\textsuperscript{43} Within DSM Anti-Infectives several plants are producing 7-ADCA from penicillin G.\textsuperscript{34}

In summary, the industrial manufacture of 7-ADCA\textsuperscript{34} involves complex chemical steps for the ring expansion of penicillin G, which are expensive and environmentally unfriendly, followed by enzymatic deacylation of the side-chain. Hence, there was a great desire to replace the chemical ring expansion with an enzymatic process, preferably during fermentation.

As mentioned before, enzymatic removal of the natural D-\(\alpha\)-aminoacidipoyl side-chain of cephalosporin C was not possible. However, it was recently found that cephalosporin C could be converted into 7-ACA via two enzymatic steps.\textsuperscript{44-47} First, D-aminoacid oxidase (EC 1.4.3.3) catalysed the oxidative deamination of cephalosporin C into the corresponding \(\alpha\)-keto acid, which underwent spontaneous decarboxylation into glutaryl-7-ACA. Next, an acylase catalysed the deacylation resulting in 7-ACA (Scheme 1.7).

\begin{center}
\begin{tikzpicture}
\node at (0,0) {\includegraphics[width=\textwidth]{Scheme_1.7.png}};
\end{tikzpicture}
\end{center}

\textbf{Scheme 1.7} Enzymatic conversion of cephalosporin C into 7-ACA.

The development of an analogous route to 7-ADCA, however, was hampered by the lack of strains producing acceptable quantities of desacetoxycephalosporin C. A key to the production of 7-ADCA was found in the cephalosporin C biosynthetic pathway. Desacetoxycephalosporinase C, better known as expandase, catalyses \textit{in vivo} the expansion of the 5-membered ring of penicillin N to the 6-membered ring of
desacetoxycephalosporin C (Scheme 1.5). Penicillin N, however, is commercially unavailable and unfortunately, expandase does not accept 6-APA, penicillin G or penicillin V as a substrate. Even genetically modified expandase could not accomplish the ring expansion. Clearly, the expandase activity is largely dependent on the structure of the substrate.

Scheme 1.8 Synthesis of cephalosporin intermediates via production of adipoyl cephalosporins.
Previously published work provided a vital clue to a biocatalytic ring-expansion process for 7-ADCA. It is known that *Penicillium chrysogenum* does not produce penicillin N or cephalosporins. However, when isopenicillin N epimerase and penicillin N expandase genes were introduced into the fungus, stable *P. chrysogenum* transformants were obtained, which produced desacetoxycephalosporin C.\(^{50}\) In addition, it had already been found in the early days of cephalosporin chemistry that when adipic acid (instead of phenylacetic acid or phenoxyacetic acid) was fed to *P. chrysogenum* fermentations the fungus synthesised adipoyl-6-APA instead of penicillin G or V respectively.\(^{51}\) These two concepts were the lead for Crawford *et. al.*\(^{52,53}\) to feed adipic acid to a recombinant penicillin producing strain *P. chrysogenum*, which also expressed an expandase gene or an expandase/hydrolase gene, resulting in the synthesis of adipoyl-cephalosporins (Scheme 1.8). Unfortunately, the traditional enzyme - penicillin acylase - used in the cleavage of penicillin G into 6-APA did not accept the adipoyl-7-ADCA substrate. Therefore a new enzyme was developed.\(^{34}\) The so-called dicarboxylic acid acylase shows a close resemblance with the glutaryl acylase used in the enzymatic synthesis of 7-ACA from Cephalosporin C.\(^{54}\)

At DSM it was found that the precursor side-chain was not restricted to adipic acid.\(^{55-58}\) However, attempts to expand penicillin G to phenylacetyl-7-ADCA (G-ADCA) were still unsuccessful. Surprisingly at DSM it was recently found that penicillin G producing *P. chrysogenum*, transformed with an expandase encoding gene, could expand penicillin G into phenylacetyl-desacetoxycephalosporanic acid.\(^{59}\) The major advantage of this process is that the side-chain can readily be removed by penicillin G amidase.

Recently, a new method was developed, based on the disruption of the expandase/hydroxylase gene of the industrial strain of *Acremonium chrysogenum* (Scheme 1.5).\(^{60}\) As a consequence, large amounts of penicillin N accumulate and further expression of the expandase gene in those disrupted strains caused *in vivo* expansion of penicillin N into desacetoxycephalosporin C. This approach avoids cross-contamination of desacetoxy-cephalosporin C with other cephalosporins. The former can be converted into 7-ADCA using the two-step enzymatic bioconversion with D-amino acid oxidase and glutaryl acylase.
Green routes to side-chains

Synthesis of side-chain building blocks

DSM Andeno developed a process for the manufacture of D-phenylglycine via diastereomeric salt crystallisation of its racemate, which is readily available from a Strecker reaction on benzaldehyde. Optically pure camphorsulfonic acid (CAS) in aqueous medium is used as resolving agent. L-Phenylglycine is racemised in a separate step and recycled (Scheme 1.9).\textsuperscript{61}

D-Phenylglycine can also be prepared by the enzymatic resolution of 5-phenylhydantoin. The racemic hydantoin is converted by a D-specific hydantoinase into D-N-carbamoyl phenylglycine. The aromatic and heterocyclic hydantoin undergo rapid and spontaneous racemisation under the (alkaline) reaction conditions, resulting in a, theoretical, yield of 100\%. N-carbamoyl-D-phenylglycine is subsequently treated with nitrous acid (but see later), resulting in D-phenylglycine. However, the latter process, developed by Kanegafuchi (nowadays Kaneka Corporation) cannot compete with the classical resolution (Scheme 1.9).\textsuperscript{61}

\begin{center}
\begin{tikzpicture}
% Diagram code here%
\end{tikzpicture}
\end{center}

Scheme 1.9 Production of D-phenylglycine.
In the manufacture of D-p-hydroxyphenylglycine all processes start from phenol, since p-hydroxybenzaldehyde is too expensive. One approach involves the classical resolution of racemic p-hydroxyphenylglycine with bromocamphorsulfonic acid as the resolving agent. In 1979 Kaneka established the industrial production of D-p-hydroxyphenylglycine using D,L-p-hydroxyhydantoin as a starting material and a D-specific hydantoinase. Decarbamoylation was again performed by treating N-carbamoyl-D-p-hydroxyphenylglycine with nitrous acid\textsuperscript{62} (Scheme 1.10).

Recordati, however, used a more elegant route that employed the microorganism \textit{Agrobacterium radiobacter} which contains a D-specific hydantoinase as well as a D-carbamoylase.\textsuperscript{63} The hydantoin underwent again rapid and spontaneous racemisation under the reaction conditions; hence, the complete conversion of the racemic hydantoin into D-p-hydroxyphenylglycine was accomplished in a one-step-two-enzyme process (Scheme 1.10).

\textbf{Scheme 1.10} Synthesis of D-p-hydroxyphenylglycine.
Recently, Kaneka Corporation cloned a D-carbamoylase gene derived from *Agrobacterium* sp. KNK712 and expressed this into *E. coli*. Subsequent mutagenesis resulted in a more stable enzyme with respect to temperature and pH.\(^{54,65}\) The immobilised mutant D-carbamoylase has been used since 1995 in the commercial production of D-p-hydroxyphenylglycine.\(^{66}\) Nowadays, all D-p-hydroxyphenylglycine is produced using the hydantionase process.

**Synthesis of activated side-chain donors**

Activated side-chain donors are required for chemical coupling to the β-lactam nuclei. Initially, D-phenylglycine was activated as its acid chloride hydrochloride. This two-step reaction was carried out in halogenated organic solvent and required the use of PCl₅, formed *in situ* from phosphorous trichloride and chlorine (Scheme 1.11).

![Scheme 1.11 Synthesis of D-phenylglycine chloride hydrochloride.](image)

By analogy, *p*-hydroxyphenylglycylic chloride hydrochloride would be the side-chain donor for amoxicillin and cefadroxil. However, in the desired purity it is not available for economically attractive prices. Therefore, the so-called Dane salt method was developed. The inexpensive Dane salt is prepared from D-*p*-hydroxyphenylglycine and methyl acetoacetate and is subsequently converted to a mixed anhydride with, *e.g.*, pivaloyl chloride or methyl chloroformate in the presence of a base (Scheme 1.12).\(^{67}\) The mixed anhydride of the Dane salt, however, cannot be isolated and can only be handled *in situ* at low temperatures. The Dane salt method was subsequently also adopted for the activation of D-phenylglycine.\(^{68}\)

The shift from traditional multi-step chemical procedures for β-lactam antibiotics to biocatalytic ones, which will be discussed later, also influenced the nature and the production method of the side-chain donors. In the chemical coupling D-phenylglycine and its *p*-hydroxy derivative are key intermediates, which are subsequently subjected to an activation step. Enzymatic coupling also requires an activated side-chain donor\(^{69,70}\) such as an ester, amide or mixed anhydride. However, preparing these from the amino
acid, which represents a minimum on the chemical energy surface would be evidently inefficient. The synthetic route should rather be designed in such a manner that the chemical energy in the primary intermediate is conserved.

Scheme 1.12 Synthesis of an activated Dane salt.

An enzymatic process for D-amino acid amides, via enantioselective hydrolysis of the Strecker-derived racemic amides (See Scheme 1.13) had already been developed by DSM in the mid-1970s. This process, which uses whole cells of *Pseudomonas putida* ATCC 12633 was readily adapted for the production of D-phenylglycine amide. The disadvantage of this resolution process is that the desired product is obtained in a maximum yield of 50%. The unwanted L-phenylglycine is racemised and added to the phenylglycine stream; its recycling via the amide would require 3 steps (racemisation, esterification and ammonolysis) and is economically (as well as energetically) unattractive.

Scheme 1.13 DSM aminopeptidase process.
A more elegant approach is an asymmetric transformation of the diastereomeric salt of D,L-phenylglycine amide with L-mandelic acid in the presence of a catalytic amount of a carbonyl compound, developed at DSM\textsuperscript{71,72} (Scheme 1.14). In this process a classical resolution is combined with an \textit{in situ} racemisation and D-phenylglycine amide is obtained in nearly quantitative yield with high \textit{ee}. The L-mandelic acid can readily be recycled.

\begin{center}
\begin{tikzpicture}
  \node [draw] (A) {\text{NH}_2} -- node [left] {L-mandelic acid} (B) {\text{NH}_2 \cdot \text{HOOC}} -- node [below] {benzaldehyde} (C) {\text{OH}};
  \node [draw] (D) at (0,0) {\text{NH}_2 \cdot \text{HOOC}};
  \node [draw] (E) at (1,0) {\text{OH}};
  \node [draw] (F) at (2,0) {\text{NH}_2 \cdot \text{HOOC}};
  \node [draw] (G) at (3,0) {\text{OH}};
\end{tikzpicture}
\end{center}

\textbf{Scheme 1.14}  Asymmetric transformation to D-phenylglycine amide.

Efficient synthesis of a D-\textit{p}-hydroxyphenylglycine-derived donor is more problematic. Present designs of enzymatic processes for amoxicillin and cefadroxil depend on D-\textit{p}-hydroxyphenylglycine methyl ester, which is to be prepared from the amino acid using two equivalents of acid and a large excess of methanol.

\textbf{Green coupling of the side-chains and the \textit{\beta}-lactam nuclei}

The traditional chemical manufacture of \textit{e.g.} ampicillin involved acylation of 6-APA with the acid chloride hydrochloride of D-phenylglycine under Schotten-Baumann conditions.\textsuperscript{73} The synthesis of the acid chloride involved hazardous chemicals as mentioned before. Moreover, this route necessitates silyl protection of the carboxyl group in 6-APA. Therefore, 6-APA is treated with a slight excess of a silylating agent, such as trimethylsilyl chloride or dimethyl dichlorosilane. Subsequently, the acid chloride is added to the silylated 6-APA at low temperature (Scheme 1.15). Ampicillin is obtained after aqueous acidic removal of the protecting group.
Scheme 1.15  Synthesis of ampicillin via Schotten-Baumann reaction.

Amoxicillin is prepared via the Dane salt method. The advantage of this route is that no carboxyl protection of 6-APA is necessary. The latter is added at low temperature to the mixed anhydride of the Dane salt, which must be prepared in situ. The product is obtained in nearly quantitative yield (Scheme 1.16). After the condensation, the pH must be adjusted to 1 in order to remove the amino protecting group.

Scheme 1.16  Synthesis of amoxicillin via the Dane salt method.
Towards green synthesis of β-lactam antibiotics

Nowadays the majority of the semi-synthetic antibiotics are produced via the Dane salt route. It has already been noted that 30-40 kg of non-recyclable waste are generated per kg of cephalixin; a large share of this amount originates in the activation, coupling and deprotection steps. The major part of this waste is not biodegradable and has to be disposed of in an environmentally and socially acceptable manner. Moreover, both routes use highly active reagents in stoichiometric amounts. Apart from the waste management problem, the energy consumed in their manufacture is lost. Moreover, due to their toxic nature, appropriate operating procedures should prevent employee exposure and (accidental) release into the environment. In conclusion an enzymatic process is highly desirable.

The kinetically controlled, enzymatic synthesis of ampicillin has been known since 1969.\textsuperscript{75} At that time 6-APA was acylated with D-phenylglycine methyl ester in the presence of penicillin acylase from \textit{E. coli}. The necessary use of an activated side-chain and the low yield prevented the enzymatic process from reaching a commercial stage. Recent studies, however, proved that thermodynamically controlled synthesis, using the free amino acid, is impossible.\textsuperscript{69,70} As mentioned above, phenylglycine represents a minimum on the chemical energy surface and consequently the N-terminal serine hydroxyl group, the catalytic site of penicillin G acylase,\textsuperscript{76} will not be acylated with phenylglycine.

Hence, the kinetically controlled synthesis came under investigation again.\textsuperscript{77-81} The desired β-lactam nucleus is acylated with the activated form of the side-chain, \textit{e.g.} an ester or amide, in water at pH 7 at ambient temperature (Scheme 1.17).

Under these conditions the amino function of the activated amino acid derivative is partly uncharged and the enzyme-substrate complex can be formed. Besides synthesis of the β-lactam antibiotic competing (primary) hydrolysis of the activated side-chain donor as well as (secondary) hydrolysis of the formed antibiotic into the free amino acid will take place. The ratio of the desired product to the unwanted hydrolysis product is expressed as the synthesis/hydrolysis ratio (S/H), which is often used to assess the economic viability of the process.\textsuperscript{82} However, the S/H and product yield can be optimised by varying the pH,\textsuperscript{83} temperature,\textsuperscript{84} substrate concentrations\textsuperscript{81} and biocatalyst immobilisation.\textsuperscript{85}

The aqueous enzymatic synthesis of β-lactam antibiotics is a rapidly evolving technique, which has proven its feasibility. The yields on the β-lactam core can be as high or even better than in the traditional chemical condensations.\textsuperscript{82} However, each process has its own different characteristics and therefore needs careful optimisation of the reaction conditions towards optimum conversion.
Scheme 1.17 Kinetically controlled enzymatic synthesis of β-lactam antibiotics.

Cephalexin
Isolation of cephalexin is hampered by the small differences, with regard to acid/base properties and solubilities, between cephalexin, 7-ADCA and d-phenylglycine. As a result, co-precipitation will occur and impure cephalexin will be obtained. The past 5-10 years of intensive research at DSM have resulted, however, in an industrial process for cephalexin whereby the chemical coupling is replaced by an enzymatic alternative. In this process the above-mentioned problems were solved by using a number of pH shifts and crystallisations. First, the immobilised enzyme was separated from the reaction mixture using a sieve-bottom reactor. The pH of the solution was increased such that only d-phenylglycine crystallised. Next, the pH of the reaction mixture was lowered and pure cephalexin crystallised. An even further decrease in pH resulted in the recovery of 7-ADCA. The introduction of biocatalysis in the cephalexin process reduced the number of reaction steps from 10 to 6 (Scheme 1.18)
Scheme 1.18  Production routes to cephalexin.

**Ampicillin**

The main problem in the enzymatic synthesis of ampicillin is its high solubility. As a consequence (secondary) hydrolysis of the product will take place, resulting in a low S/H. Moreover, 6-APA is readily susceptible to degradation (compared to 7-ADCA) over a large pH range, which hampers its recycling. Hence, 6-APA needs to be converted completely. This was accomplished by using an excess of the activated side-chain donor. Ampicillin as well as D-phenylglycine crystallised during the reaction. After the reaction the biocatalyst was removed by sieving and all the crystals were dissolved at low pH. The solution was concentrated and subsequent crystallisation at the isoelectric point gave pure ampicillin.\(^ {86}\) In a second concentration and crystallisation step, D-phenylglycine was recovered. The inherently low S/H, as well as the lagging demand for this aging therapeutic have thusfar prevented the commercialisation of an enzymatic process for ampicillin.
Amoxicillin
In contrast with ampicillin, the solubility of amoxicillin is very low. Therefore, the S/H is higher and moreover, the degradation of amoxicillin minimal. The insoluble product is separated using a sieve with small pores small enough to retain the catalyst, but to let the product pass through. The product crystals are continuously removed by filtration and subsequent centrifugation. The supernatant is passed through a feed tank filled with a slurry of the side-chain donor and the β-lactam nucleus which provides its re-saturation. Amoxicillin was obtained in 90% yield (based on 6-APA) with S/H 2.4.

Cefadroxil
The main problem in the enzymatic synthesis of cefadroxil is that the side-chain and the β-lactam nucleus are sufficiently soluble only at a completely different pH. Moreover, the solubility of cefadroxil is high, resulting in chemical degradation and enzymatic hydrolysis of the product. So, high conversions are necessary to obtain a mixture from which cefadroxil can be crystallised. The optimum pH for cefadroxil is approximately 7, but 7-ADCA is poorly soluble at this pH. The S/H is low (circa 1). The solution was found in the use of supersaturated solutions. The reaction is started with a solution of 7-ADCA, containing the catalyst, at pH 8.5. A concentrated solution of D-p-hydroxyphenylglycine methyl ester at pH 4 is added at such a rate that crystallisation does not take place. Coupling is accomplished with S/H ~5 and 85% conversion of 7-ADCA. Cefadroxil crystallises in the course of the reaction.

Outline of the thesis
In 1994 DSM Andeno and Gist-brocades started a joint venture, Chemferm, which in 1996 initiated in co-operation with 4 universities the so-called cluster project fine chemistry. One of the main goals was to develop improved or alternative clean, (bio-)catalytic, efficient routes for penicillin and cephalosporin β-lactam antibiotics and their precursors. This thesis describes environmentally friendly alternative routes to activated side-chain donors and their application for the synthesis of β-lactam antibiotics as a part of the Chemferm project. Chapter 2 is devoted to the catalytic esterification of D-phenylglycine catalysed by zeolite H-USY, thereby circumventing the formation of the stoichiometric amounts of salts associated with the traditional chemical procedures. Chapter 3 describes the Candida antarctica lipase B catalysed ammonolysis of racemic phenylglycine ester into D-phenylglycine amide. The disadvantage of a kinetic resolution, a maximum yield of 50%,
Towards green synthesis of β-lactam antibiotics

has been overcome by in situ racemisation of the substrate. Racemic phenylglycine nitrile can be hydrated to D-phenylglycine amide in the presence of nitrile hydratase/amidase systems. Chapters 4 and 5 deal with the screening and large-scale cultivation of different nitrile hydratase/amidase containing microorganisms. In Chapter 6, these microorganisms are used in the stereoretentive hydration of D-phenylglycine nitrile into the corresponding amide. In Chapter 7, the nitrile hydratase catalysed stereoretentive hydration of D-phenylglycine nitrile is combined with the penicillin acylase catalysed acylation of the in situ formed amide to give cephalaxin in a one-pot two-enzyme process.

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Towards green synthesis of β-lactam antibiotics


Chapter 2

Salt-free esterification of α-amino acids catalysed by zeolite H-USY

Abstract
A truly catalytic procedure is described for the esterification of α-amino acids, thereby circumventing the formation of stoichiometric quantities of salts associated with conventional procedures. The acid form of ultrastable zeolite Y (H-USY), a naphtha cracking catalyst, acted as a solid acid catalyst in the reaction of several α-amino acids with methanol at 100-130 °C (15-20 bar). For example, L-phenylalanine afforded the methyl ester in 83% yield after 20 h at 100 °C. Based on the (unlikely) participation of all the Al atoms of the zeolite this corresponded to a turnover number of 180. The ester product was partially racemised (52% ee).
Phenylglycine, p-hydroxyphenylglycine and homophenylalanine were similarly converted to their methyl esters. The H-USY catalyst could be recycled albeit with decreased activity after each cycle owing to the adsorption of water (formed in the reaction). Its activity was completely restored, however, after calcination.

*This chapter is in press: M.A. Wegman, J.M. Elzinga, E. Neeleman, F. Van Rantwijk, R.A. Sheldon, Green Chemistry.
Introduction

Amino acid esters are key raw materials in the manufacture of, *inter alia*, pharmaceuticals and flavours.¹ For example, the methyl ester of racemic phenylalanine is used in the enzymatic synthesis (DSM-Tosoh process) of the artificial sweetener aspartame.² Esters of D-phenylglycine and D-β-hydroxyphenylglycine are key intermediates in enzymatic routes to semi-synthetic penicillins and cephalosporins.³ Amino acids exist as zwitterions and in order to generate a free carboxyl group, for esterification, a stoichiometric amount of mineral acid, *e.g.* HCl or H₂SO₄, is required (Scheme 2.1). Consequently the product ester is formed as the corresponding salt and generation of the free amino acid ester necessitates neutralisation with a base and concomitant generation of at least one equivalent of salt, *e.g.* NaCl or H₂SO₄. For example, the synthesis of phenylglycine methyl ester (2a) involves the production of at least 0.35 kg of NaCl or 0.43 kg Na₂SO₄ per kg of product. In practice substantially larger amounts are produced owing to the use of a large excess of mineral acid.⁴

![Scheme 2.1 Conventional esterification of an α-amino acid using >1 equivalent of mineral acid.](image)

We surmised that esterification over a solid acid catalyst could, in principle, yield the ester as the free base in a salt-free, non-corrosive process. This was based on the notion that desorption of the ester from the solid catalyst into the bulk solution would enable the adsorption of more amino acid molecules. In this way a situation is created where an acid-catalysed reaction is occurring in the pores of the solid acid even though the bulk solution, containing the amino acid ester, is basic.

Yadav and Krishnan reported that the esterification of anthranilic acid is catalysed by ion-exchange resins.⁵ However, the difference in pKₐ values of the carboxyl and amino
groups in antranilic acid (ΔpKₐ = 2.4) is much smaller than that in α-amino acids (ΔpKₐ = 7). Hence, the catalytic esterification of α-amino acids over solid acids would be expected to be much more difficult. Herein we report the successful catalytic salt-free esterification of several α-amino acids over ultrastable zeolite Y (H-USY) which is used industrially on a very large scale as a naphtha cracking catalyst.

Results and discussion

Screening of several heterogeneous catalysts
For our initial screening of solid acid catalysts we studied the esterification of D-1a with methanol. Different solid acids (e.g. acid-treated clays, zeolites, ion-exchange resins and heteropolyacids) were tested in a standard esterification reaction under reflux conditions. Only zeolite H-Y and H-USY catalysed the esterification of D-1a to the corresponding methyl ester. A plausible explanation is that H-Y and H-USY are highly hydrophilic, which facilitates the adsorption of D-1a. Moreover, these zeolites consist of a three-dimensional large pore system (7.4 Å) with supercages (12 Å) which allows an efficient migration of the substrate into - and product out of - the channel system. Due to restricted accessibility of the acidic sites an acid catalysed reaction can be performed even though the bulk solution is basic owing to the accumulation of the free amino acid ester. Because of the superior results, H-USY was used in all further experiments.

Effect of the amount of H-USY
The effect of the amount of the catalyst on the reaction rate was investigated. The amount of H-USY was varied between 0.25 and 2 g and in all cases equilibrium was reached at 30 mM product concentration (35% yield). The initial rate, however, increased as more H-USY was used, as would be expected (Table 2.1).

Stability of the product
The ester 2a always racemised during the reaction, regardless of the amount of H-USY. A blank experiment showed that the racemisation occurred in the absence of the catalyst. It is known that amino acid esters undergo thermal racemisation. Free amino esters are also known to be susceptible to self-condensation to diketopiperazines and polycondensation products. Therefore, the stability of D-2a in methanol (50 mM) under reflux conditions, in the absence of the catalyst was monitored for 30 h. No degradation of 2a was observed, but the product was completely racemised.
Table 2.1 Initial rate of H-USY catalysed esterification of D-1a

<table>
<thead>
<tr>
<th>H-USY (g)</th>
<th>Initial rate (mM/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>1.3</td>
</tr>
<tr>
<td>0.5</td>
<td>3.6</td>
</tr>
<tr>
<td>1</td>
<td>5.3</td>
</tr>
<tr>
<td>2</td>
<td>9.4</td>
</tr>
</tbody>
</table>

* Reaction conditions: H-USY was added to a suspension of 6.6 mmol D-phenylglycine in 80 ml methanol. The reaction mixture was refluxed over KA molecular sieves.

**Reusability of the catalyst**

H-USY was used in a recycle experiment; after each cycle the catalyst was filtered off and reused in the next cycle without any post-treatment. The activity of the catalyst slowly decreased after each cycle as shown in Figure 2.1. However, H-USY completely recovered its activity upon calcination. The Si/Al ratio of H-USY was found to be unchanged after three consecutive esterifications consistent with no leaching of Al having occurred during the reaction.

![Graph of concentration 2a (mM) vs. time (h)](image)

**Figure 2.1** H-USY-catalysed esterification of D-phenylglycine (■) first cycle, (●) second cycle, (◆) third cycle.

**Inhibition effects**

Because hydrophilic zeolites selectively adsorb water, the possible inhibition of H-USY by this side-product was investigated. The addition of an equimolar amount of
water to the reaction mixture resulted in approximately 50% reduction of the initial rate as well as the equilibrium conversion. Refluxing the reaction mixture over KA molecular sieves only partially restored the rate, indicating that water accumulation in the highly hydrophilic H-USY could not be prevented completely (Figure 2.2). Dimethyl carbonate, which reacts with water to methanol and CO₂, was also not effective as a water scavenger (data not shown).

Since the product ester 2a is rather hydrophilic, we also studied product ester inhibition of H-USY by adding 2 mmol 2a to the reaction mixture. The initial rate decreased considerably in the presence of 2a and the additional amount of 2a formed was less than in the standard reaction (Figure 2.2).

Furthermore, we observed that 0.5 mmol of the added ester was directly adsorbed on the catalyst (1 g), which gives an estimate of the amount of accessible catalytic sites i.e. 0.5 mmol/g H-USY.

![Graph showing concentration of 2a over time](image)

**Figure 2.2** Inhibition effect on H-USY (■) standard reaction (▲) in the presence of 2 mmol phenylglycine methyl ester, (●) in the presence of 1 eq. H₂O and 2 g KA molecular sieves, (◆) in the presence of 1 eq. H₂O, no molecular sieves.

**Effect of temperature**

A drawback of the H-USY-catalysed esterification under reflux conditions (64 °C) is that equilibrium is reached at only 30 mM 2a. The reaction was performed at increased temperature⁵,¹³ in an autoclave at 100 °C and 15 bar (N₂) in order to shift the equilibrium towards product formation. The reaction rate was enhanced 10-fold and after 2 h equilibrium was reached at 60 mM 2a (73% yield) according to HPLC (Table 2.2). An isolated yield of 73% 2a was obtained after working up. To enhance the conversion even
further, the temperature was increased to 130 °C. The equilibrium conversion increased with temperature, but the racemisation rate of 2a also increased at elevated temperatures. At 130 °C equilibrium was reached at 70 mM 2a (86% yield with 51% ee).

<table>
<thead>
<tr>
<th>T (°C)</th>
<th>p (bar)</th>
<th>t (h)</th>
<th>Yield (%)&lt;sup&gt;b&lt;/sup&gt;</th>
<th>ee (%)&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>65</td>
<td>1</td>
<td>20</td>
<td>36</td>
<td>4</td>
</tr>
<tr>
<td>100</td>
<td>15</td>
<td>2.0</td>
<td>73</td>
<td>29</td>
</tr>
<tr>
<td>130</td>
<td>20</td>
<td>0.5</td>
<td>86</td>
<td>51</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reaction conditions: 1 g H-USY was added under stirring to a suspension of 6.6 mmol D-phenylglycine in 80 ml methanol.

<sup>b</sup> Analysed by HPLC.

We note, however, that the yield corresponded to the amount of amino ester present in the bulk solution and, as mentioned above, 0.5 mmol of the ester is adsorbed on the catalyst. In practice the adsorbed ester will be recovered when the catalyst is recycled and reused in a new batch. Hence, the maximum yield of ester, observable in solution, from 6.6 mmol of substrate with 1 g catalyst is 92%.

**Scope of the reaction**

In order to investigate the scope of the salt-free esterification several amino acids were subjected to H-USY-catalysed esterification with methanol at 130 °C (20 bar N₂). Phenylglycine (1a) was compared with phenylalanine (1b) and homophenylalanine (1c) as they comprise a series of three amino acids in which the phenyl group is progressively further separated from the stereogenic centre by a methylene group (Scheme 2.1).

As the chain-length increased, the equilibrium conversion declined (Figure 2.3). This is possibly due to the increasing hydrophobic character of the amino acid with increasing chain length. Alternatively, it could be due to increasing bulk of the substrate hindering access to the active site. l-2b was more stable than D-2a towards racemisation (Table 2.3). This is consistent with the higher reactivity of the benzylic C-H bond at the stereogenic centre in the latter.
Figure 2.3  H-USY catalysed esterification of (■) phenylglycine, (●) phenylalanine, (◆) homophenylalanine at 130 °C, (▲) p-hydroxyphenylglycine at 100 °C.

Table 2.3  H-USY catalysed esterification of several amino acids.a

<table>
<thead>
<tr>
<th>Substrate</th>
<th>t (h)</th>
<th>Yield (%)b</th>
<th>ee (%)c</th>
</tr>
</thead>
<tbody>
<tr>
<td>D-1a</td>
<td>0.5</td>
<td>86</td>
<td>51</td>
</tr>
<tr>
<td>L-1b</td>
<td>2.0</td>
<td>77</td>
<td>52</td>
</tr>
<tr>
<td>D,L-1c</td>
<td>2.0</td>
<td>68</td>
<td>-</td>
</tr>
<tr>
<td>D-1d</td>
<td>2.5</td>
<td>14</td>
<td>35</td>
</tr>
</tbody>
</table>

a Reaction conditions: A suspension of 1 g H-USY and 1 g of amino acid in 80 ml methanol was stirred at 130 °C and 15 bar (N₂).
b Isolated yield.
c ee analysed by HPLC.
d Reaction temperature was 100 °C.

Because racemic 2b is a key intermediate in the industrial synthesis of the artificial sweetener Aspartame,² a more detailed study was carried out. Since equilibrium is reached regardless of the amount of the catalyst, the loading of H-USY was gradually decreased. As little as 10 mg H-USY sufficed to convert 2 g L-1b at 100 °C into 2b in 83% yield after 20 h. This corresponds to a minimum Turnover Number (TON) of 180 (mol product/mol Al), based on the unlikely participation of all the aluminium atoms present in the zeolite (5.6 mmol Al/g H-USY). As noted above 1 g of H-USY adsorbed 0.5 mmol of amino ester suggesting that < 10% of the available 5.6 mmol of Al atoms are catalytically active, i.e. the actual catalytic turnover is > 1800.
We subsequently studied the salt-free esterification of D-\textit{p}-hydroxyphenylglycine (D-1d). The corresponding ester is a key intermediate in the synthesis of semi-synthetic antibiotics.\textsuperscript{3} At 130 °C (20 bar N\textsubscript{2}) 2d decomposed into several unidentified products. Therefore, the reaction was carried out at 100 °C (15 bar N\textsubscript{2}). After 2.5 h a moderate conversion equilibrium was reached of 23 mM 2d (31% yield).

We also attempted the esterification of L-proline, which differs from the other amino acids in that it contains a secondary rather than a primary amino group. However, its esterification over H-USY failed. Similarly, esterification of anthranilic acid was unsuccessful over H-USY, possibly owing to its higher hydrophobicity. Yadav and Krishnan\textsuperscript{3} similarly found that ZSM-5 was ineffective in the esterification of anthranilic acid.

Conclusions

Zeolite H-USY efficiently catalysed the esterification of aromatic \(\alpha\)-amino acids at 130 °C. The rate and equilibrium conversion declined with increasing chain-length of the amino acid. The TON for phenylalanine was 180 based on (the unlikely) participation of all Al atoms in H-USY.

Experimental section

Materials

Zeolite H-Y (Si/Al 2.5) and H-USY (Si/Al 2.5) were kindly donated by AKZO-Nobel Chemicals (Amsterdam, The Netherlands). Enantiomerically pure D-phenylglycine, D-\textit{p}-hydroxyphenylglycine, D-phenylglycine methyl ester and D-\textit{p}-hydroxyphenylglycine methyl ester were a gift from DSM (Geleen, The Netherlands). Racemic phenylglycine, racemic phenylalanine, L-phenylalanine, L-Proline, L-proline methyl ester and anthranilic acid were obtained from Acros. Racemic homophenylalanine was obtained from Sigma. Molecular sieves KA - activated at 400 °C for 24 h before use - were purchased from Aldrich. Racemic phenylglycine methyl ester, racemic phenylalanine methyl ester and racemic homophenylalanine methyl ester were chemically synthesised from the corresponding \(\alpha\)-amino acids according to the literature.\textsuperscript{14}

Activation of H-Y and H-USY was performed in a stationary oven. The catalyst was heated from room temperature to the final activation temperature (550 °C) at 1 °C/min
and held at this temperature for 7 h. The catalyst was cooled down to 200 °C and transferred to a vacuum desiccator where it was allowed to cool to room temperature.

Analysis and equipment
Inductively coupled plasma atomic emission spectroscopy (ICP-AES) was used to determine Si/Al ratios before and after reactions. ICP-AES measurements were conducted on a Perkin-Elmer plasma 2000. The reaction mixtures were analysed by chiral HPLC on a Daicel Chemical Industries Ltd. 4.6 x 150 mm 5 μ Crownpak CR (+) column using a Waters 625 LC pump and a Waters 486 UV detector. The eluent was aqueous 0.1 M HClO₄, pH 1.0 at a flow of 0.6 ml/min. The column temperature was set to 24 °C for phenylglycine and anthranilic acid, to 30 °C for phenylalanine and to 32 °C for p-hydroxyphenylglycine. For homophenylalanine the eluent was adjusted to pH 2.0 (0.01 M HClO₄) and the column temperature was 48 °C. Esterification of proline was monitored by ¹H-NMR (CDCl₃) using a 400 MHz Varian-VXR 400S spectrometer.

Esterification under reflux conditions
H-Y or H-USY (1 g, unless stated otherwise), pre-activated, was added to a suspension of 1 g α-amino acid in 80 ml methanol. The reaction mixture was refluxed over molecular sieves KA. Samples were withdrawn periodically, the catalyst was removed by centrifugation and the supernatant was analysed by HPLC. The experiments were performed in duplo and were reproducible within the margin of error. The markers in Figs. 2.1 and 2.2 indicate the margin of error.

Esterification at elevated temperatures
H-USY (1 g, unless otherwise stated), pre-activated, was added to a suspension of 1 g α-amino acid in 80 ml methanol. The reaction was performed in a 200 ml Parr stainless steel autoclave at the selected temperature under an inert atmosphere (15 bar N₂, p₀ = 10 bar). Samples were withdrawn periodically, the catalyst was removed by centrifugation and supernatant was analysed by HPLC. The experiments were performed in duplo and were reproducible within the margin of error. The markers in Fig. 2.3 indicate the margin of error.
Isolation of α-amino acid methyl ester

The catalyst and the excess α-amino acid were filtered off. Methanol was evaporated and the residue was resuspended in diethyl ether in which only the ester dissolved. The precipitate was filtered off, the ether was evaporated and the ester was obtained as a yellowish oil.

Acknowledgements

Generous donations of H-Y and H-USY by AKZO-Nobel Chemicals (Amsterdam, The Netherlands) are gratefully acknowledged. DSM Life Science Products is gratefully acknowledged for a gift of D-phenylglycine and D-β-hydroxyphenylglycine. Thanks are due to Mr. J. Padmos of Delft ChemTech who carried out the ICP-AES measurements. Also thanks are due to Dr. M.J. Verhoeof (Delft University of Technology, The Netherlands) for his helpful discussions and to Prof. Dr. A. Bruggink of DSM Life Science Products (Geleen, The Netherlands) for his encouragement. This work was financially supported by the Netherlands Ministry of Economic Affairs and coordinated by DSM Life Science Products (Geleen, The Netherlands).

References

Chapter 3

Dynamic kinetic resolution of phenylglycine esters via lipase-catalysed ammonolysis

Abstract
Ammonolysis of D,L-phenylglycine methyl ester catalysed by Novozym 435 at 40 °C in tert-butyl alcohol gave D-phenylglycine amide in 78% ee at 46% conversion, corresponding to an enantiomeric ratio (E) of 16. Lowering the temperature improved the enantioselectivity (E = 52 at -20 °C). Combination of ammonolysis with pyridoxal-catalysed in situ racemisation of the unconverted ester (dynamic kinetic resolution), at -20 °C, gave D-phenylglycine amide with 88% ee at 85% conversion. The amide racemised much slower than the ester at this low temperature.

* Performed in collaboration with M.A.P.J. Hacking.
Chapter 3

Introduction

As a result of recent developments in the manufacture of penicillin and cephalosporin antibiotics,¹ multi-step chemical procedures for the coupling of the D-phenylglycine and D-p-hydroxyphenylglycine side-chains with the β-lactam nuclei are being replaced by enzymatic alternatives (Scheme 3.1).

![Scheme 3.1 Enzyme-catalysed synthesis of pencillins.](image)

In the chemical procedure D-phenylglycine and its p-hydroxy derivative are key intermediates. Two processes are used for the commercial production of these amino acids: classical resolution via diasteromeric salt crystallisation³ or enantioselective hydrolysis of the corresponding hydantoin.⁴ In the former the L-isomer has to be racemised while the latter process is a dynamic kinetic resolution and produces the D-isomer as the sole product. The enzymatic coupling (Scheme 3.1) is only successful with an activated side-chain, e.g. an amide or an ester.⁵,⁶ Hence, when the side-chain is produced as the free acid (see above) additional steps are required to convert it to the corresponding ester or amide. Consequently, an efficient enzymatic coupling process requires a direct method for the synthesis of the D-ester or D-amide.

An enzymatic route to D-phenylglycine amide has been developed and commercialised by DSM.⁷ This process involves a kinetic resolution of the racemic amide by an L-specific aminopeptidase and consequently, suffers from the inherent disadvantage of producing L-phenylglycine as the coproduct. The latter needs to be racemised and converted, via the ester, to the D,L-amide substrate. A direct method for the enantioselective conversion of racemic ester to D-amide would have obvious advantages, especially if it could be performed with in situ racemisation, i.e. as a dynamic kinetic resolution.

Recently de Zoete et al. showed⁸ that the lipase (Novozym 435) catalysed ammonolysis of racemic phenylglycine methyl ester 1a affords D-phenylglycine amide 2 (Scheme 3.2).
This method would meet the criteria outlined above if the slow racemisation of the ester, which was observed\(^8\) (5% over 24 h) in the course of the reaction, could be sufficiently accelerated to allow a dynamic kinetic resolution. Obviously for such a scheme to be effective, the product amide should racemise much slower than the ester substrate.

\[
\begin{array}{c}
\text{NH}_2 \\
\text{O} \\
\text{NH}_2 \\
\text{O} \\
\end{array}
\xrightarrow{\text{NH}_3 \text{ Novozym 435 t-butyl alcohol}}
\begin{array}{c}
\text{NH}_2 \\
\text{O} \\
\text{NH}_2 \\
\end{array}
\]

\text{D,L-1a} \rightarrow \text{D-2}

**Scheme 3.2** Enantioselective ammonolysis of D,L-phenylglycine methyl ester.

The racemisation of amino acid esters is known to be efficiently catalysed by aromatic aldehydes such as 2-hydroxybenzaldehyde (salicylaldehyde) and 3-hydroxy-2-methyl-5-hydroxymethyl-pyridine-4-carboxaldehyde (pyridoxal) under basic conditions.\(^9,10\) The use of aldehyde-based racemisation catalysts under ammonolytic conditions would seem questionable, however, because reaction of the aldehyde group with ammonia\(^9,10\) might be expected to interfere. The problem of undesired racemisation of D-phenylglycine amide 2 would seem more manageable, because the amide 2 is racemised much slower and at high concentration it precipitates and is thus effectively removed from the reaction mixture.

Indeed we found previously\(^11\) that pyridoxal and salicylaldehyde readily racemise phenylglycine methyl ester 1a in ammonia-saturated tert-butyl alcohol, thus providing the basis for a dynamic kinetic resolution process, which we have now investigated in more detail.

**Results and discussion**

*Ammonolysis of phenylglycine esters*

Because *Candida antarctica* lipase B (Novozym 435) emerged from our initial study\(^12\) as the catalyst of choice, it was used throughout the present work. Initially we investigated the influence of the chain length of the ester group on the course of the reaction. The data in Table 3.1 show that the butyl ester 1c reacted at one third of the rate of the methyl ester.
1a. The effect on the enantiomeric ratio $E^{13}$, which varied between 16 and 19 was negligible. In view of these results all further experiments were performed with 1a.

**Table 3.1** Effect of the chain length of the ester in the ammonolysis of phenylglycine esters 1.

<table>
<thead>
<tr>
<th>Ester</th>
<th>Conversion (%)</th>
<th>$ee_{L-1a}$ (%)</th>
<th>$ee_{D-2}$ (%)</th>
<th>$E_{1a}$</th>
<th>$E_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methyl 1a</td>
<td>47</td>
<td>69</td>
<td>78</td>
<td>16</td>
<td>17</td>
</tr>
<tr>
<td>Ethyl 1b</td>
<td>36</td>
<td>47</td>
<td>84</td>
<td>18</td>
<td>18</td>
</tr>
<tr>
<td>Butyl 1c</td>
<td>16</td>
<td>17</td>
<td>89</td>
<td>19</td>
<td>20</td>
</tr>
</tbody>
</table>

*Reaction conditions: 1 mmol D,L-phenylglycine ester-HCl 1 was shaken with 50 mg Novozym 435 in 5 ml, 2.5 M ammonia-saturated tert-butyl alcohol 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 3.1). The apparent Michaelis constant $K_m$, which is approximately 830 mM for D-1a, was deduced by fitting the experimental data to the Michaelis-Menten rate equation.

![Figure 3.1](image-url)  
**Figure 3.1** Initial rate as a function of the concentration of D-1a (●) and L-1a (▲).

† The enantiomeric ratios for the ester ($E_e$) and the amide ($E_a$) were calculated from the conversion ($c$) and the enantiomeric excess 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:

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

† Experimental values for $E$ were lower than those found previously, which can be attributed to improvements in the analytical procedure.
Due to the deviation of D-1a from first order kinetics, an increased starting concentration of D,L-1a resulted in longer reaction times and a slight decrease in $E$. The concentration of ammonia, between 1.7 and 2.5 M, had no influence on either $E$ or the reaction rate (data not shown).

The $E$ value of 16 that we consistently observed in the ammonolysis of 1a in tert-butyl alcohol is rather low for an efficient kinetic resolution. We note, however, that in an ideal dynamic kinetic resolution this would result in a product ee of 88%.§

Racemisation via Schiff base intermediates

D-Methyl ester 1a and D-amide 2 were subjected to pyridoxal and salicylaldehyde catalysed racemisation under ammonolysis conditions (tert-butyl alcohol, 2.5 M NH$_3$ at 40 °C). In all cases the ee decreased to 0% according to first order kinetics. The initial racemisation rate obeyed Michaelis-Menten kinetics according to (3.1):

$$v = \frac{k_{rac} \cdot [D-PGX]}{K_{dis} + [D-PGX]} \cdot [\text{rac.cat}]$$  \hspace{1cm} (3.1)

It has generally been assumed that racemisation takes place via Schiff base formation.\textsuperscript{9,10,14} We found, however, saturation kinetics, which implies a fast pre-equilibrium with dissociation constant $K_{dis}$. We propose that racemisation takes place via a hemiaminal which undergoes slow dehydration with catalytic rate constant $k_{cat}$ followed by fast racemisation of the Schiff base (Scheme 3.3). We note, however, that racemisation of the hemiaminal (owing to increased lability of the $\alpha$-C-H bond) is a possible alternative.

The kinetic parameters were determined from Lineweaver-Burk plots (Table 3.2). It should be noted that the measured racemisation rate constant is half the kinetic rate constant $k_{cat}$. The catalytic rate constants show that both catalysts racemise 1a circa 25 times as fast as 2 under $V_{max}$ conditions. At lower concentrations this rate advantage of 1a will be less, as can be deduced from the $k_{cat}/K_{dis}$ values. Moreover, due to its lower $K_{dis}$ value 2 would be expected to compete effectively for the racemisation catalyst, which effect would be less pronounced for salicylaldehyde (Table 3.2). We tentatively

---

§ 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.
concluded that both racemisation catalysts appeared to be suitable for *in situ* racemisation.

![Scheme 3.3](image)

**Scheme 3.3** Racemisation via Schiff base intermediates.

| Table 3.2 Kinetic data of the racemisation of 1a and 2. |
|---------------------------------|--------|----------|--------------|-----------------|
| **Rac. cat.** | **Substrate** | **$K_{\text{dis}}$** (mM) | **$k_{\text{cat}}$** ($10^{-3}$ s$^{-1}$) | **$V_{\text{max}}$** (μM·s$^{-1}$) | **$k_{\text{cat}}/K_{\text{dis}}$** (M$^{-1}$·s$^{-1}$) |
| Pyridoxal | D-1a | 180 | 20.8 | 13.5 | 0.116 |
| | D-2 | 45 | 0.74 | 0.48 | 0.016 |
| Salicylaldehyde | D-1a | 44 | 7.56 | 5.36 | 0.172 |
| | D-2 | 28 | 0.25 | 0.18 | 0.009 |

*Ammonolysis with in situ racemisation*

We next combined ammonolysis and racemisation of 1a in tert-butyl alcohol (2.5 M NH$_3$ at 40 ºC). From the time-course and ee of reactant and product (Fig. 3.2) 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. The racemisation catalyst also accelerated the reaction from 50% conversion in 4 h to 60%, 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-catalysed racemisation of 2 is quite significant.
Dynamic kinetic resolution of phenylglycine esters

Figure 3.2 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: 1 mmol ester 1a was shaken with 50 mg Novozym 435 and 0.01 mmol pyridoxal in 5 ml 2.5 M ammonia-saturated tert-butyl alcohol at 40 °C.

In order to establish the optimum conditions for a dynamic kinetic resolution, 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. From the results (Table 3.3), no clear optimum with regard to concentration and ee is apparent. Racemisation catalyst concentrations exceeding 1 mM mainly effect the conversion rate. Pyridoxal and salicylaldehyde at 1 and 2 mM perform similarly with regard to ee, but in the case of pyridoxal a somewhat higher conversion is reached.

Table 3.3 Ammonolysis with in situ racemisation.a

<table>
<thead>
<tr>
<th>Concentration rac. cat.</th>
<th>pyridoxal</th>
<th>salicylaldehyde</th>
</tr>
</thead>
<tbody>
<tr>
<td>(mM)</td>
<td>conversion (%)</td>
<td>ee (%)</td>
</tr>
<tr>
<td>4.0</td>
<td>91.2</td>
<td>53.6</td>
</tr>
<tr>
<td>2.0</td>
<td>90.0</td>
<td>55.6</td>
</tr>
<tr>
<td>1.0</td>
<td>86.8</td>
<td>60.7</td>
</tr>
<tr>
<td>0.4</td>
<td>81.5</td>
<td>57.1</td>
</tr>
<tr>
<td>0.2</td>
<td>75.1</td>
<td>50.5</td>
</tr>
<tr>
<td>0.04</td>
<td>62.1</td>
<td>54.7</td>
</tr>
<tr>
<td>1.0b</td>
<td>83.1</td>
<td>60.6</td>
</tr>
</tbody>
</table>

a Reaction conditions: 1 mmol D,L-phenylglycine methyl ester 1a was shaken with 50 mg Novozym 435 and pyridoxal or salicylaldehyde in 5 ml, 2.5 M ammonia-saturated tert-butyl alcohol at 40 °C for 17 h.

b 4 mmol D,L-phenylglycine methyl ester 1a was shaken with 200 mg Novozym 435 and 0.02 mmol pyridoxal in 5 ml, 2.5 M ammonia-saturated tert-butyl alcohol at 40 °C for 17 h.
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 3.3a and b). 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 (0.04 to 2 mM, see Fig. 3.3a) the racemisation of 1a was too slow to be effective. When the pyridoxal concentration was increased to 4 mM the results improved slightly, but a further increase had no effect. For salicylaldehyde a very similar pattern was observed (Figure 3.3b). It would seem that, once the racemisation catalyst concentration exceeds a critical value, higher concentrations affect 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 and hence, no optimum becomes apparent.

Figure 3.3 Enantiomeric excess of amide 2 as a function of the conversion at different pyridoxal (3a) and salicylaldehyde (3b) concentrations. 3a: (Δ) 2, (O) 1, (□) 0.4, (●) 0.2 (■) 0.04 mM pyridoxal; 3b: (O) 4, (□) 2, (Δ) 1 mM salicylaldehyde.

Because 2 would be expected to precipitate and hence to be effectively withdrawn from the reaction mixture, we would expect that a high concentration of 1a would accelerate its racemisation relative to 2. However, when we increased the concentration of 1a and pyridoxal four-fold (to 800 and 4 mM, respectively) no effect on ee vs concentration became apparent. (Table 3.3). This is probably due to the lower $E$ value at high concentrations of 1a (see Figure 3.1). Rapid exchange of precipitated D-2 with product in solution may also contribute to this unsatisfactory result.
**Solvent effect**

To improve the results of the dynamic kinetic resolution, the racemisation rate of 1a should be increased with respect to 2. We assumed that the racemisation rate could be influenced by the reaction medium. Moreover it is known that the reaction medium can dramatically influence enantiopreferences of enzymes.\(^{15-17}\) Initially, the effect of solvent on the ammonolysis of 1a in the kinetic resolution was studied. However, little variation in the E value was observed (Table 3.4). The reaction rate, however, is strongly dependent on the reaction medium, but there was no apparent correlation with any solvent parameter such as hydrophobicity (log P), dielectric constant or dipole moment.

<table>
<thead>
<tr>
<th>Solvent</th>
<th>t (h)</th>
<th>Conversion (%)</th>
<th>ee(_{D,2}) (%)</th>
<th>E</th>
<th>Initial rate (mM/min/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tert-Butyl alcohol</td>
<td>4</td>
<td>46</td>
<td>78</td>
<td>16</td>
<td>16.7</td>
</tr>
<tr>
<td>tert-Amyl alcohol(^b)</td>
<td>3</td>
<td>48</td>
<td>76</td>
<td>17</td>
<td>14.2</td>
</tr>
<tr>
<td>1,2-Dimethoxyethane</td>
<td>6</td>
<td>40</td>
<td>85</td>
<td>21</td>
<td>8.80</td>
</tr>
<tr>
<td>tert-Butyl methyl ether(^b)</td>
<td>5</td>
<td>43</td>
<td>73</td>
<td>12</td>
<td>7.66</td>
</tr>
<tr>
<td>Dioxane</td>
<td>21</td>
<td>43</td>
<td>83</td>
<td>20</td>
<td>3.66</td>
</tr>
<tr>
<td>Acetonitrile</td>
<td>66</td>
<td>40</td>
<td>82</td>
<td>18</td>
<td>1.96</td>
</tr>
<tr>
<td>Hexane(^b)</td>
<td>15</td>
<td>&lt;5</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

\(^a\) Reaction conditions: 1 mmol D,L-phenylglycine ester 1a was shaken with 50 mg Novozym 435 in 5 ml, 2.5 M ammonia saturated solvent at 40 °C.

\(^b\) Novo SP 611, *Candida Antarcctica* lipase B on Accurel EP 100, (50 mg) was used instead of Novozym 435 to minimise hydrolysis of 1a due to traces of water in the carrier material.

Next, we studied ammonolysis with *in situ* racemisation in the various solvents at 40 °C. Experiments were carried out with pyridoxal (1 mM) as racemisation catalyst. As can be seen from Figure 3.4, the substrate is most efficiently racemised in tert-butyl alcohol and tert-amyl alcohol. In all other - non protic - solvents, the racemisation of the substrate was so slow that L-1a accumulated, which caused low yields with low ee.
Figure 3.4 ee of 2 as a function of the conversion in different organic solvents: (O) acetonitrile, (Δ) tert-amyl alcohol, (●) tert-butyl alcohol, (■) tert-butyl methyl ether, (▲) 1,2-dimethoxyethane, (□) dioxane.

Temperature effect

The reaction temperature is also known to affect $E$.\textsuperscript{18,19} Again, first temperature effect for the kinetic resolution was tested. Since the reaction rate decreases with temperature, only those solvents with a sufficiently high initial rate at 40 °C were used in the low-temperature reactions, e.g. tert-butyl alcohol, tert-amyl alcohol and tert-butyl methyl ether (Table 3.5).

Table 3.5 Ammonolysis of 1a at low temperature.\textsuperscript{a}

<table>
<thead>
<tr>
<th>Solvent</th>
<th>T (°C)</th>
<th>t (h)</th>
<th>Lipase (mg)</th>
<th>Conversion (%)</th>
<th>$ee_{D-2}$ (%)</th>
<th>$E$</th>
</tr>
</thead>
<tbody>
<tr>
<td>tert-Amyl alcohol</td>
<td>40</td>
<td>3</td>
<td>50</td>
<td>48</td>
<td>76</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>50</td>
<td>31</td>
<td>88</td>
<td>25</td>
</tr>
<tr>
<td></td>
<td>-10</td>
<td>23</td>
<td>100</td>
<td>22</td>
<td>91</td>
<td>28</td>
</tr>
<tr>
<td>tert-Butyl methyl ether</td>
<td>40</td>
<td>5</td>
<td>50</td>
<td>43</td>
<td>73</td>
<td>12</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>9</td>
<td>250</td>
<td>27</td>
<td>90</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>-10</td>
<td>9</td>
<td>600</td>
<td>22</td>
<td>90</td>
<td>24</td>
</tr>
<tr>
<td>tert-Butyl alcohol</td>
<td>40</td>
<td>4</td>
<td>50</td>
<td>46</td>
<td>78</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>11</td>
<td>100</td>
<td>39</td>
<td>89</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>-10\textsuperscript{b}</td>
<td>29</td>
<td>500</td>
<td>42</td>
<td>90</td>
<td>39</td>
</tr>
<tr>
<td></td>
<td>-20\textsuperscript{b}</td>
<td>24</td>
<td>1000</td>
<td>33</td>
<td>94</td>
<td>52</td>
</tr>
</tbody>
</table>

\textsuperscript{a} Reaction conditions: 1 mmol D,L-phenylglycine ester 1a was shaken with SP 611 in 5 ml ammonia-saturated solvent at the selected temperature.

\textsuperscript{b} tert-Butyl methyl ether was added as cosolvent (30% v/v) to avoid freezing of tert-butyl alcohol saturated with NH$_3$. 

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From the solvents tested, the upward trend in $E$ was most pronounced in tert-butyl alcohol, resulting in an $E$ value up to 52 at -20 °C. Measurements at even lower temperatures were unpractical owing to the low reaction rates.

Encouraged by our good results at low temperature we next combined the ammonolysis of 1a with in situ racemisation at -20 °C. tert-Butyl alcohol-tert-butyl methyl ether (70:30%, v/v) was used as solvent and pyridoxal (4 mM) as racemisation catalyst. The reaction was followed in time and after 66 h we obtained 85% yield with 88% ee (Figure 3.5). This result shows that at low temperature the substrate is indeed racemised much faster than the product and that a dynamic kinetic resolution is feasible whereby the product is obtained in good yield and high ee.

![Graph showing composition and ee of ester 1a and amide 2 as function of time in a dynamic kinetic resolution at -20 °C.](image)

**Figure 3.5** Composition and ee of ester 1a and amide 2 as function of time in a dynamic kinetic resolution at -20 °C: (△) ee ester 1a, (▲) amount of ester 1a, (○) ee amide 2, (●) amount of amide 2.

Reaction conditions: 1 mmol D,L-phenylglycine methyl ester 1a was shaken with 50 mg Novozym 435 and 0.02 mmol pyridoxal in 5 ml tert-butyl alcohol-tert-butyl methyl ether (70:30, % v/v) saturated with ammonia at -20 °C.

**Conclusion**

Pyridoxal or salicylaldehyde catalysed racemisation of phenylglycine methylester 1a is compatible with lipase catalysed enantioselective ammonolysis. The catalyst became more enantioselective at low temperature. At -20 °C D-phenylglycine amide 2 was obtained with 88% ee at 85% conversion.
Experimental section

Materials
Immobilised *Candida antarctica* lipase B, Novozym 435, was a gift from Novo Nordisk A/S, ( Bagsværd, Denmark). Immobilised *Candida Antarctica* lipase B on Accurel EP 100, SP 611, (ex Novo Nordisk A/S) was a gift from Uniqema (Gouda, The Netherlands). 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 synthesised from the amino acid according to the literature. Phenylglycine methyl ester was used as free base obtained by bulb to bulb distillation of a mixture of phenylglycine methyl ester hydrochloric salt and sodium methoxide (30% solution in methanol). The free base distilled at 100 °C (1 mbar) as a colorless liquid, which solidified by standing at room temperature.

Analysis and equipment
The reaction mixtures of phenylglycine methyl ester were analysed by chiral HPLC on a Daicel Chemical Industries Ltd. 4.6 × 150 mm 5 μ Crownpak CR (+) column using a Waters 625 LC pump and a Waters 486 UV detector. The eluent was aqueous 0.03 M 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 analysed by chiral HPLC on a 4.6 × 250 mm 10 μ Chiracel OD column with a Waters 510 pump, and a Shimazu SPD-6A UV detector. Hexane-*isopropl alcohol-dimethylamine (90:10:0.1, v/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 (165 mg methyl ester 1a, 216 mg ethyl ester hydrochloric salt 1b or 232 mg butyl ester hydrochloric salt 1c) and *Candida antarctica* lipase B in 5 ml ammonia saturated solvent were shaken in 40 ml reaction vessels at the selected temperature (see Table 3.1 and 3.4 for further details). Reactions were monitored in time. The partial pressure of ammonia was maintained at 1 atmosphere in all reactions, which guarantees a constant thermodynamic activity of ammonia. The reactions were
stopped by adding concentrated formic acid and the reaction mixture was dissolved by adding water. When tert-amyl alcohol or tert-butyl methyl ether was used as solvent also methanol was added, when hexane was used as solvent, ethanol was added to dissolve the reaction mixture. 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 D-1a was varied between 50 and 400 mM, with salicylaldehyde between 30 and 300 mM. The concentration of phenylglycine amide D-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 analysed by HPLC. The kinetic constants were determined by fitting the experimental data in a Lineweaver-Burk plot.21

Ammonolysis with in situ racemisation
1.0 mmol 1a, racemisation catalyst and Candida antarctica lipase B in 5 ml solvent saturated with ammonia were shaken in 40 ml reaction vessels at the selected temperature (see Table 3.5 for further details). 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 formic acid and the reaction mixture was dissolved by adding water. When tert-amyl alcohol or tert-butyl methyl ether was used as solvent also methanol was added. A sample was taken for HPLC analysis.

Acknowledgements
A generous donation of Novozym 435 (Candida antarctica lipase B) by Novo Nordisk (Bagsværd, Denmark) is gratefully acknowledged. SP 611 (Candida Antarctica lipase B, immobilised on Accurel EP 100) ex Novo Nordisk A/S was kindly donated by Uniqema (Gouda, The Netherlands). This work was supported by a grant from the Netherlands Ministry of Economic Affairs and carried out in cooperation with DSM Life Science Products (Geleen, The Netherlands).
References

Chapter 4

Hydrolysis of D,L-phenylglycine nitrile by new bacterial isolates*

Abstract
Bacterial strains were screened for nitrile hydratase/amidase activity towards phenylglycine nitrile. Various strains were obtained which harboured a non-selective nitrile hydratase and an extremely L-selective amidase. A highly active strain identified as a *Rhodococcus* sp. was cultured with different nitriles as the sole source of nitrogen. The growth rate of the cells was not influenced by the structure of the nitriles, but the effect on the activity of the nitrile hydratase was significant. The best result was obtained with the cells grown on 2-methyl-3-butenenitrile. Hydrolysis of D,L-phenylglycine nitrile catalysed by this culture gave D-phenylglycine amide in 48% yield and > 99% ee and L-phenylglycine in 52% yield and 97% ee.

* Performed in collaboration with U. Heinemann from the University of Stuttgart.
**Introduction**

D-Phenylglycine amide (D-2) is a key intermediate in the industrial enzymatic synthesis of β-lactam antibiotics.¹ A convenient method for its synthesis would be the enzymatic hydrolysis of D,L-phenylglycine nitrile (1) catalysed by a nitrile hydratase/amidase system, such as those found in *e.g.* *Rhodococcus* species.²⁻⁴ These generally comprise a non-stereoselective nitrile hydratase and an L-specific amidase.⁵ Hence, we envisioned hydration of 1 to racemic phenylglycine amide (2) followed by hydrolysis of L-2 to L-phenylglycine (L-3, see Scheme 4.1). Published reports along these lines involved hydration of α-amino nitriles at minute concentrations (0.5⁻1 mM)⁶⁻⁷ and the rate of the second step - hydrolysis of the amide - was generally quite low. Owing to the propensity of 1 for decomposition into benzaldehyde and ammonia via a retro-Strecker reaction²⁸ it is necessary to achieve high rates for the enzymatic reactions, preferably at high substrate concentrations.

![Scheme 4.1](image)

**Scheme 4.1** Hydration and subsequent hydrolysis of D,L-phenylglycine nitrile.

We reasoned that these shortcomings could be remedied by screening nitrile hydrolysing microorganisms for activity and enantioselectivity in the transformation of 1 into D-2. We now report that a *Rhodococcus* species readily converts 1 into enantiopure D-2 and L-3 in essentially quantitative yield.

**Results and discussion**

*Screening for bacterial strains with nitrile hydratase/amidase activity*

Enrichments were performed with 2-methyl-3-butenenitrile and 3-phenylpropionitrile as the sole source of nitrogen and succinate as the carbon source. From the circa. 60 cultures tested, 5 strains were able to convert D,L-phenylglycine nitrile (1). We focused our

¹ *Chemical Abstr.* name: 2-aminophenylacetonitrile

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attention on those strains with the ability to catalyse fast hydration of the substrate (to avoid undesired decomposition of 1).

Strains MAWA and MAWB synthesised a non-selective nitrile hydratase, which converted 1 under the standard conditions within 5 minutes to the corresponding amide 2. Subsequently, accumulated 2 was rapidly hydrolysed to L-acid 3 by an L-specific amidase. When other strains were used, the hydration of 1 was rather slow, which resulted in partial degradation of 1 into benzaldehyde and ammonia. Furthermore, strains MAWD and MAWE hydrolysed 2 only slowly to the corresponding acid L-3 (Table 4.1). It should be mentioned that all experiments were carried out within the scope of screening for nitrile hydratase activity; the reactions were performed with whole cell suspensions with an OD_{546 nm} of 20. Since the molar extinction coefficient of the several strains differ, no conclusions can be made about the specific activity of the different nitrile hydratase/amidase systems. Hence, no true comparison in the nitrile hydration can be made.

| Table 4.1 Nitrile hydration by several bacterial strains.\(^a\) |
|---------------------|------|------|---------------------|------|------|------|
| Strain              | Amide D-2 | Acid L-3 |                      | \(E\) |
|                     | Yield (%) | ee (%) | Yield (%) | ee (%) |      |      |
| \(R.\) globerulus MAWA | 48    | >99  | 52    | 97    | >100 |
| \(R.\) rhodochrous MAWB | 43    | 85   | 38    | >99   | >100 |
| MAWC\(^b\)           | 39    | 93   | 41    | 84    | 20   |
| MAWD\(^b\)           | 46    | 96   | 14    | >99   | >100 |
| \(R.\) rhodochrous MAWE | 40    | 35   | 24    | 88    | 20   |

\(^a\) Reaction conditions: 0.01 mmol D,L-phenylglycine nitrile 1 and resting cells (\(A_{546\ \text{nm}}\) 20) in 1 ml 50 mM phosphate buffer pH 7 were shaken at 30 °C for 4 h.

\(^b\) Strain is not identified.

HPLC analysis showed that the nitrile hydratases did not exhibit any chiral discrimination; the observed enantioselectivity was a consequence of the L-specificity of the amidase as is generally the case.\(^5\) The enantiomeric ratio \(E^{\pm,9}\) for the amidase-

\[ E = \frac{\ln[1 - c(1 + ee)]}{\ln[1 - c(1 - ee)]} \]
catalysed hydrolysis was much lower for strain MAWC and MAWE than for the other strains (Table 4.1).

*Initial taxonomic characterisation and classification of the cultures*

All strains were gram-positive organisms according to the Gram and KOH test. Strain MAWA was further identified using the Biolog test. This strain was tentatively identified by this system as *Corynebacterium pseudodiphtericum* (SIM value 0.786). For the taxonomic description the almost complete 16S rDNA was amplified by PCR; the resulting fragment was cloned and partially sequenced using the universal primers 27f and 519r. The nucleotide sequences obtained were compared with the NCBI data base using the program BLASTN. The sequence obtained from strain MAWA showed more than 99% identity (451 from 453 bp) to the corresponding sequence from the type strain of *Rhodococcus globulatus*. The strain is deposited at the Deutsche Sammlung von Mikroorganismen (DSM) (Braunschweig, Germany).

*Optimisation of growth conditions*

Because strain *R. globulerus* MAWA gave apparently the best results with regard to reaction rate and l-specificity of the amidase, further optimisation was carried out with this strain. In order to find the best nitrogen source for an optimum cell yield and for highest enzyme activity, strain *R. globulerus* MAWA was grown in mineral medium with different aliphatic as well as aromatic nitriles (80-240 mg/l, 1 mM) as the sole nitrogen source. Succinate (1.6 g/l, 10 mM) was used as the sole source of carbon and energy (Figure 4.1).

![Figure 4.1](image)

**Figure 4.1** Growth of strain MAWA on different N-sources: (□) naphthalene-2-carbonitrile, (○) benzonitrile, (△) ketoprofen-nitrile, (▲) 2-methyl-3-butenenitrile, (●) 2-methylbutanenitrile
Strain *R. globerulus* MAWA showed rapid growth with all tested nitrogen sources. The growth rate of the cells was not influenced by the structure of the nitriles used. In contrast, the activity of the nitrile hydratase and amidase was markedly affected by the addition of different nitriles (Table 4.2).

<table>
<thead>
<tr>
<th>Nitrile&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Amide D-2</th>
<th>Acid L-3</th>
<th>Relative activity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Relative activity&lt;sup&gt;d&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Yield (%)</td>
<td>ee (%)</td>
<td>Yield (%)</td>
<td>ee (%)</td>
</tr>
<tr>
<td>NN</td>
<td>27</td>
<td>83</td>
<td>28</td>
<td>98</td>
</tr>
<tr>
<td>KP</td>
<td>79</td>
<td>4</td>
<td>4</td>
<td>89</td>
</tr>
<tr>
<td>BN</td>
<td>50</td>
<td>&gt;99</td>
<td>48</td>
<td>92</td>
</tr>
<tr>
<td>MeBuN</td>
<td>71</td>
<td>27</td>
<td>21</td>
<td>76</td>
</tr>
<tr>
<td>MeBu&lt;sup&gt;3&lt;/sup&gt;N</td>
<td>48</td>
<td>&gt;99</td>
<td>52</td>
<td>97</td>
</tr>
</tbody>
</table>

<sup>a</sup> Reaction conditions: 0.01 mmol D,L-phenylglycine nitrile 1 and resting cells (*A<sub>546 nm</sub> 20) in 1 ml 50 mM phosphate buffer pH 7 were shaken at 30 °C for 4 h.


<sup>c</sup> Activity of nitrile hydratase at optical density 20 relative to 2-methyl-3-butenenitrile (MeBu<sup>3</sup>N) used as nitrogen source.

<sup>d</sup> Activity of amidase at optical density 20 relative to 2-methyl-3-butenenitrile (MeBu<sup>3</sup>N) used as nitrogen source.

After growth with naphthalene-2-carbonitrile as sole nitrogen source only a slow hydration of 1 was observed. All other nitriles used induced considerably higher rates; the best result was obtained with 2-methyl-3-butenenitrile. 1 was hydrated within 5 minutes to the corresponding amide 2 and subsequent hydrolysis of L-2 was complete within 4 hours (Figure 4.2), affording L-3 in 52% yield and 97% ee together with D-2 in 48% yield and > 99% ee. This corresponds with *E* > 100 for the amidase-catalysed hydrolysis.
Figure 4.2  Hydrolysis of D,L-phenylglycine nitrile 1 by *R. globiderus* MAWA grown on 2-methyl-3-butenenitrile (▲) phenylglycine nitrile, (■) phenylglycine amide, (□) *ee* D-phenylglycine amide, (●) phenylglycine, (○) *ee* L-phenylglycine.

**Conclusion**

Several new bacterial cultures were found, which harboured nitrile hydratase/amidase activity. Strain MAWA identified as a *Rhodococcus* sp. gave D-phenylglycine amide in 48% yield and > 99% *ee*. The different strains will be cultivated on large scale, which will be discussed in chapter 5.

**Experimental section**

**Materials**

Racemic phenylglycine and racemic phenylglycine nitrile hydrochloride (technical grade) were purchased from Acros. The latter was purified by neutralisation of the α-aminonitrile hydrochloric acid salt with base followed by an extraction with dichloromethane. The combined organic layers were acidified with 1N HCl and the nitrile hydrochloric salt was re-extracted in the aqueous phase, which was concentrated in *vacuo*. DSM (Geleen, The Netherlands) kindly donated enantiomerically pure D-phenylglycine, D-phenylglycine amide and D-phenylglycine nitrile tartaric acid salt (Alpha Drug India LTD). (S)-ketoprofenitrile was a gift from Bayer AG (Leverkusen, Germany). Benzonitrile, 2-methylbutanenitrile and 3-phenylpropionitrile were purchased from Aldrich. Naphthalene-2-carbonitrile was obtained from Lancaster Synthesis. 2-Methyl-3-butenenitrile was obtained from Fluka. All other chemicals used for mineral salts media and buffer solutions were purchased from Aldrich, E. Merck AG or Fluka.
Racemic phenylglycine amide was synthesised chemically by ammonolysis of the corresponding methylester.\textsuperscript{12}

\textbf{Analysis and equipment}

The reaction mixtures were analysed by chiral HPLC on a Daicel Chemical Industries Ltd. 4.6 x 150 mm 5 µ Crownpak CR (+) column using a Waters 625 LC pump and a Waters 486 UV detector. The eluent was aqueous HClO\textsubscript{4}, pH 1.0 at a flow of 0.6 ml/min, the column temperature was 18 °C.

\textbf{Screening of bacterial cultures}

Bacterial cultures were grown in a nitrogen-free mineral medium\textsuperscript{13} with disodium succinate (10 mM) as sole source of carbon and energy. 2-Methyl-3-butenenitrile or 3-phenylpropionitrile was added as sole source of nitrogen (80 and 130 mg/l respectively, 1 mM from a stock solution in methanol). After 1 day incubation cultures were transferred (1:33 v/v) to fresh medium with the same composition. The cells were harvested by centrifugation.

\textbf{Optimisation of growth conditions for strain MAWA}

To find the best nitrogen source for optimum cell yield, strain MAWA was grown in a mineral medium with naphthalene-2-carbonitrile (155 mg/l), benzonitrile (104 mg/l), ketoprofenitrile (235 mg/l), 2-methyl-3-butenenitrile (80 mg/l) or 2-methylbutanenitrile (85 mg/l) as nitrogen sources (1 mM each). Disodium succinate (1.6 g/l, 10 mM) was used as sole source of carbon and energy.

\textbf{Growth measurement}

The growth of bacterial cultures was either monitored with a Klett-Summerson Colorimeter (model 800-3; Klett MFG Co. Inc. New York, USA) or monitored spectrophotometrically by measuring the absorbance at 546 nm with a Kontron Uvikon 820 spectrophotometer (Kontron, Eching, Germany).

\textbf{Initial taxonomic characterisation and classification of the cultures}

Strain MAWA was characterised by using the Biolog test system (GP plates; Biolog Inc. 3938 Trust Way, Calif., USA). For a more reliable taxonomic description the almost complete 16S rDNA was amplified by PCR; the resulting fragment was cloned and partially sequenced using the universal primers 27f and 519r.\textsuperscript{11}
Standard assay for hydrolysis of D,L-phenylglycine nitrile with resting cells

10 μmol D,L-phenylglycine nitrile (10 mM stock solution in 50 mM sodium phosphate buffer pH 7) and cells with an absorbance (A_{546 \text{ nm}}) of 20 in 1 ml 50 mM sodium phosphate buffer pH 7 were shaken at 30 °C. Samples were periodically withdrawn, the cells were removed by centrifugation and the supernatant was analysed by HPLC.

Acknowledgements

Thanks due to U. Heinemann and Dr. A. Stolz (IBVT, Stuttgart) for their cooperation during this study. This work was supported by a grant from the Netherlands Ministry of Economic Affairs and carried out in cooperation with DSM Life Science Products.

References

Chapter 5

Large-scale preparation of nitrile hydratases

Abstract

*Rhodococcus* species harbouring nitrile hydratase/amidase systems were cultivated on large scale. High yields of biomass (up to 7 g/l) with good activity (up to 70 U/g) were obtained. The whole cells were entrapped in sodium alginate gel. The relative rate of nitrile hydration of the immobilised cells varied from 15 to 50% compared with suspended cells. In organic solvent, however, complete and irreversible loss of activity occurred.
Chapter 5

Introduction

Nitriles are important intermediates in organic synthesis as precursors of the corresponding amides and acids. However, the chemical transformation of nitriles has limited utility due to the severe conditions required (high temperature and strongly acidic or alkaline pH). Often this is incompatible with acid- or base labile functionalities present in the substrate. Enzymatic hydration of nitriles would overcome these shortcomings because reactions proceed under mild conditions (neutral pH and room temperature). In addition the bioconversions can be stereo- and regiospecific.\(^2\)\(^-\)\(^4\)

Unfortunately, nowadays no nitrile hydrating biocatalyst is commercially available. A few years ago, NOVO Nordisk stopped the production of their immobilised whole cell preparation (SP 361 or SP 409) derived from a *Rhodococcus* sp.\(^*\). Consequently there is a need for other biocatalysts. In chapter 4 the screening of nitrile hydratase/amidase producing strains suitable for the hydration of D,L-phenylglycine nitrile is discussed.\(^5\)

Here we report the cultivation of several of these strains on a large scale. In addition, immobilisation of these cultures was studied as well as their tolerance towards organic solvents.

Results and discussion

Growth of different strains

Four different strains from our initial study\(^5\) were grown in a nitrogen-free mineral medium. As inoculum 5 ml of a shake-flask preculture in complex medium was used. D-Fructose (18 g/l) was used as the sole source of carbon and energy and phenylacetonitrile was used as the sole source of nitrogen.

Initially, we performed comparative batch and fed-batch cultivations. In the latter, the nitrile was periodically added in aliquots (0.35 g/l - 3 mM - from a 580 g/l - 5 M- stock solution in methanol) to the culture, providing a low concentration of the potentially toxic nitrile. The fed-batch cultivation resulted in improved growth and nitrile hydratase activity (data not shown), so all further cultivations were performed using the fed-batch technique.

Under these conditions strain *R. globerulus* MAWA showed rapid growth, after a relatively long lag phase caused by the change from a rich medium to a minimum medium (Fig. 5.1). Figure 5.2 shows the parallel consumption of fructose.

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\(^*\) SP 361 and SP 409 are derived from the same *Rhodococcus* sp. strain, which contains a nitrile hydratase and an amidase. The enzyme preparations only differ by the carrier used for the immobilisation.
Figure 5.1 Growth cycle for *R. globerulus* MAWA.

Figure 5.2 Consumption of fructose for *R. globerulus* MAWA.

The other strains, however, did not grow on this medium, as became clear from the low consumption of fructose. In order to find the best source of carbon and energy for an optimum cell yield, the strains were grown in mineral medium with succinate, glycerol, lactic acid or stearate (all 10 g/l, 30-109 mM) as carbon source. Phenylacetonitrile was repeatedly added in aliquots (0.35 g/l, 3 mM) as nitrogen source. Furthermore, urea (1 g/l, 16 mM) was added as an additional N-source in order to induce nitrile hydratase activity.

In all cases glycerol gave relatively the best growth although the cell density was moderate. However, when a vitamin solution was added exponential growth was observed (Fig. 5.3).

Figure 5.3 Growth of (○) *R. rhodochrous* MAWB, (△) *R. rhodochrous* MAWE, (□) *R. erythropolis* MAWF.
All cells were harvested at the late exponential growth phase (after 16 - 48 h) and the nitrile hydratase activity was determined. Prior to the assay the whole cells were first incubated with the nonionic detergent Triton X-100, providing a gentle way of permeabilising the peptidoglycan of the gram-positive bacteria in order to minimise diffusion limitation. In the standard enzyme assay 2-phenylpropionitrile was used as substrate. The results of the growth are shown in Table 5.1.

Table 5.1 Nitrile hydratase activity of several strains.\(^a\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Culture time</th>
<th>Wet biomass</th>
<th>Total activity</th>
<th>Specific activity (^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. globerulus MAWA</td>
<td>48</td>
<td>3.0</td>
<td>213</td>
<td>71</td>
</tr>
<tr>
<td>R. rhodochrous MAWB</td>
<td>16</td>
<td>6.0</td>
<td>228</td>
<td>38</td>
</tr>
<tr>
<td>R. rhodochrous MAWE</td>
<td>16</td>
<td>7.2</td>
<td>403</td>
<td>56</td>
</tr>
<tr>
<td>R. erythropolis MAWF</td>
<td>24</td>
<td>2.7</td>
<td>59</td>
<td>22</td>
</tr>
</tbody>
</table>

\(^a\) Reaction conditions: 4 \(\mu\)mol 2-phenylpropionitrile and resting cells in 2 ml 50 mM phosphate buffer pH 7 were shaken at room temperature.

\(^b\) One unit (U) of nitrile hydratase activity is defined as the amount of enzyme (g wet biomass) that converts 1 \(\mu\)mol 2-phenylpropionitrile per min.

**Hydration of D,L-phenylglycine nitrile**

Now the specific activity of the different strains is determined, their activity can truly be compared in the hydration of D,L-phenylglycine nitrile (Table 5.2).

Table 5.2 Hydration of D,L-phenylglycine nitrile\(^a\)

<table>
<thead>
<tr>
<th>Strain</th>
<th>(v_i)NHase ((\mu)mol/min/U)</th>
<th>(v_i)amidase (10^2) (\mu)mol/min/U</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. globerulus MAWA</td>
<td>3.27</td>
<td>17</td>
</tr>
<tr>
<td>R. rhodochrous MAWB</td>
<td>2.73</td>
<td>0.70</td>
</tr>
<tr>
<td>R. rhodochrous MAWE</td>
<td>2.44</td>
<td>0.63</td>
</tr>
<tr>
<td>R. erythropolis MAWF</td>
<td>2.16</td>
<td>3.5</td>
</tr>
</tbody>
</table>

\(^a\) Reaction conditions: 0.03 mmol D,L-phenylglycine and resting cells (1 U) in 3 ml 50 mM phosphate buffer pH 7 were shaken at room temperature.

Strain *R. globerulus* MAWA afforded the highest nitrile hydratase as well as amidase activity. The other strains harboured comparable nitrile hydratase activity, but the
amidase activity was considerably lower. When the activities of the whole cells are compared with those from the screening stage for nitrile hydratase activity (Table 4.1, chapter 4) strain MAWA is the most active in both cases. For all other strains no correlation was found.

Cell disintegration
In order to release the enzymes from the whole cells as a first stage of enzyme purification, several techniques for cell disintegration were carried out. When ultrasonic disintegration or a French press were used, no nitrile hydratase activity was observed in the aqueous extract; all activity remained in the residual cells. Even in the presence of Triton X-100, no activity was found in the supernatant. Next, cell disruption was attempted by adding glass beads to a slurry of the whole cells in buffer, which were subsequently vortex-mixed. Less than 1% of the activity was found in the supernatant, whereas the residual cells retained more than 95% of the activity. Even in the presence of different detergents, no increased activity was found in the supernatant. When the suspension was vortex-mixed for a longer time no improvements were observed. Apparently these gram-positive Rhodococci tend to be resistant to breakage and more vigorous treatments (e.g. bead mill) are necessary to disrupt the whole cells. In view of the lack of suitable equipment, no further attempts were made to disintegrate the whole cells.

Immobilisation of whole cells
Because cell disintegration failed, whole cells (without further purification) were immobilised. First the whole cells were immobilised into polyurethane foam. The cell wall of the bacteria consists of peptide and sugar units. Reactive carbohydrate hydroxyl groups can react with isocyanate groups in the polyurethane prepolymer resulting in immobilisation. The method developed by Lejeune and Russel was used, but no activity in the foam was found. Since no activity was recovered from the wash liquid, the complete loss of activity might be described to diffusion limitation. Therefore, the cells were pre-incubated with Triton X-100 and subsequently immobilised, but again no activity was observed. No further investigation of the loss of activity was carried out.
Another simple and widely applicable immobilisation method for whole cells is entrapment in alginate gel. Alginate gels are block copolymers composed of β-D-mannuronate and α-guluronate residues; gelation occurs in the presence of calcium ions. The whole cells were pre-incubated with Triton X-100 and subsequently mixed with sodium alginate. The cell slurry was extruded as discrete droplets into a 150 mM
CaCl₂-solution and stirred for 2 h to harden the gel. The nitrile hydratase activity of the immobilised cells was assayed in the standard 2-phenylpropionitrile hydration test. The reaction medium was changed to Tris-buffer, because the phosphate-buffered medium has a high affinity for the cross-linked calcium ions and destabilises the gel.¹¹ In order to determine the efficiency of the entrapment method, the immobilised cells were assayed in the standard activity test. A moderate fraction of the entrapped cells were still active and yields up to 50% were reached (Table 5.3). It was not investigated whether the whole cells were deactivated during immobilisation or that diffusional limitations caused the reduced activity. However, no activity was recovered from the wash liquid, indicating that no leaching of cells from the alginate gel occurred.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Loading (U/g)</th>
<th>Found (U/g)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>R. globerulus</em> MAWA</td>
<td>5</td>
<td>0.76</td>
<td>15</td>
</tr>
<tr>
<td><em>R. rhodochrous</em> MAWB</td>
<td>5</td>
<td>2.5</td>
<td>50</td>
</tr>
<tr>
<td><em>R. rhodochrous</em> MAWE</td>
<td>5</td>
<td>1.7</td>
<td>35</td>
</tr>
<tr>
<td><em>R. erythropolis</em> MAWF</td>
<td>5</td>
<td>1.4</td>
<td>28</td>
</tr>
</tbody>
</table>

Table 5.3 Activity tests of immobilised nitrile hydratases.²

² Reaction conditions: 4 µmol 2-phenylpropionitrile and resting cells in 2 ml 50 mM phosphate buffer pH 7 were shaken at room temperature.

*Nitrile hydratase activity in low water systems*

The hydration of nitriles by either nitrile hydratases or nitrilases in organic solvents has hardly been studied. Only a few papers report the retention of activity of these enzymes in aqueous/organic systems.¹²,¹³ The immobilised whole cells were tested in various aqueous/organic solvent mixtures as well as in monophasic buffer-saturated organic solvents. In all cases no activity was detected. Moreover, when highly hydrophilic solvents were used (log P < 1), the beads shrank. Subsequently, the immobilised cells were reused in a standard buffered assay test, which confirmed the complete and irreversible loss of activity of the immobilised cells in the presence of organic solvent. Presumably, cell-free extracts (or purified enzymes) should be used in the presence of organic solvent as is described in the literature.¹²,¹³

*Conclusions*

Protocols have been developed to culture *Rhodococcus* species rapidly on multi-litre scale. Biomass yields from 3 to 7 g/l with high nitrile hydratase activity were obtained.
Because cell disruption failed, the whole cells were entrapped in alginate gel. The immobilised preparations were irreversibly deactivated in the presence of organic solvent.

**Experimental section**

**Materials**

Phenylacetonitrile, 2-phenylpropionitrile, D-fructose, disodium succinate, glycerol, D,L-lactic acid, sodium stearate and Triton X-100 were obtained from Acros. 2-Phenylpropionic acid and 2-phenylpropionamide were chemically synthesised from the corresponding nitrile.\(^4\) Nutrient Broth was obtained from Difco (Detroit, Michigan, USA). Daishin Agar was obtained from Brunschwig Chemie (Amsterdam, The Netherlands). Malt and yeast were purchased from E. Merck. All other chemicals used for mineral salts media and buffer solutions were purchased from Acros, Aldrich, E. Merck or Fluka.

**Analysis and equipment**

The reaction mixtures of the nitrile hydratase activity assays were analysed by HPLC on a custom-packed Symmetry C\(_{18}\) cartridge (Waters Radical-Pak, 8 x 100 mm, 5\(\mu\)m) contained in a Waters RCM 8 x 10 compressing unit. A Waters 590 pump and a Waters 486 UV detector were used. The eluent was acetonitrile-water 40:60, \(v/v\) with 0.1% trifluoroacetic acid at a flow of 1.0 ml/min. Fructose concentration was analysed by HPLC on a cation exchange column in the Pb\(^{2+}\) form (Phenomenex, 7.8 x 300 mm). A Waters 510 pump and a Shodex SE-51 RI detector were used. The eluent was water at a flow of 0.6 ml/min, the column temperature was 60 °C.

The optical density of the various cultures was measured using a Varian Cary 3 Bio UV/VIS spectrophotometer.

**Media**

The following components of the mineral medium were sterilised (30 min at 121 °C, 2 bar.): 0.05 g/l CaCl\(_2\), 0.02 g/l MgSO\(_4\), 0.03 g/l Fe\(^{III}\)-citrate, 2 g/l KH\(_2\)PO\(_4\), 5.6 g/l Na\(_2\)HPO\(_4\), 1 ml/l trace element solution SL 6.\(^{15}\)

Complex media were prepared by adding 5 g yeast extract and 10 g malt extract to 1 l water which was sterilised.

Solid media were prepared by adding 8 g Nutrient Broth No. 1, 5 g NaCl and 15 g Agar to 1 l water, which was sterilised.
Chapter 5

Microorganism and culture conditions
Bacterial cultures were previously isolated by enrichment from soil taken from the area of Stuttgart (Germany). For MAWA fructose (18 g/l) was added as the sole source of carbon and energy and phenylacetonitrile (1 g/l) was added as the sole source of nitrogen. The other strains were cultured with glycerol (10 g/l) as the sole source of carbon and energy. Additionally urea \(^6\) (1 g/l) and 1 ml of a vitamin solution were added. \(^7\) In all cases phenylacetonitrile (1 g/l) was added as the source of nitrogen. Cells were grown in 300-ml flasks with baffles with 50 ml medium at 25 °C with shaking (125 rpm). As inoculum 5 ml from a preculture in complex medium was used. After 24 hours incubation cultures were transferred \((1 : 20 \nu/\nu)\) to fresh medium with the same composition. The cell growth was monitored by analysing samples (2 ml) for optical density, fructose concentration and nitrile hydratase activity. After 16-48 h, the cells were, in the late exponential growth phase, harvested by centrifugation.

Strain maintenance
The different strains were maintained on agar plates using the above-described medium. Sub-culturing was performed every 12 weeks and the plates were stored at 4 °C.

Initial taxonomic characterisation and classification of the bacterial strains
For the taxonomic description the almost complete 16S rDNA was amplified by PCR; the resulting fragment was cloned and partially sequenced using the universal primers 27f and 519r. \(^16\) The nucleotide sequences obtained were compared with the NCBI database using the program BLASTN. Strains MAWB and MAWE showed the highest degree of sequence identity (more than 99% sequence identity) with strain *Rhodococcus rhodochrous* NCIMB 13064 (this strain is listed in the NCBI database as *Rhodococcus erythropolis*). A BLAST search with the sequence obtained for the strain MAWF showed the highest degree of similarity (more than 99%) with a sequence (X80618) deposited for *Nocardia calcarea*. Because *N. calcarea* is generally considered as a synonym for *Rhodococcus erythropolis*, \(^17\) this strain was designated as *R. erythropolis* MAWF. The strains are deposited at the Deutsche Sammlung von Mikroorganismen (DSM) (Braunschweig, Germany).

Enzyme assay and definition of units
An appropriate amount of whole cells (20 to 30 mg) was incubated with 2% (v/v) Triton X-100 for 30 min. After washing with 0.9% NaCl, 2 ml 50 mM sodium phosphate buffer pH 7 and 4 μmol 2-phenylpropionitrile (400 mM stock solution in ethanol) were added
and shaken at room temperature. Samples were periodically withdrawn, the cells were removed by centrifugation and the supernatant was analysed by HPLC. One unit (U) of nitrile hydratase activity is defined as the amount of enzyme (g wet biomass) that converts 1 μmol 2-phenylpropionitrile per min.

Immobilisation of whole cells
Whole cells (1 g) were suspended in tris-buffer (2 ml) and subsequently mixed with an aqueous sodium alginate solution (2.5% w/v, 10 g). The slurry was dripped via a continuous stream of air into a stirred CaCl₂ solution (200 ml, 150 mM). The beads (2 mm radius) were incubated in the CaCl₂ solution for 2 h.

Acknowledgements

Prof. Dr. L. Fischer is gratefully acknowledged for his help and helpful discussions. Thanks are due to P. Russo and A. van Uijen for sterilising all solutions and equipment. This work was financially supported by The Netherlands Ministry of Economic Affairs and coordinated by DSM Life Science Products (Geleen, the Netherlands).

References

Stereoretentive nitrile hydratase-catalysed hydration of D-phenylglycine nitrile

Abstract
The hydration of D-phenylglycine nitrile to the corresponding amide, mediated by nitrile hydratase-containing microorganisms, was studied. Batch and fed-batch reactions were compared with regard to degradation and racemisation of the chemically labile substrate. A batch process gave satisfactory results and at up to 25 mM D-phenylglycine nitrile, D-phenylglycine amide was obtained in 94% yield with 92% ee using an immobilised Rhodococcus sp. (NOVO SP 361). The enzyme could be reused, although it slowly lost its activity. When the concentration of D-phenylglycine nitrile was increased to 100 mM in a batch reaction rapid decomposition of the substrate was observed and D-phenylglycine amide was obtained in only 37% yield. A fed-batch reaction afforded an improved yield, although the decomposition of the substrate could not be avoided completely. Lowering the temperature stabilised the substrate and a fed-batch reaction at 5 °C resulted in a 96% yield of D-phenylglycine amide with 95% ee. A number of other whole-cell hydratase/amidase systems also hydrated D-phenylglycine nitrile in nearly quantitative yield and > 94% ee. Moreover, the ee was further increased to > 99% upon prolonged reaction times with minimum loss in yield due to the action of the L-specific amidase that is present in these biocatalysts.

**Chapter 6**

**Introduction**

D-Phenylglycine amide is a key intermediate in the industrial enzymatic synthesis of semisynthetic β-lactam antibiotics\(^1\) (Scheme 6.1).

\[
\begin{align*}
\text{NH}_2 & \quad \text{NH}_2 \\
\text{R} & \quad \text{H, OH} \\
& \quad 6\text{-APA} \\
& \quad \text{R} = \text{H, ampicillin} \\
& \quad \text{R} = \text{OH, amoxycillin}
\end{align*}
\]

**Scheme 6.1** Enzyme-catalysed coupling of D-phenylglycine amide and 6-aminopenicillic acid.

Several routes to D-phenylglycine amide are known, all starting with a Strecker reaction on benzaldehyde (Scheme 6.2). A route that involves an aminopeptidase catalysed L-specific hydrolysis of D,L-phenylglycine amide\(^2-4\) has been commercialised by DSM. In this kinetic resolution the yield of D-phenylglycine amide is limited to 50% and the unwanted isomer, L-phenylglycine (L-PG), has to be racemised and recycled, which requires several steps. A more elegant approach is an asymmetric transformation of the diastereomeric salt of D,L-phenylglycine amide with L-mandelic acid (MA) in the presence of a catalytic amount of a carbonyl compound.\(^5,6\) A 100% yield of enantiopure D-phenylglycine amide is theoretically possible, but the necessary recycle of L-mandelic acid complicates the process.

\[
\begin{align*}
\text{NH}_2 & \quad \text{NH}_2 \\
\text{HCN} & \quad \text{L-PG} \\
& \quad \text{L-specific aminopeptidase} \\
& \quad \text{(50% yield)}
\end{align*}
\]

**Scheme 6.2** Several routes to D-phenylglycine amide.
We considered an alternative route to D-phenylglycine amide directly from the α-amino nitrile. Enantiopure D-phenylglycine nitrile tartaric acid salt is readily accessible via an asymmetric transformation with tartaric acid (TA) as resolving agent based on described procedures. We investigated the stereoretentive nitrile hydratase catalysed hydration of D-phenylglycine nitrile (D-1) into D-phenylglycine amide (D-2), (Scheme 6.3).

\[ \text{Scheme 6.3 Hydration of D-phenylglycine nitrile.} \]

Most published work on enzymatic conversions of α-amino nitriles has been directed at the production of optically pure amino acids via a nitrilase catalysed conversion. Only a few groups reported an α-aminonitrile hydrolysis via a nitrile hydratase/amidase system, yielding D-amide and L-acid. The biocatalysis of α-amino nitriles is hampered by their spontaneous degradation into aldehyde and ammonia via a retro-Strecker reaction. This reaction takes place readily under the conditions that are optimal for the enzymatic reaction, i.e. pH 7 with low yields of amides as a result. Decomposition of the nitrile could partially be avoided by performing the reaction at concentrations of 0.5-1.5 mM, which have limited synthetic utility.

We have undertaken to improve the nitrile hydratase catalysed conversion of D-1 into D-2 by reaction engineering. As biocatalyst we selected Novo SP 361, an immobilised Rhodococcus sp. that has been widely used. Herein we report two different concepts for the SP 361 catalysed hydration of D-1 with the aim of achieving high chemical and volumetric yields of D-phenylglycine amide (D-2) by minimising competing racemisation and degradation of D-1.

**Results and discussion**

*SP 361-catalysed batch reaction at low pH*

Although pH 7 is optimal for SP 361, initial experiments were conducted at pH 5 in an attempt to minimise racemisation and degradation of D-1. Even at such a low pH D-1 was hydrated to D-2 by the non-selective nitrile hydratase and racemisation as well as
decomposition of D-1 were negligible. The amidase appeared to be slow and highly L-specific and no formation of phenylglycine was observed. However, when the enzyme was reused in a second cycle, its performance had decreased considerably. This effect was more pronounced when the substrate was used as its tartaric acid salt rather than as the free base (Table 6.1). We tentatively concluded that tartaric acid deactivated SP 361, hence further experiments were performed with D-1 as the free base.

**Table 6.1** Hydration of D-phenylglycine nitrile at pH 5.\(^a\)

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Time (h)</th>
<th>Cycle 1</th>
<th></th>
<th>Cycle 2</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Yield (_{D-2}) (%)</td>
<td>ee (_{D-2}) (%)</td>
<td>Yield (_{D-2}) (%)</td>
<td>ee (_{D-2}) (%)</td>
</tr>
<tr>
<td>Tartaric acid salt</td>
<td>3</td>
<td>65</td>
<td>95</td>
<td>11</td>
<td>86</td>
</tr>
<tr>
<td>Free base</td>
<td>3</td>
<td>70</td>
<td>99</td>
<td>31</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>77</td>
<td>99</td>
<td>38</td>
<td>92</td>
</tr>
</tbody>
</table>

\(^a\) Reaction conditions: 0.25 mmol \(\alpha\)-aminonitrile D-1 and 500 mg SP 361 in 10 ml 10 mM phosphate buffer pH 5 were shaken at room temperature.

In order to gain more insight into the deactivation of SP 361, the effect of the buffer on the enzymatic hydration of D-1 was briefly investigated. A 10 mM phosphate buffer had a slight accelerating effect compared with reaction in the absence of buffer, presumably because of the unavoidable pH excursions in the latter case. 100 mM phosphate caused a decrease in rate as well as in yield; 100 mM acetate had an even more pronounced effect (Figure 6.1).

![Figure 6.1](image)

**Figure 6.1** SP 361 catalysed hydration of D-1 (25 mM) at pH 5 in various buffers: (▲) no buffer, (●) 10 mM phosphate, (■) 100 mM phosphate, (○) 100 mM acetate.
In an attempt to reactivate the catalyst, FeCl₃ as well as CoCl₂ were added to the reaction mixture, since it is known that some nitrile hydratases contain iron or cobalt as cofactor, but no increase in enzyme activity was observed. We conclude that D-1 can be hydrated to D-2 at pH 5 without racemisation or decomposition, but recycling of the catalyst is not possible. In view of these results all further experiments were carried out at pH 7.

*SP 361-catalysed batch reaction at pH 7*

At pH 7 D-1 spontaneously racemises and decomposes, but this can be minimised by keeping its concentration low. Surprisingly we found that up to 25 mM D-1 was converted to D-2 in high yield with negligible decomposition or racemisation. Again no formation of acid was observed. The enzyme could be recycled several times, although its activity slowly declined (Figure 6.2).

![Graph showing hydration of D-1 (25 mM) at pH 7](image)

**Figure 6.2**  SP 361 catalysed batch-wise hydration of D-1 (25 mM) at pH 7 (■) cycle 1, (▲) cycle 2, (●) cycle 3.

When the substrate concentration was increased to 100 mM, the above procedure yielded only 37 mM D-2. Racemisation of D-1 was negligible (5.5%), but its decomposition into benzaldehyde and ammonia amounted to 43%. In conclusion, this simple methodology is not feasible at practically relevant concentrations.

*SP 361-catalysed fed-batch reactions at pH 7*

A fed-batch procedure would present an option to combine a low actual concentration of D-1 - to retard its decomposition - with a high over-all throughput. Accordingly, we carried out fed-batch reactions by adding a 200 mM solution of D-1 resulting in a final concentration of 100 mM reaction mixture. When the feedstock was added in 1 h, 50 mM
D-2 was obtained (whereas the batch reaction yielded only 37 mM D-2). However, attempts to increase the yield by prolonging the addition time from 1 to 5 h failed; in all cases the reaction stopped at 50 mM D-2.

A turnover effect would be a possible explanation, but when the amount of catalyst was doubled only 79 mM D-2 was obtained instead of the expected 100 mM D-2. This prompted us to investigate inhibition effects on SP 361 (Figure 6.3). First, product inhibition was examined. When the reaction was performed in the presence of 50 mM D-2, no decrease in reaction rate was observed; therefore we concluded that SP 361 is not inhibited by the product. The effect considered next was ionic strength, because in all fed-batch processes D-1 had been added to the reaction mixture as its sulfuric acid salt, whereas in the batch reactions at pH 5 the enzyme activity decreased at high buffer concentrations. Possible inhibition by sulfate was examined by performing the reaction in the presence of 100 mM Na₂SO₄, but only a small decrease in enzyme activity was observed. Moreover, when D-1 was added as the free base, the reaction also stopped at 50 mM D-2. Finally, the possible inhibition by the retro-Strecker product, benzaldehyde, was investigated. We found that benzaldehyde caused a drastic decrease in enzyme activity at concentrations as low as 0.5 mM. Hence, we conclude that the decomposition of D-1 is the main cause of deactivation of the nitrile hydratase, besides being detrimental to the yield.

![Figure 6.3](image)

**Figure 6.3** Study of inhibition effect on SP 361 in a (▲) fed-batch reaction (feeding in 1 h) of 100 mM D-1 at pH 7 in the presence of (■) 50 mM D-phenylglycine amide, (●) 100 mM sulfate, (●) 0.5 mM benzaldehyde.

**SP 361-catalysed reactions at low temperature**

Based on the above findings it is clear that in order to increase the yield of D-2, the stability of D-1 towards benzaldehyde formation via a retro-Strecker reaction must be

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increased. We found that decomposition - and racemisation - of D-1 is less at low temperatures. After 24 h at 5 °C and pH 7 spontaneous degradation of D-1 (100 mM) to benzaldehyde was 13% and the ee of the remaining D-1 was 82% compared to 75% decomposition and complete racemisation at room temperature.

A 100 mM batch reaction at 5 °C yielded 63 mM D-2, compared with 37 mM at room temperature. This prompted us to carry out a fed-batch reaction at low temperature. The feedstock (200 mM D-1 in 0.1 M H₂SO₄) was added over 3 h. The reaction was followed over time and after 7 h we obtained 96% yield of D-2 (102 mM) with 95% ee. Hence, by performing the reaction in a fed-batch mode at low temperatures decomposition is avoided and D-2 can be obtained in nearly quantitative yield.

Other nitrile hydratase/amidase systems

In order to demonstrate the general applicability of our fed-batch procedure at low temperature for the conversion of chemically labile D-1, other nitrile hydratase/amidase systems were also tested in our set-up. We selected several microorganisms which hydrated D,L-phenylglycine nitrile 1 efficiently. The bacterial cultures were grown on defined media as described in chapter 5; the whole cells were permeabilised by incubation with Triton X-100 and used without further treatment in the fed-batch hydration of D-1.

<table>
<thead>
<tr>
<th>Entry</th>
<th>Enzyme (U)</th>
<th>Time (h)</th>
<th>Yield (%)</th>
<th>ee (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>SP 361 (-)</td>
<td>7</td>
<td>96.0</td>
<td>95.0</td>
</tr>
<tr>
<td>2</td>
<td><em>R. globulus</em> MAWA (69)</td>
<td>4</td>
<td>98.7</td>
<td>94.6</td>
</tr>
<tr>
<td>3</td>
<td><em>R. erythropolis</em> MAWF (44)</td>
<td>7</td>
<td>95.6</td>
<td>97.4</td>
</tr>
<tr>
<td>4</td>
<td><em>R. rhodochrous</em> MAWE (56)</td>
<td>7</td>
<td>98.6</td>
<td>96.8</td>
</tr>
<tr>
<td>5</td>
<td><em>R. rhodochrous</em> MAWB (38)</td>
<td>6</td>
<td>95.4</td>
<td>98.3</td>
</tr>
</tbody>
</table>

22 95.1 99.5

*Reaction conditions: 1 mmol α-amino nitrile D-1 in 5 ml 0.1 M H₂SO₄ was added in 3 h to the microorganism in 5 ml 10 mM phosphate buffer. The reaction mixture was shaken at 5 °C.*

In all cases D-1 was hydrated to D-2 with high yield and ee (Table 6.2). Moreover, the enantiomeric purity of D-2 could be increased - with a minimum loss in yield - by running the reaction for a longer time (Table 6.2, entry 5). The amidase present in the
microorganism converted the unwanted L-amide slowly into the corresponding L-acid resulting in 95% yield of D-2 with > 99% ee.

Conclusions

Notwithstanding the instability of D-phenylglycine nitrile D-1, its hydration to the corresponding amide D-2 is feasible in high yield. Up to 25 mM D-1, a batch reaction at room temperature gives D-2 in 94% yield and 92% ee. At higher substrate concentrations a fed-batch mode of operation at lower temperatures is necessary to achieve high yields and enantiopurities, e.g. at 5 °C and a final concentration of 100 mM, D-2 was obtained in 95% yield and 94% ee. This concept appeared to be a generally applicable method, as other nitrile hydratase/amidase systems gave similar results. Moreover, the L-amidase yielded an increased ee up to 99.5% with minimum loss in yield.

Experimental section

Materials

Immobilised *Rhodococcus* sp. SP 361 was a gift from Novo Nordisk A/S (Bagsværd, Denmark). Enantiomerically pure D-phenylglycine, D-phenylglycine amide and D-phenylglycine nitrile tartaric acid salt (Alpha Drug India LTD) were kindly donated by DSM (Geleen, The Netherlands). Racemic phenylglycine nitrile hydrochloride (technical grade), racemic phenylglycine and phenylpropionitrile were obtained from Acros. Phenylglycine nitrile was purified by neutralisation of the α-aminonitrile hydrochloric acid salt with base, followed by an extraction with dichloromethane. The combined organic layers were acidified with 1 M HCl and the nitrile hydrochloric acid salt was re-extracted in the aqueous phase, which was concentrated *in vacuo*, to give racemic phenylglycine nitrile hydrochloride as a white solid. Racemic phenylglycine amide was chemically synthesised *via* ammonolysis of the corresponding methyl ester. D-Phenylglycine nitrile was used as the free base. Therefore the pH of a 25 mM solution of D-phenylglycine nitrile tartaric acid in water was adjusted to pH 5 with 2 M sodium hydroxide. The aqueous phase was extracted with CH₂Cl₂, dried over MgSO₄ and concentrated *in vacuo* to give D-1 as a yellowish oil, which was used directly.

During the enzymatic reactions there is a potential risk of producing cyanide *via* retro-Strecker reaction of D-phenylglycine nitrile. Care must be taken by performing all reactions in a fume cupboard provided with an efficient draught.
**Analysis and equipment**

The reaction mixtures of the stereoretentive hydration were analysed by chiral HPLC on a Daicel Chemical Industries Ltd. 4.6 x 150 mm 5 µ Crownpak CR (+) column using a Waters 625 LC pump and a Waters 486 UV detector. The eluent was aqueous 0.1 M HClO₄, pH 1.0 at a flow of 0.6 ml/min, the column temperature was 18 °C.

**Batch-wise hydration of d-phenylglycine nitrile**

0.25 mmol d-phenylglycine nitrile (33 mg) and 0.5 g SP 361 in 10 ml 10 mM sodium phosphate buffer were shaken in 50 ml reaction vessels at room temperature at a selected pH, which was kept constant using an automatic titrator (Metrohm 665 Dosimat and a Metrohm 614 Impulsomat). The reactions were monitored by periodically withdrawing 0.1 ml samples, which were analysed by HPLC.

**Fed-batch hydration of d-phenylglycine nitrile**

1.0 mmol d-phenylglycine nitrile (132 mg) in 5 ml 0.1 M H₂SO₄ was added over 1 to 5 h to - unless mentioned otherwise - 0.5 g SP 361 in 5 ml 10 mM sodium phosphate buffer pH 7. The reaction mixture was shaken at the selected temperature. The pH was kept constant using an automatic titrator (Metrohm 665 Dosimat and a Metrohm 614 Impulsomat). The reactions were monitored by periodically withdrawing 0.1 ml samples which were analysed by HPLC.

**Acknowledgements**

Generous donations of nitrile hydratase/amidase SP 361 by Novo Nordisk A/S (Bagsvaerd, Denmark) are gratefully acknowledged. DSM Life Science Products is acknowledged for a gift of d-phenylglycine nitrile tartaric acid salt. Thanks are due to Dr. P. Porskamp of DSM Deretil (Almeria, Spain) for his encouragement. This work was financially supported by the Netherlands Ministry of Economic Affairs and coordinated by DSM Life Science Products (Geleen, The Netherlands).

**References**

Chapter 7

A two-step-one-pot synthesis of cephalalexin from D-phenylglycine nitrile

Abstract
A cascade of two enzymatic transformations is employed for the one-pot synthesis of cephalalexin, thereby circumventing an intermediate purification step. The nitrile hydratase (from R. rhodochrous MAWE) catalysed hydration of D-phenylglycine nitrile to the corresponding amide was combined with the penicillin G acylase (penicillin amidohydrolase, E.C. 3.5.1.11) catalysed acylation of 7-ADCA, with the in-situ formed amide, to afford a two-step-one-pot synthesis of cephalalexin. When 1,5-dihydroxynaphthalene was added to the reaction mixture, a complex with cephalalexin was formed, which precipitated. Consequently, cephalalexin was protected against chemical as well as enzymatic degradation and was obtained in 79% yield with a synthesis/hydrolysis ratio (S/H) of 7.7. The enhanced S/H was not only due to employing a complexing agent during the enzymatic reaction. D-Phenylglycine nitrile appeared to have a remarkable selective inhibitory effect on penicillin G acylase, resulting in a 3-fold increase in S/H.

* This chapter has been submitted for publication: M.A. Wegman, L.M. Van Langen, F. Van Rantwijk, R.A. Sheldon, Biotechnol. Bioeng.
Chapter 7

Introduction

The traditional chemical procedures for the synthesis of β-lactam antibiotics consume reagents and auxiliaries in stoichiometric amounts and, consequently, produce copious amounts of waste that somehow must be disposed of. The replacement of these procedures by more efficient biocatalytic ones is, to an increasing degree, perceived as the solution to the waste problem.¹

Thus, we recently reported the nitrile hydratase catalysed stereoretentive hydration of d-phenylglycine nitrile (D-1) into d-phenylglycine amide (D-2),² which is a key intermediate in the industrial synthesis of cephalixin (3). A key issue in this procedure is to avoid the decomposition of D-1 into benzaldehyde via a retro-Strecker reaction. This reduces the yield; moreover, benzaldehyde deactivates the biocatalyst. However, when D-1 was slowly added to the reaction mixture - to keep its concentration low - at 5 °C, D-2 was obtained in nearly quantitative yield.

Synthesis using cascades of enzymatic reactions may be even more efficient than the usual stepwise approach, because the intermediate isolation and purification stages are avoided. Hence, multi-enzyme methodologies are receiving increasing attention.³⁶ An elegant example of cascade catalysis is the multi-enzymatic synthesis of the β-lactam antibiotic cefazolin,³⁴ which encompasses three consecutive biotransformations in aqueous medium, starting from cephalosporin C.

![Scheme 7.1](image)

Scheme 7.1 Two-step-one-pot synthesis of cephalixin.
We now report the combination of enzymatic stereoretentive nitrile hydration and coupling of the intermediately formed amide D-2 with 7-aminodesacetoxy-cephalosporanic acid (7-ADCA) to cephalexin in a dual enzyme catalytic cascade (Scheme 7.1).

The effect of the reaction parameters on the outcome of the procedure will be discussed in detail.

Results and discussion

Screening of nitrile hydratases
In order to find the best nitrile hydratase/amidase system for the two-step-one-pot synthesis, the different strains used in our previous work were tested in a standard one-pot reaction. Although SP 361 gave satisfactory results, it was not used in further experiments because of the uncertainty regarding its future commercial availability. Apart from R. globulerus MAWA, which was strongly inhibited by 7-ADCA, all other strains gave good results. However, R. rhodochrous MAWE was used in all further experiments since it showed the highest initial rate in the nitrile hydration. Assemblase 7500 (a preparation of E. coli penicillin acylase covalently attached to a gelatin-based carrier) was used in the coupling of the in-situ formed amide D-2 with 7-ADCA to cephalexin.

Optimisation of the two-step-one-pot synthesis
An important parameter in this study is the synthesis/hydrolysis ratio (S/H). Besides synthesis of cephalexin, competitive (primary) hydrolysis of amide D-2 and (secondary) hydrolysis of cephalexin into D-phenylglycine should also be expected. The molar ratio of the formed product to the hydrolysis product is expressed as S/H, which is often used to assess the economic viability of the process.\(^1\)

In all reactions nitrile D-1 was fed over time to the reaction mixture at 5 °C and pH 7 in order to minimise its decomposition into benzaldehyde, ammonia and cyanide via a retro-Strecker reaction.\(^2\) A factorial experimental design was used to optimise the reaction procedure with regard to the yield of cephalexin and S/H. Eight experiments were set up according to a \(2^3\) factorial design, which allows the estimation of the main effects of the variables of interest. Therefore, the amounts of Assemblase 7500 and 7-ADCA, and the feeding time of nitrile D-1 were varied. The amounts of D-1 and nitrile hydratase were kept constant. The results are compiled in Table 7.1.
Table 7.1 Two-step-one-pot synthesis of cephalixin 3

<table>
<thead>
<tr>
<th>Feeding time (h)</th>
<th>7-ADCA (mmol)</th>
<th>Assemblase 7500 (U)</th>
<th>Yield 3 (%)</th>
<th>S/H</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1</td>
<td>315</td>
<td>29</td>
<td>0.9</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>630</td>
<td>23</td>
<td>0.6</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>315</td>
<td>45</td>
<td>1.8</td>
</tr>
<tr>
<td>1</td>
<td>3</td>
<td>630</td>
<td>32</td>
<td>1.0</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>315</td>
<td>23</td>
<td>0.6</td>
</tr>
<tr>
<td>3</td>
<td>1</td>
<td>630</td>
<td>17</td>
<td>0.3</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>315</td>
<td>32</td>
<td>0.8</td>
</tr>
<tr>
<td>3</td>
<td>3</td>
<td>630</td>
<td>28</td>
<td>0.7</td>
</tr>
<tr>
<td>-d</td>
<td>3</td>
<td>315</td>
<td>48</td>
<td>1.9</td>
</tr>
</tbody>
</table>

*a Reaction conditions: 1 mmol D-phenylglycine nitrile in 5 ml 0.1 M H₂SO₄ was added in time to 7-ADCA, 40 U R. rhodochrous MAWE and Assemblase 7500 in 5 ml 50 mM phosphate buffer pH 7. The reaction mixture was stirred at 5 °C.

*b Represents the 2^2 factorial design matrix.

*c Represents the response vector.

*d D-Phenylglycine nitrile was added at once to the reaction mixture (10 ml).

Doubling the amount of Assemblase 7500 had no influence on the initial rate of cephalixin 3 formation (data not shown). However, at high Assemblase concentration the yield of 3 and the S/H were reduced, irrespective of the other conditions. This effect is presumably caused by increased product hydrolysis. Upon increasing the amount of 7-ADCA the initial rate of 3 synthesis was enhanced slightly. Moreover the yield of 3 as well as the S/H increased. We ascribe this to the suppression of the competitive product hydrolysis at high 7-ADCA concentrations resulting in an enhanced S/H. As the feeding time was prolonged from 1 to 3 h, the yield of 3 and the S/H decreased. Presumably, a slow addition of nitrile D-1 to the reaction mixture resulted in a lower concentration of the intermediately formed amide D-2. As a consequence, hydrolysis of 3 took place, resulting in a decreased yield of 3 and a low S/H. Moreover, the response surface plot (Figure 7.1) shows that a maximum yield of 3 and S/H can be reached when D-1 is added at once to the reaction mixture.
**Figure 7.1** Response surface plot of the two-step-one-pot synthesis.

This would seem questionable, since nitrile D-1 is readily susceptible to decomposition under these conditions. However, the hydration of D-1 to D-2 in the two-step-one-pot synthesis is approximately twice as fast as the separate enzymatic hydration of D-1 to D-2. In the latter, amide D-2 accumulates in the course of the reaction, whereas in the two-step-one-pot synthesis D-2 is directly converted to cephalixin (Scheme 7.1). This prompted us to investigate the inhibition of *R. rhodochrous* MAWE by D-2. A standard activity test - the hydration of 2-phenylpropionitrile to the corresponding amide - showed that in the presence of D-2 (20 mM) the activity of *R. rhodochrous* MAWE was reduced to 72%.

However, as explained above in the two-step-one-pot synthesis the inhibition of amide D-2 is of minor concern and a low concentration of D-1 is provided due to its fast hydration (thus minimising decomposition). Hence D-1 was added in one portion to the reaction mixture. No decomposition was observed and cephalixin was obtained in 48% yield with an S/H of 1.9. In all further experiments D-1 was added directly to the reaction mixture since it afforded the highest yield and S/H.

**Effect of biocatalyst formulation on S/H in the one-pot synthesis**

It is known that the S/H is dependent on reaction conditions such as pH and co-solvents. However, the biocatalyst immobilisation method also influences the S/H. Diffusion limitations of both the substrate and the product resulted in a decreased S/H. A possibility to overcome this problem, is the use of cross-linked
enzyme aggregates (CLEAs), which catalysed the synthesis of ampicillin with an enhanced S/H approaching that of the dissolved enzyme. Consequently, we used T-CLEA (tert-butyl alcohol was used as precipitant, followed by cross-linking the aggregate with glutaraldehyde to form the insoluble T-CLEA) in the two-step-one-pot synthesis of cephalaxin. The S/H increased to 2.7 and 3 was obtained in 60% yield (compared with S/H 1.9 and 48% yield when Assemblase 7500 was used).

Effect of a complexing agent in the one-pot synthesis
In order to improve the S/H even further the effect of a complexing agent was studied. It is known that cephalaxin selectively co-crystallises with e.g. β-naphthol in aqueous solution. The formed complex precipitates; hence the β-naphthol complex of cephalaxin is less susceptible to hydrolysis, resulting in a higher yield and S/H. Schroën et al. (private communication) showed that penicillin G acylase was inhibited by β-naphthol. Although 1,5-dihydroxy-naphthalene is less effective as complexing agent, it is not an inhibitor and was therefore used in our reactions. Assemblase 7500 as well as T-CLEA were used in the one-pot synthesis of 3 in the presence of 1,5-dihydroxy-naphthalene. Irrespective of the biocatalyst formulation cephalaxin was obtained in 79% yield with an S/H of 7.7. Hence, the diffusion limitation that is inherent in the use of Assemblase-type carriers seems to be less of an issue if the product is effectively removed from the reaction mixture.

We surprisingly found that the synthetic efficiency of penicillin acylase in the one-pot synthesis (S/H 7.7) was significantly higher than that in the direct synthesis of cephalaxin from amide D-2 under otherwise identical conditions (S/H 6.4; the amount of catalyst was reduced to 80 U, Figure 7.2a). This result prompted us to study the effect of nitrile D-1 on the S/H in more detail. Therefore 7-ADCA was acylated with D-2 in the presence 1,5-dihydroxy-naphthalene and D-1, while the nitrile hydratase was omitted. (Figure 7.2b).

In the presence of D-1 315 U of Assemblase 7500 were necessary to compensate the inhibition of penicillin acylase (see above). However the hydrolysis of D-2 was more severely inhibited. Consequently, cephalaxin was formed in 80% yield with S/H 19.3, which is 3 times the value observed in the absence of D-1.
Figure 7.2 Coupling of D-phenylglycine amide with 7-ADCA to cephalaxin: (□) D-phenylglycine nitrile, (○) D-phenylglycine amide, (●) cephalaxin, (■) D-phenylglycine. 7.2a: no D-phenylglycine nitrile was added. 7.2b: 1 mmol D-phenylglycine nitrile was added to the reaction mixture.

The observed selective inhibition could be caused by D-1 itself or by the decomposition products HCN or benzaldehyde, since decomposition of D-1 could not be avoided completely. To investigate these possibilities we performed the acylation of 7-ADCA with D-2 in the presence of 2.5 mol% HCN or benzaldehyde and 1,5-dihydroxynaphthalene. No effect of HCN was observed, but benzaldehyde showed selective inhibition, similar to nitrile D-1. However, an attempt to clarify whether the effect of D-1 could be attributed entirely to benzaldehyde was inconclusive. Clearly more work is necessary to elucidate the details of this selective inhibition.

Effect of 7-ADCA/nitrile D-1 molar ratio in the presence of a complexing agent
Up to this point 7-ADCA was taken in a 3-fold excess in all reactions, since as mentioned earlier, this resulted in enhanced S/H in the factorial design. Although 7-ADCA can be efficiently recovered from the reaction mixture, we surmised that in the presence of a complexing agent the amount of 7-ADCA could be reduced without a significant loss in S/H. Thus, the amount of 7-ADCA was reduced to a 2-fold excess and, in addition, an extra batch of D-1 (together with 1,5-dihydroxynaphthalene), resulting in an equimolar ratio was added to the reaction mixture when the original one had been converted (Figure 7.3).
Figure 7.3 Two-step-one-pot synthesis to cephalexin in the presence of a complexing agent. (□) D-phenylglycine nitrile, (○) D-phenylglycine amide, (●) cephalexin, (■) D-phenylglycine, (▲) 7-ADCA.

Figure 7.3 shows that the first portion of D-1 was hydrated within 2 h, whereas the second batch was not even completely hydrated after 3.5 h. The deactivation of the nitrile hydratase might be a result of inhibition by amide D-2 or by traces of benzaldehyde, although no other possible inhibitors were examined. The S/H was 7.6 in the first stage of the reaction. However, after the addition of the second batch of D-1, the S/H was further enhanced to 8.1. We ascribe this again to the selective inhibition effect of D-1 or benzaldehyde. The two-step-one-pot synthesis gave cephalexin in 73% yield with an S/H of 8.1.

Conclusions

A dual enzyme two-step-one-pot synthesis of cephalexin from D-phenylglycine nitrile in the presence of a complexing agent resulted in 79% yield with an S/H of 7.7. The α-amino nitrile had an unexpected selective inhibitory effect on Assemblase 7500, which resulted in enhanced S/H ratios.

Experimental section

Materials

Immobilised *Rhodococcus* sp. SP 361 was a gift from Novo Nordisk A/S (Bagsværd, Denmark). The nitrile hydratase/amidase systems were grown as described before. Enantiomerically pure D-phenylglycine, D-phenylglycine amide, D-phenylglycine nitrile tartaric acid salt (Alpha Drug India LTD), 7-ADCA and cephalexin were kindly
Two-step-one-pot-synthesis to cephalexin

donated by DSM (Geleen, The Netherlands). Assemblase 7500 was a gift of DSM Life Science Products (Delft, The Netherlands). Penicillin acylase T-CLEA was prepared by Dr. L. Cao. 12 1,5-Dihydroxynaphthalene was obtained from ACROS.

D-Phenylglycine nitrile was used as the free base, which was prepared as follows: The pH of a 25 mM aqueous solution of D-phenylglycine nitrile tartrate was adjusted to pH 5 with 2 M sodium hydroxide. The aqueous phase was extracted with CH₂Cl₂, the organic layer was dried over MgSO₄ and concentrated in vacuo to give D-phenylglycine nitrile as a yellowish oil, which was used directly.

During the enzymatic reactions there is a potential risk of producing cyanide via a retro-Strecker reaction of D-phenylglycine nitrile. Care must be taken by performing all reactions in a fume cupboard provided with an efficient draught.

Analysis and equipment
The reaction mixtures were analysed by HPLC on a custom-packed 10 μm Nucleosil C18 column (Waters Radial-Pak 8 x 100 mm) using a Waters 590 pump and a Waters 486 UV detector. The eluent was acetonitrile-water (68:32, v/v) containing 5 mM phosphate buffer pH 3.2 and 1.3 g/l dodecyl sulfate sodium salt, at a flow of 0.3 ml/min.

Two-step-one-pot synthesis
In a typical experiment 1 mmol D-phenylglycine nitrile (132 mg) in 5 ml 0.1 M H₂SO₄ was added over 1 h to 1 mmol 7-ADCA (214 mg), 40 U R. rhodochrous MAWE nitrile hydratase and 315 U penicillin G acylase (1 g, Assemblase 7500) in 5 ml 10 mM sodium phosphate buffer pH 7. The reaction mixture was stirred at 5 °C. The pH was kept constant using an automatic titrator (Metrohm 665 Dosimat and a Metrohm 614 Impulsomat). The reactions were monitored by periodically withdrawing 0.25 ml samples to which 75 ml water (pH 2) was added to dissolve all components. The samples were centrifuged to remove the enzymes and subsequently analysed by HPLC.

Two-step-one-pot synthesis in the presence of a complexing agent
1 mmol D-phenylglycine nitrile (132 mg), 3 mmol 7-ADCA (642 mg) 1 mmol 1,5-dihydroxynaphthalene (160 mg), 40 U R. rhodochrous MAWE nitrile hydratase and 315 U Assemblase 7500 (1 g) in 5 ml 10 mM sodium phosphate buffer pH 7 were stirred at 5 °C. The pH was kept constant using an automatic titrator (Metrohm 665 Dosimat and a Metrohm 614 Impulsomat). The reactions were monitored by periodically withdrawing 0.25 ml samples. In order to break the complex and dissolve
all components 65 ml water (pH 2) and 10 ml acetonitrile were added. The samples were centrifuged to remove the enzymes and subsequently analysed by HPLC.

_Cephalexin via acylation of 7-ADCA with D-phenylglycine amide_
1 mmol D-phenylglycine amide (150 mg), 3 mmol 7-ADCA (642 mg) and 80 U Assemblase 7500 (250 mg) in 10 ml 10 mM phosphate buffer pH 7 were stirred at 5 °C. The pH was kept constant using an automatic titrator (Metrohm 665 Dosimat and a Metrohm 614 Impulsomat). The reactions were monitored by periodically withdrawing 0.25 ml samples to which 75 ml water (pH 2) was added to dissolve all components. The samples were centrifuged to remove the enzyme and subsequently analysed by HPLC.

_Cephalexin via acylation of 7-ADCA with D-phenylglycine amide in the presence of a complexing agent_
The same procedure was followed as described above, but 1 mmol 1,5-dihydroxynaphthalene was added to the reaction mixture. The reaction was followed by periodically withdrawing 0.25 ml samples. Again decomplexation was effected at low pH in a two-phase system, as mentioned earlier.

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**References**

Summary

Alternative routes to activated side-chain donors of \( \beta \)-lactam antibiotics

In Chapter 1 an overview is given of the development of environmentally friendly processes for the manufacture of \( \beta \)-lactam antibiotics. The traditional chemical routes, as applied in 1985, have been taken as a starting-point. The introduction of biocatalysis made it possible to replace several "stoichiometric" chemical steps by enzymatic alternatives. In addition, major breakthroughs were reached in fermentation using genetic engineering. These new processes resulted not only in shorter routes to semi-synthetic penicillins and cephalosporins, but are, moreover, environmentally more friendly.

In Chapter 2 a catalytic procedure is described, using ultrastable zeolite Y, for the esterification of several aromatic \( \alpha \)-amino acids. In this route the formation of stoichiometric quantities of salts associated with conventional methods is circumvented. H-USY could be recycled although its activity decreased after each cycle owing to the adsorption of water. After calcination, however, its activity was completely restored.

All ester products underwent partial thermal racemisation during the reaction.

In Chapter 3 the enantioselective ammonolysis of D,L-phenylglycine methyl ester to D-phenylglycine amide, catalysed by Candida Antarctica lipase B, is described. The ammonolysis was combined with pyridoxal-catalysed in-situ racemisation of the unconverted ester (dynamic kinetic resolution) resulting in enhanced yields of the amide. The process could be further optimised since the enantioselectivity of the catalyst was enormously improved at low temperatures.

The two transformations described in Chapter 2 and 3 could be combined, in principle, to recycle L-phenylglycine, which is produced as a side-product in the DSM aminopeptidase process for D-phenylglycine amide. Otherwise, recycling of L-phenylglycine to the racemic amide involves 3 chemical steps (racemisation, esterification and ammonolysis).

In Chapter 2 the conversion of L-phenylglycine into the corresponding racemic ester via H-USY catalysed salt-free esterification is described. Subsequently, (Chapter 3) the racemic ester can be subjected to lipase catalysed ammonolysis with in-situ racemisation at low temperature yielding D-phenylglycine amide. The latter can be directly used in the enzymatic coupling to \( \beta \)-lactam antibiotics.

Chapter 4 is devoted to the screening of bacterial strains, which harboured nitrile hydratase/amidase activity towards phenylglycine nitrile. Various strains were found
which contained both a non-selective nitrile hydratase and an extremely L-selective amidase, yielding D-phenylglycine amide and L-phenylglycine.

In Chapter 5 the nitrile hydratase/amidase containing microorganisms (all *Rhodococci*) were cultivated on a multi-litre scale. The whole cells were entrapped in sodium alginate gel. However, when the immobilised preparations were used in organic solvents irreversible deactivation took place.

In Chapter 6 the stereoretentive hydration of D-phenylglycine nitrile to the corresponding amide, catalysed by nitrile hydratase containing microorganisms, is described. Batch and fed-batch reactions were compared with respect to degradation and racemisation of the chemically labile substrate. Good results were obtained in a batch process up to 25 mM substrate concentration. When the concentration was increased to 100 mM, however, the simple batch reaction resulted in decomposition and racemisation of the substrate. A fed-batch reaction afforded an improved yield, although racemisation and decomposition could not be avoided completely. However, lowering the temperature stabilised the substrate and D-phenylglycine amide was obtained in nearly quantitative yield with high ee.

In Chapter 7 the stereoretentive nitrile hydratase catalysed hydration of D-phenylglycine nitrile is combined with the penicillin G acylase catalysed acylation of 7-ADCA with the *in-situ* formed amide to cephalaxin. This cascade of two enzymatic steps in one-pot circumvents an intermediate purification step. In the presence of a complexing agent (1,5-dihydroxynaphthalene) cephalaxin is precipitated from the reaction mixture as its naphthalene complex. As a consequence the product is protected from chemical as well as enzymatic degradation and high conversions and synthesis/hydrolysis ratios are easily reached. D-Phenylglycine nitrile appeared to have a remarkable selective inhibitory effect on penicillin G acylase resulting in a 3-fold enhanced synthesis/hydrolysis ratio.
Samenvatting

Alternatieve routes naar geactiveerde zijketendonoren van β-lactam antibiotica

In Hoofdstuk 1 wordt een overzicht gegeven van de ontwikkeling van milieuvriendelijke processen voor de productie van β-lactam antibiotica. De traditionele routes, zoals in 1985 toegepast, zijn hierbij als uitgangspunt genomen. De introductie van biokatalyse heeft het mogelijk gemaakt verschillende chemische, stoïchiometrische stappen te vervangen door enzymatische stappen, en daarbij organische oplosmiddelen te vervangen door water. Bovendien is een grote doorbraak bereikt in de fermentatie, waarbij gebruik wordt gemaakt van genetisch gemodificeerde micro-organismen. Deze nieuwe processen hebben tot kortere routes naar semi-synthetische penicillines en cephalosporines geleid en zijn tevens milieubesparend.

In Hoofdstuk 2 wordt een katalytische procedure beschreven voor de verestering van diverse α-aminozuren, waarbij gebruik wordt gemaakt van ultra stabiel zeoliet Y (H-USY). In deze route wordt de vorming van stoïchiometrische hoeveelheden zouten, die gepaard gaat met de conventionele methoden, vermeden. H-USY kan meerdere keren worden gebruikt, alhoewel de activiteit na elke cyclus afneemt als gevolg van de adsorptie van water. Na calcineren van de katalysator is de activiteit echter geheel hersteld.

In Hoofdstuk 3 wordt de enantioselectieve ammonolysie van D,L-phenylglycine methylester naar D-phenylglycine amide, gekatalyseerd door Candida antarctica lipase B, beschreven. De ammonolysie is gecombineerd met in situ racemisatie van de niet omgezette ester, gekatalyseerd door pyridoxal. Deze dynamisch kinetische resolutie leidt tot verhoogde opbrengsten van D-phenylglycine amide. Dit proces kon verder worden geoptimiseerd aangezien bij lage temperaturen de enantioselectiviteit van het enzym verbeterd kon worden.

De twee omzettingen die in Hoofdstuk 2 en 3 beschreven zijn, kunnen in principe gecombineerd worden om L-phenylglycine te recyclen. L-Phenylglycine wordt in het DSM aminopeptidase proces voor D-phenylglycine amide als bijproduct gevormd. De recycling van L-phenylglycine naar het racemisch amide omvat 3 stappen (racemisatie, veresterende ammonolysie). Uit Hoofdstuk 2 volgt dat L-phenylglycine kan worden omgezet in de racemische ester via H-USY gekatalyseerde zoutvrije verestering. Vervolgens (Hoofdstuk 3) kan de racemische ester worden onderworpen aan lipase gakatalyseerde ammonolysie met in situ racemisatie bij lage temperatuur waarbij D-
phenylglycine amide wordt gevormd. Deze laatste kan direct worden gebruikt in de enzymatische koppeling naar β-lactam antibiotica.

In Hoofdstuk 4 wordt de screening van bacteriële stammen, die nitril hydratase/amidase activiteit bezitten voor phenylglycine nitrol beschreven. Diverse stammen zijn gevonden, die zowel een niet selectief nitrol hydratase als een zeer L-specifiek amidase bevatten, waarbij D-phenylglycine amide en L-phenylglycine werden gevormd.

In Hoofdstuk 5 wordt de kweek op multi-liter schaal van alle nitrol hydratase/amidase bevattende micro-organismen (allen Rhodococci) beschreven. De hele cellen zijn ingesloten in natrium alginaat gel. Echter, wanneer de gelimmobiliseerde preparaten in organisch oplosmiddel worden gebruikt, treedt irreversibele deactivatie op.

In Hoofdstuk 6 wordt de stereore Ventura hydratie van D-phenylglycine nitrol tot het overeenkomstige amide beschreven. Deze reactie wordt gekatalyseerd door nitrol hydratase bevattende micro-organismen. Batch-en fedbatch reacties zijn vergeleken met betrekking tot ontleding en racemisatie van het chemisch labiele substraat. In een batchproces wordt tot 25 mM substraatconcentratie goede resultaten behaald. Echter, wanneer de concentratie wordt verhoogd naar 100 mM leidde de eenvoudige batchreactie tot ontleding en racemisatie van het substraat. Een fed-batchreactie leidde tot een verbeterde opbrengst, alhoewel de ontleding en racemisatie niet volledig vermeden konden worden. Verlaging van de temperatuur stabiliseerde het substraat en D-phenylglycine amide werd in een bijna kwantitatieve opbrengst met hoge ee verkregen.

In Hoofdstuk 7 is de stereore Ventura nitrol hydratase gekatalyseerde hydratie van D-phenylglycine nitrol gecombineerd met penicilline G acylase gekatalyseerde acylering van 7-ADCA met het in situ gevormde amide naar cephalexine beschreven. Deze cascade van twee enzymatische stappen in één pot vermijdt een tussentijdse zuiveringsstap. In de aanwezigheid van een complexant (1,5-dihydroxy naftaleen) precipiteert cephalexine uit het reactiemengsel als zijn naftaleencomplex. Als gevolg hiervan is het product beschermd tegen zowel chemische als enzymatische degradatie en kunnen hoge conversies en synthese/hydrolyse verhoudingen worden behaald. D-Phenylglycine nitrile bleek bovendien een opmerkelijk selectief inhibitie-effect te hebben op penicilline G acylase waardoor 3 keer zo hoge synthese/hydrolyse ratio's werden verkregen.
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Marguth
List of publications


Curriculum Vitae

Sinds 1 januari 2001 is zij werkzaam bij Avantium Technologies in Amsterdam.