Synthesis and applications of unsaturated non-proteinogenic α-H-α-amino acids.
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CHAPTER 3

ENZYMATIC RESOLUTION OF UNSATURATED α-H-α-AMINO ACID AMIDES
Development of an Improved Biocatalytic System Using a Genetically Modified Organism

3.1 Introduction

Biocatalysis has gradually become more important in organic synthesis over the past two decades or so. In the beginning, the use of enzymes was restricted to the wild type enzymes, that were simply screened on their ability to effect specific transformations on ‘organic’ substrates. Nevertheless, this concept has made a large impact in organic synthesis and led to a large variety of useful reactions in which often the enantioselective properties of the enzymes are exploited. In the past years, however, it has become clear that molecular biology techniques can be utilized to enhance the scope of enzymatic transformations even further. As a first approach, identifying the gene coding for the desired activity and expression of that gene in a suitable host organism can be used to increase the catalytic activity and may also result in improved selectivity because unwanted side reactions caused by other enzymes present in the wild type strain are absent. Recently it was shown that also the properties of enzymes could be improved using newly developed molecular biology techniques. One of the most prominent examples was published by the group of Reetz, who showed that by applying a random mutagenesis protocol, the enantioselective hydrolysis of an ester by a lipase could be significantly improved by producing a modified lipase. This principle is now generally known as ‘directed evolution’ and also applied onto other types of enzymes. Thus, Arnold and coworkers have shown that the stereoselectivity of a hydantoinase could be reversed from D-selective into L-selective. Recently, an even more powerful tool has been developed, called gene shuffling.

In this Chapter we describe the application of the most simple molecular biology approach to construct an improved whole cell catalyst based on the versatile aminopeptidase from Pseudomonas putida.

Non-proteinogenic amino acids are ideal substrates for enzymatic conversions due to their resemblance to proteinogenic amino acids, which are the natural substrates for a seemingly infinite number of enzymes. This is the reason that amino acids can be resolved in a variety of ways with enzymatic methods. Among the most widely applied enzymatic routes are the acylase, esterase, hydantoinase and aminopeptidase based ones. The first industrial application was the acylase-based resolution of N-acyl-amino acids developed by Tanabe and Degussa. In the mid 1970s, at DSM a different track was followed, utilizing aminopeptidases
and amidases to resolve racemic amino acid amides. The essentials of this process are depicted in Scheme 1. Subjection of a (proteinogenic or non-proteinogenic) racemic amino acid amide to the amidase present in the organism *Pseudomonas putida* ATCC 12633 leads to a mixture of the (S)-acid 3 and the (R)-amide 2 in generally high enantioselectivity. It is important to emphasize that permeabilized whole cells of the *Pseudomonas putida* strain are used, so all enzymes expressed in these cells come into contact with substrates and products of the amidase reaction. The (R)-amide 2 can be separated from the (S)-acid 3 by adding one equivalent (relative to the amide) of benzaldehyde to the reaction mixture, thus converting the amide into the corresponding Schiff base 4. The Schiff base precipitates and can be filtered off or can be extracted from the water layer with an organic solvent. At this point, racemization of the Schiff base with NaOH, followed by imine hydrolysis will yield a racemic amide that can again be subjected to the biocatalytic system. In this way, eventually a 100% yield of the (S)-acid can be obtained. Alternatively, mild acid hydrolysis of the Schiff base 4 regenerates the (R)-amide, which can be converted into the corresponding (R)-acid at 90 °C in 6 N HCl. An elegant alternative to this rather harsh chemical method is enzymatic hydrolysis using a non-selective amidase produced by *Rhodococcus erythropolis* NCIMB 11540. This reaction proceeds under very mild conditions (pH 8, 37 °C) and is especially useful if the side chain contains a reactive functionality.

**Scheme 3.1**

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{CO} & \quad \text{NH}_2 \\
\text{R} & & \\
\text{O} & & \\
1 & & \\
\text{Pseudomonas putida} & \quad \text{ATCC 12633} & \quad \text{R} & \quad \text{H}_2\text{N} & \quad \text{CO} & \quad \text{NH}_2 \\
& & & & & & \\
2 & & & & & & \\
\text{PhCHO} & \quad \text{H}^+ & \quad \text{H}_2\text{O} & \quad \text{Ph} & \quad \text{N} & \quad \text{O} & \quad \text{H}_2\text{N} & \quad \text{CO} & \quad \text{NH}_2 \\
& & & & & & \\
3 & & & & & & \\
\end{align*}
\]

Already, more than 100 different amino acid amides have been successfully resolved via this method. It has been shown that a broad structural variety of α-H-α-amino acid amides (small and large groups) are accepted by the biocatalyst without losing its enantioselective properties. Aryl or alkyl side chains containing heteroatoms such as sulfur, nitrogen and oxygen are also accepted. Cyclic amino acid amides can also be resolved, but in some cases product inhibition is observed.
Although the majority of examples concern amino acid amides, some other substrates have been resolved as well successfully with the same enzyme system. The methoxy-substituted amides 5 appeared suitable substrates that were hydrolyzed in an enantioselective manner (eq 3.1) albeit at a significantly lower rate.\textsuperscript{13} In addition, recently the amino acid-derived azido amides 7 were also resolved leading to the corresponding products with good selectivity.\textsuperscript{14} The (S)-aminopeptidase originating form\textit{ P. putida} was used as the biocatalyst.

\[
\begin{align*}
\text{H}_2\text{N} & \quad \text{H}_2\text{N} \\
\text{R} & \quad \text{R} \\
\text{NH} & \quad \text{OH} \\
\text{OMe} & \quad \text{OMe} \\
\end{align*}
\]

\[
\begin{align*}
\text{Pseudomonas putida} & \\
5 & \quad 6 \text{ (R)-methoxyamide} \quad 3 \text{ (S)-acid} \\
R = \text{allyl, isopropyl, cyclopentyl} & \\
\text{aminopeptidase} & \\
\end{align*}
\]

The high acceptance of a variety of substrates was a strong impetus for us and DSM to further explore the scope and limitations of this biocatalytic system and, more importantly, investigate whether its selectivity and catalytic properties could be improved. A particular drive into such research were the relative poor results with methionine amide, but also with allylglycine amide. In both cases, excellent ee's were obtained for the amide, whereas under standard conditions the ee of the corresponding acid was significantly lower. The concern was raised that the wild type strain contained undesired enzyme activities that would be responsible for these anomalous results. Therefore, it was our goal to identify the amidase in the wild type organism, clone the genetic information and investigate whether this sequence could be expressed in a suitable host organism, which would lack any undesirable activity.

In previous studies, the purification and characterization of the most important leucine aminopeptidase from the\textit{ Pseudomonas putida} ATCC 12633 strain has been carried out,\textsuperscript{8c,15} and resulted in the following insight. Divalent cations sometimes have a positive effect (Mg\textsuperscript{2+} and Co\textsuperscript{2+} 2- to 3-fold; Mn\textsuperscript{2+} 12-fold), but can also have a negative influence (Cu\textsuperscript{2+} and Ca\textsuperscript{2+} 70\% and 40\% inhibition, respectively) on the activity of the enzyme. Hydrolyzing activity of the (S)-aminopeptidase was observed between pH 7 and 11, with the highest activity at pH 9.0-9.5 and at 40 °C. The enzyme displayed activity on several dipeptides so that the enzyme eventually was designated as an (S)-aminopeptidase.

To construct a potentially more efficient biocatalytic system, the gene coding for the (S)-aminopeptidase from\textit{ Pseudomonas putida} ATCC 12633 was cloned and brought to overexpression in an\textit{ E. coli} K-12 host micro-organism. This bacterium was chosen, due to its
favorable fermentation properties and the availability of a large number of specialized expression vectors. This procedure has been detailed in a recent publication and therefore will not be subject of this chapter.\textsuperscript{15b} Thus, E. coli DH5α/pTrpLAP whole cells were obtained, which will be referred to as GMO (Genetically Modified Organism) cells. Resolution of some simple alkyl-substituted α-H-amino acid amides by E. coli DH5α/pTrpLAP and P. putida ATCC 12633 showed on average a 25-fold increase of activity by the genetically modified organism (GMO). This is caused by the improved expression of the P. putida pepA gene, due to the use of a strong promoter in combination with a multicopy system. The strategy used in this case for the cloning and heterologous expression of P. putida pepA, did not result in any mutation on protein level. Therefore, the wild type P. putida enzyme will be formed in the recombinant E. coli strain. This means that all intrinsic properties of the (S)-aminopeptidase (e.g. pH optimum, substrate range, enantiospecificity) are unaltered in comparison with the properties of the enzyme found in P. putida.

In this chapter, an overview will be given from the results that were obtained by comparing both biocatalytic systems in the resolution of a range of unsaturated amino acid amides. The racemic amino acid amides that were described in Chapter 2 (Scheme 3.2) were subjected to the whole cells of the wild type P. putida strain and the GMO and a comparison was made between the two different biocatalytic systems.

\textbf{Scheme 3.2}

\begin{align*}
\text{H}_2\text{N} &\quad \text{X} &\quad \text{O} &\quad \text{X} &\quad \text{H}_2\text{N} &\quad \text{X} &\quad \text{O} &\quad \text{X} &\quad \text{H}_2\text{N} &\quad \text{X} &\quad \text{O} &\quad \text{X} &\quad \text{H}_2\text{N} &\quad \text{X} &\quad \text{O} &\quad \text{X} \\
\text{14} &\quad \text{a: } &\quad X &\quad = &\quad \text{NH}_2, &\quad \text{b: } &\quad X &\quad = &\quad \text{OH} \\
\text{15} &\quad \text{16} &\quad \text{17} &\quad \text{18}
\end{align*}

3.2 Resolutions

The enzymatic resolutions were conducted with both systems at 40 °C and pH 9.2 in the absence of Na\textsuperscript{+} and Cl\textsuperscript{−} ions, which are known to inhibit the aminopeptidase activity.
Initially, the experiments were run at one gram scale to determine the rate of the reaction and establish the time at which 50% conversion was reached. In a later stage, batches on a preparative scale (up to 30 g of the amino acid amide) were resolved. In most cases, a 10% solution (by weight) of the amide in water was treated with whole cells from *Pseudomonas putida* ATCC 12633 (substrate/enzyme ratio 10:1) and from the GMO (substrate/enzyme ratio 500:1). During the resolution, small samples (0.5 mL) were taken from the reaction mixture, quenched with 1 M H$_3$PO$_4$ (1 mL, to stop the enzyme activity), after which the conversions and the ee's of the acid and the amide were determined in a single run via chiral HPLC analysis (Sumichiral OA 5000, 150 mm x 4.6 mm, eluant: 2mM CuSO$_4$ in H$_2$O/MeOH).

In addition to the HPLC method, the conversions were also determined via measuring the concentration of liberated NH$_3$ using an ion-selective NH$_4^+$-electrode (Orion 95-12). Thus, control measurements were carried out in each resolution experiment. Furthermore, blanco experiments were conducted in a parallel fashion to assure that basic hydrolysis did not take place at pH 9.2.

The results of the resolutions of the racemic amides are shown in Table 1. The ee's shown in the Table for the (S)-acid and the (R)-amide were determined at the time (generally around 21 h) that the maximum theoretical conversion (50%) was reached. However, in all cases the reactions were allowed to react longer (until 62 h) to get a better idea of the catalytic activity of the cell's (this will be discussed later in this chapter). The yields were not determined in these reactions, since the goal was to find out when 50% conversion had been obtained for a given enzyme/substrate ratio. In contrast with the other substrates, the racemic amides 12a and 15a were subjected to the GMO in a 2.5% weight solution (0.5 g substrate in 20 g water) due to the small amount of the amide that was available (entries 7 and 12).

In general, the performance of both biocatalytic systems was reasonably good, in particular in terms of enantioselectivity. Precipitation of the (S)-acids during the enzymatic reaction of amides 11a and 17a made it more difficult to take homogeneous samples. This complication could explain the relatively moderate ee of the (S)-acid 11b (entries 5 and 6) and the longer reaction time. The solid amino acid present in the reaction mixture might hinder the enzyme to reach the substrate. Precipitation of the (S)-acid 16a was also observed although in a smaller degree. The difference between both biocatalysts is clear from the table: first of all, there is a significant difference in reaction time in some entries (3 and 4, 5 and 6, 13 and 14 and 15 and 16). The resolution with the GMO is faster, although the amount of cells is 50 times less. Secondly, especially in the resolutions of amides 14a and 16a a large difference in the ee's of the (S)-acids was observed (entries 10, 11: 91% ee vs. 97% ee and entries 13, 14: 70 ee%, vs. 99% ee). These latter remarkable differences in enantioselectivity encouraged us to further investigate these results.
Table 3.1

<table>
<thead>
<tr>
<th>entry</th>
<th>amide</th>
<th>substrate solution</th>
<th>enzyme</th>
<th>time (h)</th>
<th>ee&lt;sup&gt;d&lt;/sup&gt; (R)-amide</th>
<th>(S)-acid</th>
<th>ee&lt;sup&gt;d&lt;/sup&gt; (S)-acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>9a</td>
<td>10 %</td>
<td>P. putida</td>
<td>21 h</td>
<td>&gt; 99 %</td>
<td>9b</td>
<td>99 %</td>
</tr>
<tr>
<td>2</td>
<td>9a</td>
<td>10 %</td>
<td>GMO</td>
<td>21 h</td>
<td>99 %</td>
<td>9b</td>
<td>99 %</td>
</tr>
<tr>
<td>3</td>
<td>10a</td>
<td>10 %</td>
<td>P. putida</td>
<td>21 h</td>
<td>97 %</td>
<td>10b</td>
<td>97 %</td>
</tr>
<tr>
<td>4</td>
<td>10a</td>
<td>10 %</td>
<td>GMO</td>
<td>9 h</td>
<td>&gt; 99 %</td>
<td>10b</td>
<td>&gt; 99 %</td>
</tr>
<tr>
<td>5</td>
<td>11a</td>
<td>10 %</td>
<td>P. putida</td>
<td>21 h</td>
<td>93 %</td>
<td>11b</td>
<td>93 %</td>
</tr>
<tr>
<td>6</td>
<td>11a</td>
<td>10 %</td>
<td>GMO</td>
<td>9 h</td>
<td>99 %</td>
<td>11b</td>
<td>93 %</td>
</tr>
<tr>
<td>7</td>
<td>12a</td>
<td>2.5 %</td>
<td>GMO</td>
<td>21 h</td>
<td>98%</td>
<td>12b</td>
<td>98 %</td>
</tr>
<tr>
<td>8</td>
<td>13a</td>
<td>10 %</td>
<td>P. putida</td>
<td>10 h</td>
<td>&gt; 99 %</td>
<td>13b</td>
<td>96 %</td>
</tr>
<tr>
<td>9</td>
<td>13a</td>
<td>10 %</td>
<td>GMO</td>
<td>10 h</td>
<td>&gt; 99 %</td>
<td>13b</td>
<td>97 %</td>
</tr>
<tr>
<td>10</td>
<td>14a</td>
<td>10 %</td>
<td>P. putida</td>
<td>21 h</td>
<td>&gt; 99 %</td>
<td>14b</td>
<td>91 %</td>
</tr>
<tr>
<td>11</td>
<td>14a</td>
<td>10 %</td>
<td>GMO</td>
<td>21 h</td>
<td>&gt; 99 %</td>
<td>14b</td>
<td>97 %</td>
</tr>
<tr>
<td>12</td>
<td>15a</td>
<td>2.5 %</td>
<td>GMO</td>
<td>45 h</td>
<td>98 %</td>
<td>15b</td>
<td>98 %</td>
</tr>
<tr>
<td>13</td>
<td>16a</td>
<td>10 %</td>
<td>P. putida</td>
<td>45 h</td>
<td>&gt; 99 %</td>
<td>16b</td>
<td>70 %</td>
</tr>
<tr>
<td>14</td>
<td>16a</td>
<td>10 %</td>
<td>GMO</td>
<td>45 h</td>
<td>&gt; 99 %</td>
<td>16b</td>
<td>99 %</td>
</tr>
<tr>
<td>15</td>
<td>17a</td>
<td>10 %</td>
<td>P. putida</td>
<td>45 h</td>
<td>&gt; 99 %</td>
<td>17b</td>
<td>98 %</td>
</tr>
<tr>
<td>16</td>
<td>17a</td>
<td>10 %</td>
<td>GMO</td>
<td>21 h</td>
<td>99 %</td>
<td>17b</td>
<td>98 %</td>
</tr>
<tr>
<td>17</td>
<td>18a</td>
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<td>&gt; 99 %</td>
<td>18b</td>
<td>98 %</td>
</tr>
<tr>
<td>18</td>
<td>18a</td>
<td>10 %</td>
<td>GMO</td>
<td>21 h</td>
<td>&gt; 99 %</td>
<td>18b</td>
<td>99 %</td>
</tr>
</tbody>
</table>

* 10:1 mass percentage of amide in H2O, b enzymes were added as permeabilized whole cells in mass ratio: P. putida : amide 1:10; GMO : amide 1:500, c time after which complete conversion (50%) is reached, d ee's were determined via chiral HPLC.

Figure 3.1 shows the conversion of allylglycine amide 9a and the progress development of the ee's of the products in time with the two different organisms. As soon as the reaction starts, the ee of the (S)-acid is > 98%, while the ee of the amide is increasing as the conversion proceeds. It is evident that the rate is faster in case of the GMO, which is even more so taken into account that a 50-fold higher amount of the P. putida cells was used. This is a strong indication that the GMO cells contain more then 50 times the amount of amidase than the wild type P. putida cells.

This general trend was observed in all of these resolution experiments. In some cases, somewhat different behavior was encountered.
Enzymatic resolution of amino acid amides

Figure 3.1

Allylglycine 9, P. putida

Allylglycine 9, GMO

Figure 3.2 shows the conversion of propargylglycine amide 13a and the progress development of the ee's in time with the two different organisms. At the start of the reactions, a sample was taken every hour, while the last sample was taken after 48 hours. The figures nicely illustrate the difference in rate, and also why the ee of the acid does not reach values higher than 96%. In the beginning of the reaction, the ee of the (S)-acid is only ca. 91%, which is probably due to a small amount of racemic acid that is present in the substrate before the resolution starts. Due to this contamination, the ee of the (S)-acid can never reach 100% anymore.

Figure 3.2

Propargylglycine 13, P. putida

Propargylglycine 13, GMO

More drastic deviations were observed for the homopropargylglycine amide series (Figure 3.3). A significantly larger decrease of the ee for the (S)-acid 14b was found. With the
P. putida cells, after 2 hours the ee was 96% and after 48 hours the ee dropped to 80% for the (S)-acid. Again, in the racemic amide some acid was already present, which explains the lower ee of the (S)-acid in the beginning of the resolution. Inversely, the ee of the (R)-amide was >98% after 48 hours and appeared stable. In contrast, with the GMO cells, no decrease in ee was observed for the (S)-acid after 48 hours. The difference obviously cannot be the result of basic hydrolysis, the blanco experiments – without an enzyme – did not show any racemization.

Figure 3.3

A similar pattern was observed for homoallylglycine 10 (the ee of the (S)-acid dropped from 99% to 92%) and for methylhomopropargylglycine 16b (from 95% to 57% after 69 hours). During the resolution of amide 16a, the (S)-acid 16b partially precipitated, which made it more difficult to obtain homogeneous samples of the reaction mixture.

Methionine amide 18, which is structurally 'related' to the homoallylglycine and homopropargylglycine derivatives was also subjected to the resolution conditions. Interestingly, the same trend – excellent ee of the amide, decreasing ee of the acid with progressing reaction times - was observed. Figure 4 summarizes the ee’s of the structurally related (S)-acids 10b, 14b, 16b and 18b during the resolution with the P. putida cells (left graphic) and the GMO cells (right graphic). The superior properties of the GMO-whole cell system are evident: virtually no decrease in ee was observed.

Since the (S)-aminopeptidase responsible for the enantioselective hydrolysis of the racemic amides is identical in both systems, another enzyme, which is only present in the P. putida ATCC 12633 cells has to be the cause of the difference in ee, This could either be an amino acid racemase or a second, non-specific amidase. Such activity is not present in the cells
of the E. coli K-12 strain (E. coli DH5α / pTrpLAP) since there is no decrease in ee when these cells are used.

Figure 3.4

(S)-acids from resolution with P. putida

(S)-acids from resolution with GMO

3.3 Tests with enantiomerically pure homopropargylglycine

To identify the type of enzyme that causes the poor results a number of experiments were carried out. In order to verify the presence of an amino acid racemase, both types of cells were used in combination with enantiomerically pure (>98%) (S)- homopropargylglycine 14b under exactly identical conditions (10% solution, pH 9.2, 40 °C, 70 hours, enzyme: substrate; 1:10 (P. putida:substrate) 1:500 (GMO:substrate).

Figure 3.5

In the case of the P. putida cells, the ee dropped from > 98% to 80% in 70 hours, which means that the (R)-acid is formed during the reaction. This does not happen with the GMO.
cells; the ee remained >98% during the whole experiment. Therefore, it could be concluded that there must be an amino acid racemase present in the *P. putida* ATCC 12633 cells, which is absent in the *E. coli* K12 cells.

The aforementioned experiment, however, cannot exclude that there is also a non-specific amidase in the *P. putida* cells. A similar experiment, with enantiomerically pure (R)-homopropargylglycine amide 14a was conducted and gave rather unexpected results.

**Figure 3.6**

In both experiments the (R)-amide was slowly hydrolyzed to the (R)-acid. The initially low ee (t = 5 h) is probably because a small amount of the (S)-acid was already present due to imperfect separation after the first resolution. In the case of the *P. putida* cells, the ee of the (R)-acid remained approximately 50% during the whole experiment, meaning that both (R)- and (S)-acid were formed. The formation of (S)-acid was due to the presence of the amino acid racemase in the whole cells of the *P. putida*. The total amount of acid increased during the experiment although the exact amount could not be determined. With the GMO cells, the ee of the (R)-acid clearly increased during the course of the experiment up to the value of 88%. Considering that the initial low value was a result of a small amount of (S)-acid already present, it can be concluded that there is largely (R)-acid produced in this reaction and virtually no (S)-acid. These experiments show that the (in principle) (S)-specific aminopeptidase in both systems hydrolyzed the (R)-amides to some extent. Another possibility would be the presence of a non-specific enzyme in both cell systems.

This seems in contradiction with the earlier results of the racemic homopropargyl amide resolution, where the formation of the (R)-acid was not observed. Eventually, we set up another experiment to imitate the reaction conditions at the time 50% conversion was obtained: a solution containing 0.5 gram of (R)-homopropargyl amide (14a) and 0.5 gram of
(S)-homopropragyl acid (14b) was subjected to the resolution conditions with the GMO cells (Figure 3.7)

**Figure 3.7**

![Graph](image)

(R)-14a + (S)-14b with GMO

In this case, hardly any (R)-acid was formed and both ee's remained high (>97%) during the reaction. Only a decrease of 1% ee was observed after 48 hours for the (S)-acid. A probable explanation could be that with the 5 weight% (S)-acid present in the reaction mixture the undesired hydrolysis is slowed down by competitive inhibition of the enzyme. Only under "extreme conditions" (with only the unfavored (R)-amide present) this conversion has a chance to take place, but under the regular resolution conditions only the (S)-amide is hydrolyzed.

In summary, from these control experiments it can be concluded that in the cells of *P. putida* ATCC 12633 an amino acid racemase is present which is not present in the cells of the *E. coli* DH5α/ pTrpLAP. This racemase seems to have a narrow substrate specificity and recognizes methionine and the structurally and electronically related amino acids homopropargylglycine, methylhomopropargylglycine and homoallylglycine.¹⁷

### 3.4 Preparative scale resolutions

Preparative scale experiments were carried out with the GMO cells because of its higher activity and selectivity. In all resolutions, a wet cell mass:substrate ratio of 1:250 was used to ensure that the reaction would be finished in the course of a night. The reactions were worked-up as follows: the cell mass was filtrated or centrifuged off the solution. The resulting clear solution was treated with 0.5 equiv of benzaldehyde and stirred vigorously for a few hours to convert the (R)-amide into their corresponding Schiiff base. The Schiff base was separated from the water layer by filtration or by extraction with an organic solvent and (after
concentration) hydrolyzed with one equiv of HCl in acetone to give the HCl salt of the (R)-amides after filtration. The (S)-acid was obtained upon lyophilization of the aqueous layer followed by purification with ion exchange chromatography.

The (R)-amides were hydrolyzed to their corresponding (R)-acids with a non-specific amidase from whole cells of *Rhodococcus erythropolis* NCIMB 11540 containing an non-specific amidase in a phosphate buffer of pH 8 (dry cell mass : substrate ratio 1:2) in 3 hours.

**Table 3.2**

<table>
<thead>
<tr>
<th>entry</th>
<th>enzyme</th>
<th>time (h)</th>
<th>yield (S)-acid</th>
<th>ee (S)-acid</th>
<th>yield (R)-amide HCl</th>
<th>ee (R)-acid yield (R)-acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>GMOa</td>
<td>48</td>
<td>9b 31%</td>
<td>&gt;98%</td>
<td>9a 47%</td>
<td>&gt;99% 9b 86%</td>
</tr>
<tr>
<td>2</td>
<td>GMOa</td>
<td>48</td>
<td>10b 41%</td>
<td>&gt;99%</td>
<td>10a 40%</td>
<td>&gt;99% 10b 93%</td>
</tr>
<tr>
<td>3</td>
<td>GMO</td>
<td>24</td>
<td>11b nd</td>
<td>&gt;98%</td>
<td>11a 45%</td>
<td>&gt;99% 11b 89%</td>
</tr>
<tr>
<td>4</td>
<td><em>P. putida</em></td>
<td>60</td>
<td>12b 44%</td>
<td>80%</td>
<td>12a 35%</td>
<td>&gt;99% 12b 88%</td>
</tr>
<tr>
<td>5</td>
<td>GMO</td>
<td>21</td>
<td>13b nd</td>
<td>&gt;99%</td>
<td>13a 41%</td>
<td>&gt;99% 13b 80%</td>
</tr>
<tr>
<td>6</td>
<td>GMO</td>
<td>21</td>
<td>14b 40%</td>
<td>&gt;98%</td>
<td>14a 42%</td>
<td>&gt;99% 14b 99%</td>
</tr>
<tr>
<td>7</td>
<td>GMO</td>
<td>19</td>
<td>15b 43%</td>
<td>&gt;99%</td>
<td>15a 33%</td>
<td>&gt;99% 15b 92%</td>
</tr>
<tr>
<td>8</td>
<td>GMO</td>
<td>21</td>
<td>16b 32%</td>
<td>98%</td>
<td>16a 30%</td>
<td>&gt;99% 16b 86%</td>
</tr>
<tr>
<td>9</td>
<td>GMO</td>
<td>21</td>
<td>17b nd</td>
<td>96%</td>
<td>17a 37%</td>
<td>&gt;99% 17b 63%</td>
</tr>
</tbody>
</table>

*AAfter 24 h an additional amount of the GMO cells was added.*

In Table 3.2 the yields of the resolutions are shown. The yields of the (S)-acids in the table are the yields after purification via ion exchange chromatography. The ee's of the (S)-acids are > 98% unless mentioned otherwise. The yields of the (R)-amides refer to the corresponding HCl salts; the yields of the (R)-acids were obtained after hydrolysis and subsequent purification by ion exchange chromatography (ee's were in all cases >99%).

Because the racemic amides 9a and 10a (entries 1 and 2) reacted slower than expected (40% conversion after 24 h), an additional amount of enzyme was added (eventually, the cell mass:substrate ratio was 1:125). After 48 hours the reaction was finished, which had no negative influence on the ee’s. Amide 12a was only subjected to the *P. putida* cells; under identical reaction conditions (cell mass:substrate ratio of 1:10) the ee of the (S)-acid was only 80%.
Enzymatic resolution of amino acid amides

The yields of the (S)-acids 11b and 17b were not determined due to the poor water solubility. During the resolution the acids precipitated in the reaction mixture, which made separation of the enzyme, acid and amide more difficult and probably some of the acid was lost in the work-up. During ion exchange chromatography the (S)-acid also precipitated to some extent, and was lost.

The yield of (S)-13b was not determined due to the presence of H₃PO₄ salts. After the resolution was finished the reaction mixture was neutralized with phosphoric acid (1M) which resulted in a large amount of salts which made it very difficult to purify the amino acid by ion exchange chromatography. After these problems we were more careful with adding acids to the mixture, instead of phosphoric acid hydrochloric acid or sulfuric acid was used.

Although work-up and solubility caused some problems, this is a very nice method to obtain enantiopure (S)-amino acids and (R)-amides in relatively large quantities (up to 30 g).

3.5 Conclusions

In this chapter, several enantiopure unsaturated amino acids and amino acid amides were obtained via enzymatic resolution. For this resolution an aminopeptidase was used from Pseudomonas putida ATCC 12633 both present in the wild type cells and in E. coli K12 upon overexpression (GMO genetically modified organism). Whole cells were used for the enzymatic resolution. Both biocatalytic systems could be used to hydrolyze enantioselectively (S)-amides to (S)-acids. In the resolution with P. putida for some amino acid amides (10a, 14a, 16a and 18a) a progressive decrease in ee of the (S)-acid was observed. After several experiments it was concluded that the whole cells of P. putida contained an amino acid racemase with a narrow substrate specificity. In the whole cells of the GMO this racemase was not observed. At preparative scale the enantiopure (S)-acids and (R)-amides were separated and isolated. The (R)-amides were hydrolyzed by a non specific amidase from Rhodoccus erythropolis NCIMB 11540 in high yields.

3.6 Acknowledgements

DSM is gratefully acknowledged for hosting me in their research lab. T. Sonke (DSM Research) is gratefully acknowledged for his large contribution to this chapter, many fruitful discussions and the molecular biology work which led to the GMO. J.M.M. Boesten (DSM Research) and K.C.M.F. Tjen are gratefully acknowledged for the HPLC analyses of the enantiopure amino acids and amino acid amides.
3.7 Experimental section

General information.
Purification of the amino acids by ion exchange chromatography using a strongly acidic Dowex 50W×4 resin involved the following sequence: the resin was treated with the HCl salt and washed with water until no more HCl was detected. Then the resin was eluted with 2 N NH₄OH, the ninhydrin positive fractions were collected and concentrated to give the free amino acid. The ee’s of the free amino acids were determined by HPLC on a Crownpak CR(+) column (aqueous HClO₄, at 0–7 °C). The ee’s were determined by HPLC on a Chiralcel OD column (10–20% iPrOH in heptane). The amino acid amide HCl-salts were obtained as the free amino acids via ion exchange (Dowex 50W×4 H⁺-form, 20-50 mesh, Fluka).

Resolution of amino acid amides.

Curve determinations.
The enzymes were added as permeabilized whole cells in a HEPES Buffer (20 mM, NaOH, pH 7.67), which is commercially available. The amino acid amide (3.05 g) was dissolved in distilled water. With KOH or H₂SO₄ the solution was brought to pH 9.2. A solution of MnSO₄ (0.38 mL of 80mM solution which is 1 mM MnSO₄ in total) was added. Distilled water was added (29.5 mL) to the solution to bring the end solution at 10% amide concentration in H₂O. The solution was divided in 3 equal amounts. To the first reaction flask 0.5 mL HEPES was added. To the second one Pseudomonas putida ATCC 12633 (0.1 g, 1:10 enzyme : substrate ratio) in 0.5 mL HEPES was added. To the third one E. coli DH5α/pTrpLAP (2 mg, 1:500 enzyme : substrate ratio) in 0.5 mL HEPES. The reaction mixtures were stirred at 40 °C for several days. Samples were taken as follows: 0.5 mL of the reaction mixtures was poured into 1 mL 1M HPO₄ and the cell masses were filtrated of. The mixtures were then analyzed by HPLC analysis/NH₃ detection. The conversion was determined using the formula:

\[ c = \frac{\text{ee}_s}{\text{ee}_s + \text{ee}_p} \]

in which \( \text{ee}_s \) is the enantiomeric excess of the substrate and \( \text{ee}_p \) the enantiomeric excess of the product.

General procedure A for preparative scale:
A solution of the HCl salt of the racemic amide in distilled H₂O was brought to pH 9.2 with KOH or H₂SO₄ followed by addition of a 80 mM solution of MnSO₄ (1mM MnSO₄ in the final solution) With distilled H₂O to solution was brought to a 10% solution of the amide. The enzyme was added as a solution in a HEPES buffer. The reaction was stirred at 40 °C for 24 h and then brought to a pH of 6 with H₂SO₄. The enzyme was filtered or centrifuged of the
solution. Base (NaOH) was added to bring the pH at 8-9, followed by addition of benzaldehyde (0.51 equivalent), the reaction mixture was stirred for 2 hours at 20 °C. The reaction mixture was extracted CH₂Cl₂ (3 x). The combined organic layers containing the Schiff base of the (R)-amide were extracted with H₂O (3 x), dried with MgSO₄ filtered and concentrated in vacuo. The combined aqueous layers containing the (S)-acid were lyophilized and purified by ion exchange chromatography. The Schiff base of the (R)-amide was dissolved in acetone and concentrated HCl (1 equivalent) was added. The reaction mixture was stirred for 2 hours. The HCl-salt of the (R)-amide was filtered of the solvent.

General procedure B for the conversion of (R)-amides to (R)-acids.
The (R)-amino acid amides are dissolved in buffer (concentration 5%) (buffer: pH 8: 500 mL 0.1 M NaH₂PO₄ + 467 mL 0.1 M NaOH). Dried whole cells of Rhodococcus erythropolis NCIMB 11540 (0.5 equivalent in weight) were added and the reaction mixture was stirred at 37°C for 3 hours. The reaction could be monitored on TLC (CHCl₃, MeOH, NH₃; ninhydrine). The reaction mixture was centrifuged and the solvent was separated from the enzyme. The enzyme was washed with water and centrifuged again this was repeated 2 times. The combined water layers were lyophilized. After ion exchange chromatography the (R)-acids were obtained salt free.

(R)-2-Amino-pent-4-enoiic acid amide (9a). Following the general procedure for preparative scale: to a solution of the racemic amide (20.0 g, 0.136 mol) in H₂O, KOH (pH 9.2), MnSO₄ (2.5 g, 80 mM), H₂O (120 g, 10% solution) and finally GMO cells (81 mg in 1 mL Buffer) were added. Work up using benzaldehyde (7.6 mL, 0.075 mol) and conc. HCl (6.0 mL, 0.075 mol) afforded the HCl salt of 9a (9.5 g, 0.063, 47% mol) as a white solid. ee >99% (HPLC), Mp 230-232°C, [α]D = +4.3 (c = 1, H₂O), ¹H NMR (400 MHz, D₂O) δ 5.87-5.76 (m, 1H, H-4), 5.36-5.32 (m, 2H, H-5), 4.14 (t, J = 6.3 Hz, 1H, H-2). ¹³C NMR (100 MHz, D₂O) δ 176.37 (C-1), 134.92 (C-4), 126.32 (C-5), 57.17 (C-2), 40.00 (C-3), IR (KBr) ν 3250, 3450, 2733, 2641, 1981, 1716 cm⁻¹, HRMS (FAB) calculated for C₅H₁₀N₂O (MH⁺) 115.0871, found 115.0861. Anal. Calcd. for C₅H₁₀N₂O: C 39.87, H 7.36, N 18.60. Found: C 39.88, H 7.27, N 18.52. To a solution of 9a (1.0 g, 6.66 mmol) in buffer (20 mL) enzyme (0.2 g) was added. Work up afforded 9b (0.66 g, 5.70 mol, 86%) as a white solid. [α]D = +9.6 (c = 0.5, 1M HCl).

(S)-2-Amino-pent-4-enoiic acid (9b). The (S)-acid was obtained after lyophilization and ion exchange chromatography (4.8 g, 0.042 mol, 31%). ee >98% (HPLC), Mp 235-237°C, [α]D = -35.5 (c = 1, H₂O) ¹H NMR (400 MHz, D₂O) δ 5.82-5.72 (m, 1H, H-4), 5.29-5.25 (m, 2H, H-5), 3.80 (t, J = 5.0 Hz, 1H, H-2), 2.70-2.56 (m, 1H, H-3), ¹³C NMR (100 MHz, D₂O) δ 176.85 (C-1), 134.13 (C-
(R)-2-Amino-hept-5-enoic acid amidine (10a). Following the general procedure for preparative scale: to a solution of the racemic amidine (19.9 g, 0.12 mol) in H$_2$O, KOH (pH 9.2), MnSO$_4$ (2.49 g, 80 mM), H$_2$O (199 g, 10% solution) and finally GMO cells (80 mg in 1 mL buffer) were added. After 18 h an additional amount of enzyme (320 mg) was added. Work up using benzaldehyde (6.80 mL, 0.067) and conc. HCl (5.39 mL, 0.067 mol) afforded the HCl salt of the 10a (7.95 g, 0.048, 40% mol) as a white solid. ee >99% (HPLC), Dec 256-258°C, [α]$^D$ = -17.2 (c = 1, H$_2$O), $^1$H NMR (400 MHz, D$_2$O) δ 5.95-5.85 (m, 1H, H-5), 5.21-5.21 (m, 2H, H-6), 4.06 (t, J = 6.5 Hz, 1H, H-2), 2.25-2.20 (m, 2H, H-4), 2.08-1.98 (m, 2H, H-3), $^{13}$C NMR (100 MHz, D$_2$O) δ 174.94 (C-1), 139.34 (C-5), 119.22 (C-6), 55.52 (C-2), 32.81 (C-4), 31.08 (C-3), IR (KBr) v 3224, 2828, 2759, 2350, 1981, 1668 cm$^{-1}$, HRMS (EI) calculated for C$_6$H$_{12}$N$_2$O 128.0950, found 128.0957. Anal. Calcd. for C$_6$H$_{12}$N$_2$O: C 43.77, H 7.96, Cl 21.53, N 17.02, O 9.72, found: C 43.86, H 7.87, Cl 21.49, N 17.13, O 9.77. To a solution of 10a (1.0 g, 6.10 mmol) in buffer (20 mL) enzyme (0.2 g)) was added. Work up afforded 10b (0.73 g, 5.67 mmol, 93%) as a white solid. [α]$^D$ = -28.8 (c = 0.5, 2M HCl).

(S)-2-Amino-hept-5-enoic acid (10b). The (S)-acid was obtained after lyophilization and ion exchange chromatography (6.44 g, 0.05 mol, 41%). ee >99% (HPLC), Dec >270°C, [α]$^D$ = +8.0 (c = 1, H$_2$O), $^1$H NMR (400 MHz, D$_2$O) δ 5.93-5.86 (m, 1H, H-5), 5.21-5.21 (m, 2H, H-6), 4.06 (t, J = 6.5 Hz, 1H, H-2), 2.18 (dd, J = 7.5, 15 Hz, 2H H-4), 2.04-1.91 (m, 2H, H-3), $^{13}$C NMR (100 MHz, D$_2$O) δ 177.50 (C-1), 139.88 (C-5), 118.71 (C-6), 57.15 (C-2), 32.50 (C-4), 31.42 (C-3), IR (KBr) v 3220, 3250-2500, 2357, 2125, 1640 cm$^{-1}$, HRMS (EI) calculated for C$_6$H$_{12}$NO 129.0790, found 129.0793. Anal. Calcd. for C$_6$H$_{12}$NO: C 55.80, H 8.58, N 10.84, O 24.78, found: C 55.88, H 8.57, N 10.95, O 24.68.

(R)-2-Amino-hept-5-enoic acid amidine (11a). Following the general procedure for preparative scale: to a solution of the racemic amidine (19.2 g, 0.12 mol) in H$_2$O, KOH (pH 9.2), MnSO$_4$ (2.49 g, 80 mM), H$_2$O (191 g, 10% solution) and finally GMO cells (80 mg in 1 mL buffer) were added. Work up using benzaldehyde (6.03 mL, 0.06 mol) and conc. HCl (5.0 mL, 0.06 mol) afforded the HCl salt of the 11a (8.66 g, 0.053 mol, 45%) as a white solid. ee >99% (HPLC), Dec >245°C (HCl salt), [α]$^D$ = -14.3 (c = 1, H$_2$O), $^1$H NMR (400 MHz, D$_2$O) δ 5.96-5.86 (m, 1H, H-6), 5.18-5.02 (m, 2H, H-7), 3.43 (t, J = 6.6 Hz, 1H, H-2), 2.14-2.08 (m, 2H, H-5), 1.73-1.58 (m, 2H, H-4), 1.52-1.42 (m, 2H, H-3), $^{13}$C NMR (100 MHz, D$_2$O) δ 182.44 (C-1), 141.13 (C-6), 116.68 (C-7), 56.04 (C-2), 35.66 (C-5), 34.55 (C-4), 26.02 (C-3), IR (KBr) v 3434, 3307, 2999, 1686 cm$^{-1}$, HRMS
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(R)-2-Amino-hept-5-enoi acid (11b). ee >98% (HPLC), Dec >248 Mp °C, [α]D = -24.9 (c = 1, 2M HCl), 1H NMR (400 MHz, D2O) δ 5.92-5.84 (m, 1H, H-6), 5.07 (ddd, J = 7.1, 14.1 Hz, 2H, H-5), 1.91-1.83 (m, 2H, H-4), 1.53-1.43 (m, 2H, H-3), 13C NMR (100 MHz, D2O) δ 177.65 (C-1), 141.38 (C-6), 117.80 (-7), 57.50 (C-2), 35.24 (C-5), 32.62 (C-4), 26.29 (C-3), IR (KBr) v 3250-2500, 1833, 1581, 1516 cm⁻¹, HRMS (FAB) calculated for C7H15N02 (MH⁺) 143.1184, found 143.1189. To a solution of 11a (1.0 g, 5.61 mmol) in buffer (20 mL) enzyme (0.2 g) was added. Work up afforded 11b (0.71g, 4.99 mmol, 89%) as a white solid. [α]D = -24.9 (c = 1, 2M HCl).

(R)-2-Amino-4,5-hexadienoi acid (12a). Following the general procedure for preparative scale: to a solution of the racemic amide (3.60 g, 0.02 mol) in H2O, KOH (pH 8.5), MnSO4 (0.36 g, 80 mM), H2O (36 g, 10% solution) and finally P. putida cells (1.0 g) were added. Work up using benzaldehyde (1.30 mL, 0.01 mol) and conc. HCl (0.8 mL, 0.01 mol) afforded the HCl salt of the 12a (1.12 g, 6.89 mmol, 35% mol) as a white solid. ee >99% (HPLC), Mp 210-211°C, [α]D = +9.3 (c = 1, H2O), 1H NMR (400 MHz, CD3OD) δ 5.13 (q, J = 7.0 Hz, 1H, H-4), 4.86-4.84 (m, 2H, H-6), 3.94 (dd, J = 5.2, 7.4 Hz, 1H, H-2), 2.64-2.61 (m, 1H, H-3), 2.57-2.53 (m, 1H, H-3), 13C NMR (100 MHz, CD3OD) δ 211.35 (C-5), 171.63 (C-1), 84.08 (C-4), 76.38 (C-6), 53.88 (C-2), 32.18 (C-3), IR (KBr) v 3431, 3282, 3200, 1661 cm⁻¹, HRMS (FAB) calculated for C6H10N2O0 (MH⁺) 128.0712, found 128.0703.

(R)-2-Amino-4,5-hexadienoi acid amide (12b). The (S)-acid was obtained after lyophilization and ion exchange chromatography (1.13 g, 8.80 mmol, 44%). ee, 80%, (HPLC), Mp 231-235°C, [α]D = -36.4. (c = 0.5 in H2O), 1H NMR (400 MHz, CD3OD) δ 5.13 (q, J = 7.0 Hz, 1H, H-4), 4.82-4.79 (m, 2H, H-6), 3.58 (dd, J = 4.2, 7.8 Hz, 1H, H-2), 2.64-2.62 (m, 1H, H-3), 2.52-2.47 (m, 1H, H-3), 13C NMR (100 MHz, CD3OD) δ 211.39 (C-5), 175.82 (C-1), 85.50 (C-4), 77.85 (C-6), 56.04 (C-2), 31.72 (C-3), IR (KBr) v 3850-2600, 1661 cm⁻¹, HRMS (FAB) calculated for C6H10N2O2 (MH⁺) 128.0712, found 128.0703.

(R)-2-Amino-pent-4-ynoi acid amid e (13a). Following the general procedure for preparative scale: to a solution of the racemic amide (8.25 g, 0.07 mol) in H2O, KOH (pH 9.2), MnSO4 (1.03 g, 80 mM MnSO4), H2O (81.53 g, 10% solution) and finally G. putida cells (16.5 mg in 1 mL buffer) were added. Work up using benzaldehyde (3.89 mL, 0.037 mol) and conc. HCl (2.95 mL, 0.037
mol) afforded the HCl salt of 13a (4.29 g, 0.029 mol, 41%) as a white solid. ee 99% (HPLC), Mp 237-239 °C, [α]₀ = -0.4 (c = 1, H₂O). ¹H NMR (400 MHz, D₂O) δ 4.37 (t, J = 5.75 Hz, 2H, H-2), 3.06-3.05 (m, 2H, H-3), 2.75 (d, J = 2.09 Hz, 1H, H-5), ¹³C NMR (100 MHz, D₂O) δ 173.18 (C-1), 79.58 (C-4), 77.67 (C-5), 54.20 (C-2), 24.14 (C-3), IR (KBr) v 3430, 3282, 3200, 2750, 1677 cm⁻¹. Anal. Calcd. for C₅H₈ClN₂O: C 40.42, H 6.11, Cl 23.86, N 18.85, O 10.77, found: C 40.31, H 5.90, Cl 23.81, N 18.93, O 10.82.

Following the general procedure B: To a solution of 13a (5.0 g, 0.034 mol) in buffer (100 mL) enzyme (2.0 g) was added. Work-up afforded 13b (3.1 g, 0.027 mol, 80%) as a white solid. [α]₀ = +29.6 (c = 0.5, H₂O).

(S)-2-Amino-4-pentynoic acid (13b). The (S)-acid was obtained after lyophilization and ion exchange chromatography. ee >99% (HPLC), Dec >216 °C, [α]₀ = -25.3 (c = 0.5, D₂O). ¹H NMR (400 MHz, D₂O) δ 3.90 (t, J = 5.5 Hz, 1H, H-2), 2.85-2.84 (m, 2H, H-3), 2.52 (t, J = 4.0 Hz, 1H, H-5), ¹³C NMR (100 MHz, D₂O) δ 175.51 (C-1), 77.07 (C-4), 76.30 (C-5), 55.67 (C-2), 23.29 (C-3), IR (KBr) v 3286, 3250-2600, 2091, 1651 cm⁻¹. Anal. Calcd. for C₅H₇N₀₂: C 53.09, H 6.24, N 12.38, O 28.29, found: C 52.92, H 6.21, N 12.47, O 28.37.

(R)-2-Amino-hex-5-ynoic acid amide (14a). Following the general procedure for preparative scale: to a solution of the racemic amide (15.5 g, 0.122 mol) in H₂O, KOH (pH 9.2), MnSO₄ (1.94 g, 80 mM MnSO₄), H₂O (155 g, 10% solution) and finally GMO cells (62 mg in 1 mL buffer) were added. Work-up with benzaldehyde (6.81 mL, 0.067 mol) and conc. HCl (5.4 mL, 0.067 mol) followed by purification afforded the HCl salt of 14a (8.23 g, 0.051 mol, 42%) as a white solid. ee >99% (HPLC), Mp 241-242°C, Mp (free amine) 55-57 °C, [α]₀ = -50.3 (c = 1, D₂O). ¹H NMR (400 MHz, D₂O) δ 4.21 (t, J = 6.6 Hz, 1H, H-2), 2.52-2.51 (m, 1H, H-6), 2.46 (dt, J = 7.0, 2.6 Hz, 2H, H-3), 2.17-2.15 (m, 2H, H-4), 2.11-2.03 (m, 1H, H-3), ¹³C NMR (100 MHz, D₂O) δ 174.44 (C-1), 89.37 (C-5), 74.26 (C-6), 55.14 (C-2), 32.30 (C-3), 16.87 (C-4), IR (KBr) v 3416, 3270, 3013, 2125, 1677 cm⁻¹. Anal. Calcd. for C₆H₁₀N₂O: C 57.12, H 7.99, N 22.21, found: C 56.97, H 8.10, N 22.10. To a solution of 14a (1.0 g, 6.17 mmol) in buffer (20 mL) enzyme (0.5 g) was added. Work up afforded 14b (0.78 g, 6.14 mmol, 99%) as a white solid. [α]₀ = +23.6 (c = 1.1, 2 M HCl).

(S)-2-Amino-hex-5-ynoic acid (14b). The (S)-acid was obtained after lyophilization and ion exchange chromatography (6.2 g, 0.049 mol, 40%). ee >98% (HPLC), Dec >214 °C, [α]₀ = +26.6 (c = 1, 1 M HCl), [α]₀ = +73.0 (c = 0.5, 2M HCl). ¹H NMR (400 MHz, D₂O) δ 3.88-3.86 (m, 1H, H-2), 2.46-2.36 (m, 3H, H-4, H-6), 2.20-2.13 (m, 1H, H-3), 2.11-2.03 (m, 1H, H-3), ¹³C NMR (100 MHz, D₂O) δ 176.94 (C-1), 85.00 (C-5), 73.62 (C-6), 56.76 (C-2), 32.01 (C-3), 17.18 (C-4), IR (KBr) v 3288, 3100-2500, 2749, 2591, 2100, 1611 cm⁻¹. Anal. Calcd. for C₆H₁₀N₂O: C 56.68, H 7.13, N 11.02, found C 56.53, H 7.02, N 11.09.
(R)-2-Amino-hex-4-ynoic acid amide (15a). Following the general procedure for preparative scale: to a solution of the racemic amide (12 g, 0.073 mol) in H$_2$O, KOH (pH 9.2), MnSO$_4$ (1.5 g, 80 mM MnSO$_4$), H$_2$O (119 g, 10% solution) and finally GMO cells (48 mg in 1 mL buffer) were added. Work up using benzaldehyde (4.09 mL, 0.036 mol) and conc. HCl (2.8 mL, 0.036 mol) afforded the HCl salt of 15a (4.01 g, 0.024 mol, 33%) as a white solid. ee >99% (HPLC), Dec (HCl salt) > 243 °C, [α]$_D$ = +10.5 (c = 1, H$_2$O), $^1$H NMR (400 MHz, D$_2$O) δ 3.55 (t, J = 5.8 Hz, 1H, H-2), 2.55-2.53 (m, 2H, H-3), 1.79 (t, J = 2.5 Hz, 3H, H-6), $^{13}$C NMR (100 MHz, D$_2$O) δ 173.55 (C-1), 85.38 (C-4), 54.49 (C-2), 24.32 (C-3), 5.42 (C-6), IR (KBr) v 3321, 3246, 2986, 2360, 1701 cm$^{-1}$, HRMS (EI) calculated for C$_6$H$_{10}$N$_2$O 126.0793, found 126.0793. Anal. Calcd. for C$_6$H$_{10}$N$_2$O: C 44.32, H 6.82, Cl 21.80, N 17.23, O 9.84. To a solution of 15a (2.0 g, 0.024 mol) in buffer (40 mL) enzyme (0.4 g) was added. Work up afforded 15b (1.44 g, 0.01 mol, 92%) as a white solid. [α]$_D$ = +48.8 (c = 1, H$_2$O), Enzymatic resolution of amino acid amides

(S)-2-Amino-hex-4-ynoic acid amide (15b). The (S)-acid was obtained after lyophilization and ion exchange chromatography (4.0 g, 0.032 mol, 43%). ee >99% (HPLC), Mp 238-240 °C, [α]$_D$ = -29.3 (c = 1, H$_2$O), $^1$H NMR (400 MHz, D$_2$O) δ 3.85-3.83 (m, 1H, C-2), 2.77-2.76 (m, 2H, H-3), 1.77 (t, J = 2.34 Hz, 3H, H-6), $^{13}$C NMR (100 MHz, D$_2$O) δ 175.92 (C-1), 84.37 (C-4), 74.76 (C-5), 56.12 (C-2), 23.63 (C-3), 5.25 (C-6), IR (KBr) v 3100-2600 (b), 2359, 2048, 1608 cm$^{-1}$. Anal. Calcd. for C$_6$H$_{12}$N$_2$O: C 56.68, H 7.13, N 11.02, found: C 56.33, H 6.95, N 10.77.

(R)-2-Amino-hept-5-ynoic acid amide (16a). Following the general procedure for preparative scale: to a solution of the racemic amide (10.6 g, 0.06 mol) in H$_2$O, KOH (pH 9.2), MnSO$_4$ (1.32 g, 80 mM), H$_2$O (106 g, 10% solution) and finally GMO cells (106 mg in 1 mL buffer) were added. Work up using benzaldehyde (7.28 mL, 0.068 mol) conc. HCl (5.48 mL, 0.068 mol) afforded the HCl salt of 16a (3.19 g, 0.018 mol, 30%) as a white solid. ee 99% (HPLC), Dec (HCl salt) >256 °C, [α]$_D$ = -31.6 (c = 1, H$_2$O), $^1$H NMR (400 MHz, D$_2$O) δ 4.16 (t, J = 6.5 Hz, 1H, H-2), 2.39-2.35 (m, 2H, H-4), 2.14-2.03 (m, 2H, H-3), 1.78 (t, J = 2.5 Hz, 3H, H-7), $^{13}$C NMR (100 MHz, D$_2$O) δ 176.00 (C-1), 82.03 (C-5), 79.53 (C-6), 55.13 (C-2), 32.63 (C-3), 17.00 (C-4), 5.18 (C-7), IR (KBr) v 3433, 3307, 2999, 2467, 1995, 1865, 1582, 1485 cm$^{-1}$, HRMS (EI) calculated for C$_7$H$_{11}$N$_2$O$_2$: C 47.60, H 7.42, N 15.86, found: C 47.51, H 7.36, N 15.72. To a solution of 16a (1.0 g, 5.68 mmol) in buffer (20 mL) enzyme (0.2 g) was added. Work up afforded 16b (0.69 g, 4.88 mmol, 86%) as a white solid. [α]$_D$ = -30.5 (c = 0.55, 1M HCl).

(S)-2-Amino-hept-5-ynoic acid (16b). The (S)-acid was obtained after lyophilization and ion exchange chromatography (2.67 g, 0.019 mol, 31%). ee 98% (HPLC) Mp 223-227 °C, [α]$_D$ = +27.0 (c = 0.5, 1 M HCl), $^1$H NMR (400 MHz, D$_2$O) δ 3.84 (dd, J = 5.2, 7.4 Hz, 1H, H-2), 2.40-2.22
(m, 2H, H-4), 2.19-2.07 (m, 1H, H-3), 2.05-1.94 (m, 1H, H-3), 1.77 (t, J = 2.32 Hz, 3H, H-7), $^{13}$C NMR (100 MHz, D$_2$O) δ 177.14 (C-1), 81.57 (C-5), 80.22 (C-6), 56.92 (C-2), 32.39 (C-3), 17.37 (C-4), 5.15 (C-7), IR (KBr) v 3200-2700, 2744, 2097, 1650, 1585, 1415, 1342 cm$^{-1}$, HRMS (EI) calculated for C$_7$H$_{15}$NO, 142.0868 found 142.0610. Anal. Calcd. for C$_7$H$_{15}$NO: C 59.56, H 7.85, N 9.94; found: C 59.46, H 7.77, N 9.96.

(R)-2-Amino-oct-6-ynoic acid amide (17a). Following the general procedure for preparative scale: to a solution of the racemic amide (37 g, 0.195 mol) in H$_2$O, KOH (pH 9.2), MnSO$_4$ (4.62 mL, 80 mM), H$_2$O (370 g, 10% solution) and finally (370 mg GMO in buffer) were added. Work up using benzaldehyde (22.8 mL, 0.21 mol) and conc. HCl (15.7 mL, 0.21 mol) afforded the HCl salt of 17a (13.7 g, 0.072 mol, 37%) as a white solid. ee >99% (HPLC), Dec (HCl salt) >251 °C, [α]$_D$ = -18.0 (c = 1, H$_2$O), $^1$H NMR (400 MHz, D$_2$O) δ 4.07 (t, J = 6.5 Hz, 1H, H-2), 2.26-2.23 (m, 2H, H-5), 2.03-1.98 (m, 2H, H-4), 1.77 (t, J = 2.5 Hz, 3H, H-8), 1.62-1.57 (m, 2H, H-3), $^{13}$C NMR (100 MHz, D$_2$O) δ 174.87 (C-1), 81.73 (C-6), 80.79 (C-7), 55.53 (C-2), 32.72 (C-3), 26.27 (C-4), 20.31 (C-5), 5.12 (C-8), IR (KBr) v 3427, 3304, 3006, 1996, 1687, 1584, 1480 cm$^{-1}$. Anal. Calcd. for C$_7$H$_{15}$NO: C 50.39, H 7.93, N 14.69; found: C 50.41, H 7.87, N 14.58. To a solution of 17a (1.0 g, 5.23 mmol) in buffer (20 mL) enzyme (0.2 g) was added. Work up afforded 17b (0.52 g, 3.30 mmol, 63%) as a white solid. [α]$_D$ = -38.4 (c = 0.53, 2M HCl).

(S)-2-Amino-oct-6-ynoic acid (17b). The (S)-acid was obtained after lyophilization. During the reaction the acid precipitated, so that during the work up some of the acid was lost. Ion exchange chromatography failed due to the insolubility of the acid. The (S)-acid was not purified on large scale. ee 96% (HPLC), Mp 240-244 °C, [α]$_D$ = +11.2 (c = 0.25, 2M HCl), $^1$H NMR (100 MHz, D$_2$O) δ = 3.76 (t, J = 6.1 Hz, 1H, H-2), 2.25-2.21 (m, 2H, H-5), 2.11-1.88 (m, 2H, H-4), 1.77 (t, J = 2.5 Hz, 3H, H-8), 1.68-1.48 (m, 2H, H-3), $^{13}$C NMR (100 MHz, D$_2$O) δ 177.43 (C-1), 82.00 (C-6), 80.60 (C-7), 57.29 (C-2), 32.46 (C-3), 26.64 (C-4), 20.38 (C-5), 5.10 (C-8), IR (KBr) v 3250-2600, 2740, 2093, 1655, 1581, 1415, 1334 cm$^{-1}$, HRMS (FAB) calculated for C$_7$H$_{14}$NO (MH$^+$) 156.1025 found 156.0609. Anal. Calcd. for C$_7$H$_{14}$NO: C 61.91, H 8.44, N 9.03; found: C 61.55, H 8.39, N 9.12.

3.8 References and notes


7. For references see chapter 1 ref: 22-26.
12. Sonke, T.; Schoemaker, H. E. Personal communication.

16. Racemic methionine amide was a kind gift of DSM Research at Geleen.