A Biocatalytic Route to Enantiomerically Pure Unsaturated α-H-α-Amino Acids

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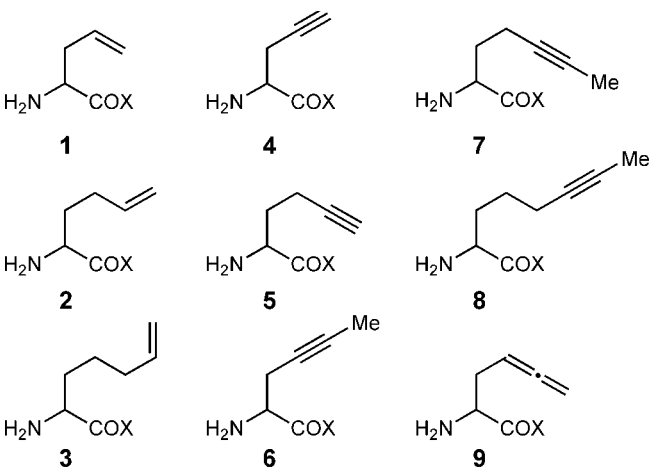
Abstract: A set of both enantiomeric forms of non-proteinogenic, unsaturated α-H-α-amino acids was efficiently synthesized using a biocatalytic pathway. This route involved the straightforward synthesis of the required unsaturated amino acid amides, followed by resolution with an aminopeptidase present in *Pseudomonas putida* ATCC 12633 and/or a genetically modified organism, leading to the (*S*)-acids and (*R*)-amides. Undesired amino acid racemase activity was identified in the wild-type strain, which was absent in the newly developed organism. The (*R*)-amides were hydrolyzed under mild conditions using an amidase present in whole cells from *Rhodococcus erythropolis* NCIMB 11540 to the (*R*)-acids. The viability of this procedure was demonstrated with the multi-gram synthesis of a variety of unsaturated amino acids in excellent enantiopurity.

Keywords: amidase; aminopeptidase; biocatalysis; enzymatic resolution; unsaturated amino acids;

Introduction

Amino acids have a long-standing history as readily available, enantiomerically pure starting materials from the chiral pool that can be used in the widest sense for organic synthesis purposes.[1] This use, however, has been mainly restricted to the well-known twenty so-called proteinogenic amino acids, which have their restrictions in terms of side chain functionality and availability of the non-natural enantiomer. We envisaged that the chemistry of amino acids could be greatly extended by ensuring a general and relatively facile access to non-proteinogenic amino acids containing specifically designed reactive functional groups in the side chain such as olefins, acetylenes, and allenes (**1a – 9a**, Scheme 1).[2] It is interesting to emphasize at this point, that although non-proteinogenic, not all of these amino acids are non-natural since a majority occurs in Nature. Unfortunately, they are not available in quantities that would be required for use in the chiral pool. To mention a few examples, both (*S*)-allylglycine (**1a**) and



Scheme 1. The unsaturated amino acids.

Supporting information for this article is available on the WWW under http://www.wiley-vch.de/home/asc/ or from the author.

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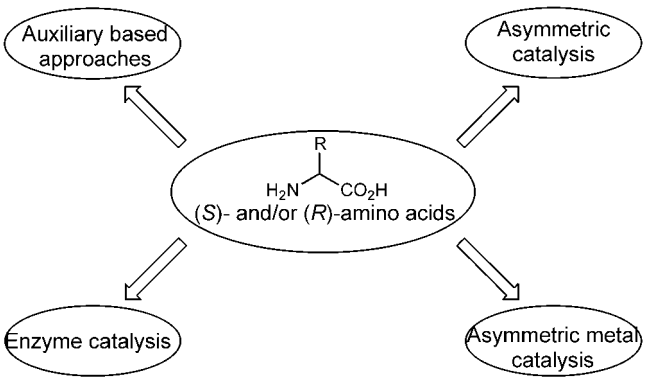


Scheme 2. Approaches towards enantiopure amino acids.

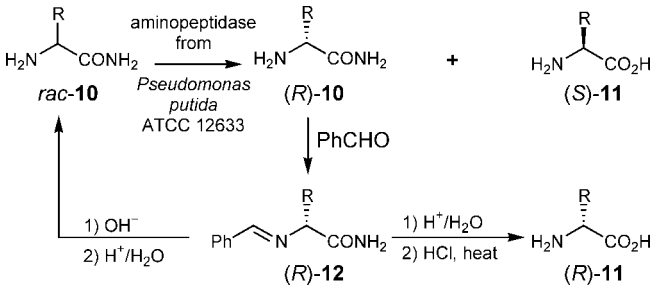
(*S*)-homoallylglycine (**2a**) were isolated from mushrooms;[3] (*S*)-propargylglycine (**4a**)[4] and (*S*)-homopropargylglycine (**5a**)[5] have been isolated, (*S*)-**6a** has been described,[6] also the allene-containing amino acid (**9a**) is known.[7]

Some of these compounds have been previously prepared in enantiomerically pure form. Enantiopure allylglycine (**1a**) has been synthesized in many ways, either using chiral auxiliaries[8] or using (bio)catalysts.[9] In contrast, syntheses of enantiopure homoallylglycine (**2a**) and its homologue bishomoallylglycine (**3a**) are less abundant.[9b,10] A similar picture is encountered for the acetylene-containing amino acids. Enantiopure syntheses of propargylglycine (**4a**) exist,[9b,11] but to the best of our knowledge, entries into homopropargylglycine (**5a**) in enantiopure form have not been published. In addition, an enantioselective synthesis of 2-amino-4-hexynoic acid (**6a**) is known,[12] whereas syntheses of its homologues are not. Finally, one synthetic route to enantiopure 2-amino-4,5-hexadienoic acid (**9**) has been reported.[13]

To meet the general demand for α-amino acids with specific, non-proteinogenic functional groups, over the years a large number of methods has been developed to synthesize such amino acids in enantiomerically pure form (Scheme 2).[14]

The approaches can be roughly divided in four different strategies: (i) approaches employing a stoichiometric amount of a chiral auxiliary,[15] (ii) asymmetric catalytic processes without metals,[16] (iii) asymmetric processes catalyzed by (transition) metals,[17] and (iv) biocatalytic procedures involving enzymatic transformations. Among the latter methods,[18] enzyme classes that have been frequently applied to resolve a broad range of amino acid derivatives include esterases or proteases,[19] acylases,[9b,20] hydantoinases,[21] and aminopeptidases.[22]

An important aspect of the synthesis of the targeted amino acids is the scaling up of the process, which is relatively straightforward for the enzymatic pathways. Furthermore, it would be advantageous if all these amino acids could be synthesized in enantiopure from via a single method.



Scheme 3. The general biocatalytic pathway.

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. In the mid 1970's, at DSM a process was developed, utilizing aminopeptidases to resolve racemic amino acid amides. The essentials of this process are depicted in Scheme 3. Subjection of a (proteinogenic or non-proteinogenic) racemic amino acid amide (**10**) to the aminopeptidase present in the organism *Pseudomonas putida* ATCC 12633 leads to a mixture of the (*R*)-amide **10** and the (*S*)-acid **11** in generally high enantioselectivity.[22] It is important to emphasize that permeabilized whole cells of the *P. putida* strain are used, so that all enzymes expressed in these cells come into contact with substrates and products of the amidase reaction. The (*R*)-amide **10** can be readily separated from the (*S*)-acid **11** by adding one equivalent (relative to the amide) of benzaldehyde to the reaction mixture, thus converting the amide into the corresponding Schiff base **12**. 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 **12** regenerates the (*R*)-amide **10**, which can be converted into the corresponding (*R*)-acid at 90 °C in 6 N HCl.

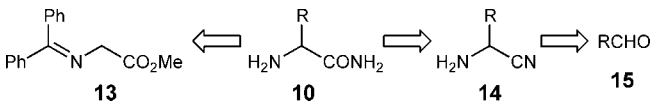
Up till now, a vast number of different amino acid amides has been successfully resolved via this method. It has been shown that a broad structural variety of α-H-α-amino acid amides (small and large groups) is accepted by the biocatalyst without losing its enantioselective properties. Aryl or alkyl 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. The high acceptance of a variety of sub-

strates was a strong impetus for us 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 this research were the relatively poor results with methionine amide, but also with allylglycine amide upon prolonged subjection to the enzyme.[9a] 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, we identified the amidase in the wild type organism, cloned the genetic information and investigated whether this sequence could be expressed in a suitable host organism, which would lack any undesirable activity.

In this article, it was our goal to investigate whether the existing process could also be applied to these unsaturated amino acids and whether the enzymatic properties of the whole system could be improved. This requires an efficient and straightforward entry into the racemic amino acid amides **1b — 9b**.

Results and Discussion

Allylglycine amide (**1b**) was synthesized according to a known procedure.[9a] The other amino acids were either synthesized via alkylation of methyl *N*-(diphenylmethylene)glycinate (**13**),[23] or via a Strecker reaction and subsequent partial hydrolysis of the cyanide to the amide (Scheme 4).

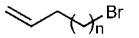
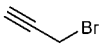
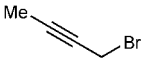
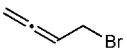


Scheme 4. Retrosynthesis of amino acid amides.

Alkylation Reactions

The amino acid amides **2b — 4b**, **6b**, and **9b** were prepared by an extension of the O'Donnell protocol[23] involving alkylation of methyl *N*-(diphenylmethylene)-glycinate (**13**)[24] with the appropriate unsaturated bromides. Since the ketimine function is hydrolyzed under mildly acidic conditions, decomposition of the side chain is circumvented in this pathway. The bulky diphenyl group also prevents dialkylation. Alkylation of **13** either proceeded via deprotonation with a phase-transfer catalyst (K_2CO_3, *n*-Bu_4NBr, MeCN, reflux; method A), or via deprotonation with NaH (THF, reflux; method B) followed by reaction with the alkylating agent (Table 1). The somewhat milder method

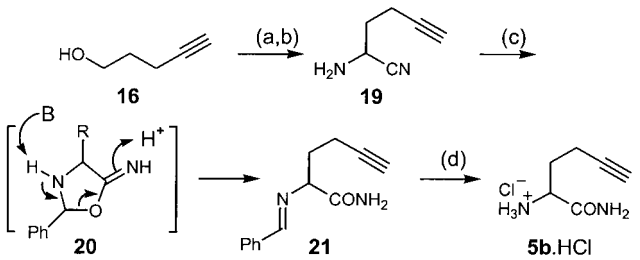
Table 1. Synthesis of amino acids via alkylation.

entry	bromide	method	amino acid amide	overall yield
1	 n = 2	A	**2b**·HCl: n = 2	62%
2	n = 3	A	**3b**·HCl: n = 3	54%
3		B	**4b**·HCl	66%
4		B	**6b**·HCl	66%
5		B	**9b**·HCl	55%

A appeared especially useful when sensitive alkylating agents were used. The unsaturated bromides were commercially available except for 4-bromo-1,2-butadiene,[25] which was prepared from 2,3-butadienol[26] using Br_2PPh_3 and imidazole. In the case of long reaction times, (with method A reaction times were in the order of days), additional alkylating agent was added during the reaction to compensate for the loss of the alkylating agent due to evaporation or decomposition. The alkylated Schiff bases were then selectively hydrolyzed in a two-phase system (1 equivalent of 2 M HCl in Et_2O at ambient temperature) to afford the HCl salts of the corresponding esters. Treatment with concentrated ammonia (25% in water) then gave the desired amides. During this conversion, partial hydrolysis of the ester took place so that separation of the amide and acid became necessary. Therefore, the residue was dissolved in aqueous NaOH (pH ~10) and treated with benzaldehyde to react the amide selectively to the corresponding Schiff base. Extraction with CH_2Cl_2 from the water layer, followed by dissolving the concentrated residue in acetone and hydrolysis via the addition of an equimolar amount of concentrated HCl then gave the HCl salts of the desired amides, which precipitated from the solution in pure form. It should be stressed that with this single purification step at the end of the whole sequence the amides were synthesized in reasonable amounts (up to 25 g) in overall yields (starting from **13**) ranging from 54 to 66% (Table 1).

Strecker Reactions

Amino acid amides **5b**, **7b**, and **8b** were synthesized via an alternative method. In this case, the aforementioned pathways failed as a result of β-elimination of the alkylating agent under the basic conditions of the alkylation step. Instead, these amides were synthesized via a modified Strecker reaction. The starting material, the acetylenic alcohol **16** is commercially available, while **17** and **18** can be readily prepared from the terminal acetylenes via alkylation using an excess of $LiNH_2$ in liquid ammonia, followed by addition of one equivalent of methyl iodide.[27] The amino



Reagents and conditions: (a) $(COCl)_2$ (2 equiv), DMSO (3 equiv), –78 °C, 1 h, then Et_3N (5 equiv), –78 °C to rt, 1.5 h; (b) NaCN (1 equiv), NH_4Cl (1 equiv), 25% NH_3 in H_2O, H_2O, rt, 1 h, then 4-pentynal, 40 °C, 4 h; (c) NaOH (1 equiv), PhCHO (1 equiv), 25% NH_3 in H_2O, H_2O, rt, 4 h; (d) acetone, concentrated HCl (1 equiv), rt, 3 h, 36% starting from **16**.

Scheme 5. The Strecker synthesis of amino acid amides.

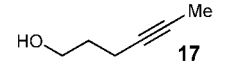
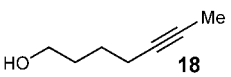
acid syntheses commenced with oxidation of the alcohol under Swern conditions to the corresponding aldehyde. The crude aldehyde was subjected to the Strecker conditions (HCN prepared *in situ* from equimolar amounts of NH_4Cl and NaCN in concentrated ammonia) to produce the aminonitrile **19** (Scheme 5), which, after extraction and concentration, was directly further reacted with benzaldehyde in the presence of NaOH (pH ~10).[28] Thus, the cyanide function was partially hydrolyzed to the corresponding amide under formation of the benzaldimine **21**, which probably proceeded via the five-membered ring *N,O*-acetal **20**. An advantage of the latter transformation is that undesired hydrolysis to the amino acid is avoided, which usually is a side reaction in conversions of cyanides to amides. Subsequent hydrolysis of the Schiff base in acetone with one equivalent of concentrated HCl then provided the HCl salt of the desired amide.

This sequence was routinely carried out on a 0.3 – 0.6 mol scale for the three alcohols to give the corresponding HCl salts of the amides **5b**, **7b**, and **8b** in reasonable to good overall yields from the alcohol (Table 2). Again, the only purification step was the final precipitation and filtration of the HCl salt of the amides after the hydrolysis step in acetone.

Enzymatic Resolutions

The characteristics of the most important leucine aminopeptidase from the *Pseudomonas putida* ATCC 12633 strain have been previously determined after purification of the enzyme.[22b,29] The most prominent features are as follows. 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 was finally designated as an (*S*)-aminopeptidase. Divalent cations sometimes have a positive effect (Mg^{2+} and Co^{2+} 2- to 3-fold; Mn^{2+} 12-fold), but can also have a negative influence (Cu^{2+} and Ca^{2+} 70% and 40% inhibition, respectively) on the activity of the enzyme.

Table 2. Synthesis of amino acid amides via the Strecker reaction.

entry	amino ester	amino acid amide	overall yield
1	 **17**	**7b**·HCl	65%
2	 **18**	**8b**·HCl	59%

To construct a potentially more efficient biocatalytic system, the gene coding for the (*S*)-aminopeptidase from *P. putida* ATCC 12633 was cloned and brought to overexpression in an *E. coli* K-12 host microorganism. This bacterium was chosen because of its favorable fermentation properties and the availability of a large number of specialized expression vectors. The procedure itself has been detailed in a recent publication and therefore will not be described in this article.[22b] Thus, *E. coli* DH5α/pTrpLAP whole cells were obtained, which will be referred to as *GMO* (genetically modified organism) cells. Resolution of some natural 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 (based on the crude weight of the cell mass) by the genetically modified organism (*GMO*).[22b] This is to a large extent most probably caused by the improved expression of the *P. putida pep*A 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 pep*A did not result in any mutation on the 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*.

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 **1b – 9b** 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. The enzymatic resolutions were conducted with both systems at 40 °C and pH 9.2 in the absence of Na^+ and Cl^- ions, which are known to inhibit the aminopeptidase activity. Initially, the experiments were run on a 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 *P. putida* (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_3PO_4 (1 mL, to stop the enzyme activity), after which the conversions and the ee's of the acids and amides were determined in a single run via chiral HPLC analysis (Sumichiral OA 5000, 150 mm × 4.6 mm, eluant: 2 mM $CuSO_4$ in H_2O/MeOH).

The results of the resolutions of the racemic amides are shown in Table 3. The ee's of 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 obtain a better impression of the catalytic activity of the cells. The yields were not determined in these reactions, since the goal was to find out at what time approximately 50% conversion had been obtained for a given enzyme/substrate ratio. In contrast with the other substrates, the racemic amides **6b** and **9b** 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 11 and 16). Furthermore, methionine amide (**22b**, entries 17 and 18) was subjected to both enzyme preparations.

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 **3b** and **8b** made it more difficult to take homogeneous samples.

Table 3. Comparing the resolution results with *P. putida* and *GMO*.

entry	*rac*-amide	substr[a] soln (m%)	cells[b]	time (h)[c]	ee (%)[d] (*R*)-amide	(*S*)-acid	ee (%)[d]
1	**1b**	10	*P. putida*	21	>99	**1a**	99
2	**1b**	10	*GMO*	21	99	**1a**	99
3	**2b**	10	*P. putida*	21	97	**2a**	97
4	**2b**	10	*GMO*	9	>99	**2a**	>99
5	**3b**	10	*P. putida*	21	93	**3a**	93
6	**3b**	10	*GMO*	9	99	**3a**	93
7	**4b**	10	*P. putida*	10	>99	**4a**	96
8	**4b**	10	*GMO*	10	>99	**4a**	97
9	**5b**	10	*P. putida*	21	>98	**5a**	91
10	**5b**	10	*GMO*	21	>99	**5a**	97
11	**6b**	2.5	*GMO*	45	98	**6a**	98
12	**7b**	10	*P. putida*	45	>99	**7a**	70
13	**7b**	10	*GMO*	21	99	**7a**	99
14	**8b**	10	*P. putida*	45	>99	**8a**	98
15	**8b**	10	*GMO*	21	99	**8a**	98
16	**9b**	2.5	*GMO*	21	98	**9a**	98
17	**22b**	10	*P. putida*	21	>99	**22a**	98
18	**22b**	10	*GMO*	21	>99	**22a**	99

[a] 1:10 mass percentage of amide in H_2O.

[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%) was reached.

[d] ee's were determined via chiral HPLC.

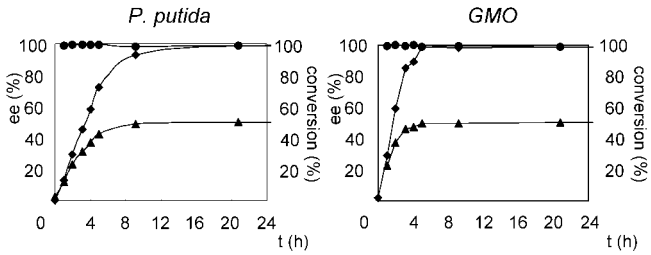


Figure 1. Resolution of amide **1b**. —♦— (*R*)-amide; —·— (*S*)-acid; —▲— conversion.

This complication could explain the relatively moderate ee of the (*S*)-acid **3a** (entries 5 and 6) and the longer reaction time. Precipitation of the (*S*)-acid **7a** was also observed although in a smaller degree. The difference between both biocatalysts is clear from Table 3: first of all, there is a significant difference in reaction time in some entries (3 and 4, 5 and 6, 12 and 13, and 14 and 15). The resolution with the *GMO* is faster, although the amount of cells is approximately 50 times less. Secondly, especially in the resolutions of amides **5b** and **7b** a large difference in the ee of the corresponding (*S*)-acids was observed (entries 9, 10: 91 vs. 97% ee; entries 12, 13: 70 vs. 99% ee). These latter remarkable differences in enantioselectivity encouraged us to further investigate these results.

Figure 1 shows the conversion of allylglycine amide **1b** and the progress development of the ee of the products over 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 than 50 times the amount of amidase as the wild-type *P. putida* cells.

This general trend was observed in all of these resolution experiments. In some cases, however, more drastic deviations were observed such as in the homopropargylglycine amide (**5b**) series (Figure 2). A relatively large decrease of the ee for the (*S*)-acid **5a** was found. In case of the *P. putida* cells, after 2 hours the ee of (*S*)-**5a** was 96%, but after 48 hours had dropped to 80%. As can be seen from the curves, 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. Gratifyingly, in case of the *GMO* cells no decrease in ee was observed for the (*S*)-acid after 48 hours. The difference cannot be the result of base-induced hydrolysis of the racemic amide since the blank experiments – reactions without an enzyme – did not show any racemization.

A similar pattern was observed for homoallylglycine **2a** [the ee of the (*S*)-**2a** dropped from 99% to

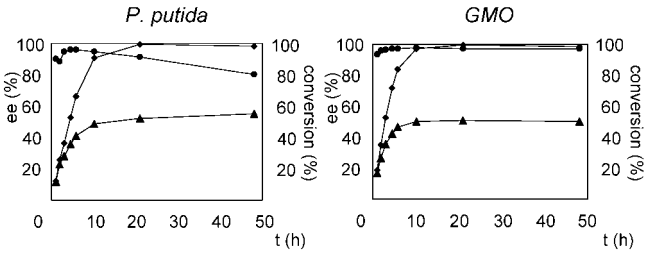


Figure 2. Resolution of amide **5a**. —♦— (*R*)-amide; —·— (*S*)-acid; —▲— conversion.

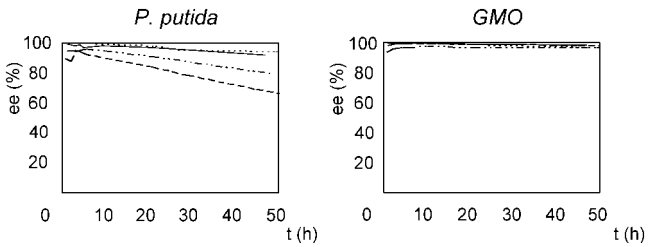


Figure 3. Decreasing ee of some (*S*)-acids. —— **2a**; – ·· – **5a**; – – – **7a**; ···· **22a**.

92%] and for **7a** [the ee of (*S*)-**7a** dropped from 95% to 57% after 69 hours]. Methionine amide (**22b**), which is structurally 'related' to the homoallylglycine and homopropargylglycine derivatives,[30] 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 3 summarizes the ee's of the structurally related (*S*)-acids **2a**, **5a**, **7a**, and **22a** during the resolution with the *P. putida* cells (left) and the *GMO* cells (right). 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* 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* strain (*E. coli* DH5α/pTrpLAP) because there is no decrease in ee when these cells are used. A number of experiments was carried out to identify the type of enzyme that caused the poor results. 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 **5a** under exactly identical conditions [10% solution, pH 9.2, 40 °C, 70 h, enzyme:substrate ratio: 1:10 (*P. putida*), 1:500 (*GMO*), results are shown in Figure 4). 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* cells, which is absent in the *E. coli* 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 **5b** was conducted and gave rather unexpected results (Figure 5). 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.

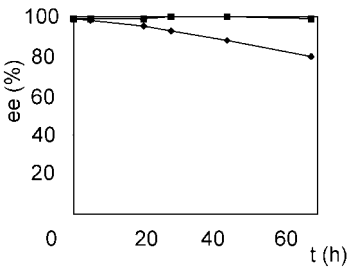


Figure 4. Decrease in the ee of (*S*)-**5a**. —♦— *P. putida*; —■— *GMO*.

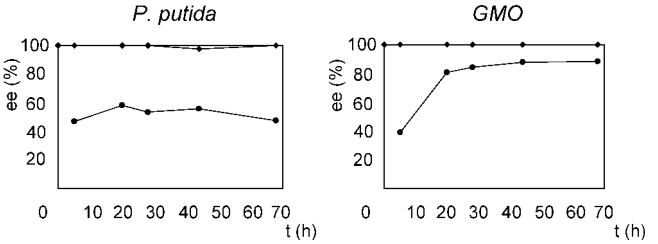


Figure 5. Change in the ee of (*R*)-**5b**. —♦— (*R*)-amide; —·— (*R*)-acid.

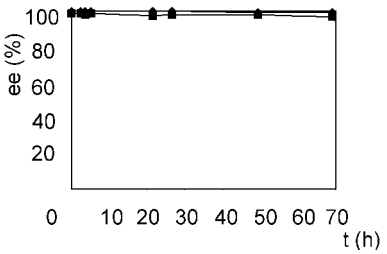
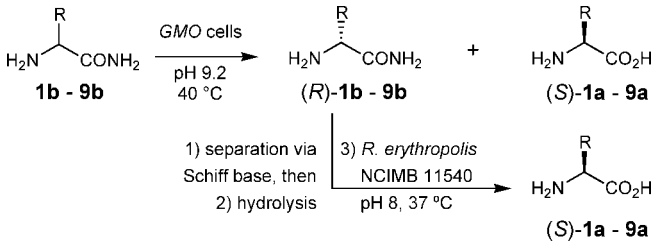


Figure 6. Change in the ee with acid and amide present. —♦— (*R*)-amide; —■— (*S*)-acid.



Scheme 6. The optimized biocatalytic pathway.

This seems to be in contradiction with the earlier results on the racemic homopropargylglycine amide resolution, where the formation of the (*R*)-acid was not observed. Finally, we set up another experiment to imitate the reaction conditions at the time at which 50% conversion was reached: a solution containing 0.5 g of (*R*)-homopropargylglycine amide (**5b**) and 0.5 g of (*S*)-homopropargylglycine (**5a**) was subjected to the resolution conditions with the *GMO* cells (Figure 6).

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 in the mixture the undesired hydrolysis is slowed down by competitive inhibition of the enzyme. Only under "extreme conditions" [with only the unfavored (*R*)-amide present] does this conversion have a chance to take place, but under the regular resolution conditions only the (*S*)-amide is hydrolyzed.

In summary, these control experiments led us to conclude 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 homoallylglycine (**2a**), homopropargylglycine (**5a**), and its methylated homologue **7a**.[30]

Preparative scale experiments (Scheme 6) were carried out with the *GMO* cells because of the 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 filtered or centrifuged off the solution. The resulting clear solution was treated with 0.5 equivalent of benzaldehyde and stirred vigorously for a few hours to convert the (*R*)-amide into the corresponding Schiff base. The Schiff base was separated from the water layer by extraction with an organic solvent and (after concentration) hydrolyzed with one equivalent 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.

Next, hydrolysis of the (*R*)-amides was called for. Because hydrolysis under the harsh acidic conditions (6 N HCl, 90 °C) led to decomposition of some of the side chains, an alternative method was sought. We found that a non-selective amidase produced by *Rhodococcus erythropolis* NCIMB 11540[31] could effect this transformation under very mild conditions (pH 8, 37 °C, dry cell mass:substrate ratio 1:2, 3 h) in excellent yields.

The results of these reactions are shown in Table 4. The yields of the (*S*)-acids are the isolated yields after purification via ion exchange chromatography. The ee's of the (*S*)-acids were all >98% unless mentioned otherwise. The yields of the (*R*)-amides refer to the corresponding HCl salts, while the yields of the (*R*)-acids were obtained after hydrolysis and subsequent purification by ion exchange chromatography. In all cases, the ee of the (*R*)-acids appeared to be excellent.

Because the racemic amides **1b** and **2b** (entries 1 and 2) reacted slower than expected (40% conversion after 24 h), an additional amount of enzyme was added (finally, 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 **9b** was only subjected to the *P. putida* cells; under identical reaction conditions (cell mass:substrate ra-

Table 4. Results of the preparative scale reactions.

entry	time (h)	(*S*)-acid yield (%)	ee (%) (*S*)-acid	(*R*)-amide[c] yield (%)	ee (%) (*R*)-amide	(*R*)-acid yield (%)
1	48[a]	**1a** 31	>98	**1b** 47	>99	**1a** 86
2	48[a]	**2a** 41	>99	**2b** 40	>99	**2a** 93
3	24	**3a** nd	>98	**3b** 45	>99	**3a** 89
4	21	**4a** 39	>99	**4b** 41	>99	**4a** 80
5	21	**5a** 40	>98	**5b** 42	>99	**5a** 99
6	19	**6a** 43	>99	**6b** 33	>99	**6a** 92
7	21	**7a** 32	98	**7b** 30	>99	**7a** 86
8	21	**8a** nd	96	**8b** 37	>99	**8a** 63
9	60[b]	**9a** 44	80	**9b** 35	>99	**9a** 88

[a] After 24 h, an additional amount of the *GMO* cells was added.
[b] Whole cells from *P. putida* were used.
[c] Obtained as the HCl salt.

tio of 1:10) the ee of the (*S*)-acid was only 80%. The yields of the (*S*)-acids **3a** and **8a** could not be 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 partly lost. Considering these results – in virtually all cases excellent ee's, combined with good yields – this pathway offers a useful and efficient access to these unsaturated amino acids at the multigram level.

Conclusion

A biocatalytic procedure for the synthesis of non-proteinogenic, unsaturated amino acids has been developed, based on the enantioselective hydrolysis of amino acid amides. First of all, this has led to straightforward and efficient synthetic routes to unsaturated amino acid amides. These routes are also amenable to larger scale production due to the minimum of purification steps (a single crystallization at the end suffices). Comparison of the resolution results of *Pseudomonas putida* cells and those of a *genetically modified organism* (GMO) containing an identical aminopeptidase clearly showed the superior properties of the latter system, which lacked the undesired amino acid racemase activity that was encountered in the *P. putida* cells. Furthermore, a novel mild hydrolysis method for the resulting (*R*)-amides was discovered involving the use of whole cells from a *Rhodococcus erythropolis* strain. Finally, it was shown that the aforementioned results could be successfully applied for the synthesis of enantiopure unsaturated amino acids in multi-gram quantities.

Experimental Section

General Information

All reactions were carried out under an inert atmosphere of dry nitrogen, unless stated otherwise. Standard syringe techniques were applied for transfer of Lewis acids and dry solvents. Infrared (IR) spectra were obtained from KBr pellets or neat, using a Bruker IFS 28FT-spectrometer and wavelengths (ν) are reported in cm^{-1}. Proton nuclear magnetic resonance (^1H NMR) spectra were determined in $CDCl_3$ (unless stated otherwise) using a Bruker AC 200 (200 MHz) and a Bruker ARX 400 (400 MHz) spectrometer. These systems were also used for ^{13}C NMR (APT) spectra (50 MHz and 100 MHz) in $CDCl_3$ (unless stated otherwise). Chemical shifts (δ) are given in ppm downfield from tetramethylsilane. Mass spectra and accurate mass measurements were carried out using a JEOL JMS-SX/SX 102A tandem mass spectrometer, a Varian NIAT 711, or a VG Micromass ZAB-HFQQ instrument. Elemental analysis were performed by Dornis u. Kolbe Mikroanalytisches Laboratorium, Mülheim an der Ruhr, Germany. R_f values were obtained by using thin layer chromatography (TLC) on silica gel-coated plastic sheets (Merck silica gel 60 F_{254}) with the indicated solvent (mixture). Chromatographic purification refers to flash column chromatography[32] using the indicated solvent (mixture) and Acros Organics silica gel (0.035 – 0.070 mm). Melting and boiling points are uncorrected. Melting points were determined with Büchi melting point B-545. Dry THF and Et_2O were distilled from sodium benzophenone ketyl prior to use. Dry DMF, CH_2Cl_2 and MeCN were distilled from CaH_2 and stored over 4 Å MS under a dry nitrogen atmosphere. Triethylamine was dried from KOH pellets. Et_2O, EtOAc, PE (60 – 80 °C) were distilled prior to use. All commercially available reagents were used as received, unless indicated otherwise. Purification of the amino acids by ion exchange chromatography using a strongly acidic resin (Dowex 50W×4, H^+ form, 20 – 50 mesh, Fluka) 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 NH_4OH, 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 $HClO_4$, at 0 – 7 °C). The ee's during the reaction were determined by HPLC on a Sumichiral OA 5000 column, 150 mm × 4.6 mm, eluant: 2 mM $CuSO_4$ in H_2O/MeOH).

Curve Determinations

The enzymes were added as whole cells[33] 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 H_2SO_4 the solution was brought to pH 9.2. A solution of $MnSO_4$ (0.38 mL of 80 mM solution which is 1 mM $MnSO_4$ in total) was added. Distilled water was added (29.5 mL) to the solution to bring the end solution at 10% amide concentration in H_2O. 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 1 M H_3PO_4 and the cell masses were filtered off. The mixtures were then analyzed by HPLC analysis/NH_3 detection. The conversion was determined using the formula: conversion = $ee_s/(ee_s + ee_p)$, in which ee_s is the enantiomeric excess of the substrate and ee_p the enantiomeric excess of the product.

General Procedure A for Preparative Scale Resolutions

A solution of the HCl salt of the racemic amide in distilled H_2O was brought to pH 9.2 with KOH or H_2SO_4 followed by addition of a 80 mM solution of $MnSO_4$ (1 mM $MnSO_4$ in the final solution) With addition of distilled H_2O the solution was

brought to a 10% solution of the amide. The enzyme was added as a solution in a HEPES buffer. The reaction mixture was stirred at 40 °C for 24 h and then brought to a pH of 6 with H_2SO_4. The enzyme was filtered or centrifuged from the solution. Base (NaOH) was added to bring the pH to 8 – 9, followed by addition of benzaldehyde (0.51 equivalent), the reaction mixture was stirred for 2 h at 20 °C. The reaction mixture was extracted CH_2Cl_2 (3 ×). The combined organic layers containing the Schiff base of the (*R*)-amide were extracted with H_2O (3 ×), dried with $MgSO_4$ filtered, and concentrated under vacuum. 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 h. The HCl salt of the (*R*)-amide was filtered from the solvent.

General Procedure B for the Conversion of (*R*)-Amides into (*R*)-Acids

The (*R*)-amino acid amides were dissolved in a buffer (concentration 5%, buffer: pH 8: 500 mL 0.1 M NaH_2PO_4 + 467 mL 0.1 M NaOH). Dried whole cells of *Rhodoccocus erythropolis* NCIMB 11540 (0.5 equivalent in weight) were added and the reaction mixture was stirred at 37 °C for 3 h. The reaction was monitored on TLC (eluent: $CHCl_3$, MeOH, NH_3). 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 free (*R*)-acids were obtained.

(*R*)-2-Amino-4-pentenoic Acid Amide (1b)

Following general procedure A: to a solution of the racemic amide (20.0 g, 0.136 mol) in H_2O, KOH (pH 9.2), $MnSO_4$ (2.5 g, 80 mM), H_2O (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 concentrated HCl (6.0 mL, 0.075 mol) afforded (*R*)-**1b**·HCl as a white solid; yield: 9.5 g (0.063 mol, 47% mol). (*R*)-**1b**: ee >99% (HPLC); mp 230 – 232 °C; ^{1}H NMR (400 MHz, D_2O): δ = 5.87 – 5.76 (m, 1H), 5.36 – 5.32 (m, 2H), 4.14 (t, *J* = 6.3 Hz, 1H), 2.77 – 2.63 (m, 1H); ^{13}C NMR (100 MHz, D_2O): δ = 176.3, 134.9, 126.3, 57.1, 40.0; IR (KBr): ν = 3250, 3450, 2733, 2641, 1981, 1716 cm^{-1}; anal. calcd. for $C_5H_{11}ClN_2O$: C 39.87, H 7.36, N 18.60; found: C 39.88, H 7.27, N 18.52.

(*S*)-2-Amino-4-pentenoic Acid (1a)

The (*S*)-acid was obtained after lyophilization and ion exchange chromatography; yield: 4.8 g (0.042 mol, 31%). (*S*)-**1a**: ee >98% (HPLC); mp 235 – 237 °C; ^{1}H NMR (400 MHz, D_2O): δ = 5.82 – 5.72 (m, 1H), 5.29 – 5.25 (m, 2H), 3.80 (t, *J* = 5.0 Hz, 1H), 2.70 – 2.56 (m, 1H); ^{13}C NMR (100 MHz, D_2O) δ 176.8, 134.1, 123.2, 56.7, 37.5; IR (KBr): ν = 3250 – 2500, 2104, 1610 cm^{-1}; anal. calcd. for $C_5H_9NO_2$: C 52.16, H 7.88, N 12.17; found: C 52.23, H 7.76, N 12.17.

(*R*)-2-Amino-4-pentenoic Acid (1a)

Hydrolysis of (*R*)-**1b** according to general procedure B: to a solution of (*R*)-**1b** (1.0 g, 6.66 mmol) in the buffer (20 mL) enzyme (0.2 g) was added. Work-up afforded (*R*)-**1a** as a white solid; yield: 0.66 g (5.70 mol, 86%).

(*R*)-2-Amino-5-hexenoic Acid Amide (2b)

Following general procedure A: to a solution of the racemic amide (19.9 g, 0.12 mol) in H_2O, KOH (pH 9.2), $MnSO_4$ (2.49 g, 80 mM), H_2O (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 mol) and concentrated HCl (5.39 mL, 0.067 mol) afforded (*R*)-**2b**·HCl as a white solid; yield: 7.95 g (0.048 mol, 40% mol). (*R*)-**2b**: ee >99% (HPLC); mp 256 – 258 °C; ^{1}H NMR (400 MHz, D_2O): δ = 5.95 – 5.85 (m, 1H), 5.21 – 5.21 (m, 2H), 4.06 (t, *J* = 6.5 Hz, 1H), 2.25 – 2.20 (m, 2H), 2.08 – 1.98 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ = 174.9, 139.3, 119.2, 55.5, 32.8, 31.0; IR (KBr): ν = 3224, 2828, 2759, 2350, 1981, 1668 cm^{-1}; anal. calcd. for $C_6H_{13}ClN_2O$: C 43.77, H 7.96, N 17.02; found: C 43.86, H 7.87, N 17.13.

(*S*)-2-Amino-5-hexenoic Acid (2a)

The (*S*)-acid was obtained after lyophilization and ion exchange chromatography; yield: 6.44 g, (0.05 mol, 41%). (*S*)-**2a:** ee >99% (HPLC); mp >270 °C (dec.); ^{1}H NMR (400 MHz, D_2O): δ = 5.93 – 5.86 (m, 1H), 5.18 – 5.08 (m, 2H), 3.76 (t, *J* = 6.10 Hz, 1H), 2.18 (dd, *J* = 7.5, 15 Hz, 2H), 2.04 – 1.91 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ = 177.5, 139.8, 118.7, 57.1, 32.5, 31.4; IR (KBr): ν = 3200, 3250 – 2500, 2357, 2125, 1640 cm^{-1}; anal. calcd. for $C_6H_{11}NO_2$: C 55.80, H 8.58, N 10.84; found: C 55.88, H 8.57, N 10.95.

(*R*)-2-Amino-5-hexenoic Acid (2a)

Hydrolysis of (*R*)-**2b** according to general procedure B: to a solution of (*R*)-**2b** (1.0 g, 6.10 mmol) in the buffer (20 mL) enzyme (0.2 g) was added. Work-up afforded (*R*)-**2a** as a white solid; yield: 0.73 g (5.67 mmol, 93%).

(*R*)-2-Amino-6-heptenoic Acid Amide (3b)

Following general procedure A: to a solution of the racemic amide (19.2 g, 0.12 mol) in H_2O, KOH (pH 9.2), $MnSO_4$ (2.49 g, 80 mM), H_2O (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 concentrated HCl (5.0 mL, 0.06 mol) afforded (*R*)-**3b**·HCl as a white solid; yield: 8.66 g (0.053 mol, 45%). (*R*)-**3b**: ee >99% (HPLC); mp (HCl salt) >245 °C (dec.); ^{1}H NMR (400 MHz, D_2O): δ = 5.96 – 5.86 (m, 1H), 5.18 – 5.02 (m, 2H), 3.43 (t, *J* = 6.6 Hz, 1H), 2.14 – 2.08 (m, 2H), 1.73 – 1.58 (m, 2H), 1.52 – 1.42 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ = 182.4, 141.1, 116.6, 56.0, 35.6, 34.5, 26.0; IR (KBr): ν = 3434, 3307, 2999, 1686 cm^{-1}; HRMS (FAB): calcd for $C_7H_{15}N_2O$ (MH^+): 143.1184; found: 143.1189.

(*S*)-2-Amino-6-heptenoic Acid (3a)

The (*S*)-acid was obtained after lyophilization and ion exchange chromatography. **3a**: ee >98% (HPLC); mp >248 °C (dec.); ^{1}H NMR (400 MHz, D_2O): δ = 5.92 – 5.84 (m, 1H), 5.18 – 5.03 (m, 2H), 3.74 (t, *J* = 6.1 Hz, 1H), 2.12 (dd, *J* = 7.1, 14.1 Hz, 2H), 1.91 – 1.83 (m, 2H), 1.53 – 1.43 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ = 177.6, 141.3, 117.8, 57.5, 35.2, 32.6, 26.2; IR (KBr): ν = 3250 – 2500 (b), 1833, 1581, 1516 cm^{-1}; anal. calcd. for $C_7H_{14}NO_2$: C 58.72, H 9.15, N 9.78; found: C 58.62, H 9.02, N 9.85.

(*R*)-2-Amino-6-heptenoic Acid (3a)

Hydrolysis of (*R*)-**3b** according to general procedure B: to a solution of (*R*)-**3b** (1.0 g, 5.61 mmol) in the buffer (20 mL) enzyme (0.2 g) was added. Work-up afforded (*R*)-**3a** as a white solid; yield: 0.71 g (4.99 mmol, 89%).

(*R*)-2-Amino-4-pentynoic Acid Amide (4b)

Following general procedure A: to a solution of the racemic amide (8.25 g, 0.07 mol) in H_2O, KOH (pH 9.2), $MnSO_4$ (1.03 g, 80 mM $MnSO_4$), H_2O (81.5 g, 10% solution) and finally *GMO* cells (16.5 mg in 1 mL buffer) were added. Work-up using benzaldehyde (3.89 mL, 0.037 mol) and concentrated HCl (2.95 mL, 0.037 mol) afforded (*R*)-**4b**·HCl as a white solid; yield: 4.29 g (0.029 mol, 41%). (*R*)-**4b**: ee 99% (HPLC); mp 237 – 239 °C; ^{1}H NMR (400 MHz, D_2O): δ = 4.37 (t, *J* = 5.7 Hz, 1H), 3.06 – 3.05 (m, 2H), 2.75 (t, *J* = 2.0 Hz, 1H); ^{13}C NMR (100 MHz, D_2O): δ = 173.1, 79.5, 77.6, 54.2, 24.1; IR (KBr): ν = 3430 ,3282, 3200, 2750, 1677 cm^{-1}; anal. calcd. for $C_5H_9ClN_2O$: C 40.42, H 6.11, N 18.85; found: C 40.31, H 5.90, N 18.93.

(*S*)-2-Amino-4-pentynoic Acid (4a)

The (*S*)-acid was obtained after lyophilization and ion exchange chromatography; yield: 3.05 g (0.027 mol, 39%). **4a**: ee >99% (HPLC); mp >216 °C (dec.); ^{1}H NMR (400 MHz, D_2O): δ = 3.90 (t, *J* = 5.5 Hz, 1H), 2.85 – 2.84 (m, 2H), 2.52 (t, *J* = 2.0 Hz, 1H); ^{13}C NMR (100 MHz, D_2O): δ = 175.5, 77.0, 76.3, 55.6, 23.2; IR (KBr): ν = 3286, 3250 – 2600, 2091, 1651 cm^{-1}; anal. calcd. for $C_5H_7NO_2$: C 53.09, H 6.24, N 12.38; found: C 52.92, H 6.21, N 12.47.

(*R*)-2-Amino-4-pentynoic Acid (4a)

Hydrolysis of (*R*)-**4b** according to general procedure B: to a solution of (*R*)-**4b** (5.0 g, 0.034 mol) in the buffer (100 mL) enzyme (2.0 g) was added. Work-up afforded (*R*)-**4a** as a white solid; yield: 3.1 g (0.027 mol, 80%).

(*R*)-2-Amino-5-hexynoic Acid Amide (5b)

Following general procedure A: to a solution of the racemic amide (15.5 g, 0.122 mol) in H_2O, KOH (pH 9.2), $MnSO_4$ (1.94 g, 80 mM $MnSO_4$), H_2O (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 concentrated HCl (5.4 mL, 0.067 mol) followed by purification afforded (*R*)-**5b**·HCl as a white solid; yield: 8.23 g (0.051 mol, 42%). (*R*)-**5b**: ee >99% (HPLC); mp (HCl salt) 241 – 242 °C; mp (free amine) 55 – 57 °C; ^{1}H NMR (400 MHz, D_2O): δ = 4.21 (t, *J* = 6.6 Hz, 1H), 2.51 (t, *J* = 2.6 Hz, 1H), 2.46 (dt, *J* = 7.0, 2.6 Hz, 2H), 2.17 – 2.15 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ = 174.4, 89.3, 74.2, 55.1, 32.3, 16.8; IR (KBr): ν = 3416, 3270, 3013, 2125, 1677 cm^{-1}; anal. calcd. for $C_6H_{10}N_2O$: C 57.12, H 7.99, N 22.21; found: C 56.97, H 8.10, N 22.10.

(*S*)-2-Amino-5-hexynoic Acid (5a)

The (*S*)-acid was obtained after lyophilization and ion exchange chromatography; yield: 6.2 g (0.049 mol, 40%). (*S*)-**5a**: ee >98% (HPLC); mp >214 °C (dec.); $[\alpha]_D$: +26.6 (*c* 1, 1 M HCl); ^{1}H NMR (400 MHz, D_2O): δ = 3.88 – 3.86 (m, 1H), 2.46 – 2.36 (m, 3H), 2.20 – 2.13 (m, 1H), 2.11 – 2.03 (m, 1H); ^{13}C NMR (100 MHz, D_2O): δ = 176.9, 85.0, 73.6, 56.7, 32.0, 17.1; IR (KBr): ν = 3288, 3100 – 2500, 2749, 2591, 2100, 1611 cm^{-1}; anal. calcd. for $C_6H_9NO_2$: C 56.68, H 7.13, N 11.02; found: C 56.53, H 7.02, N 11.09.

(*R*)-2-Amino-5-hexynoic Acid (5a)

Hydrolysis of (*R*)-**5b** according to general procedure B: to a solution of (*R*)-**5b** (1.0 g, 6.17 mmol) in the buffer (20 mL) enzyme (0.5 g) was added. Work-up afforded (*R*)-**5a** as a white solid; yield: 0.78 g (6.14 mmol, 99%).

(*R*)-2-Amino-4-hexynoic Acid Amide (6b)

Following general procedure A: to a solution of the racemic amide (12 g, 0.073 mol) in H_2O, KOH (pH 9.2), $MnSO_4$ (1.5 g, 80 mM $MnSO_4$), H_2O (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 concentrated HCl (2.8 mL, 0.036 mol) afforded (*R*)-**6b**·HCl as a white solid; yield: 4.01 g (0.024 mol, 33%). (*R*)-**6b**: ee >99% (HPLC); mp (HCl salt) >243 °C (dec.); ^{1}H NMR (400 MHz, D_2O): δ = 3.55 (t, *J* = 5.8 Hz, 1H), 2.55 – 2.53 (m, 2H), 1.79 (t, *J* = 2.5 Hz, 3H); ^{13}C NMR (100 MHz, D_2O): δ = 173.5, 85.3, 73.7, 54.4, 24.3, 5.4; IR (KBr): ν = 3321, 3246, 2986, 2360, 1701 cm^{-1}; anal. calcd. for $C_6H_{12}ClN_2O$: C 44.32, H 6.82, N 17.23; found: C 44.10, H 6.77, N 17.13.

(*S*)-2-Amino-4-hexynoic Acid (6a)

The (*S*)-acid was obtained after lyophilization and ion exchange chromatography; yield: 4.0 g (0.032 mol, 43%). (*S*)-**6a**: ee >99% (HPLC); mp 238 – 240 °C; ^{1}H NMR (400 MHz, D_2O): δ = 3.85 – 3.83 (m, 1H), 2.77 – 2.76 (m, 2H), 1.77 (t, *J* = 2.3 Hz, 3H); ^{13}C NMR (100 MHz, D_2O): δ = 175.9, 84.3, 74.7, 56.1, 23.6, 5.2; IR (KBr): ν = 3100 – 2600 (b), 2359, 2048, 1608 cm^{-1}; anal. calcd. for $C_6H_9NO_2$: C 56.68, H 7.13, N 11.02; found: 56.33, H 6.95, N 10.77.

(*R*)-2-Amino-4-hexynoic Acid (6a)

Hydrolysis of (*R*)-**6b** according to general procedure B: to a solution of (*R*)-**6b** (2.0 g, 0.024 mol) in buffer (40 mL) enzyme (0.4 g) was added. Work-up afforded (*R*)-**6a** as a white solid; yield: 1.44 g (0.01 mol, 92%).

(*R*)-2-Amino-5-heptynoic Acid Amide (7b)

Following general procedure A: to a solution of the racemic amide (10.6 g, 0.06 mol) in H_2O, KOH (pH 9.2), $MnSO_4$ (1.32 g, 80 mM), H_2O (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) and concentrated HCl (5.48 mL, 0.068 mol) afforded (*R*)-**7b**·HCl as a white solid; yield: 3.19 g (0.018 mol, 30% mol). (*R*)-**7b**: ee >99% (HPLC); mp >256 °C (dec.); ^{1}H NMR (400 MHz, D_2O): δ = 4.16 (t, *J* = 6.5 Hz, 1H), 2.39 – 2.35 (m, 2H), 2.14 – 2.03 (m, 2H), 1.78 (t, *J* = 2.5 Hz, 3H); ^{13}C NMR (100 MHz, D_2O): δ = 176.0, 82.0, 79.5, 55.1, 32.6, 17.0, 5.1; IR (KBr): ν = 3433, 3307, 2999, 2467, 1995, 1685, 1582, 1485 cm^{-1}; anal. calcd. for $C_7H_{15}N_2OHCl$: C 47.60, H 7.42, N 15.86; found: C 47.51, H 7.36, N 15.72.

(*S*)-2-Amino-5-heptynoic Acid (7a)

The (*S*)-acid was obtained after lyophilization and ion exchange chromatography; yield: 2.67 g (0.019 mol, 32%). (*S*)-**7a**: ee >98% (HPLC); mp 223 – 227 °C; ^{1}H NMR (400 MHz, D_2O): δ = 3.84 (dd, *J* = 5.2, 7.4 Hz, 1H), 2.40 – 2.22 (m, 2H), 2.19–2.07 (m, 1H), 2.05 – 1.94 (m, 1H), 1.77 (t, *J* = 2.3 Hz, 3H); ^{13}C NMR (100 MHz, D_2O): δ = 177.1, 81.5, 80.2, 56.9, 32.3, 17.3, 5.1; IR (KBr): ν = 3200 – 2700, 2744, 2097, 1650, 1585, 1415, 1342 cm^{-1}; anal. calcd. for $C_7H_{11}NO_2$: C 59.56, H 7.85, N 9.94; found: C 59.46, H 7.77, N 9.96.

(*R*)-2-Amino-5-heptynoic Acid (7a)

Hydrolysis of (*R*)-**7b** according to general procedure B: to a solution of (*R*)-**7b** (1.0 g, 5.68 mmol) in the buffer (20 mL) enzyme (0.2 g) was added. Work-up afforded (*R*)-**7a** as a white solid; yield: 0.69 g (4.88 mmol, 86%).

(*R*)-2-Amino-6-octynoic Acid Amide (8b)

Following general procedure A: to a solution of the racemic amide (37 g, 0.195 mol) in H_2O, KOH (pH 9.2), $MnSO_4$ (4.62 mL, 80 mM), H_2O (370 g, 10% solution) and finally *GMO* cells (370 mg in 1 mL buffer) were added. Work-up using benzaldehyde (22.8 mL, 0.21 mol) and concentrated HCl (15.7 mL, 0.21 mol) afforded (*R*)-**8b**·HCl as a white solid; yield: 13.7 g (0.072 mol, 37%). (*R*)-**8b**: ee >99% (HPLC); mp >251 °C (dec.); ^{1}H NMR (400 MHz, D_2O): δ = 4.07 (t, *J* = 6.5 Hz, 1H), 2.26 – 2.23 (m, 2H), 2.03 – 1.98 (m, 2H), 1.77 (t, *J* = 2.5 Hz, 3H), 1.62 – 1.57 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ = 174.8, 81.7, 80.7, 55.5, 32.7, 26.2, 20.3, 5.1; IR (KBr): ν = 3427, 3304, 3006, 1996, 1687, 1584, 1480 cm^{-1}; anal. calcd. for $C_8H_{15}N_2O$: C 50.39, H 7.93, N 14.69; found: C 50.41, H 7.87, N 14.58.

(*S*)-2-Amino-6-octynoic Acid (8a)

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. Therefore, the (*S*)-acid was not purified on large scale. (*S*)-**8a**: ee 96% (HPLC); mp 240 – 244 °C; ^{1}H NMR (100 MHz, D_2O): δ = 3.76 (t, *J* = 6.1 Hz, 1H), 2.25 – 2.21 (m, 2H), 2.11 – 1.88 (m, 2H), 1.77 (t, *J* = 2.5 Hz, 3H), 1.68 – 1.48 (m, 2H); ^{13}C NMR (100 MHz, D_2O): δ = 177.4, 82.0, 80.6, 57.2, 32.4, 26.6, 20.3, 5.1; IR (KBr): ν = 3250–2600, 2740, 2093, 1655, 1581, 1415, 1334 cm^{-1}; anal. calcd. for $C_8H_{14}NO_2$: C 61.91, H 8.44, N 9.03; found: C 61.55, H 8.39, N 9.12.

(*R*)-2-Amino-6-octynoic Acid (8a)

Hydrolysis of (*R*)-**8b** according to general procedure B: to a solution of (*R*)-**8b** (1.0 g, 5.23 mmol) in the buffer (20 mL) enzyme (0.2 g) was added. Work-up afforded (*R*)-**8a** as a white solid; yield: 0.52 g (3.30 mmol, 63%).

(*R*)-2-Amino-4,5-hexadienoic Acid Amide (9b)

Following general procedure A: to a solution of the racemic amide (3.60 g, 0.02 mol) in H_2O, KOH (pH 8.5), $MnSO_4$ (0.36 g, 80 mM), H_2O (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 concentrated HCl (0.8 mL, 0.01 mol) afforded (*R*)-**9b**·HCl as a white solid; yield: 1.12 g (6.89 mmol, 35% mol). (*R*)-**9b**: ee >99% (HPLC); mp 210 – 211 °C; ^{1}H NMR (400 MHz, CD_3OD): δ = 5.13 (q, *J* = 7.0 Hz, 1H), 4.86 – 4.84 (m, 2H), 3.94 (dd, *J* = 5.2, 7.4 Hz, 1H), 2.64 – 2.61 (m, 1H), 2.57–2.53 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ = 211.3, 171.6, 84.0, 76.3, 53.8, 32.1; IR (KBr): ν = 3431, 3282, 3200, 2750, 1661 cm^{-1}; HRMS (FAB): calcd for $C_6H_{11}ClN_2O$ (MH^+): 163.0638; found: 163.0629.

(*S*)-2-Amino-4,5-hexadienoic Acid (9a)

The (*S*)-acid was obtained after lyophilization and ion exchange chromatography; yield: 1.13 g (8.80 mmol, 44%). (*S*)-**9a**: ee 80% (HPLC); mp 231 – 235 °C; ^{1}H NMR (400 MHz, CD_3OD): δ = 5.13 (q, *J* = 7.0 Hz, 1H), 4.82 – 4.79 (m, 2H), 3.58 (dd, *J* = 4.2, 7.8 Hz, 1H), 2.64 – 2.62 (m, 1H), 2.52 – 2.47 (m, 1H); ^{13}C NMR (100 MHz, CD_3OD): δ = 211.3, 175.8, 85.5, 77.8, 56.0, 31.7; IR (KBr): ν = 3850 – 2600, 1651 cm^{-1}; HRMS (FAB): calcd for $C_6H_{10}NO_2$ (MH^+): 128.0712; found: 128.0703.

(*R*)-2-Amino-4,5-hexadienoic Acid (9a)

Hydrolysis of (*R*)-**9b** according to general procedure B: to a solution of (*R*)-**9b** (1.0 g, 5.61 mmol) in the buffer (20 mL) enzyme (0.2 g) was added. Work-up afforded (*R*)-**9a** as a white solid; yield: 0.78 g (6.06 mmol, 88%).

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