Towards a modified hydantoinase process for the chemoenzymatic production of chiral β-amino acids

Substrate synthesis, screening, and characterization of novel biocatalysts

zur Erlangung des akademischen Grades eines

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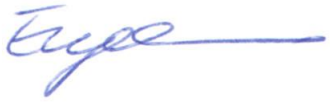

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Contents

Nomenclature

βPhe	β-phenylalanine
CH_3-IMH	D,L-5-(3-Indolylmethyl)-3-*N*-methylhydantoin
DMSO	Dimethyl sulfoxide
DSMZ	German Collection of Microorganisms and Cell Cultures
ee	enantiomeric excess
HPLC	High-Performance Liquid Chromatography
IMH	Indolylmethyl hydantoin
IUPAC	International Union of Pure and Applied Chemistry
*N*CβPhe	*N*-Carbamoyl-β-phenylalanine
*N*CTrp	*N*-Carbamoyl-α-tryptophan
NMR	Nuclear Magnetic Resonance (spectroscopy)
ORF	Open Reading Frame
*p*Cl*N*CβPhe	*para*-Chloro-*N*-carbamoyl-β-phenylalanin
*p*ClPheDU	*para*-Chlor-D,L-6-phenyl-5,6-dihydrouracil
PCR	Polymerase Chain Reaction
PheDU	6-Phenyl-5,6-dihydrouracil
PheHyd	Phenylhydantoin

Abstract

The aim of this thesis was to examine the applicability of a modified hydantoinase process to the production of chiral aromatic β-amino acids. The hydantoinase process employs up to three enzymes for the dynamic kinetic resolution of 5'-monosubstituted hydantoins via *N*-carbamoyl-α-amino acids to α-amino acids: a hydantoin racemase, a hydantoinase and a carbamoylase. In order to produce β-amino acids it had to be studied whether these enzymes also show activity towards the substrates of a modified hydantoinase process: dihydropyrimidines instead of hydantoins and *N*-carbamoyl-β-amino acids instead of *N*-carbamoyl-α-amino acids.

In the first part of this work (Bretschneider *et al.*, 2010, see chapter 4) a screening for biocatalysts able to cleave unnatural dihydropyrimidines via *N*-carbamoyl β-amino acids to β-amino acids was conducted. Therefore 6-phenyl-5,6-dihydrouracil (PheDU), the model substrate for this project, was chemically prepared and the synthesis route was successfully scaled-up to several hundred grams. Subsequently, an HPLC method for the chiral analysis of PheDU and its corresponding *N*-carbamoyl-β-amino acid *N*-carbamoyl-β-phenylalanine (*N*CβPhe) was developed. For the screening experiment, microorganisms with known activity towards aryl-substituted hydantoins were employed. Most of these isolates were able to hydrolyze the model substrate: Eight of 17 tested strains converted significant amounts (>10 %) of PheDU to *N*CβPhe, six isolates showed only basal activity towards the substrate (<10 %) and two isolates were not able to hydrolyze PheDU. However, a further conversion of *N*CβPhe to β-phenylalanine (βPhe) was not detected for any of the strains.

All microorganisms applied in these biotransformations were grown in medium both with and without the potential inducer D,L-5-(3-Indolylmethyl)-3-*N*-methylhydantoin (CH_3-IMH). For a few strains the addition of CH_3-IMH seemed to induce the expression of dihydropyrimidinases. Other isolates showed dihydropyrimidinase activity in inducer-free medium only. For a third group of strains the presence or absence of CH_3-IMH had no influence on the enzyme activity.

In order to examine the stereoselectivity of the detected dihydropyrimidinase activities long-term biotransformation reactions were conducted with four isolates (725, 728, 731, *Bacillus* sp. A16) showing high activity towards PheDU. Chiral HPLC analysis of these experiments gave a first hint at the stereoselective conversion of PheDU to *N*CβPhe by the strain *Bacillus* sp. A16.

In a second screening (Engel *et al.*, 2011, see chapter 5) for dihydropyrimidine hydrolyzing enzymes various bacterial isolates and three recombinantly expressed D-hydantoinases were applied in biotransformation reactions with PheDU, *N*CβPhe and *para*-chloro-D,L-6-phenyl-5,6-dihydrouracil (*p*ClPheDU). For the analysis of the latter substrate and its product *para*-chloro-*N*-carbamoyl-β-phenylalanine (*p*Cl*N*CβPhe) an HPLC method was developed. Additionally, L-*N*CβPhe was chemically synthesized and the HPLC method for the chiral analysis of both *N*CβPhe enantiomers was significantly improved. Thus, a detailed exami-

nation of the stereoselective conversion of PheDU was feasible for the first time.

The three recombinantly expressed D-hydantoinases originating from *Arthrobacter crystallopoietes* DSM20117, *Ochrobactrum* sp. DSM18828 and *Delftia* sp. DSM18833 showed dihydropyrimidinase activity towards PheDU. While the two latter enzymes exhibited no stereoselectivity towards PheDU an L-selectivity was detected for the *A. crystallopoietes* DSM20117 enzyme. These results confirmed the hypothesis that hydantoinases are not only able to hydrolyze 5'-monosubstituted hydantoins, heterocyclic five-ring systems but also 6'-monosubstituted dihydropyrimidines, heterocyclic six-ring systems.

For the bacterial isolates biotransformation experiments with PheDU, *p*ClPheDU and *N*CβPhe were conducted. For this screening eleven strains already analyzed in the first screening and with four additional isolates were applied. Eight of these microorganisms *Arthrobacter polychromogenes* DSM20136, *Arthrobacter polychromogenes* DSM342, *Arthrobacter* sp. (E7) DSM24883, *Bacillus* sp. (A16) DSM25052, *Ochrobactrum* sp. (F21) DSM25042, *Aminobacter* sp. (728) DSM24754, *Aminobacter* sp. (735) DSM24755 and *Rhizobium* sp. (NA04-01) DSM24917 showed activity towards PheDU. Besides *Aminobacter* sp. DSM24754 which exhibited no stereoselectivity, all of these strains preferably hydrolyzed the D-PheDU enantiomer. This was the first time that a stereoselective conversion of a 6'-monosubstituted dihydropyrimidines by bacterial isolates was detected.

Due to its highly stereoselective conversion of PheDU *A. polychromogenes* DSM342 was applied in a long-term biotransformation experiment with a high starting concentration of PheDU. It was shown that the D-PheDU enantiomer was hydrolyzed much faster than the L-PheDU enantiomer.

The second dihydropyrimidine *p*ClPheDU was converted by the three recombinantly expressed D-hydantoinases and by all strains which showed activity towards PheDU except for the two *A. polychromogenes* strains. Within this study the enzymatic hydrolysis of this substrate was detected for the first time. As the chlorine substituent of *p*ClPheDU is a good leaving group the products of this substrate, achieved within a modified hydantoinase process, could serve as building blocks in numerous follow-up reactions.

The two recombinantly expressed D-hydantoinases of *Ochrobactrum* sp. DSM18828 and *Delftia* sp. DSM18833 and the isolates *A. polychromogenes* DSM342, *Bacillus* sp. DSM25052 and *Rhizobium* sp. DSM24917 were subjected to further biotransformation reactions with the hydantoins phenylhydantoin (PheHyd) and indolylmethyl hydantoin (IMH). *A. polychromogenes* DSM342 exhibited no activity towards the tested hydantoins. The other four biocatalysts showed hydantoinase activity towards PheHyd and IMH. When the conversion of PheHyd and IMH was compared to the conversion of the dihydropyrimidines PheDU and *p*ClPheDU the hydantoin PheHyd was the best substrate for all of the four biocatalysts. Based on these data it can be hypothesized that at least the enzymatic activities of *Bacillus* sp. DSM25052 and *Rhizobium* sp. DSM24917 result from one enzyme able to accept hydantoins and dihydropyrimidines as substrates.

For all wild-type strains biotransformation experiments with *N*CβPhe were conducted as well. But none of the strains showed any carbamoylase activity. This finding was unexpected as hydantoinases are often found in gene clusters together with carbamoylases. At the beginning of the study it was assumed that most of the strains with activity towards 6'-monosubstituted dihydropyrimidines are also able to catalyze the follow-up reaction by

hydrolyzing the produced *N*-carbamoyl-β-amino acid to the corresponding β-amino acids. In order to find carbamoylases with activity towards *N*-carbamoyl-β-amino acids a screening for microorganisms able to use PheDU as sole source of nitrogen was performed. However, out of 65 strains isolated only one strain exhibited dihydropyrimidinase activity towards PheDU (*Rhizobium* sp. DSM24917) and none of the strains showed any carbamoylase activity towards *N*CβPhe.

In the third part of this thesis (Engel *et al.*, 2012, see chapter 6) the amidase activities of the two novel *Aminobacter* strains *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 were examined in more detail. First of all biotransformation experiments with hydantoinase/dihydropyrimidinase substrates PheDU, *p*ClPheDU, PheHyd, IMH as well as with the carbamoylase substrates *N*CβPhe and *N*-carbamoyl-α-tryptophan (*N*CTrp) were conducted. Both strains showed activity towards all applied hydantoins and dihydropyrimidines but no carbamoylase activity was detected for any of the isolates. The best substrates for *Aminobacter* sp. DSM24754 were PheHyd and PheDU, while the best substrate for *Aminobacter* sp. DSM24755 was PheHyd. These results again support the hypothesis that the enzymatic activities detected with hydantoins and dihydropyrimidines result from one enzyme in each strain.

By PCR several amidase gene sequences of the two *Aminobacter* strains were successfully amplified for the first time. For *Aminobacter* sp. DSM24754 a DNA fragment containing two complete and one incomplete open reading frames (ORFs) was elucidated. The first ORF coded for a putative D-carbamoylase exhibiting highest identity (85 %) to the well-studied and very thermostable *N*-carbamoyl-D-amino acid amidohydrolase of *Pseudomonas* sp. KNK003A (AB007369). The second ORF comprised a putative dihydropyrimidinase showing highest identity (83 %) to a putative dihydropyrimidinase of *Mesorhizobium* sp. BNC1. As the third ORF was incomplete and displayed only similarities to hypothetical proteins its potential function remains unclear. For *Aminobacter* sp. DSM24755 a DNA fragment also comprising two complete and one incomplete ORF was amplified. The first ORF coded for a putative dihydropyrimidinase showing highest identity (90 %) to a putative dihydropyrimidinase of *Mesorhizobium loti* MAFF303099. The two other ORFs displayed highest similarities to putative ABC transporter proteins of *Mesorhizobium loti* MAFF303099 and *Mesorhizobium amorphae* CCNWGS0123. The two genes coding for putative dihydropyrimidinases of the *Aminobacter* sp. strains DSM24754 and DSM24755 had an overall gene sequence identity of 74 %. The identity of the deduced amino acid sequences was 68 %. When compared to other non putative hydantoinases and dihydropyrimidinases both deduced protein sequences showed a high identity to the dihydropyrimidinase of *Sinorhizobium meliloti* CECT4114. For the latter, enzyme activity towards unnatural hydantoins and dihydropyrimidines is reported. This further supports the hypothesis that the gene products of the detected dihydropyrimidinase genes are enzymes able to accept hydantoins and dihydropyrimidines as substrates. For that reason, these enzymes are interesting candidates for an application in a modified hydantoinase process.

Within this thesis, it was shown that several well-studied hydantoinases have dihydropyrimidinase activity towards PheDU and *p*ClPheDU. Additionally, a variety of microorgan-

isms exhibiting activity towards these substrates was detected. Most of them displayed a D-selective conversion of PheDU. Although there are still tasks to be addressed, e.g. the discovery of suitable carbamoylases and racemases, this thesis is a first step towards a modified hydantoinase process applicable to the production of chiral β-amino acids.

Zusammenfassung

Ziel der vorliegenden Arbeit war es, die Eignung eines modifizierten Hydantoinase-Prozesses für die Synthese chiraler aromatischer β-Aminosäuren zu untersuchen. Im Hydantoinase-Prozess werden bis zu drei Enzyme für die dynamisch kinetische Racematspaltung 5'-monosubstituierter Hydantoine über *N*-Carbamoyl-α-Aminosäuren zu α-Aminosäuren eingesetzt: eine Hydantoinracemase, eine Hydantoinase und eine Carbamoylase. Für den Einsatz dieser Enzyme zur Synthese von β-Aminosäuren musste überprüft werden, ob sie auch Aktivität mit den Substraten eines modifizierten Hydantoinase-Prozesses zeigen: Mit Dihydropyrimidinen an Stelle von Hydantoinen bzw. *N*-Carbamoyl-β-Aminosäuren an Stelle von *N*-Carbamoyl-α-Aminosäuren.

Im ersten Teil dieser Arbeit (Bretschneider *et al.*, 2010, siehe Kapitel 4) wurde ein Screening nach Biokatalysatoren mit der Fähigkeit zur Hydrolyse unnatürlicher Dihydropyrimidine zu *N*-Carbamoyl-β-Aminosäuren bzw. weiter zu β-Aminosäuren durchgeführt. Dafür wurde zunächst 6-Phenyl-5,6-dihydrouracil (PheDU), das Modellsubstrat dieser Arbeit, chemisch synthetisiert und die Synthese erfolgreich auf den Maßstab von mehreren hundert Gramm hochskaliert. Anschließend wurde eine HPLC-Methode für die chirale Analyse von PheDU und dessen korrespondierender *N*-Carbamoyl-β-Aminosäure *N*-Carbamoyl-β-Phenylalanin (*N*CβPhe) entwickelt. Für das Screening wurden Mikroorganismen eingesetzt, für die bekannt war, dass sie Aktivität für Aryl-substituierte Hydantoine aufweisen. Die meisten der untersuchten Isolate waren fähig, das eingesetzte Modellsubstrat zu hydrolysieren: Acht von 17 getesteten Stämmen setzten eine signifikante Menge PheDU zu *N*CβPhe um (>10 %), sechs Isolate zeigten nur basale Aktivität (<10 %) und zwei Stämme waren nicht in der Lage PheDU zu hydrolysieren. Allerdings wurde in keinem Fall ein weiterer Umsatz von *N*CβPhe zu β-Phenylalanin beobachtet.

Alle für diese Biotransformationen eingesetzten Mikroorganismen wurden jeweils in Medium mit und ohne den potenziellen Induktor D,L-5-(3-Indolylmethyl)-3-*N*-methylhydantoin (CH_3-IMH) kultiviert. Bei einigen Stämmen schien der Einsatz des Induktors die Enzymaktivität zu induzieren. Für andere Isolate konnte nur Aktivität bestimmt werden, wenn sie in induktorfreiem Medium kultiviert worden waren. Bei einer dritten Gruppe von Mikroorganismen schien die An- oder Abwesenheit von CH_3-IMH im Medium keinen Einfluss auf die Enzymaktivität zu haben.

Um die Stereoselektiviät der gefunden Dihydropyrimidinaseaktivität genauer zu untersuchen, wurden mit vier Isolaten (725, 728, 731, *Bacillus* sp. A16), die eine hohe Aktivität mit PheDU zeigten, Langzeitbiotransformationen durchgeführt. Eine Analyse mittels chiraler HPLC gab erste Hinweise auf einen stereoselektiven Umsatz von PheDU zu *N*CβPhe durch den Stamm *Bacillus* sp. A16.

In einem zweiten Screening (Engel *et al.*, 2011, siehe Kapitel 5) nach Dihydropyrimidin-hydrolysierenden Enzymen wurden verschiedene Bakterienstämme und drei rekombinant exprimierte D-Hydantoinasen in Biotransformationsexperimenten mit PheDU, *N*CβPhe und

para-Chlor-D,L-6-phenyl-5,6-dihydrouracil (*p*ClPheDU) eingesetzt. Für die Untersuchung des letztgenannten Substrates und seines Produktes *para*-chloro-*N*-carbamoyl-β-phenylalanin (*p*Cl*N*CβPhe) wurde eine HPLC-Methode neu entwickelt. Außerdem wurde L-*N*CβPhe chemisch synthetisiert und eine verbesserte HPLC-Methode für die Analyse der *N*CβPhe-Enantiomere erarbeitet. Mit dieser Methode war erstmals eine detaillierte Auswertung des stereoselektiven PheDU-Umsatzes möglich.

Die drei rekombinant exprimierten D-Hydantoinasen aus *Arthrobacter crystallopoietes* DSM20117, *Ochrobactrum* sp. DSM18828 und *Delftia* sp. DSM18833 zeigten Enzymaktivität mit PheDU. Während für die beiden letztgenannten Enzyme keine Stereoselektivität für PheDU beobachtet wurde, konnte für das Enzym aus *Arthrobacter crystallopoietes* DSM20117 eine L-Selektivität detektiert werden. Diese Ergebnisse bestätigen die Hypothese, dass Hydantoinasen nicht nur 5'-monosubstituierte Hydantoine, heterozyklische Fünf-Ring-Systeme, sondern auch 6'-monosubstituierte Dihydropyrimidine, heterozyklische Sechs-Ring-Systeme, als Substrate akzeptieren.

Für die Bakterienstämme wurden Biotransformationen mit PheDU, *p*ClPheDU und *N*CβPhe durchgeführt. Dafür wurden elf Isolate, die bereits im ersten Screening untersucht wurden, und vier zusätzliche Isolate eingesetzt. Für acht dieser Mikroorganismen *Arthrobacter polychromogenes* DSM20136, *Arthrobacter polychromogenes* DSM342, *Arthrobacter* sp. (E7) DSM24883, *Bacillus* sp. (A16) DSM25052, *Ochrobactrum* sp. (F21) DSM25042, *Aminobacter* sp. (728) DSM24754, *Aminobacter* sp. (735) DSM24755 und *Rhizobium* sp. (NA04-01) DSM24917 wurde Aktivität mit PheDU gemessen. Außer für den Stamm *Aminobacter* sp. DSM24754, der keinen stereoselektiven PheDU-Umsatz zeigte, wurde für alle anderen Isolate eine D-Selektivität beobachtet. Dies ist das erste Mal, dass der stereoselektive Umsatz eines 6'-monosubstitutierten Dihydropyrimidines gezeigt werden konnte.

Wegen seiner hohen Enantioselektivität gegenüber PheDU wurde der Stamm *A. polychromogenes* DSM342 für ein Langzeit-Biotransformationsexperiment mit einer höheren PheDU-Konzentration eingesetzt. Dabei wurde gezeigt, dass das D-Enantiomer wesentlich schneller hydrolysiert wird als das L-Enantiomer.

Das zweite Dihydropyrimidin *p*ClPheDU wurde von allen drei rekombinant exprimierten D-Hydantoinasen und von allen Stämmen, die auch Aktivität mit PheDU zeigten, außer von den beiden *A. polychromogenes* Stämmen, umgesetzt. Im Rahmen dieser Arbeit konnte somit erstmals die enzymatische Hydrolyse dieses Substrates gezeigt werden. Da der Chlorsubstituent von *p*ClPheDU eine gute Abgangsgruppe ist, könnten die aus einem modifizierten Hydantoinase-Prozess hervorgehenden Produkte dieses Substrates als Bausteine in zahlreichen Folgereaktionen dienen.

Die zwei rekombinant exprimierten D-Hydantoinasen aus *Ochrobactrum* sp. DSM18828 und *Delftia* sp. DSM18833 sowie die Isolate *A. polychromogenes* DSM342, *Bacillus* sp. DSM25052 und *Rhizobium* sp. DSM24917 wurden für weitere Biotransformationen mit den Hydantoinen Phenylhydantoin (PheHyd) und Indolylmethylhydantoin (IMH) eingesetzt. Dabei zeigte der Stamm *A. polychromogenes* DSM342 keine Aktivität mit diesen Substraten. Die anderen vier Biokatalysatoren dagegen waren in der Lage PheHyd und auch IMH zu hydrolysieren. Beim Vergleich der für diese Hydantoine erhaltenen Ergebnisse mit den für die Dihydropyrimidine PheDU und *p*ClPheDU gewonnen Daten zeigte sich, dass PheHyd für alle vier Biokatalysatoren das beste Substrat war. Diese Ergebnisse legen nahe, dass

zumindest die für *Bacillus* sp. DSM25052 und *Rhizobium* sp. DSM24917 gemessenen Enzymaktivitäten jeweils von einem Enzym stammen, das in der Lage ist, sowohl Hydantoine als auch Dihydropyrimidine als Substrate zu akzeptieren.

Für alle Wildtypstämme wurden auch Biotransformationen mit *N*CβPhe durchgeführt. Allerdings zeigte keiner der Stämme Aktivität mit diesem Substrat. Dieses Ergebnis war unerwartet, da Hydantoinasen oftmals in gemeinsamen Genclustern mit Carbamoylasen auftreten. Daher wurde zu Beginn des Projektes angenommen, dass Stämme, die Aktivität mit 6'-monosubstituierten Dihydropyrimidinen zeigen, sehr wahrscheinlich auch in der Lage sind die Folgereaktion, ausgehend von der *N*-Carbamoyl-β-Aminosäure hin zur entsprechenden β-Aminosäure, zu katalysieren. Um dennoch Carbamoylasen mit Aktivität für *N*-Carbamoyl-β-Aminosäuren zu finden, wurde ein Screening nach Mikroorganismen, die in der Lage sind PheDU als einzige Stickstoffquelle zu nutzen, durchgeführt. Allerdings konnte für keinen der Stämme Carbamoylaseaktivität detektiert und nur für einen Mikroorganismus (*Rhizobium* sp. DSM24917) von etwa 65 isolierten Stämmen Aktivität mit PheDU nachgewiesen werden.

Im dritten Teil dieser Arbeit (Engel *et al.*, 2012, siehe Kapitel 6) wurde die Amidaseaktivität der beiden neuen *Aminobacter* Stämme *Aminobacter* sp. DSM24754 und *Aminobacter* sp. DSM24755 genauer untersucht. Zuerst wurden Biotransformationen mit den Hydantoinase- bzw. Dihydropyrimidinasesubstraten PheHyd, IMH, PheDU, *p*ClPheDU sowie mit den Carbamoylasesubstraten *N*CβPhe und *N*-Carbamoyl-α-tryptophan (*N*CTrp) durchgeführt. Für beide Stämme wurde Aktivität mit allen eingesetzten Hydantoinase- und Dihydropyrimidinasesubstraten detektiert, aber für keines der beiden Isolate konnte Carbamoylaseaktivität nachgewiesen werden. Die besten Substrate für *Aminobacter* sp. DSM24754 waren PheHyd und PheDU, während das beste Substrat für *Aminobacter* sp. DSM24755 PheHyd war. Diese Ergebnisse unterstützen wieder die These, dass die für Hydantoine und Diyhdropyrimidine gemessenen Aktivitäten eines Stammes jeweils von einem Enzym ausgehen.

Mittels PCR konnten mehrere für Amidasen codierende Gene der beiden *Aminobacter* Stämme zum ersten Mal amplifiziert werden. Für das Isolat *Aminobacter* sp. DSM24754 wurde ein DNA-Fragment, welches zwei komplette und einen unvollständigen offenen Leserahmen (ORF) codiert, amplifiziert. Der erste ORF codierte für eine putative D-Carbamoylase, die höchste Übereinstimmung (85 %) mit der gut untersuchten und sehr thermostabilen *N*-Carbamoyl-D-Aminosäure-Amidohydrolase von *Pseudomonas* sp. KNK003A aufweist. Der zweite ORF enthielt die Gensequenz für eine putative Dihydropyrimidinase, die größte Sequenzübereinstimmung (83 %) mit einer putativen Dihydropyrimidinase von *Mesorhizobium* sp. BNC1 hatte. Da der dritte offene Leserahmen unvollständig war und nur Ähnlichkeiten zu hypothetischen Proteinen zeigte, blieb dessen potenzielle Funktion unklar. Für *Aminobacter* sp. DSM24755 wurde ebenfalls ein DNA-Fragment, das zwei komplette und einen unvollständigen ORF enthielt, amplifiziert. Der erste ORF codierte für eine putative Dihydropyrimidinase, welche höchste Übereinstimmung (90 %) zu einer putativen Dihydropyrimidinase von *Mesorhizobium loti* MAFF303099 hatte. Die beiden anderen ORFs wiesen höchste Ähnlichkeiten zu putativen ABC-Transporter-Proteinen von *Mesorhizobium loti* MAFF303099 und *Mesorhizobium amorphae* CCNWGS0123 auf.

Die beiden für putative Dihydroopyrimidinasen codierenden Gene von *Aminobacter* sp.

DSM24754 und DSM24755 hatten eine Gesamtsequenzgleichheit von 74 %. Die Übereinstimmung der davon abgeleiteten Proteinsequenzen lag bei 68 %. Bei einem Vergleich mit anderen gut untersuchten Hydantoinasen und Dihydropyrimidinasen zeigten die beiden abgeleiteten Proteinsequenzen höchste Ähnlichkeit mit der Dihydropyrimidinase von *Sinorhizobium meliloti* CECT4114. Für dieses Enzym ist Aktivität sowohl mit Hydantoinen als auch mit Dihydropyrimidinen beschrieben. Dieser Befund stützt wiederum die Hypothese, dass die gefunden Dihydropyrimidinasegene sehr wahrscheinlich für Enyzme codieren, die in der Lage sind sowohl Hydantoine als auch Dihydropyrimdine als Substrate zu akzeptieren. Daher sind diese Enzyme potenziell geeignete Kandidaten für den Einsatz in einem modifizierten Hydantoinase-Prozess.

Im Rahmen dieser Arbeit konnte gezeigt werden, dass einige gut untersuchte Hydantoinasen Dihydropyrimidinaseaktivität mit PheDU und *p*ClPheDU aufweisen. Zusätzlich konnte eine Vielzahl von Mikroorganismen mit Enzymaktivität gegenüber diesen Substraten gefunden werden. Für die meisten von ihnen war ein D-stereoselektiver Umsatz von PheDU nachweisbar. Auch wenn es noch immer Herausforderungen zu lösen gibt, wie z.B. die Entdeckung geeigneter Carbamoylasen und Racemasen, stellt diese Arbeit einen ersten Schritt hin zu einem modifiziertem Hydantoinase-Prozess dar.

Part I

Introduction

1 β-amino acids

1.1 Characteristics, occurrence in nature and applications

In contrast to α-amino acids, the amino group of β-amino acids is attached to the β-carbon atom. Depending on the number and position of the substituents, β-amino acids are classified as β^2-, β^3- or $\beta^{2,3}$-amino acids. Compared to α-amino acids, β-amino acid are weaker acids and stronger bases (Sewald, 1996).

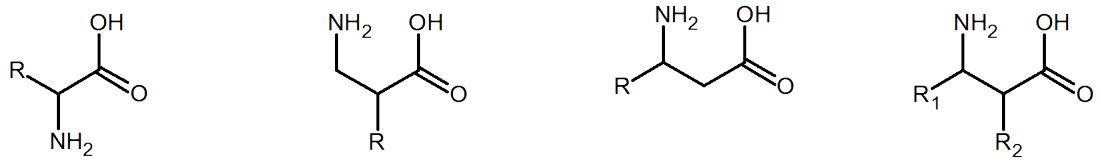

Figure 1.1 Comparison of an α-amino acid with β-amino acids.

Due to its second carboxyl group the proteinogenic α-amino acid aspartate can also be considered a β-amino acid. Other frequently occurring β-amino acids are β-alanine and β-aminoisobutyric acid. They are products of the reductive degradation of uracil and thymine within the pyrimidine degradation pathway found in mammals (Vogels & van der Drift, 1976). More often β-amino acids are found as part of biologically active secondary metabolites. One example is the phenylisoserine moiety, a β-amino α-hydroxy acid, of the anti-tumor drug paclitaxel (Taxol ™, see figure 1.2), isolated from the pacific yew tree (*Taxus brevifolia*) (Parness *et al.*, 1982). Another example is the β-tyrosine moiety of jaspamide (see figure 1.2), a cyclodepsipeptide with antifungal, anthelmintic and anti-tumor activities isolated from the marine sponge *Japsis splendens* (Crews *et al.*, 1986; Senderowicz *et al.*, 1995).

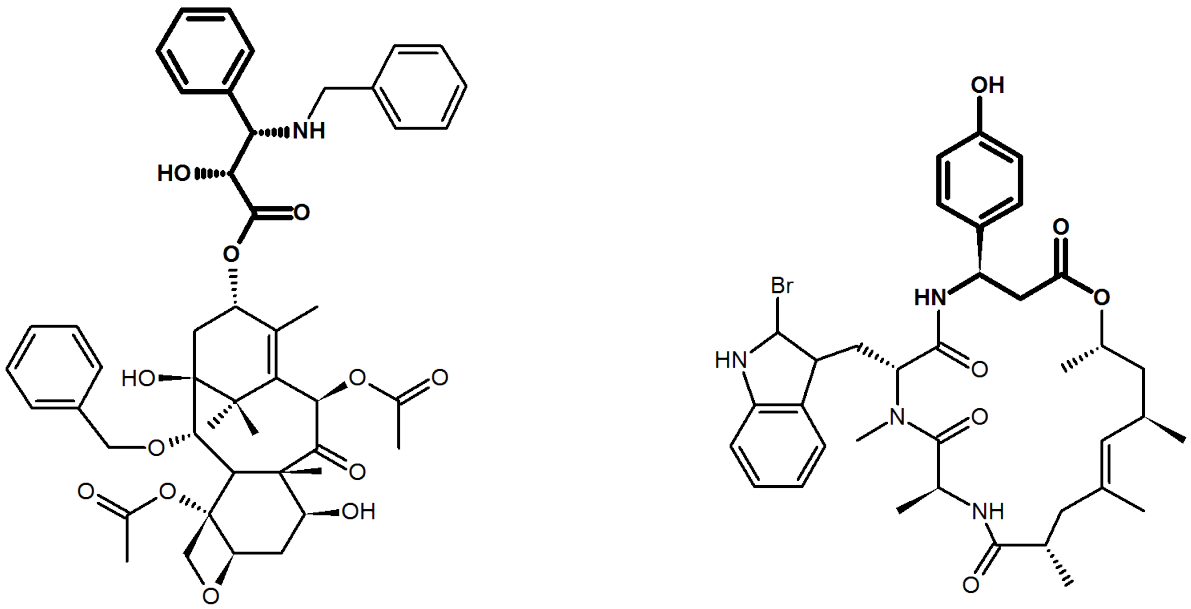

Figure 1.2 The β-amino acid containing biologically active secondary metabolites paclitaxel and jaspamide, modified after Cardillo & Tomasini, 1996.

Peptides exclusively consisting of β-amino acids are called β-peptides. Their peptide backbone contains an additional methylene group between the amino group and the carboxyl group due to the chemical structure of β-amino acids. This leads to a higher conformational variability of β-peptides compared to α-peptides. It is reported that β-peptides can adopt very stable secondary structures like helices, sheets and reverse turns (Cheng *et al.*, 2001; Seebach & Gardiner, 2008). Another important feature of β-peptides is their remarkable stability against standard proteases and peptidases (Frackenpohl *et al.*, 2001). Until now, only five enzymes, so called β-peptidyl aminopeptidases, are described to be capable of the N-terminal cleavage of β-peptides (Geueke & Kohler, 2007; Fuchs *et al.*, 2011). Because of their structural variety and their biological stability, the application of β-peptides as peptidomimetics is extensively studied. In literature, antimicrobial (Porter *et al.*, 2002; Godballe *et al.*, 2011) and antifungal β-peptides (Moradi *et al.*, 2009) are described as well as β-peptides mimicking protein-protein, peptide-protein and protein-nucleic acid interactions (Seebach & Gardiner, 2008).

1.2 Synthesis of chiral β-amino acids

β-amino acids are building blocks for fine chemicals and pharmaceuticals. Especially for the production of drugs enantiopure compounds are of vital importance. In the following section, different methods for the preparation of chiral β-amino acids and their advantages and disadvantages are discussed.

1.2.1 Chemical routes to chiral β-amino acids

In general, there are three main strategies for the production of enantiopure β-amino acids: (i) The chiral pool approach, (ii) asymmetric synthesis and (iii) racemic resolutions.

(i) The chiral pool approach

Natural occurring α-amino acids are commercially available and provide a reservoir of chiral educts for the synthesis of enantiopure β-amino acids. The most common method for β-amino acid synthesis starting from chiral α-amino acid is the Arndt-Eistert homologation (Seebach *et al.*, 1996, see figure 1.3). In the course of this reaction, a carboxylic acid is extended by one carbon atom.

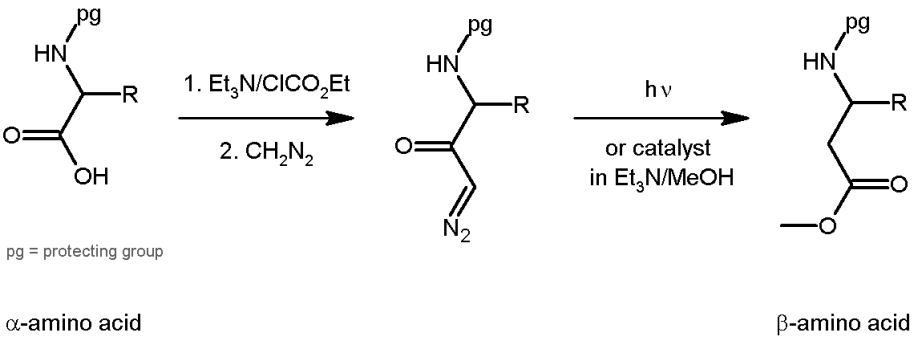

Figure 1.3 Synthesis of β-amino acids by applying the Arndt-Eistert-homologation, modified after Seebach *et al.*, 1996.

The disadvantages of this strategy are the limited number of naturally occurring α-amino acids, the toxicity and the difficult handling of the methylation agent diazomethan and the high cost caused by the catalyst (Liu & Sibi, 2002). For these reasons this method is not applicable for a large scale production of β-amino acids.

(ii) Asymmetric synthesis

A huge variety of asymmetric syntheses of β-amino acids starting from diverse compounds is described in literature and discussed in detail by several reviews (Cardillo & Tomasini, 1996; Liu & Sibi, 2002; Ma, 2003; Juaristi & Soloshonok, 2005; Weiner *et al.*, 2010). For the preparation of chiral compounds from achiral educts either chiral auxiliaries or catalysts are applied. According to Weiner *et al.* (2010) who reviewed only catalytic asymmetric synthesis

the most promising reactions are rhodium- and ruthenium-catalyzed hydrogenations. These syntheses require only low amounts of catalysts and show high enantioselectivities (see figure 1.4).

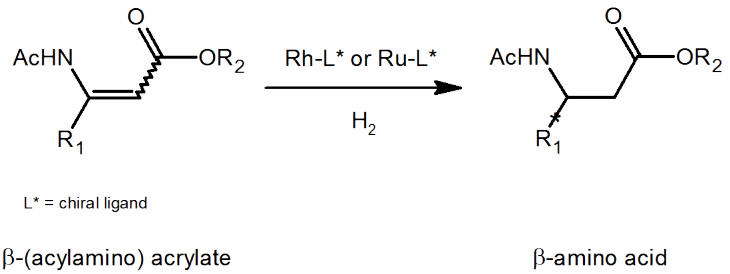

Figure 1.4 β-amino acids synthesized via asymmetric hydrogenation, modified after Juaristi *et al.*, 2005.

Besides the enormous progress in the development of asymmetric syntheses, the costs for catalysts or chiral auxiliaries are still the main challenge towards an efficient and economic large scale production process of β-amino acids.

(iii) Racemic Resolutions

For this strategy, the racemate of the desired β-amino acid is synthesized and converted into its diastereomeric salt by employing a chiral base. The diastereomers are subsequently separated by fractional crystallization. Finally the diastereomerically pure salt is retransformed to the free chiral β-amino acid. Fractional crystallization often requires multiple steps and is time consuming. Therefore it is not suitable for large scale production of enantiopure β-amino acids. Another disadvantage of racemic resolutions is that the maximum achievable yield is limited to 50 %.

1.2.2 Biocatalytic routes to chiral β-amino acids

Another strategy for the production of chiral β-amino acids is the application of enzymes or whole cells as catalysts. Most chemoenzymatic methods are kinetic resolutions of racemic β-amino acids or their derivatives. In the following section, some methods are discussed.

(i) Hydrolases

In the majority of cases hydrolytic enzymes are employed for kinetic resolutions of racemic β-amino acids or their derivatives. Among them lipases are best studied and reviewed in detail by Liljeblad & Kanerva, 2006. Especially the lipase B of *Candida antarctica* (CAL-B) which is commercially available as Novozym 435 is often applied in biocatalysis. One example is reported by Forro *et al.*, 2006 who employed CAL-B for the enantioselective ring opening of 4-aryl-substituted β-lactams (see figure 1.5 A). The reaction yielded the corresponding (*R*)-β-amino acids and the remaining (*S*)-β-lactam in good enantiomeric excess. It is also

reported that both products are easy to separate. Two years later the same group reported a similar process with comparable good yields and high enantioselectivities using *Pseudomonas cepacia* lipase (lipase PS). This enzyme catalyzed the hydrolytic cleavage of racemic β-aryl-β-amino acid ethyl esters to their corresponding β-amino acids (Tasnádi *et al.*, 2008, see figure 1.5 B).

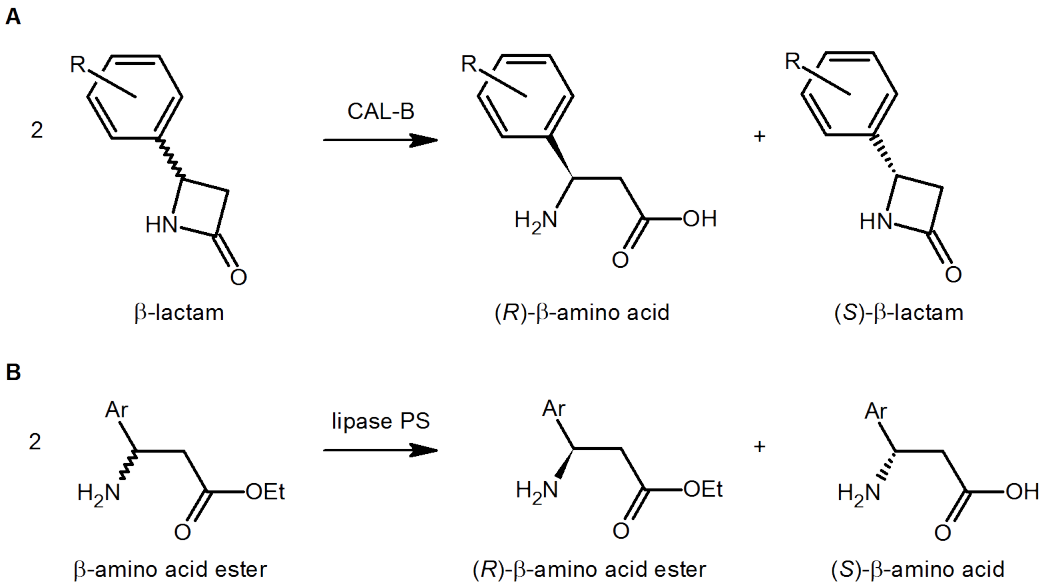

Figure 1.5 Chiral β-amino acids produced with lipase B of *Candida antarctica* (CAL-B) and *Pseudomonas cepacia* lipase (lipase PS), modified after A: Forro *et al.*, 2006, and B: Tasnádi *et al.*, 2008.

Ogawa *et al.*, 2009 recently described a β-phenylalanine ester hydrolase. This enzyme is reported to produce (*S*)-β-phenylalanine with high enantioselectivity starting from racemic β-phenylalanine esters (see figure 1.6). Furthermore, it showed peptidase activity yielding β-phenylalanyl-β-phenylalanine ester when high educt concentrations were used. As this was the first report about such an enzyme, further work has to be carried out in order to evaluate its potential for the application in biocatalytic production processes.

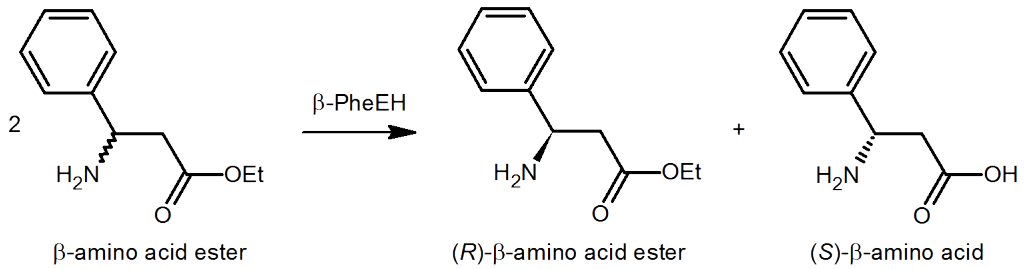

Figure 1.6 β-amino acids produced with β-phenylalanine ester hydrolase (β-PheEH), modified after Ogawa *et al.*, 2009.

Other hydrolases applicable for the production of β-amino acids are aminoacylases (Liljeblad & Kanerva, 2006). In 2004, Gröger *et al.* reported the synthesis of chiral aromatic β-amino acids catalyzed by porcine kidney acylase I (see figure 1.7). The products were prepared with high enantiomeric excess and good yields starting from *N*-chloroacetylated β-amino acids. Additionally, the isolation of the product is reported to be uncomplicated.

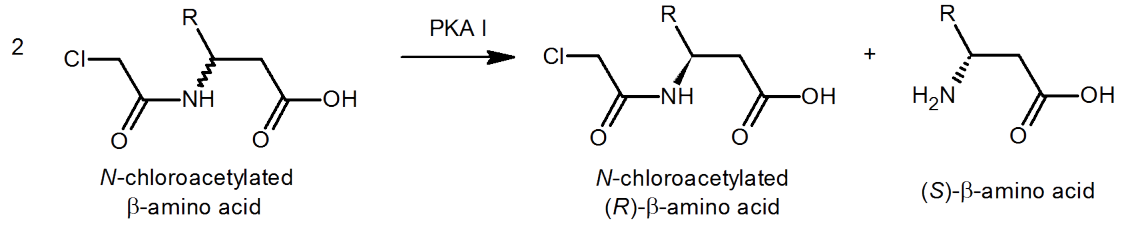

Figure 1.7 Chiral β-amino acids produced with porcine kidney acylases I (PKA I), modified after Gröger *et al.*, 2004.

(ii) Phenylalanine aminomutases (PAM)

In course of the elucidation of the paclitaxel biosynthetic pathway, Walker & Floss, 1998 detected phenylalanine aminomutase activity in crude extracts of *Taxus brevifolia*. This enzyme activity led to the transformation of α-phenylalanine to β-phenylalanine. In 2004, the same group reported the successful cloning and characterization of this enzyme (Walker *et al.*, 2004). In the following years, the application of PAMs from different *Taxus* sp. for biocatalysis was intensively studied. Wu *et al.*, 2009 and Szymanski *et al.*, 2009 employed the PAM of *Taxus chinensis* for the synthesis of several non-natural α- and β-amino acids in high enantiomeric excess starting from substituted cinnamic acids (see 1.8). As this method yields a mixture of an α- and a β-amino acid Verkuijl *et al.*, 2010 successfully developed an approach for selective extraction of the α-amino acid by addition of a palladium phosphine complex.

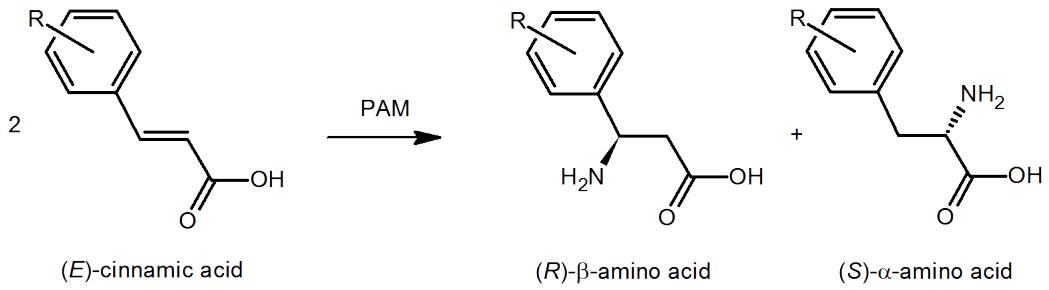

Figure 1.8 Chiral β-amino acids produced with phenylalanine aminomutase (PAM), modified after Wu *et al.*, 2009; Szymanski *et al.*, 2009.

Wu *et al.*, 2010 applied the *T. chinensis* PAM together with a phenylalanine ammonia lyase (PAL) for the racemic resolution of β-amino acids. The latter enzyme is responsible for the reversible addition of ammonia to cinnamic acid (see figure 1.9). The PAL reaction

was used to convert the (*S*)-phenylalanine produced by the PAM to cinnamic acid in order to shift the equilibrium of the process. For this method, a high enantiomeric excess and a yield of about 50 % is reported.

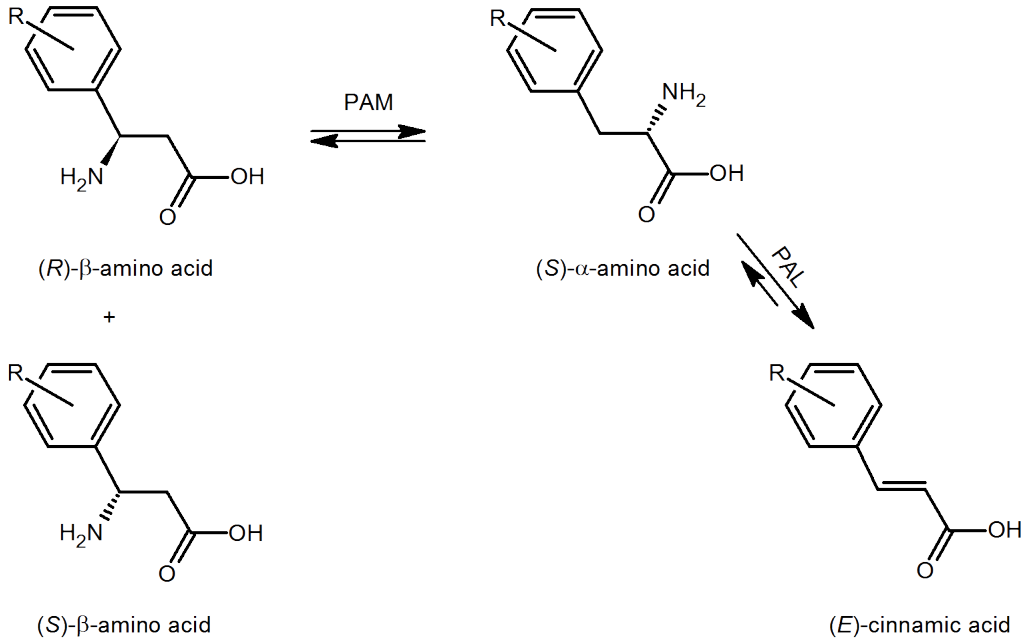

Figure 1.9 Chiral β-amino acids produced with phenylalanine aminomutase (PAM) and phenylalanine ammonia lyase (PAL), modified after Wu *et al.*, 2010.

A first step towards a dynamic kinetic resolution process leading to a yield above 50 % was carried out by Cox *et al.*, 2009. In this process, the alanine racemase from *Pseudomonas putida* was coupled with the PAM of *Taxus canadensis* for the production of (*R*)-β-arylalanines starting from racemic α-arylalanines (see figure 1.10). With this method, the yield was increased moderately and the enantiomeric excess remained very high.

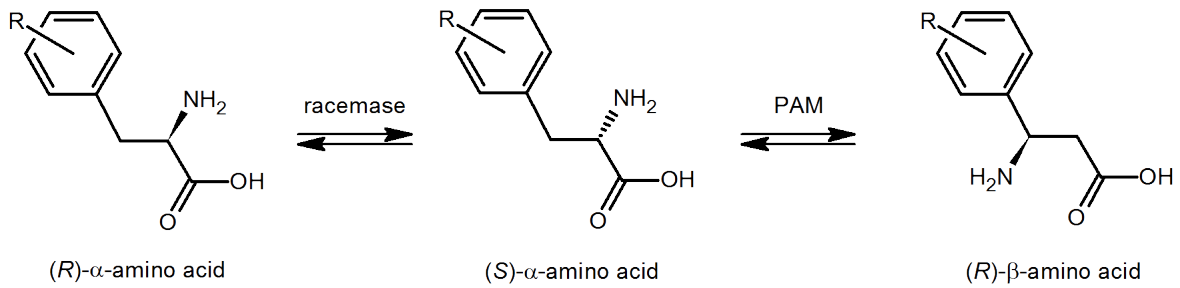

Figure 1.10 Dynamic kinetic resolution of α-amino acids using alanine racemase and phenylalanine aminomutase (PAM) for the production of chiral β-amino acids, modified after Cox *et al.*, 2009.

(iii) Transaminases

Brucher *et al.*, 2010 described several bacterial isolates with stereoselective transaminase activity towards β-phenylalanine. The authors postulated that a reversion of this transaminase reaction could be used for the enantioselective production of (*S*)-β-phenylalanine starting from the corresponding β-keto acid precursor. The latter had to be freshly synthesized prior to the reaction due to its thermodynamical instability.

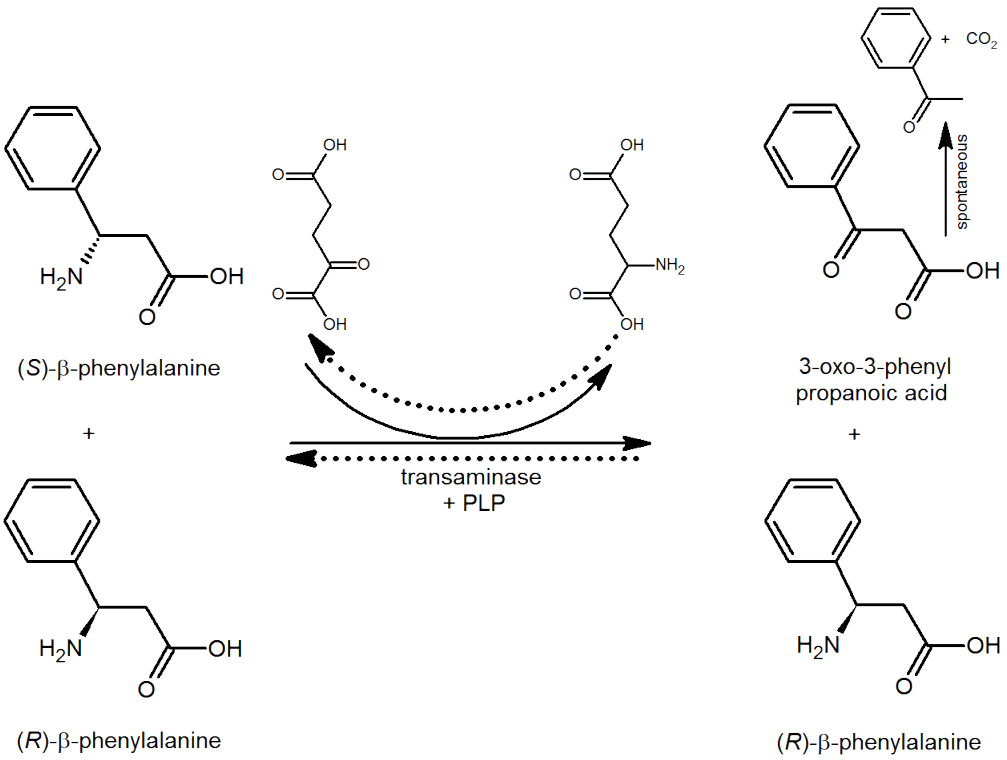

Figure 1.11 Postulated transaminase reaction applied for the production of optical pure (*S*)-β-phenylalanine starting from a β-keto acid, modified after Brucher *et al.*, 2010. dotted line = postulated reaction, continuous line = natural reaction, cosubstrates are α-ketoglutarate for the natural reaction and glutamate for the reversed reaction, PLP = pyridoxal phosphate

In conclusion, most biocatalytic syntheses for the production of β-amino acids are kinetic resolutions. The advantages of biocatalytic reactions in general are the high reaction-, stereo- and regiospecificity, the mild reaction conditions and thus the lesser production of toxic waste (Leuenberger, 1990). The major drawback of today's biocatalytic processes for the production of β-amino acids is that the maximum achievable yield is limited to 50 %. First studies are conducted trying to overcome this problem by establishing dynamic kinetic resolution processes applying enzyme cascades.

2 The hydantoinase process and the enzymes involved

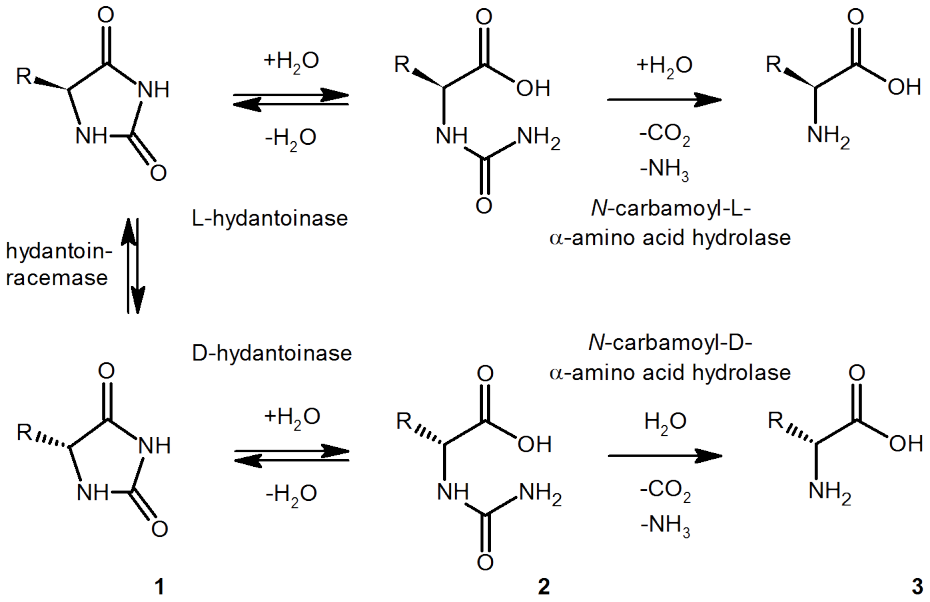

Figure 2.1 Dynamic kinetic resolution of 5'-monosubstituted hydantoins (1) via *N*-carbamoyl amino acids (2) to α-amino acids (3) within the hydantoinase process.

2.1 The hydantoinase process – Biocatalytic production of chiral α-amino acids

The hydantoinase process employs up to three enzymes for the dynamic kinetic resolution of 5'-monosubstituted hydantoins to α-amino acids: a hydantoin racemase [EC 5.1.99.5], a hydantoinase [EC 3.5.2.2] and a carbamoylase [EC 3.5.1.77, EC 3.5.1.87] (see figure 2.1). Depending on the enantioselectivity of the applied hydantoinase and/or carbamoylase, L- or D-α-amino acids can be synthesized. This process is well established in industry because most educts are easy to synthesize from inexpensive compounds, hydantoinases and carbamoylases have a wide substrate specificity and the application of a racemase or alternatively the self-racemization of the particular hydantoin derivatives leads to a potential yield of 100 %. Even if no appropriate carbamoylase is available for the decarbamoylation of a distinct *N*-carbamoyl amino acid the enzymatic reaction can be replaced by a chemical decarbamoylation step. Due to its versatility, this process has become a platform process in chemical industry and is applied for the production of various optically pure D- or L-amino acids (Becker *et al.*, 2008). Initially, it was mainly used for the production of

D-*p*-hydroxyphenyl alanine, a building block for several β-lactam antibiotics (Cecere *et al.*, 1977; Dinelli *et al.*, 1976; Yamada *et al.*, 1978). But since the 1970s the process was continuously improved and numerous further patent applications were filed (for a review see Clemente-Jiménez *et al.*, 2008).

2.2 Hydantoin cleaving enzymes

2.2.1 Overview

In 1861 Adolf Baeyer (Baeyer, 1861) studied the *hyd*rogenation of all*antoin* and thereby synthesized a compound he called hydantoin. The offical IUPAC name for this compound is imidazolidine-2,4-dione. First experiments on the metabolism of hydantoin were conducted by Lewis, 1912 and Gaebler & Keltch, 1926 by injecting or feeding animals with hydantoin and controlling the urine for remaining hydantoin and its degradation products. Bernheim & Bernheim, 1946 observed the degradation of hydantoin by different animal tissues and concluded that "a specific enzyme is involved" in this reaction. Consequently, he published a paper entitled "The specificity of hydantoinase" one year later (Bernheim, 1947). Today, six enzymes with different physiological functions able to cleave hydantoin or its derivatives are known: allantoinase, carboxymethylhydantoinase, carboxyethylhydantoinase, maleimide hydrolase, *N*-methylhydantoinase and hydantoinase.

- *Allantoinase* [EC 3.5.2.5] is a key enzyme for the purine degradation pathway of microorganisms, plants and some animals (Vogels *et al.*, 1966). This enzyme catalyzes the hydrolytic cleavage of allantoin to allantoate. While some allantoinases show an enantioselectivity for (*S*)-allantoin others are non-selective (Vogels, 1969). Little is known about alternative substrates for allantoinases. Vogels *et al.* (1966) compared several allantoinases purified from bacteria, plants and animal tissues and demonstrated that methylolallantoin and 5-amino-hydantoin were also hydrolyzed by some of the enzymes. Kim *et al.*, 2000b reported that hydantoin, isopropyl hydantoin and 5-bromouracil are substrates for *Escherichia coli* allantoinase but with poor activities. In contrast, the two proteins with allantoinase activity detected in *Phaseolus vulgaris* showed no conversion of hydantoin or 5-bromouracil (Raso *et al.*, 2007).

- *Carboxymethylhydantoinase* [EC 3.5.2.4] catalyzes the hydrolysis of carboxymethyl hydantoin to *N*-carbamoyl-L-aspartate. Enzyme activity is only reported for *Clostridium oroticum* (Lieberman & Kornberg, 1954) and gram negative soil bacteria (Akamatsu, 1960). Besides these two studies, no further information about these enzymes is available in literature.

- *Carboxyethylhydantoinase* (not classified in EC nomenclature) is an enzyme different from carboxymethylhydantoinase and able to cleave carboxyethyl hydantoin to *N*-carbamoyl glutamate (Akamatsu, 1960). This enzyme is thought to be part of a side route of the histidine degradation pathway (Syldatk *et al.*, 1999).

- *Imidase* [EC 3.5.2.16] is presumed to conduct the first step in the degradation of cyclic imides (Ogawa *et al.*, 1996). This enzyme was first isolated from *Blastobacter* sp. A17p-4 (Ogawa *et al.*, 1995a). In contrast to dihydropyrimidinases with imidase activity (Yang *et al.*, 1993; Huang & Yang, 2003), the *Blastobacter* sp. A17p-4 imidase hydrolyzed cyclic imides with a higher activity and affinity than dihydropyrimidines (Ogawa *et al.*, 1997). Similar kinetic parameter were reported for the imidase of *Pseudomonas putida* YZ-26. This enzyme showed much higher activity and hydrolysis velocity with succinimde and maleimide than with hydantoin or dihydrouracil (Shi *et al.*, 2007).

- *N-Methylhydantoinase* [EC 3.5.2.14] hydrolyzes *N*-methylhydantoin to *N*-carbamoyl sarcosine and is part of the creatinine degradation pathway. This enzymatic reaction is ATP-dependent. *N*-Methylhydantoinase derived from *Pseudomonas putida* 77 is the only *N*-Methylhydantoinase described in literature. Besides its natural substrate, the hydrolysis of cyclic imides, hydantoin and 5-methylhydantoin is reported. The dihydropyrimidines dihydrouracil and dihydrothymine were no substrates but accelerated the hydrolysis of ATP to ADP (Ogawa *et al.*, 1995b,d).

- *Dihydroorotase* [EC 3.5.2.3] catalyzes the reversible ring closure of *N*-L-carbamoyl aspartate to L-dihydroorotate in the pyrimidine biosynthesis pathway. Alternative substrates for dihydroorotase are thioorotate for the enzyme of *E. coli* (Porter *et al.*, 2004) and L-dihydroorotate methyl ester for *Pseudomonas putida* IFO 12996 dihydroorotase. For the latter, dihydrouracil, dihydrothymine as well as hydantoin and 5'-monosubstituted hydantoins were no substrates (Ogawa & Shimizu, 1995).

- *Hydantoinase* [EC 3.5.2.2] is used synonymously with the term dihydropyrimidinase for enzymes able to cleave 5'-monosubstituted hydantoins and/or dihydropyrimidines to the corresponding *N*-carbamoyl amino acids. This enzyme class is discussed in detail in the following section.

2.2.2 Hydantoinases and dihydropyrimidinases

Definition of terms

The terms hydantoinase and dihydropyrimidinase are used as synonyms in EC nomenclature. This is due to the results reported by Wallach & Grisolia, 1957 for the dihydropyrimidinase from calf liver which was able to cleave hydantoin and the natural occurring dihydropyrimidines dihydrouracil and dihydrothymine. The latter two are intermediate products of the reductive pyrimidine degradation pathway and the natural substrates of dihydropyrimidinases (see figure 2.2). However, some hydantoinases only cleave hydantoins but lack the ability of hydrolyzing dihydrouracil and dihydrothymine. Runser & Meyer, 1993 reported that the hydantoinase of *Agrobacterium* sp. IP I-671 cleaves 5'-monosubstituted hydantoins but has no activity towards the assumed natural substrates. Morin, 1993 compared crude extracts of several legume hydantoinases and detected hydantoinase activity in all enzyme preparations tested but four extracts showed no activity towards dihydrouracil (*Archis hypogaea*, *Macrotyloma uniflorum*, *Phaesolus lunatus* and *Vicia faba*). In 2000 Kim *et al.* described the

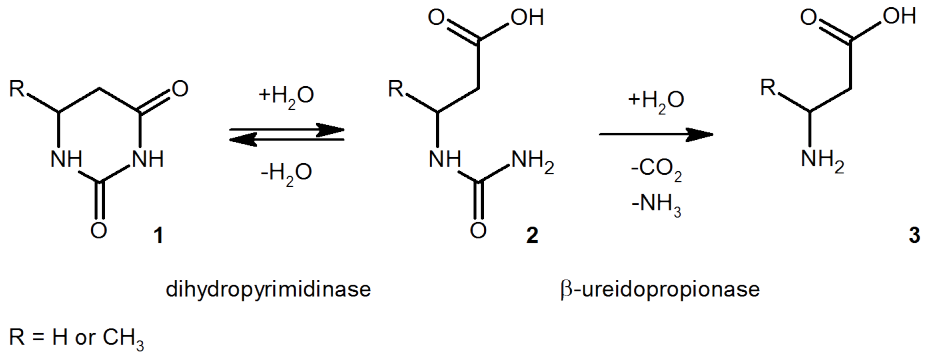

Figure 2.2 Degradation of dihydrouracil (R=H)/dihydrothymine (R=CH_3) (**1**) via *N*-carbamoyl-β-alanine/*N*-carbamoyl-β-aminobutyric acid (**2**) to β-alanine/β-aminobutyric acid (**3**).

hydantoinase of *Escherichia coli* K-12 which displayed activity towards 5'-monosubstituted hydantoins but was not capable of hydrolyzing dihydrouracil or dihydrothymine. Similar results were reported for the hydantoinase of the archaeon *Methanococcus jannaschii* DSM2661 (Chung *et al.*, 2002). The recombinantly expressed enzyme exhibited activity towards substituted hydantoins but not towards the natural substrates. Already in 1999 Syldatk *et al.* "concluded that hydantoinases and dihydropyrimidinases are not necessarily the same enzymes". The authors requested not to use the terms hydantoinase and dihydropyrimidinase synonymously. Instead the term dihydropyrimidinase should be used for the enzymes of the reductive pyrimidine degradation pathway able to hydrolyze dihydrouracil and dihydrothymine and hydantoins. The term hydantoinase should be applied for enzymes catalyzing the degradation of hydantoins but are not involved in pyrimidine degradation. The physiological function of hydantoinases and their natural substrate is still unknown.

Distribution of hydantoinases and dihydropyrimidinases in nature

Eukaryotic dihydropyrimidinases were isolated from animals (Kikugawa *et al.*, 1994; Brooks *et al.*, 1983; Kautz & Schnackerz, 1989), plants (Morin, 1993), fungi and slime mold (Gojkovic *et al.*, 2003). Besides the natural substrates eukaryotic enzymes are often also capable of hydrolyzing hydantoins. Bovine liver dihydropyrimidinase was reported to show activity towards hydantoin and 5-methylhydantoin (Brooks *et al.*, 1983). For the calf liver enzyme an enantioselective cleavage of several 5'-monosubstituted hydantoins was demonstrated by Cecere *et al.*, 1975. Arcuri *et al.*, 2004 showed the use of *Vigna angularis* dihydropyrimidinase for the D-selective cleavage of several phenylhydantoins. In contrary, Gojkovic *et al.*, 2003 reported that the dihydropyrimidinases of *Saccharomyces kluyveri* and *Dictyostelium discoideum* are not accepting hydantoin as substrate. Eukaryotic hydantoinases, defined as enzymes not able to hydrolyze dihydrouracil and dihydrothymine are not explicitly described in literature. Only Morin, 1993 mentioned four legume extracts displaying hydantoinase but no dihydropyrimidinase activity (see above).

In general, bacterial hydantoinases/dihydropyrimidinases are widespread in nature (Dürr

et al., 2006). However, it is often difficult to discriminate the terms hydantoinase and dihydropyrimidinase for the bacterial enzymes because the activity towards the natural substrates dihydrouracil and dihydrothymine is not always tested. This is due to the fact that most reported enzymes are derived from screenings for novel hydantoin cleaving enzymes applicable in the hydantoinase process. But, as already mentioned above, the enzymes of *Escherichia coli* K-12, *Agrobacterium* sp. IP I-671 can clearly be defined as hydantoinases.

Characteristics

Hydantoinases and dihydropyrimidinases belong to the amidohydrolase superfamily and are acting on carbon-nitrogen bonds of cyclic amides (Holm & Sander, 1997). To date several crystal structures of dihydropyrimidinases and hydantoinases are solved and listed in the Protein Data Bank (www.PDB.org, Berman *et al.* 2000).

The subunits of these enzymes consist of two domains: a β-sandwich domain and a distorted $(\alpha/\beta)_8$ barrel domain harboring the catalytic core. This active site contains a binuclear metal center and thus explains the dependence on divalent metal ions of hydantoinases and dihydropyrimidinases. Except for the D-hydantoinase from a *Bacillus* sp. AR9 all described enzyme crystal structures accommodate a posttranslational carboxylated lysine residue in the active site (Seibert & Raushel, 2005; Kishan *et al.*, 2005). Another feature is the highly conserved GXXDXHXH-sequence motif which is involved in the coordination of the metal ions in the active site (May *et al.*, 1998a; Holm & Sander, 1997).

The substrate recognition and enantioselectivity of hydantoinases/dihydropyrimidinases is assumed to be determined by three so called stereochemistry gate loops (SGLs). These SGLs are structurally conserved among hydantoinases/dihydropyrimidinases and form a hydrophobic pocket suggested to recognize the side chain of 5'-monosubstituted hydantoins. The specific amino acid composition of these SGLs defines the unique substrate specificity of each enzyme (Cheon *et al.*, 2002, 2003, 2004; Lo *et al.*, 2009).

The C-terminus of hydantoinases/dihydropyrimidinases is reported to be nonessential for catalysis and to be nonconserved among the known eukaryotic and prokaryotic enzymes (Kim & Kim, 1998). However, Martínez-Rodríguez *et al.*, 2010a found that the C-terminal regions of hydantoinases/dihydropyrimidinases of *α-Proteobacteria* are highly conserved. The C-termini are assumed to influence the oligomeric structure of the enzyme (Kim & Kim, 1998). Most hydantoinases and dihydropyrimidinases are homotetramers or homodimers (Syldatk *et al.*, 1999; Schnackerz & Dobritzsch, 2008). It was shown by Yoon *et al.*, 2003 that a mutation of several C-terminal residues of the hydantoinase from *Bacillus stearothermophilus* SD1 led to a change of the oligomeric structure of the enzyme from a homotetramer to a homodimer.

Classification

Hydantoinases are commonly subdivided in D-, L- or non-selective enzymes (LaPointe *et al.*, 1994). This system is not reliable because several hydantoinases were shown to have a substrate dependent enantioselectivity. Yokozeki & Kubota, 1987 reported for the enzyme

of *Flavobacterium* sp. AJ-3912 an L-selective cleavage of indolylmethyl hydantoin but a D-selective conversion of benzyloxymethyl hydantoin. The hydantoinase of *Arthrobacter aurescens* DSM3745 exhibited no selectivity for methylthioethyl hydantoin but showed an L-selectivity for indolylmethyl hydantoin (May *et al.*, 1998b). For the enzyme of *Brevibacillus agri* NCHU1002 a D-specific conversion of methylthioethyl hydantoin and *p*-hydroxyphenyl-hydantoin was demonstrated in contrast to a non-selective cleavage of homophenylalanyl hydantoin (Kao *et al.*, 2008).

2.3 *N*-Carbamoyl amino acid cleaving enzymes

Three enzymes classes are known to catalyze the hydrolytic degradation of *N*-carbamoyl amino acids to amino acids: β-Ureidopropionases [EC 3.5.1.6], *N*-Carbamoyl L-amino acid hydrolases (L-carbamoylases) [EC 3.5.1.87] and *N*-Carbamoyl D-amino acid hydrolases (D-carbamoylases) [EC 3.5.1.77].

2.3.1 β-ureidopropionases and L-carbamoylases

β-Ureidopropionases were detected in human, calf and rat liver (Kvalnes-Krick & Traut, 1993; Vreken *et al.*, 1999; Waldmann *et al.*, 2005), insects and slime mold (Gojkovic *et al.*, 2001) as well as in plants (Walsh *et al.*, 2001), yeast (Lundgren *et al.*, 2003) and bacteria (Ogawa & Shimizu, 1994; Martínez-Gómez *et al.*, 2009). The natural function of these enzymes is the hydrolysis of *N*-carbamoyl β-alanine and *N*-carbamoyl β-aminobutyric acid to β-alanine and β-aminobutyric acid in the last step of the reductive pyrimidine degradation pathway (Vogels & van der Drift, 1976, see figure 2.2). Besides a wide substrate specificity for several *N*-carbamoyl α-, *N*-carbamoyl β- and *N*-carbamoyl γ-amino acids was reported for the bacterial enzymes from *Agrobacterium tumefaciens* C58 and from *Pseudomonas putida* IFO 12996 (Ogawa & Shimizu, 1997; Martínez-Gómez *et al.*, 2009). The latter also showed activity towards *N*-acetyl α-amino acids and *N*-formyl α-amino acids.

Until now L-carbamoylases were solely found in bacteria (Martínez-Rodríguez *et al.*, 2010b). These enzymes exhibit activity towards *N*-carbamoyl-L-α-amino acids but show no activity towards the natural substrates of β-ureidopropionases *N*-carbamoyl β-alanine and *N*-carbamoyl β-aminobutyric acid. Some L-carbamoylases were reported to hydrolyze *N*-acetyl α-amino acids and *N*-formyl α-amino acids (Ogawa *et al.*, 1995c; Wilms *et al.*, 1999; Batisse *et al.*, 1997; Martínez-Rodríguez *et al.*, 2005; Pozo-Dengra *et al.*, 2010). The natural function of these enzymes is not yet clear.

Despite their different biochemical properties, β-ureidopropionases and L-carbamoylases are structurally related. Except for the enzyme of *Pseudomonas* sp. ON-4, all bacterial β-ureidopropionases and L-carbamoylases were reported to be homodimers and to depend on divalent metal ions (Ohmachi *et al.*, 2004; Martínez-Rodríguez *et al.*, 2010b). Within their studies of the L-carbamoylases from *Bacillus stearothermophilus* CECT43 and from *Sinorhizobium meliloti* CECT 4114, Martínez-Rodríguez *et al.*, 2008 and Martínez-Rodríguez *et al.*,

2006a found that a bimetal center is conserved for carbamoylases. The authors concluded from their results that L-carbamoylases are members of the peptidase family M20/M25/M40. Enzymes belonging to this family have a typical subunit structure build up of two domains: a catalytic domain harboring the metal ions and a lid domain.

2.3.2 D-carbamoylases

All D-carbamoylases described in literature originate from bacteria (Martínez-Rodríguez *et al.*, 2010b). They show activity towards a broad range of *N*-carbamoyl-D-α-amino acids but are not capable of hydrolyzing the natural substrates of β-ureidopropionases, *N*-carbamoyl-L-α-amino acid or *N*-acetyl- or *N*-formyl-α-amino acids. Like for L-carbamoylases, the natural function of D-carbamoylases is still unknown.

D-Carbamoylases are not structurally related to L-carbamoylases or β-ureidopropionases. In contrast to the latter two enzyme classes, D-carbamoylases are not metal dependent. The subunit composition varies between the enzymes described in literature. For example, the D-carbamoylase of *Pseudomonas* sp. KNK003A was assumed to be a dimer, the *Blastobacter* sp. A17-4 was suggested to be a trimer while the D-carbamoylase of *Sinorhizobium morelense* S-5 was reported to be a homotetramer (Ikenaka *et al.*, 1998; Ogawa *et al.*, 1994; Wu *et al.*, 2006). Already in 1979 Olivieri *et al.* described the inactivation of the enzyme of *Agrobacterium radiobacter* NRRL B11291 by sulphydryl agents which hinted at the involvement of cysteine residues in the catalytic mechanism. By solving the crystal structures of two *Agrobacterium* D-carbamoylases it was shown that a catalytic triad consisting of Glu-Lys-Cys, and thus a cysteine residue, seemed to be essential for the catalytic activity (Nakai *et al.*, 2000; Wang *et al.*, 2001). Due to the detection of the catalytic triad and the structural framework Martínez-Rodríguez *et al.*, 2010b concluded that D-carbamoylases belong to the nitrilase superfamily.

2.4 Hydantoin racemases

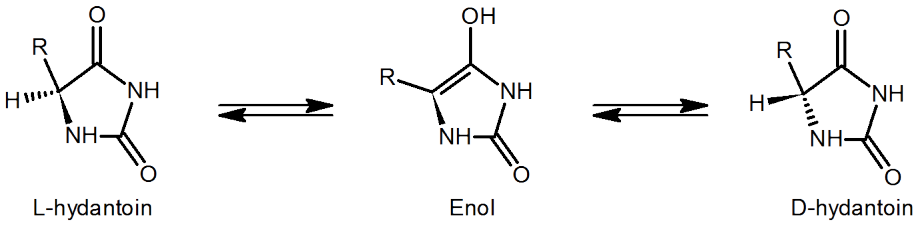

Figure 2.3 The keto-enol tautomerism of 5'-monosubstituted hydantoins

5'-Monosubstituted hydantoins racemize chemically under alkaline conditions via a keto-enol tautomerism (Ware, 1950, see figure 2.3). Depending on the substituent especially its bulkiness and charge the racemization proceeds more or less slowly. The racemization velocity for most hydantoins is rather slow. For benzylhydantoin the half-life-time ($t_{1/2,rac}$) for the

chemical racemization is 5.00 h and for methylhydantoin $t_{1/2,rac}$ is 33.98 h. Only for D,L-5-phenylhydantoin ($t_{1/2,rac}$=0.12 h) and D,L-5-*p*-hydroxyphenyl hydantoin ($t_{1/2,rac}$=0.27 h) the racemization velocity is fast enough to use these substrates in a hydantoinase process without the application of a hydantoin racemase (Lazarus, 1990; Pietzsch & Syldatk, 1995).

Hydantoin racemases are isomerases and are classified as [EC 5.1.99.5]. These enzymes catalyze the racemization of 5'-monosubstituted D- or L-hydantoins but their natural substrates are yet unknown. To date hydantoin racemases were solely detected in bacteria and only six enzymes are characterized and described in literature. These are the two hydantoin racemases from *Agrobacterium tumefaciens* C58, the racemase from *Arthrobacter aurescens* DSM 3747, the enzyme from *Microbacterium liquefaciens* AJ 3912, the hydantoin racemase from *Pseudomonas* sp. NS 671 and the enzyme from *Sinorhizobium meliloti* CECT4114 (Heras-Vázquez *et al.*, 2003; Martínez-Rodríguez *et al.*, 2004a; Wiese *et al.*, 2000; Suzuki *et al.*, 2005; Watabe *et al.*, 1992; Martínez-Rodríguez *et al.*, 2004b).

Most hydantoin racemases are reported to be tetramers. Exceptions are the enzyme from *A. aurescens* DSM 3747 which was described to be either a hexa-, hepta- or octamer and the racemase from *Pseudomonas* sp. NS 671 which was shown to be a hexamer. The enzymatic reaction also occurs under alkaline conditions and for most of the enzymes at an optimal temperature of 55 °C (see table 2.1). It was reported for all these hydantoin racemases that the metal chelating agent EDTA had no significant effect on enzyme activity. On the other hand its is described that some divalent metal ions like Cu^{2+} and Hg^{2+} can act as enzyme inhibitors (see table 2.1). Despite this fact Heras-Vázques *et al.*, 2009 assume that these enzymes are not metalloenzymes. All hydantoin racemases showed different substrate specificities. For example, a higher activity towards hydantoins with aromatic substituents rather than apolar aliphatic substituents is reported for the *A. aurescens* DSM 3747 enzyme. By contrast, the enzymes of *A. tumefaciens* C58 and *S. meliloti* CECT4114 showed a preference for hydantoins with short aliphatic side chains. Furthermore, a substrate dependent enantioselectivity is reported for some hydantoin racemases. The *M. liquefaciens* AJ 3912 enzyme exhibited a selectivity for the L-enantiomer of benzylhydantoin. For the *S. meliloti* CECT4114 enzyme racemization of L- but not D-ethylhydantoin and a conversion of both isomers of benzyl- and isobutyl hydantoin was described. First studies on the reaction mechanism propose a two-base mechanism. When one substrate enantiomer enters the catalytic core a proton is abstracted by one residue acting as base and the enolate of the substrate enantiomer is generated. Subsequently, this enolate retrieves a proton from another residue acting as an acid and thus the other enantiomer is formed (Pietzsch *et al.*, 1992; Martínez-Rodríguez *et al.*, 2006b).

Table 2.1 Comparison of the six hydantoin racemases described in literature. The given values of pH and T (= temperature) are for the optimal hydantoin racemase activity towards the corresponding substrate in boldface. IMH = indolylmethyl hydantoin, CH_3-IMH = 5-(3-indolylmethyl)-3-*N*-methylhydantoin, HBzH = hydroxybenzyl hydantoin, BzH = benzyl-hydantoin, iBuH = isobutyl hydantoin, MeTEH = methylthioethyl hydantoin, EH = ethyl-hydantoin, iPrH = isopropyl hydantoin, IAA = iodoacetamide, NEM = *N*-ethylmaleimide, 2-ME = 2-mercaptoethanol, 8-OH-Q = 8-hydroxy-quinoline, EGTA = ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid

Strain	pH	T	Substrates	Inhibitors	None Inhibitors
A. aurescens DSM3747 (Pietzsch *et al.*, 1992; Wiese *et al.*, 2000)	8.5	55 °C	IMH, CH_3-IMH, HBzH, **BzH**, iBuH, MeTEH	$HgCl_2$, $Hg(Ac)_2$, IAA, $CuSO_4$, cystine, acetone	EDTA, DTT, SDS, 2-ME, 8-OH-Q, cysteine
A. tumefaciens C58 hyuA1 (Heras-Vázquez *et al.*, 2003)	7.5	55 °C	**EH**, iBuH, MeTEH, BzH	Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}	EDTA, DTT, Mn^{2+}
A. tumefaciens C58 hyuA2 (Martínez-Rodríguez *et al.*, 2004a)	7.5	55 °C	**EH**, iBuH, MeTEH, BzH	Cu^{2+}, Hg^{2+}	EDTA, DTT
M. liquefaciens AJ3912 (Suzuki *et al.*, 2005)	8.2	55 °C	**BzH**	NEM, IAA, $CuSO_4$	MeOH
Pseudomonas sp. NS671 (Watabe *et al.*, 1992)	9.5	45 °C	**MeTEH**, iBuH, MeH	Cu^{2+}, Zn^{2+}, D-MeH, iPrH	Co^{2+}, Mn^{2+}, Ca^{2+}, Mg^{2+}, Ni^{2+}, EDTA, EGTA,
S. meliloti CECT 4114 (Martínez-Rodríguez *et al.*, 2004b)	8.5	40 °C	BzH, iBuH, L-EH	Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Hg^{2+}, Zn^{2+}, Pb^{2+}	DTT, EDTA

2.5 Natural function of hydantoinases, D- and L-carbamoylases and hydantoin racemases

The natural function of neither hydantoinases nor D- and L-carbamoylases nor hydantoin racemases is known today. But these enzymes are often found together in gene clusters (Dürr *et al.*, 2008). One hypothesis is that *N*-carbamoyl-α-amino acids and hydantoins may have served as carbon and nitrogen sources for the first microorganisms developing on earth. This was concluded by Syldatk *et al.*, 1999 from the results published by Taillades *et al.*, 1998 who found that *N*-carbamoyl-α-amino acids and hydantoins are more likely to be the precursors of first peptides rather than α-amino acids. Consequently, the hydantoinases and carbamoylases found today could be the successors of the enzymes responsible for hydantoin and carbamoyl amino acid cleavage on the early earth (Syldatk *et al.*, 1999). Another hypothesis is that L-carbamoylases are detoxifying enzymes (Martínez-Rodríguez *et al.*, 2010b).

2.6 Enzyme engineering for the "hydantoinase process"

Although the hydantoinase process is well established in industry there are still problems to be solved and improvements to be made.

In general, the process is more frequently used to produce chiral D-amino acids, because much more hydantoinases/dihydropyrimidinase with D-specificity for 5'-monosubstituted hydantoins where detected and characterized. Hydantoinases/dihydropyrimidinase with good selectivities for L-enantiomers are less available (Syldatk *et al.*, 1999). To overcome this problem, May *et al.*, 2000 successfully inverted the enantioselectivity of *Arthrobacter* sp. DSM 9771 hydantoinase towards the L-enantiomer of methionine by applying directed evolution. Another strategy for employing the hydantoinase process for the production of L-amino acids is the combination of unselective and enantiospecific enzymes. For the production of L-homophenylalanine Kao *et al.*, 2008 coexpressed the dihydropyrimidinase from *Brevibacillus agri* NCHU1002, which is non-enantiospecific for homophenylalanyl hydantoin, together with the L-carbamoylase of *Bacillus kaustophillus* CCRC11223 in order to produce L-homophenylalanine. In addition, the screening for novel strains showing L-selective conversion of substituted hydantoins to *N*-carbamoyl L-amino acids or L-amino acids is continuing (Mei *et al.*, 2008, 2009).

The efficiency of the hydantoinase process is also strongly dependent on the stability of the enzymes used. Especially carbamoylases are susceptible against thermal and oxidative stress (Ogawa *et al.*, 1994; Kim & Kim, 1995; Grifantini *et al.*, 1996). At first random mutagenesis was used to enhance the thermal stability of carbamoylases (Ikenaka *et al.*, 1999). The determination of the crystal structures of the D-carbamoylases from *Agrobacterium* sp. KNK 712 and from *Agrobacterium radiobacter* CCRC14924 led to a better understanding of the mechanisms influencing enzyme stability (Nakai *et al.*, 2000; Wang *et al.*, 2001). Since then, these results were used for the improvement of oxidative and thermal stability of several carbamoylases by directed evolution (Oh *et al.*, 2002; Chien *et al.*, 2002; Chiang *et al.*, 2008; Zhang *et al.*, 2011). Other strategies for enhancing the stability of hydantoinases and carbamoylases are the construction of hydantoinase-carbamoylase fusion proteins (Kim *et al.*, 2000a) or enzyme immobilization (Foster *et al.*, 2003; Aranaz *et al.*, 2009).

In addition to enzyme engineering, the hydantoinase process can also be improved by adjusting other process parameters. For example, the use of wild-type strains grown on inexpensive nitrogen and carbon sources (Jiang *et al.*, 2007), the influence of different carbon sources, nitrogen sources and inducers on enzyme activities (Hartley *et al.*, 2001; Kirchmann *et al.*, 2007), the co- and separate expression of hydantoinase, carbamoylase and hydantoin racemase in *E. coli* (Martínez-Rodríguez *et al.*, 2002; Nozaki *et al.*, 2005) and the optimum ratio of hydantoinase and carbamoylase are studied (Chao *et al.*, 2000).

3 Research Proposal

Chiral β-amino acids are valuable building blocks for fine chemicals and pharmaceuticals (Cheng *et al.*, 2001; Pilsl & Reiser, 2011). However, their chemical synthesis is inefficient and costly (Weiner *et al.*, 2010). Today's established biocatalytic synthesis routes are based on kinetic resolutions of racemates thus producing a maximal yield of 50 %. In contrast, the biocatalytic production of optically pure α-amino acids is well established in industry e.g. by the application of the hydantoinase process (see figure 2.1) and leads up to 100 % yield. This reaction employs up to three enzymes, a hydantoin racemase, a hydantoinase and an *N*-carbamoylase for the production of either D- or L-amino acid starting from 5'-monosubstituted hydantoins. The aim of this thesis was to examine the applicability of a modified hydantoinase process to the production of chiral β-amino acids starting from cheap bulk chemicals. Especially the following main questions had to be addressed:

- Are hydantoinases/ dihydropyrimidinases able to hydrolyze unnatural 6'-monosubstituted dihydropyrimidines?
- Are carbamoylases able to cleave the resulting unnatural *N*-carbamoyl-β-amino acids?
- If there are such hydantoinase/dihydropyrimidinase and carbamoylases, are their enzymatic reactions enantioselective?

In order to answer these questions the following tasks had to be processed:

▷ Synthesis of model substrates from cheap bulk chemicals in gram scale.

▷ Establishing of appropriate HPLC analytics.

▷ Screening for novel microorganisms with enzymatic activity towards unnatural 6'-monosubstituted dihydropyrimidines and *N*-carbamoyl-β-amino acids.

▷ Isolation and characterization of novel strains.

▷ Elucidation of the genetic code of novel isolated hydantoinase/dihydropyrimidinase and carbamoylases.

▷ Recombinant expression and characterization of the novel enzymes.

Part II

Results published in or submitted to journals

4 Synthese aromatischer β-Aminosäuren mit neuen cyclischen Amidasen

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Publiziert in:
Chemie Ingenieur Technik
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Chirale β-Aminosäuren sind bedeutende Bausteine für zahlreiche Feinchemikalien und Pharmazeutika, für deren chemische Synthese es momentan keine wirtschaftliche Methode gibt. Daher sollte untersucht werden, ob ein modifizierter Hydantoinase-Prozess für die biokatalytische Herstellung von β-Aminosäuren eingesetzt werden kann. Dafür wurde im Rahmen dieser Arbeit ein Aryl-substituiertes Dihydrouracil chemisch synthetisiert. Das hergestellte Phenyldihydrouracil wurde dann als Substrat in Ganzzell-Biotransformationen eingesetzt. Ziel war es, cyclische Amidasen zu finden, die die Umsetzung zur entsprechenden *N*-Carbamoyl-β-Aminosäure katalysieren.

Schlagwörter: β-Aminosäuren, Biotransformationen, Hydantoinase

Problemstellung

In den letzten Jahren ist das Interesse an β-Aminosäuren, die man in der Natur viel seltener findet als ihre α-Analoga, ständig gewachsen. Für freie β-Aminosäuren sind u. a. antimykotische (Mittendorf *et al.*, 2003) und interessante pharmakologische Eigenschaften beschrieben (Shinagawa *et al.*, 1987). Oft sind β-Aminosäuren auch struktureller Bestandteil potenter Naturstoffe, wie z. B. β-Phenylalanin als Teil des hochwirksamen Chemotherapeutikums Paclitaxel (Juaristi & Soloshonok, 2005). Peptide mit inkorporierten β-Aminosäuren bilden wohldefinierte Sekundärstrukturen (Cheng *et al.*, 2001) und sind gegen einen Abbau durch menschliche Proteasen außerordentlich stabil (Frackenpohl *et al.*, 2001). Entsprechend groß ist das Interesse an der Erforschung effizienter Synthesewege für die Herstellung enantiomerenreiner β-Aminosäuren. Für die Synthese chiraler α-Aminosäuren ist der Hydantoinase-Prozess etabliert. Dabei werden mithilfe einer Hydantoin-Racemase, einer Hydantoinase und einer Carbamoylase racemische Hydantoine zu enantiomerenreinen D- oder L-α-Aminosäuren umgesetzt (Syldatk *et al.*, 1999; Martínez-Gómez *et al.*, 2009). Im Rah-

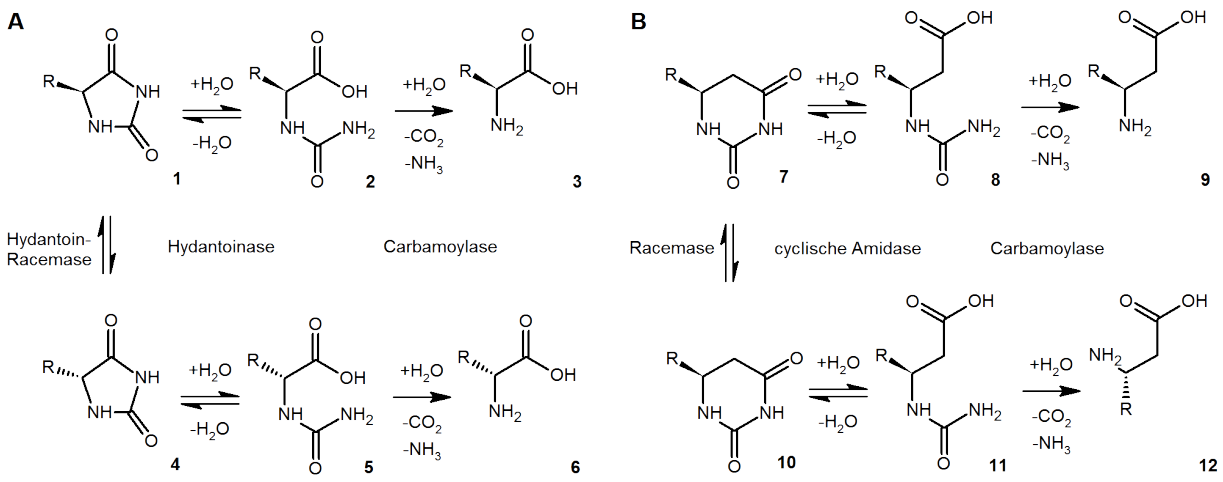

Figure 4.1 (A) Hydantoinase-Prozess zur Synthese chiraler D- oder L-α-Aminosäuren (modifiziert nach Syldatk *et al.*, 1999); R = Aryl; 1 = (*S*)-Aryl-Hydantoin, 2 = (*S*)-Aryl-Carbamoylaminosäure, 3 = (*S*)-Aryl-Aminosäure, 4 = (*R*)-Aryl-Hydantoin, 5 = (*R*)-Aryl-Carbamoylaminosäure, 6 = (*R*)-Aryl-Aminosäure. (B) Möglicher modifizierter Hydantoinase-Prozess zur Herstellung chiraler β-Aminosäuren; R = Aryl; 7 = (*R*)-Aryl-Dihydrouracil, 8 = (*R*)-Aryl-Carbamoyl-β-Aminosäure, 9 = (*R*)-Aryl-β-Aminosäure, 10 = (*S*)-Aryl-Dihydrouracil, 11 = (*S*)-Aryl-Carbamoyl-β-Aminosäure, 12 = (*S*)-Aryl-β-Aminosäure.

men dieses Projektes sollte getestet werden, ob dieser Prozess auf die Synthese von β-Aminosäuren übertragbar ist (Abb. 4.1). Dafür mussten zunächst aus kostengünstigen Rohstoffen entsprechende Substrate, Aryl-substituierte Dihydrouracile, chemisch synthetisiert werden. Anschließend sollten in der Stammsammlung der Arbeitsgruppe vorhandene cyclische Amidasen und Mikroorganismen auf ihre Fähigkeit zum Umsatz dieser Dihydrouracile zur entsprechenden Carbamoylaminosäure bzw. weiter zur β-Aminosäure untersucht werden.

Material und Methoden

Synthese von Phenyldihydrouracil

Phenyldihydrouracil (PheDU) wurde modifiziert nach (Fischer & Roeder, 1901; Vuano *et al.*, 2000) aus Zimtsäure und Harnstoff synthetisiert und mehrfach in Methanol umkristallisiert. Die Reinheit wurde mit ^{1}H-NMR und HPLC nachgewiesen.

Stammanzucht

Die verwendeten Bakterienstämme gehören zur Stammsammlung der Technischen Biologie (Dürr *et al.*, 2006). Das Wachstumsmedium GMi wurde modifiziert nach (Dürr *et al.*, 2006) mit 0,2 g/L D,L-5-(3-Indolylmethyl)-3-*N*-methylhydantoin (CH_3-IMH) hergestellt. Das Medium GM enthält im Gegensatz zu GMi kein CH_3-IMH. LB-Medium wurde nach Fluka, 2002 hergestellt. Das Medium LBi enthält zusätzlich 0,1 g/L CH_3-IMH.

Biotransformationen

Für Biotransformationen wurden Zellen in entsprechendem Medium angezogen, in der späten exponentiellen Phase geerntet und zweimal mit Kaliumphosphatpuffer (0,2 M, pH 8) gewaschen. Schließlich wurden die Zellen im selben Puffer resuspendiert (Biotrockenmasse etwa 30 mg/mL). Für Biotransformationen wurden ein Volumen Zellsuspension und ein Volumen in Kaliumphosphatpuffer (0,2 M, pH 8) gelöstes PheDU (4 mM) zusammengegeben und für 24 h bei 40 C im Thermomixer geschüttelt. Anschließend wurden die Zellen abzentrifugiert und die Überstände mittels HPLC analysiert (Dürr *et al.*, 2006). Für einige Isolate wurden zusätzlich Langzeit-Biotransformationen durchgeführt. Dabei wurden die Ansätze für bis zu 200 h bei 40 C im Thermomixer inkubiert und zu bestimmten Zeitpunkten jeweils Proben für die HPLC-Analyse abgenommen.

HPLC-Analytik

Achirale HPLC-Analysen wurden mit einer NUCLEODUR-Säule (100-5 C18, 200 x 4) an einer Agilent 1100 HPLC-Anlage nach (Dürr *et al.*, 2006) durchgeführt. Für die chirale Analytik wurde eine NUCLEODEX β-OH Säule (150 x 4, 5 µm) eingesetzt.

Ergebnisse

Synthese

PheDU konnte als einfachstes Modell für Arylsubstituierte Dihydrouracile modifiziert nach (Fischer & Roeder, 1901; Vuano *et al.*, 2000) aus Zimtsäure und Harnstoff hergestellt werden. Die Synthese wurde im Maßstab von mehreren hundert Gramm in Kooperation mit dem Fraunhofer Institut für Chemische Technologie durchgeführt.

Table 4.1 Ergebnisse der Ganzzell-Biotransformationen von PheDU zu *N*CβPhe mit Wildstämmen innerhalb von 24 h. (-) nicht nachweisbar; (+) Umsatz < 10 %; (++) 10 % < Umsatz < 50 %; (+++) Umsatz > 50 %; GM, Wachstumsmedium; LB, Lysogeny Broth Medium; i, Medium enthält CH_3-IMH

Stamm	Medium	PheDu Umsatz zu *N*CβPhe
Microbacteriacae sp. K3[a]	GM	-
	GMi	+
Burkholderia sp. M3[a]	GM	-
	GMi	-
Flavobacterium sp. F8[a]	GM	+
	GMi	-
Bacillus sp. A16[a]	GM	-
	GMi	++
Bacillus sp. G18[a]	GM	+
	GMi	-
Pseudomonas sp. G7[a]	GM	+
	GMi	-
Pseudomonas sp. M18[a]	GM	-
	GMi	-
Ochrobactrum sp. C15[a]	GM	+
	GMi	-
Ochrobactrum sp. F21[a]	GM	++
	GMi	++
157[b]	GM	++
	GMi	-
158[b]	GM	++
	GMi	-
222[b]	GM	+
	GMi	-
225[b]	GM	+
	GMi	-
725[b]	LB	+++
	LBi	+++
728[b]	LB	+++
	LBi	++
731[b]	LB	+++
	LBi	+++
735[b]	LB	+
	LBi	++

[a] vgl. Dürr *et al.*, 2006

[b] Stammsammlung Technische Biologie Universität Karlsruhe.

Neue cyclische Amidasen

Auf der Suche nach cyclischen Amidasen mit der Fähigkeit, das ausgewählte Beispielsubstrat PheDU in *N*-Carbamoyl-β-Phenylalanin (*N*CβPhe) zu hydrolysieren, wurden Stämme mit bekannter Aktivität für Aryl-substituierte Hydantoine untersucht (Dürr *et al.*, 2006) und jeweils in Medium mit und ohne CH_3-IMH angezogen. Anschließend wurden die Überstände der Ganzzell-Biotransformationen mittels achiraler HPLC analysiert. Die Ergebnisse dieser Versuche sind in Tabelle 4.1 zusammengefasst. Von den 17 untersuchten Mikroorganismen waren nur zwei Stämme, *Burkholderia* sp. M3 und *Pseudomonas* sp. M18, nicht in der Lage, PheDU zu *N*CβPhe zu hydrolysieren. Keiner der Stämme mit Aktivität für dieses Aryl-substituierte Dihydrouracil war in der Lage, die entstandene *N*-Carbamoyl-β-Aminosäure weiter zur β-Aminosäure umzusetzen. Ein anderer Faktor, der beeinflusst, ob die Stämme PheDU umsetzen, ist die Zusammensetzung des Wachstumsmediums. Für alle Stämme außer 725 und 731 gab es signifikante Unterschiede hinsichtlich des Umsatzes von PheDU, je nachdem ob CH_3-IMH im Medium enthalten war oder nicht. Während für einige Stämme nur nach Kultivierung in CH_3-IMH-haltigem Medium Aktivität für PheDU detektiert wurde, konnten andere Isolate nur dann PheDU umsetzen, wenn sie in CH_3-IMH-freiem Medium angezogen wurden (vgl. Tabelle 4.1). Generell wurde das Substrat PheDU in den durchgeführten Ganzzell-Biotransformationen innerhalb von 24 h von allen Mikroorganismen in verschiedenem Maße umgesetzt. Einige Stämme zeigten nur basale Aktivität, während andere bis zu 50 % des vorgelegten Substrates zur Carbamoylaminosäure hydrolysierten. Schließlich waren drei Isolate sogar fähig, mehr als 50 % der PheDU-Menge umzusetzen.

Untersuchungen zur Stereospezifität

In einem zweiten Schritt wurde eine Methode für die chirale HPLC-Analyse von PheDU und *N*CβPhe entwickelt. Da keine enantiomerenreinen Standards für diese beiden Verbindungen erhältlich sind, konnte die absolute Konfiguration der jeweils eluierenden Enantiomere nicht eindeutig einem Stereoisomer zugeordnet werden. Deswegen wurden die Enantiomere nach ihrer Elutionsreihenfolge mit A und B für *N*CβPhe und C und D für PheDU bezeichnet. Die Überstände der Biotransformationen wurden mittels chiraler HPLC erneut untersucht (Abb. 4.2). Dabei zeigte sich, dass das Isolat *Bacillus* sp. A16 bevorzugt das PheDU-Enantiomer C zum *N*CβPhe-Enantiomer A umsetzt. Für die Isolate 725, 728 und 731 wurden die Proben der Langzeit-Biotransformationen mit chiraler HPLC analysiert. Dabei wurde festgestellt, dass beide PheDU-Enantiomere (C und D) von Anfang an gleichmäßig zu den Enantiomeren A und B von *N*CβPhe umgesetzt werden.

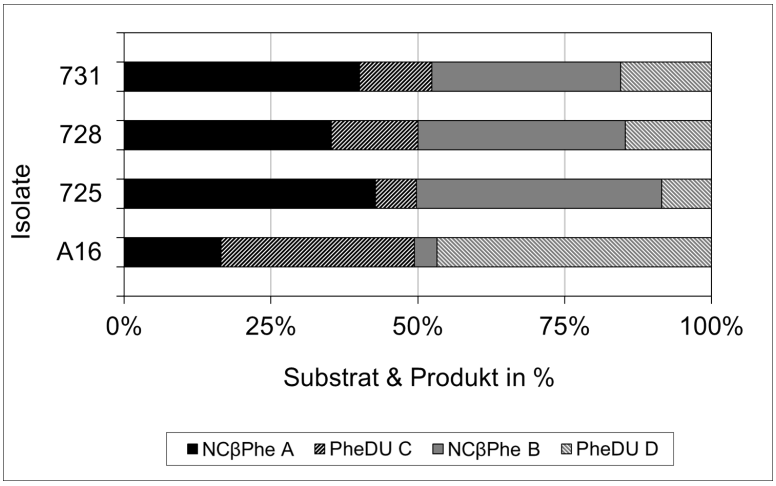

Figure 4.2 Untersuchungen zum stereospezifischen Umsatz der Enantiomere C und D von PheDU zu den entsprechenden *N*CβPhe-Enantiomeren A und B durch die Isolate *Bacillus* sp. A16, 725, 728 und 731 (vgl. Tabelle 4.1) innerhalb von 24 h.

Diskussion

Im Rahmen dieser Arbeit wurde untersucht, ob man für die Synthese enantiomerenreiner β-Aminosäuren einen modifizierten Hydantoinase-Prozess einsetzen kann. Es war möglich, aus den kostengünstigen Bulkchemikalien Zimtsäure und Harnstoff das Substrat PheDU in ausreichender Menge und Qualität zu synthetisieren. Damit stand ein neuartiges Substrat für cylische Amidasen zur Verfügung. Auf der Suche nach Mikroorganismen, die die gewünschten Enzyme besitzen, wurde auf die Stammsammlung der Arbeitsgruppe zurückgegriffen. Da Hydantoine und Dihydrouracile sich in ihrer chemischen Struktur ähneln, wurden gezielt Isolate ausgesucht, für die schon in früheren Arbeiten eine Hydantoinase-Aktivität für Aryl-substituierte Hydantoine detektiert wurde. Außerdem gab es für einige Stämme bereits Voruntersuchungen (Dürr *et al.*, 2006) bezüglich ihrer Fähigkeit, PheDU umzusetzen. Zusätzlich wurde untersucht, ob die Expression der entsprechenden Gene durch Zugabe von CH_3-IMH, einem Aryl-substituierten Hydantoin, induzierbar ist. In einer früheren Arbeit (Syldatk *et al.*, 1990) wurde gezeigt, dass dieses Hydantoin zur Induktion von Hydantoinasen während der Stammkultivierung führte, aber selbst nicht als Substrat genutzt werden konnte.

Die vorliegenden Ergebnisse zeigen deutlich, dass fast alle untersuchten Stämme das unnatürliche Substrat PheDU zu *N*CβPhe umsetzen können. Je nach Kultivierungsbedingungen gibt es aber große Unterschiede zwischen den einzelnen Isolaten. Während scheinbar bei einigen Stämmen die Expression der Gene erst durch Zugabe von CH_3-IMH induziert werden muss, wird bei anderen Isolaten gerade durch CH_3-IMH-Zusatz die Expression unterdrückt. Daneben hat bei einigen Mikroorganismen die An- oder Abwesenheit von CH_3-IMH im Kultivierungsmedium keinen Einfluss auf die Expression der gewünschten Enzyme. Daraus lässt sich schließen, dass die Isolate sehr unterschiedliche cyclische Amidasen besitzen.

Außerdem unterscheiden sich alle untersuchten Stämme sehr stark darin, wie viel der vorgelegten PheDU-Menge sie in Ganzzell-Biotransformationen innerhalb von 24 h umsetzen können. Interessant für weitere Untersuchungen sind sowohl Stämme, die einen nahezu voll-

ständigen Umsatz zeigten, als auch Isolate, die nur bis zu 50% des Substrates hydrolysieren. Letztere könnten eine stereoselektive cyclische Amidase besitzen.

Mit der für die chirale HPLC-Analytik von PheDU und *N*CβPhe entwickelten Methode wurden erste Untersuchungen zum stereoselektiven Umsatz durchgeführt. Für das Isolat *Bacillus* sp. A16 konnte gezeigt werden, dass bevorzugt ein PheDU-Enantiomer zu einem *N*CβPhe-Enantiomer umgesetzt wird. Die cyclische Amidase von *Bacillus* sp. A16 ist daher wahrscheinlich stereoselektiv. Alternativ könnte allerdings auch ein stereoselektiver Transportmechanismus vorliegen, so dass nur eines der PheDU-Enantiomere ins Zellinnere gelangt und zur Carbamoylaminosäure hydrolysiert werden kann. Für die Isolate 725, 728 und 731 wurden zusätzlich Langzeit-Biotransformationen durchgeführt. Mittels chiraler HPLC-Analytik konnte festgestellt werden, dass beide PheDU-Enantiomere in gleichem Maße zu den entsprechenden *N*CβPhe-Enantiomeren hydrolysiert werden. Vermutlich besitzen die Stämme 725, 728 und 731 cyclische Amidasen, die nicht zwischen den beiden PheDU-Enantiomeren differenzieren können. Festzuhalten ist auch, dass in keinem Fall Carbamoylase-Aktivität detektiert werden konnte, obwohl während der Untersuchungen immer mit ganzen Zellen gearbeitet wurde.

Es konnte im Rahmen der Arbeit gezeigt werden, dass es einige Mikroorganismen gibt, die das eingesetzte Aryl-substituierte Dihydrouracil zur entsprechenden *N*-Carbamoyl-β-Aminosäure umsetzen können. Aber bis jetzt wurde noch keine Carbamoylase gefunden, die den weiteren Umsatz zur entsprechenden β-Aminosäure katalysieren kann.

Ausblick

In weiteren Arbeiten sollen die Ganzzell-Biotransformationen wiederholt und hinsichtlich der Stereoselektivität des Umsatzes genauer untersucht werden. Außerdem sollen weitere Aryl-substituierte Dihydrouracile synthetisiert werden. Des Weiteren wird derzeit ein Screening nach Carbamoylasen, die in der Lage sind, *N*-Carbamoyl-β-Aminosäuren zu β-Aminosäuren umzusetzen, durchgeführt.

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5 Stereoselective hydrolysis of aryl-substituted dihydropyrimidines by hydantoinases

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In this study, we investigated the possibility of using a modified hydantoinase process for the production of optically pure β-amino acids. Two aryl-substituted dihydropyrimidines D,L-6-phenyl-5,6-dihydrouracil (PheDU) and *para*-chloroD,L-6-phenyl-5,6-dihydrouracil (*p*ClPheDU) were synthesized. Hydrolysis of these novel substrates to the corresponding *N*-carbamoyl-β-amino acids by three recombinant D-hydantoinases and several bacterial strains was tested. All applied recombinant D-hydantoinases and eight bacterial isolates catalyzed the conversion of PheDU to *N*-carbamoyl-β-phenylalanine (*N*CβPhe). Some of these biocatalysts showed an enantioselectivity for either the D- or the L-PheDU enantiomer. The second dihydropyrimidinase substrate *p*ClPheDU was hydrolyzed by all three recombinant D-hydantoinases and six of the wild-type strains. To our knowledge, this is the first dihydropyrimidinase activity reported with this aryl-substituted dihydropyrimidine. For selected biocatalysts, hydantoinase activity towards aryl-substituted hydantoins was demonstrated as well. However, none of the bacterial strains tested so far exhibited any carbamoylase activity towards *N*CβPhe.

Keywords: Beta-amino acid – Hydantoinase – Dihydropyrimidinase – Carbamoylase

Introduction

β-Amino acids are important building blocks for pharmaceuticals and fine chemicals. Some β-amino acids occur in nature in free form like β-alanine as a product of the reductive degradation of pyrimidines, a metabolic pathway observed in animals, plants, yeast, and bacteria (Schnackerz & Dobritzsch, 2008; Vogels & van der Drift, 1976). More often, β-amino acids are found as essential components of bioactive compounds. One example is the antitumor agent paclitaxel (Taxol ™) which was isolated from the pacific yew (*Taxus brevifolia*) and carries a β-phenylalanine subunit (Fleming *et al.*, 1993). Furthermore β-amino acids can serve as precursors for β-peptides. Numerous studies have demonstrated that these peptides form very stable and well-defined secondary structures (Cheng *et al.*, 2001, Seebach & Matthews, 1997) and moreover are extraordinary resistant to proteolytic enzymes (Frackenpohl *et al.*, 2001). Therefore, the application of β-peptides as peptidomimetics is extensively studied (Seebach & Gardiner, 2008; Steer *et al.*, 2002). Various methods for the chemical synthesis of chiral β-amino acids have been described, in general there are three main strategies: (1) the resolution of racemic β-amino acids derivatives, (2) the use of naturally occurring chiral α-amino acids as starting material and (3) asymmetric synthesis. All of these strategies have their limitations when applied in an industrial scale as resolutions of racemic mixtures are complex and time-consuming procedures, the chiral pool of natural α-amino acids is limited and catalysts or chiral auxiliaries cause high costs (Liu & Sibi, 2002; Weiner *et al.*, 2010). Thus, biocatalytic paths are a promising alternative for the preparation of chiral β-amino acids. For example, the application of aminomutases (Wu *et al.*, 2009), Baeyer–Villiger monooxygenases (Rehdorf *et al.*, 2010), or lipases (Tasnádi *et al.*, 2008) is described. In the majority of cases, hydrolytic enzymes are applied for the resolution of racemic mixtures of β-amino acids or their derivatives. Biocatalytic reactions employing lipases enantioselectivly cleaving racemic β-amino acid esters or *N*-acetylated β-amino acids are best studied (Liljeblad & Kanerva, 2006). However, racemic resolutions are limited to a maximum yield of 50 %.

In this study, we tested the possibility of using a modified hydantoinase process for the production of optically pure β-amino acids. The hydantoinase process has a theoretical yield of 100 % and is industrially applied for the biocatalytic synthesis of enantiopure α-amino acids, e.g., α-D-phenylglycine and α-D-*p*-hydroxyphenylglycine (Martínez-Gómez *et al.*, 2007; Cai *et al.*, 2009). For this purpose, a hydantoin racemase, an enantioselective hydantoinase, and an enantioselective *N*-carbamoyl-α-amino acid hydrolase are employed to produce optically pure α-amino acids starting from racemic hydantoins (see Fig. 5.1a). As our intention is the production of β-amino acids, substituted dihydropyrimidines serve as substrates. For the stereoselective hydrolysis, an appropriate racemase, a cyclic amidase able to cleave these six-ring systems, and an appropriate carbamoylase have to be applied (see Fig. 5.1b). Cyclic amidases acting on unsubstituted dihydropyrimidines are often described in literature, e.g., dihydropyrimidinases catalyzing the second step of reductive pyrimidine degradation pathway; and most hydantoinases can hydrolyze dihydrouracil (summarized in EC 3.5.2.2.). Carbamoylases able to act on carbamoyl groups of unsubstituted *N*-carbamoyl-β-amino acids are also reported, e.g., the β-ureidopropionases (EC 3.5.1.6) responsible for the third step of reductive pyrimidine degradation pathway (Martínez-Gómez *et al.*, 2009; Ogawa

& Shimizu, 1994) and a few *N*-carbamoyl-D-amino-acid hydrolases (EC 3.5.1.77) are able to degrade β-aminoisobutyric acid or β-ureidopropionic acid (Louwrier & Knowles, 1996; Yokozeki & Kubota, 1987). Here, two aryl-substituted dihydropyrimidines were synthesized and their degradation by three well-studied D-hydantoinases and by bacterial strains with known hydantoinase activity from our in-house strain collection was tested.

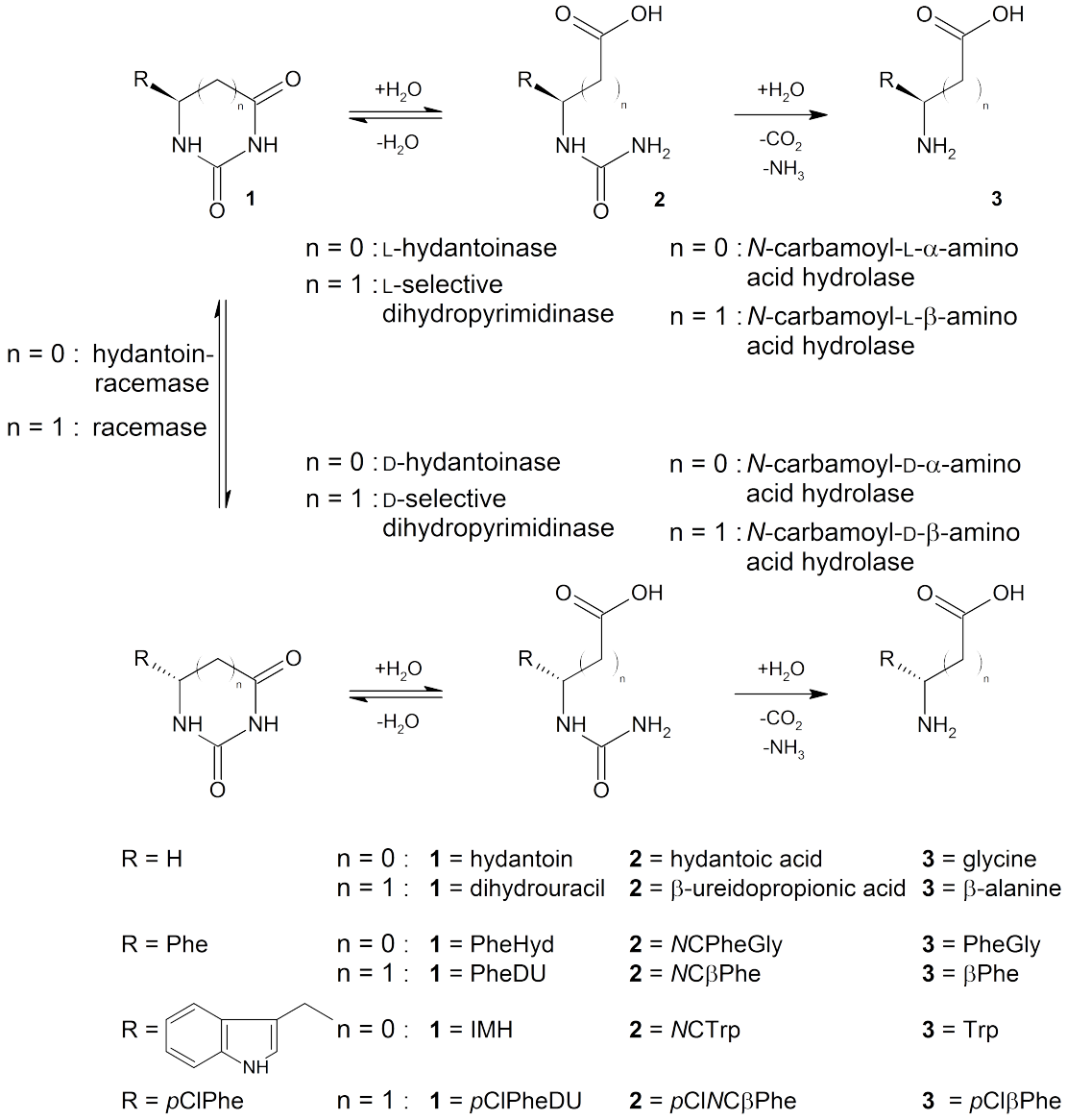

Figure 5.1 *n = 0*: hydantoinase process for the synthesis of chiral D- or L-α-amino acids **(3)** starting from racemic 5'-monosubstituted hydantoins **(1)** via *N*-carbamoyl-α-amino acids **(2)** applying a hydantoin racemase, an enantioselective hydantoinase and an enantioselective *N*-carbamoyl-α-amino acid hydrolase; *n =1*: Proposed modified hydantoinase process for the synthesis of chiral D- or L-β-amino acids **(3)** starting from racemic 6'-monosubstituted dihydropyrimidines **(1)** via *N*-carbamoyl-β-amino acids **(2)** applying a racemase, an enantioselective dihydropyrimidinase and an enantioselective *N*-carbamoyl-β-amino acid hydrolase

Experimental

Chemicals

Chemicals were of reagent grade and obtained from commercial sources if not stated otherwise. D,L-5-Indolylmethyl-*N*-3-methyl hydantoin (CH_3-IMH) was supplied by former Degussa AG (now Evonik Industries AG). D-β-Phenylalanine and L-β-phenylalanine (βPhe) were obtained from Pep-Tech Corporation (Burlington, USA). Phenylhydantoin (PheHyd), 5-indolylmethyl hydantoin (IMH), and *N*-carbamoyl-α-tryptophan (*N*CTrp) were synthesized according to Stark & Smyth, 1963 and Suzuki *et al.*, 1973. Phenyldihydrouracil (PheDU), *N*-rac-carbamoyl-β-phenylalanine (*N*CβPhe), and L-*N*CβPhe as standard for HPLC analysis were prepared according to Dakin & Dudley, 1914 and Dürr, 2007. The purity was proven by HPLC and ^{1}H-NMR. For biotransformation and screening experiments, PheDU was prepared from cinnamic acid and urea. In contrast to the original procedure by Fischer & Roeder, 1901 and Vuano *et al.*, 2000 the scale was extended to several hundred grams in cooperation with the Fraunhofer Institute for Chemical Technology (FhG-ICT; Pfinztal Germany). The purity was proven by HPLC and ^{1}H-NMR. *para*-Chloro-D,L-phenyldihydrouracil *p*ClPheDU was provided from Fraunhofer ICT and the purity was proven by ^{1}H-NMR. *p*ClPheDU was hydrolyzed under alkaline conditions in order to prepare *para*-chloro-*N*-carbamoyl-β-phenylalanine (*p*Cl*N*CβPhe) as HPLC standard. Therefore, *p*ClPheDU was dissolved to concentration of 400 mM in DMSO due to its poor water solubility. Subsequently, 1 volume of the 400 mM *p*ClPheDU solution was added to 79 volumes of water so that *p*ClPheDU dropped out as white crystals. Alkaline hydrolysis was started by adding 10 volumes of 1 M NaOH to the suspension, which turned immediately into a clear solution. After 30-min incubation at 40 °C the solution was neutralized with 10 volumes of 1 M HCl and applied as standard solution for HPLC analysis.

Media

The following media were used: Lysogeny broth (LB) medium modified after Bertani, 1951 was used for cultivation of strains for biotransformation assays: 10 g/L bacto-tryptone, 5 g/L yeast extract, and 5 g/L NaCl. The pH was adjusted to 7.2 with NaOH. For LBi medium, 0.1 g/L CH_3-IMH was added to LB. Growth medium with 0.2 g/L CH_3-IMH (GMii) modified after Syldatk *et al.*, 1990 was used for cultivation of strains for biotransformation assays: 10 g/L glucose, 6.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MgSO_4$*7 H_2O, 0.02 g/L $MnCl_2$*4 H_2O, 0.02 g/L $FeSO_4$*7 H_2O, 0.02 g/L $CaCl_2$*2 H_2O, 0.28 g/L citrate, 335 mL potassium sodium phosphate buffer (with K_2HPO_4 and NaH_2PO_4, 0.2 M; pH 6.8). Growth medium (GMi) was prepared as GMii but with 0.1 g/L CH_3-IMH. Screening medium (ScM) consisted of 0.19 g/L PheDU, 0.21 g/L glucose, 0.01 g/L yeast extract, 0.02 g/L $MgSO_4$*7 H_2O, 0.01 g/L $FeSO_4$*7 H_2O, 0.01 g/L $CaCl_2$*2 H_2O, 0.05 g/L citrate, 100 mL potassium sodium phosphate buffer (with K_2HPO_4 and NaH_2PO_4, 0.2 M; pH 7). For ScM plates, 15 g/L agarose was added to ScM medium.

Bacterial strains

The bacterial strains used within this study were described elsewhere: *Escherichia coli* JM109 carrying the plasmid pMW11 coding for the D-hydantoinase of *Arthrobacter crystallopoietes* DSM20117 (Werner *et al.*, 2004), *E. coli* JM109 strain carrying pJOE5702.1 coding for the D-hydantoinase of *Ochrobactrum* sp. G21 DSM18828 and *E. coli* JM109 strain carrying pJOE5704.1 coding for the D-hydantoinase of *Delftia* sp. I24 DSM18833 (Dürr *et al.*, 2008). The wild-type strains *Arthrobacter* sp. E7, *Bacillus* sp. A16, *Bacillus* sp. G18, *Burkholderia* sp. M3, *Flavobacterium* sp. F8, *Pseudomonas* sp. G7, *Pseudomonas* sp. M18, *Microbacteriacae* sp. K3, *Ochrobactrum* sp. C15, and *Ochrobactrum* sp. F21 were described by Dürr *et al.*, 2006. The strains 728 and 735 were isolated from marine sediments and kindly provided by Dr. A. Puñal. The strains *Arthrobacter polychromogenes* DSM20136 and *A. polychromogenes* DSM342 were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ). The recombinantly expressed D-hydantoinases of *Ochrobactrum* sp. G21 DSM18828 and *Delftia* sp. I24 DSM18833 (Dürr *et al.*, 2008), the bacterial strains isolated by Dürr *et al.*, 2006 and the *A. polychromogenes* strains DSM20136 and DSM342 were chosen for this study due to their ability to hydrolyze aryl-substituted hydantoins, DU, and/or PheDU (according to first experiments conducted by Dürr in course of his doctoral thesis, Dürr, 2007). The isolates provided by Dr. A. Puñal were known to show activity towards IMH and DU (personal communication).

Screening and isolation of strains with dihydropyrimidinase and carbamoylase activity

Soil samples originating from the Namib desert (Namibia) were used for the screening experiments. To screen for strains able to use PheDU as sole nitrogen source, 10 mL ScM and small amount of soil sample were inoculated in a test tube and incubated at 30 °C and 140 rpm. After one week of incubation, 1 mL of the inoculum was sterilely transferred in a new test tube containing 9 mL of fresh ScM and incubated for another week at 30 °C and 140 rpm. This procedure was repeated four times. Afterwards, the inoculum was diluted and plated on ScM plates and incubated at room temperature until colonies were visible. Single colonies were isolated by plating on LB-plates. To test for dihydropyrimidinase and/or carbamoylase activity a single colony of a newly isolated strain was inoculated into 4 mL LB and incubated at 30 °C and 140 rpm overnight. 0.5 mL of this preculture were added to 20 mL ScM in a 100-mL shaking flask and incubated at 30 °C and 140 rpm for one week. Finally, the culture was centrifuged and the supernatants were analyzed by HPLC.

Cultivation conditions

E. coli JM109 carrying the plasmid pMW11 was cultivated as described by Werner *et al.*, 2004 in LB medium supplemented with ampicillin 100 µg/mL and ZnSO4 0.1 mol/L. *E. coli* JM109 strain carrying plasmid pJOE5702.1, *E. coli* JM109 strain carrying pJOE5704.1, and *E. coli* JM109 were grown as described by Dürr *et al.*, 2008 but induction time was 16 h. Wild-type strains were cultivated as follows: A bacterial colony was inoculated in 4 mL LB

at 30 °C, 140 rpm overnight. The resulting preculture was added to 100 mL of appropriate medium in a 1 L shaking flask and incubated at 30 °C and 140 rpm. The following media were used for the main cultures: GMii for the wild-type strains described by Dürr *et al.*, 2006 and for *A. polychromogenes* DSM20136; GMi for *A. polychromogenes* DSM342, LB for strain 728 and NA04-01 and LBi for strain 735.

Assay of enzyme activity

Cells were harvested by centrifugation (8000 x g, 10 min, 12 °C) after the appropriate induction time (*E. coli* strains) or in the late exponential growth phase (wild-type strains). The supernatant was discarded and the cells were washed twice with potassium phosphate buffer (0.1 M, pH 8) followed by centrifugation. Finally resting cells were obtained by resuspending the cells in a small volume of the same buffer. Preparation of substrate solutions: the assay substrates PheDU, PheHyd, and IMH were dissolved in potassium phosphate buffer (0.1 M, pH 8) to a concentration of 4 mM assisted by heating to 70 °C for 30 min. *p*ClPheDU was dissolved in potassium phosphate buffer (0.1 M, pH 8) to a concentration of 2 mM assisted by heating to 70 °C for 30 min. In later experiments, *p*ClPheDU was dissolved in DMSO to a concentration of 400 mM due to its poor water solubility. *N*CβPhe and *N*CTrp were dissolved to a concentration of 4 mM in potassium phosphate buffer (0.1 M, pH 8). Biotransformation reactions with PheDU, IMH, PheHyd, *N*CTrp, or *N*CβPhe were started by the addition of 500 µL substrate solution to 500 µL suspension of resting cells. For biotransformation reactions with the recombinantly expressed hydantoinases, a 2 mM solution of *p*ClPheDU in potassium phosphate buffer was applied. In later biotransformation reactions, *p*ClPheDU was dissolved to a concentration of 400 mM in DMSO and 5 µL of this substrate solution were added to 495 µL of potassium phosphate buffer (0.1 M, pH 8) and subsequently biocatalysis was started by adding 500 µL suspension of resting cells. All assays were carried out in a thermomixer at 40 °C at 1400 rpm for 24 h. Reactions were stopped by centrifugation (13.000 x g, 1 min). Supernatants were harvested and stored at -28 °C until analysis. For long-term biotransformation assays cells were grown and harvested as described above. The substrate PheDU was weighted into a 15 mL falcon tube and incubated with 1 volume of phosphate buffer (0.1 M, pH 8) at 40 °C, 700 rpm for 0.5 h. Subsequently 1 volume of suspension of resting cell was added. The final substrate concentration of the assay was 25 mM. A blank sample with 1 volume of phosphate buffer instead of the cell suspension was prepared as described above. Due to its low solubility, the substrate was not completely solved when the reaction was started. The assays were incubated at 40 °C and 700 rpm. At different time points, samples were taken, centrifuged (13.000 x g, 1 min), and the supernatants were harvested and stored at -28 °C until analysis.

Analysis

All substrate and product concentrations were analyzed by HPLC on an Agilent 1100 system (Agilent Technologies, Santa Clara, USA) using a Nucleodur 100-5 C18 ec column (Macherey-Nagel, Germany). The mobile phase for the analysis of PheDU, *N*CβPhe and βPhe, IMH, *N*CTrp, tryptophan (Trp), PheHyd, *N*-carbamoyl-α-phenylglycine (*N*CPheGly) and phenyl-

glycine (PheGly) consisted of 20 % MeOH/80 % (0.1 % (v/v) H_3PO_4 (pH 3.0, NaOH)). The flow rate was 0.8 mL/min, the temperature 30 °C and the detection wavelength 210 nm. For analysis of *p*ClPheDU and *p*Cl*N*CβPhe the mobile phase was composed of 40 % MeOH/60 % 0.04 M potassium phosphate buffer (pH 6.5). The flow rate was 0.8 mL/min, the temperature 30 °C and the detection wavelength 210 nm. Chiral analysis of *N*CβPhe was carried out with a Chiralpak QN-AX column (Daicel, Chiral Technologies Europe; France). The mobile phase was prepared according to manufacturer's instructions and consisted of 90 % MeOH/10 % acetic acid 0.2 M with an apparent pH of 6 adjusted with concentrated aqueous ammonia. The flow rate was 0.8 mL/min, the temperature 25 °C and the detection wavelength 210 nm.

16SrDNA preparation and sequencing

To extract genomic DNA from bacterial isolates, the ZR Soil Microbe DNA Kit ™was applied according to the manufacturer's instructions. Quality and quantity of genomic DNA was controlled by agarose gel electrophoresis using 1 % agarose in TBE buffer. The gel was stained with ethidium bromide solution and analyzed under UV light. The polymerase chain reaction to amplify the 16SrRNA gene was performed in a total volume of 25 µL containing the appropriate reaction buffer and reagents, the universal bacterial primer sequences 27f (5'-AGAGTTTGATC(AC)TGGCTCAG-3') and 1385r (5'-CGGTGTGT(AG)CAAGGCCC-3') (Lane 1991) and the HotStarTaq from Qiagen following the manufacturer's instructions. A Mastercycler gradient (Eppendorf) was used, and the conditions were as follows: initial denaturation (15 min, 95 °C), followed by 30 cycles of denaturation (1 min, 94 °C), annealing (1 min, 50 °C) and primer extension (1 min, 72 °C) and final primer extension (10 min, 72 °C). The PCR products were sent to sequencing (GATC Biotech AG, Germany) and were identified by comparison with the GenBank database (Benson *et al.*, 2006).

Results

Taxonomical identification of new strains

The isolates 728, 735, and NA04-01 were identified by amplification and sequencing of the 16SrDNA gene and subsequent comparison with the GenBank database (see Table 5.3). The strains 728 and 735 showed highest identity with strains belonging to the genus *Aminobacter*. Isolate NA04-01 displayed highest identity with a strain from the genus *Rhizobium*. All three isolates are members of the phylum *α-Proteobacteria*. The 16SrDNA gene sequences of the novel isolates and of the strains with dihydropyrimidinase activity isolated by Dürr *et al.*, 2006 were submitted to the EMBL Nucleotide Sequence Database, and these strains were deposited at the DSMZ. The strains and the 16SrDNA data are deposited under the following numbers: *Rhizobium* sp. NA04-01 = *Rhizobium* sp. DSM24917 (HE578129), *Aminobacter* sp. 728 = *Aminobacter* sp. DSM24754 (HE578126), *Aminobacter* sp. 735 = *Aminobacter* sp. DSM24755 (HE578127), *Arthrobacter* sp. E7 = *Arthrobacter* sp. DSM24883 (HE578128), *Bacillus* sp. A16 = *Bacillus* sp. DSM25052 (HE578130), *Ochrobactrum* sp. F21 = *Ochrobactrum* sp. DSM25042 (HE578131).

Table 5.1 Results of biotransformation experiments after incubating resting cells with the substrates PheDU, *p*ClPheDU and *N*CβPhe (see Fig. 5.1) for 24 h.

Biocatalyst	Hydrolysis of PheDU to *N*CβPhe	Selectivity for PheDU	Hydrolysis of *p*ClPheDU to *p*Cl*N*CβPhe	Hydrolysis of *N*CβPhe to βPhe	Source of biocatalyst
pMW11; D-hydantoinase of *Arthrobacter crystallopoietes* DSM20117	+	L	+		Werner *et al.*, 2004
pJOE5702.1; D-hydantoinase of *Ochrobactrum* sp. G21 DSM18828	+	D,L	+		Dürr *et al.*, 2008
pJOE5704.1; D-hydantoinase of *Delftia* sp. I24 DSM18833	+	D,L	+		Dürr *et al.*, 2008
Arthrobacter polychromogenes DSM20136	+	D	-	-	DSMZ
A. polychromogenes DSM342	+	D	-	-	DSMZ
Arthrobacter sp. E7 DSM24883	+	D	+	n.t.	Dürr *et al.*, 2006
Bacillus sp. A16 DSM25052	+	D	+	-	Dürr *et al.*, 2006
Bacillus sp. G18	-	n.t.	n.t.	-	Dürr *et al.*, 2006
Burkholderia sp. M3	-	n.t.	n.t.	-	Dürr *et al.*, 2006
Flavobacterium sp. F8	-	n.t.	n.t.	-	Dürr *et al.*, 2006
Microbacteriacae sp. K3	-	n.t.	n.t.	-	Dürr *et al.*, 2006
Pseudomonas sp. G7	-	n.t.	n.t.	-	Dürr *et al.*, 2006
Pseudomonas sp. M18	-	n.t.	n.t.	-	Dürr *et al.*, 2006
Ochrobactrum sp. C15	-	n.t.	n.t.	-	Dürr *et al.*, 2006
Ochrobactrum sp. F21 DSM25042	+	n.t.	+	-	Dürr *et al.*, 2006
Aminobacter sp. 728 DSM24754	+	D,L	+	-	this study
Aminobacter sp. 735 DSM24755	+	D	+	-	this study
Rhizobium sp. NA04-01 DSM24917	+	D	+	-	this study

The three recombinantly expressed D-hydantoinases were not tested for carbamoylase activity with *N*CβPhe

"+" hydrolysis product detected, "-" no significant amount of hydrolysis product was detected, *n.t.* not tested, *D,L* unselective hydrolysis of PheDU-enantiomers, *D* selective for D-PheDU-enantiomer, *L* selective for L-PheDU-enantiomer

Biotransformation experiments

In first biotransformation experiments, three D-hydantoinases and several bacterial isolates with known activity towards aryl-substituted hydantoins were screened for their ability to hydrolyze the aryl-substituted dihydropyrimidines PheDU and *p*ClPheDU (see Fig. 5.1b). The results are summarized in Table 5.1.

Table 5.2 Chiral analysis of produced *N*CβPhe after biotransformation experiments with resting cells and 2 mM PheDU for 24 h

Biocatalyst	Cultivation medium	Cell dry weight g/L	Conversion %	Enantiomeric excess (of D- or L-*N*CβPhe)
pMW11; D-hydantoinase of *Arthrobacter crystallopoietes* DSM20117	LB	6	27	61 (L)
pJOE5702.1; D-hydantoinase of *Ochrobactrum* sp. G21 DSM18828	LB	1	85	5 (D)
pJOE5704.1; D-hydantoinase of *Delftia* sp. I24 DSM18833	LB	1	50	6 (D)
Arthrobacter polychromogenes DSM20136	GMii	6	12	90 (D)
A. polychromogenes DSM342	GMi	5	24	96 (D)
Arthrobacter sp. E7 DSM24883	GMii	9	47	45 (D)
Bacillus sp. A16 DSM25052	GMii	15	29	51 (D)
Aminobacter sp. 728 DSM24754	LB	3	78	3 (D)
Aminobacter sp. 735 DSM24755	LBi	9	44	61 (D)
Rhizobium sp. NA04-01 DSM24917	LB	9	30	20 (D)

Dihydropyrimidinase activity of D-hydantoinases

Three D-hydantoinases recombinantly expressed in *E. coli* JM109 and as a negative control the same *E. coli* JM109 without a plasmid were applied to resting cell biotransformation reactions with the two unnatural dihydropyrimidines. No dihydropyrimidinase activity was detected with *E. coli* JM109 as expected. The D-hydantoinase from *A. crystallopoietes* DSM20117 (pMW11) showed activity towards both dihydropyrimidines. Chiral analysis of the produced *N*CβPhe revealed that the enzyme preferably hydrolyzed L-PheDU to L-*N*CβPhe (see Table 5.2). The D-hydantoinases from *Ochrobactrum* sp. G21 DSM18828 (pJOE5702.1) and from *Delftia* sp. I24 DSM18833 (pJOE5704.1) were able to hydrolyze PheDU and *p*ClPheDU to the corresponding *N*-carbamoyl-β-amino acids without displaying a significant enantioselectivity (see Table 5.2).

Dihydropyrimidinase activity of wild-type strains

For the wild-type strains described by Dürr *et al.*, 2006 and *A. polychromogenes* DSM20136 growth curves in GMii were determined. *A. polychromogenes* DSM342 growth was measured in GMi. Subsequently, the strains were grown in their appropriate medium until they reached the late exponential growth phase and were then applied to biotransformation experiments with PheDU, *p*ClPheDU, and *N*CβPhe, respectively. No carbamoylase activity towards *N*CβPhe was detected for any of the tested strains. But the isolates *A. polychromogenes* DSM20136, *A. polychromogenes* DSM342, *Arthrobacter* sp. DSM24883, *Bacillus* sp. DSM25052, and *Ochrobactrum* sp. DSM25042 showed dihydropyrimidinase activity towards PheDU. Chiral HPLC analysis of the *N*CβPhe enantiomers revealed that strain *Arthrobacter* sp. DSM24883, *Bacillus* sp. DSM25052 and the two *A. polychromogenes* strains preferably hydrolyzed the D-PheDU enantiomer to D-*N*CβPhe (see Table 5.2). Additionally, dihydropyrimidinase activity towards *p*ClPheDU was detected for the isolates *Arthrobacter* sp. DSM24883, *Bacillus* sp. DSM25052, and *Ochrobactrum* sp. DSM25042.

Since no growth in GMi was detected for the strains *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755, growth in LB and LBi was tested and biotransformation experiments with PheDU were conducted. For *Aminobacter* sp. DSM24754, a slightly higher activity towards PheDU when grown in LB and for *Aminobacter* sp. DSM24755, a higher activity towards PheDU when grown in LBi was detected (data not shown). Consequently, these media were used in further experiments with these strains. As already stated, both isolates were able to hydrolyze PheDU to *N*CβPhe. Chiral HPLC analysis showed that strain *Aminobacter* sp. DSM24754 was not able to differentiate between the two PheDU-enantiomers while strain *Aminobacter* sp. DSM24755 preferably converted D-PheDU to D-*N*CβPhe (see Table 5.2). Both strains were also able to hydrolyze *p*ClPheDU but none of the strains displayed carbamoylase activity towards *N*CβPhe.

Rhizobium sp. DSM24917 was isolated by screening for bacteria with amidase activity towards PheDU and *N*CβPhe (see below). It was applied in biotransformation experiments with PheDU, *p*ClPheDU and *N*CβPhe. The isolate exhibited dihydropyrimidinase activity towards *p*ClPheDU, and PheDU but carbamoylase activity towards *N*CβPhe was not detected. Chiral HPLC revealed that *Rhizobium* sp. DSM24917 had a slight enantioselectivity for the D-enantiomer of PheDU (see Table 5.2).

Dihydropyrimidinase activity compared with hydantoinase activity

For some biocatalysts, further biotransformation experiments using the hydantoins PheHyd and IMH and the *N*-carbamoyl-α-amino acid *N*CTrp were conducted and compared with the experiments with the dihydropyrimidine substrates (see Fig. 5.2). While none of the tested strains showed any carbamoylase activity, most strains were able to hydrolyze the hydantoin substrates. Strain *A. polychromogenes* DSM342 solely cleaved PheDU to *N*CβPhe while all other biocatalysts catalyzed the hydrolysis of all applied aryl-substituted hydantoins and aryl-substituted dihydropyrimidines. Under the used conditions, phenylhydantoin was the best substrate for the isolates *Bacillus* sp. DSM25052 and *Rhizobium* sp. DSM24917 and for the two recombinantly expressed D-hydantoinases *E. coli* JM109 (pJOE5702.1) and *E. coli* JM109 (pJOE5704.1). Interestingly, the latter two enzymes hydrolyzed the dihydro-

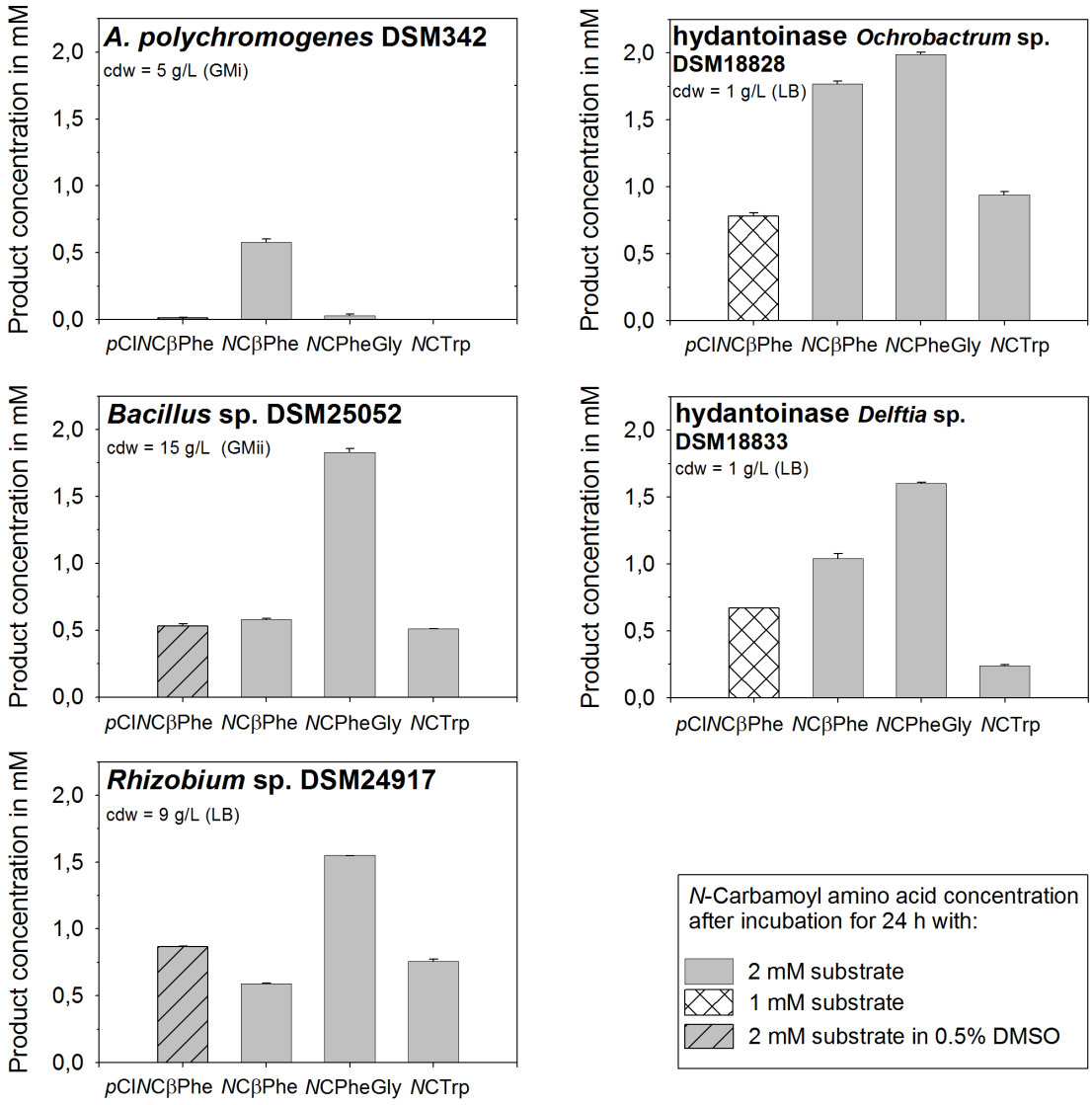

Figure 5.2 Product concentrations measured after 24 h of resting cell biotransformation experiments with the wild-type strains *A. polychromogenes* DSM342, *Bacillus* sp. DSM25052 and *Rhizobium* sp. DSM24917 and the two recombinantly expressed hydantoinases *E. coli* JM109 pJOE5702.1 (originating from *Ochrobactrum* sp. G21 DSM18828) and *E. coli* JM109 pJOE5704.1 (originating from *Delftia* sp. I24 DSM18833) with four different substrates. *para*-Chloro-phenyldihydrouracil (*p*ClPheDU) is converted to *para*-chloro-*N*-carbamoyl-β-phenylalanine (*p*ClNCβPhe), phenyldihydrouracil (PheDU) is converted to *N*-carbamoyl-β-phenylalanine (*N*CβPhe); phenylhydantoin (PheHyd) is converted to *N*-carbamoyl-α-phenylglycine (*N*CPheGly) and indolylmethyl hydantoin (IMH) is converted to *N*-carbamoyl-α-tryptophan (*N*CTrp). The substrate concentrations were 2 mM except for *p*ClPheDU, which was applied in biotransformation experiments with the recombinantly expressed hydantoinases at a concentration of 1 mM.

pyrimidine PheDU more efficiently than the hydantoin IMH. Additionally, the conversion of *p*ClPheDU was 78 % for pJOE5702.1 and 67 % for pJOE5704.1 taking into account that the initial concentration of this substrate was only 1mM.

Due to the high ee-value for the conversion of PheDU, *A. polychromogenes* DSM342 cells were applied in a long-term biotransformation experiment with a concentration of 25 m M PheDU. The results are illustrated in Fig. 5.3. After 2 h of reaction, conversion of racemic PheDU was only 2 % but enantiomeric excess of D-*N*CβPhe was 96 %. The final conversion of the racemic PheDU was 35 % but with regard to the enantiomers conversion of D-PheDU was about 57 % while only 13 % of L-PheDU was hydrolyzed. This corresponds to an enantiomeric excess of D-*N*CβPhe of 64 %.

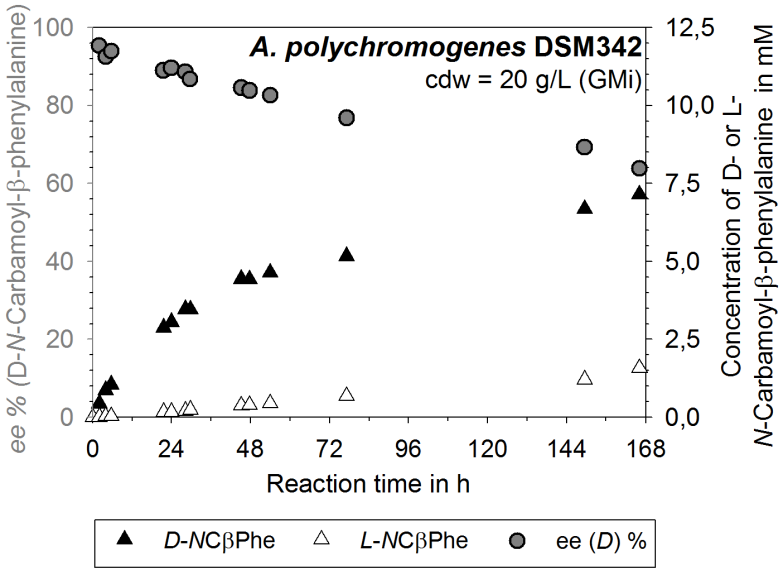

Figure 5.3 Stereoselective conversion of phenyldihydrouracil to *N*-carbamoyl-β-phenylalanine by resting cells of *A. polychromogenes* DSM342. The assay with a volume of 10 mL and an initial substrate concentration of 25 mM was incubated at 40 °C for several days. With chiral HPLC the amount of produced D- (▲) and L-*N*CβPhe (△) was measured and the enantiomeric excess of D-*N*CβPhe (●) was calculated.

Screening and isolation of strains with dihydropyrimidinase and carbamoylase activity

As none of the tested strains of our in-house strain collection was able to hydrolyze *N*CβPhe, a screening for bacteria able to use PheDU as sole source of nitrogen was conducted. Out of about 65 strains isolated, only one strain exhibited dihydropyrimidinase activity towards PheDU (*Rhizobium* sp. DSM24917, see Table 5.1) and none of the strains showed any carbamoylase activity towards *N*CβPhe. All isolated strains were also able to grow in screening medium without PheDU.

Table 5.3 Results of 16SrDNA sequencing

Isolate	Deposited at DSMZ as	EMBL accession number	Closest relative in GenBank	%[a]/bp
728	*Aminobacter* sp. DSM24754	HE578126	*Aminobacter* sp. COX (AY307924.1)	99/1103
735	*Aminobacter* sp. DSM24755	HE578127	*Aminobacter* sp. MSH1 (DQ401867.1)	99/1033
NA04-01	*Rhizobium* sp. DSM24917	HE578129	*Rhizobium* sp. RK22 (EF437256.1)	99/1071

DSMZ = German Collection of Microorganisms and Cell Cultures, *EMBL accession number* = accession number for the 16SrDNA sequence data at the EMBL nucleotide sequence archive, *number in brackets* = GenBank accession number, *bp* = sequence length in base pairs
[a]% = % similarity with closest relative in GenBank

Discussion

Dihydropyrimidinase activity of D-hydantoinases

Dihydropyrimidinase activity of three well-studied D-hydantoinases and of several wild-type strains was demonstrated by biotransformation experiments. The recombinantly expressed enzymes of *A. crystallopoietes* DSM20117 (pMW11), *Ochrobactrum* sp. G21 DSM18828 (pJOE5702.1), and *Delftia* sp. I24 DSM18833 (pJOE5704.1) hydrolyzed the aryl-substituted dihydropyrimidines PheDU and *p*ClPheDU to the corresponding *N*-carbamoyl-β-amino acids. For D-hydantoinase of *A. crystallopoietes* DSM20117, a wide substrate specificity for 5'-monosubstituted hydantoins with aliphatic and aromatic side chains and with the unsubstituted dihydrouracil is described while no activity towards dihydroorotic acid is reported. Moreover, the *A. crystallopoietes* DSM20117 hydantoinase is described to be strictly D-selective for IMH and methylthioethyl hydantoin (MeTH) (Siemann *et al.*, 1999; Werner *et al.*, 2004). This is contrary to the finding that the enzyme hydrolyzes both PheDU enantiomers but with preference for L-PheDU. A substrate-dependent stereoselectivity is reported for several hydantoinases. *Arthrobacter aurescens* DSM 3745 hydantoinase is described to be strictly L-selective for IMH conversion but is able to hydrolyze both enantiomers of MeTH with a slight preference for D-MeTH (May *et al.*, 1998). The dihydropyrimidinase of *Brevibacillus agri* NCHU1002 is reported to hydrolyze *para*-hydroxyphenyl hydantoin (HPH) strictly D-specific but to catalyze the conversion of homophenylalanyl hydantoin (HPAH) without displaying any enantioselectivity (Kao *et al.*, 2008).

For the D-hydantoinases from *Ochrobactrum* sp. G21 DSM18828 (pJOE5702.1) and *Delftia* sp. I24 DSM18833 (pJOE5704.1), conversion of IMH and benzylhydantoin (BnH) was demonstrated (Dürr *et al.*, 2008). For the wild-type strains of these recombinant enzymes, the degradation of DU was described and a D-selectivity for BnH conversion was detected (Dürr *et al.*, 2006). In this study, both enzymes exhibited activity towards PheDU and *p*ClPheDU. But neither pJOE5702.1 nor pJOE5704.1 showed any enantioselectivity for PheDU.

To our knowledge, this is the first hydrolysis of the aryl-substituted dihydropyrimidines

PheDU and *p*ClPheDU by D-hydantoinases to be reported. Solely for the dihydropyrimidinase from *Sinorhizobium meliloti* CECT4114 activity towards several alkyl-substituted dihydropyrimidines is described (Martínez-Rodríguez *et al.*, 2010). For this enzyme, D-selectivity for 5'-monosubstituted hydantoins was shown but the selectivity towards the tested dihydropyrimidines is not reported.

Dihydropyrimidinase activity of wild-type strains

In this study, 8 of 15 tested wild-type strains exhibited significant activity towards PheDU (see Table 5.1). When the stereoselectivity of PheDU hydrolysis was examined, the strains *Aminobacter* sp. DSM24755, *A. polychromogenes* DSM20136, *A. polychromogenes* DSM342, *Arthrobacter* sp. DSM24883, *Bacillus* sp. DSM25052, and *Rhizobium* sp. DSM24917 were found to preferably convert the D-PheDU enantiomer to D-*N*CβPhe. For *Arthrobacter* sp. DSM24883 and *Bacillus* sp. DSM25052, a strict D-specificity for the conversion of BnH was reported (Dürr *et al.*, 2006), while for the other strains, no such data are available. The second substrate *p*ClPheDU was hydrolyzed by all tested strains except for the two *A. polychromogenes* strains. This indicates that the enzymes responsible for the detected dihydropyrimidinase activity towards PheDU in the *A. polychromogenes* strains are quite different from the enzymes causing the activities towards PheDU and *p*ClPheDU in the other isolates.

Dihydropyrimidinase activity compared with hydantoinase activity

Five biocatalysts were subjected to further biotransformations with several hydantoinase substrates (see Fig. 5.2). Beside *A. polychromogenes* DSM342 all tested biocatalysts hydrolyzed the aryl-substituted hydantoins PheHyd and IMH. Interestingly, the recombinantly expressed hydantoinases converted the dihydropyrimidine PheDU to a higher degree than the hydantoin IMH. All of the three recombinantly expressed hydantoinases exhibited hydantoinase activity and dihydropyrimidinase activity and for the dihydropyrimidinase from *S. meliloti* CECT4114 also both enzyme activities are described (Martínez-Rodríguez *et al.*, 2010). For this reason, we hypothesize that the enzymatic activities towards hydantoins and dihydropyrimidines in the wild-type strains also originate from one enzyme able to show activity towards both substrate types respectively.

For *A. polychromogenes* DSM342 only activity towards PheDU was detected whereas *p*ClPheDU and the hydantoins PheHyd and IMH were not hydrolyzed. Despite its narrow substrate range, this isolate was subjected to further experiments due to its high selectivity towards the D-enantiomer of PheDU. In a longterm biotransformation experiment with a higher PheDU concentration, the enzyme displayed a good enantioselectivity towards PheDU (see Fig. 5.3). However, the L-enantiomer was converted also but only to a very small extent. This raises the question whether *A. polychromogenes* DSM324 (1) harbors one enzyme enantiopreference for the D-enantiomer or (2) whether there are two different enzymes with different specificities or (3) whether a disparate transport across the cytoplasmic membrane led to the detected enantioselectivity. Further experiments with the purified enzyme have to be performed to answer this question, because examples for all three possibilities were

described in literature. (1) Several hydantoinases are reported to convert hydantoins enantioselectivly meaning that one enantiomer is hydrolyzed much faster than the other one; e.g., for D-hydantoinase of *Agrobacterium tumefaciens* BQL9 it was demonstrated that the enzyme preferably converts the D-enantiomers of ethylhydantoin and MeTH but to also have a slight activity towards the corresponding L-enantiomers (Andújar-Sánchez *et al.*, 2006). (2) On the contrary other hydantoinases are reported to be strictly D- or L-selective for certain substrates, e.g., the D-hydantoinase of *A. crystallopoietes* DSM20117 for D-IMH and D-MeTH or the L-hydantoinase of *A. aurescens* DSM 3747 for L-IMH. Moreover, *Pseudomonas* sp. NS671 is described to harbor two distinct genes displaying hydantoinase activity (Watabe *et al.*, 1992). (3) The transport of dihydropyrimidines across bacterial cell membranes is poorly studied. The structural similar hydantoins are assumed to diffuse more or less easily through membranes depending on the lipophilicity which is influenced by their side chains (Scholl *et al.*, 1999). Recently, a hydantoin transport protein was described for the first time. The preferred substrates of this protein from *Microbacterium liquefaciens* were IMH and BnH. Interestingly the transporter showed an enantioselectivity for the L-enantiomer respectively (Suzuki & Henderson, 2006).

Carbamoylase activity

Bacterial hydantoinase genes are often organized in clusters together with a hydantoin racemase and a carbamoylase (Dürr *et al.*, 2008). For that reason, the wild-type strains were also tested for carbamoylase activity towards *N*CβPhe. However, no activity towards this *N*-carbamoyl-β-amino acid was detected with any of the strains. Additional biotransformation experiments with *N*CTrp were conducted with the strains *A. polychromogenes* DSM342, *Bacillus* sp. DSM25052, and *Rhizobium* sp. DSM24917. This *N*-carbamoyl-α-amino acid was not hydrolyzed by one of these isolates either. The reasons for these results may be that none of the strains possesses a carbamoylase gene or that the gene is not expressed or that the carbamoylase has no activity towards the two tested substrates. Similar results were also reported by Gokhale *et al.*, 1996 for three Pseudomonas strains. These bacteria showed hydantoinase activity towards PheHyd, but no carbamoylase activity towards the further hydrolysis of the produced *N*CPheGly was detected.

In order to find bacteria possessing dihydropyrimidinase and carbamoylase activity towards the complete conversion of PheDU via *N*CβPhe to βPhe a screening with PheDU as sole source of nitrogen was conducted. But only one strain (*Rhizobium* sp. DSM24917) exhibiting dihydropyrimidinase with PheDU was detected. None of the 65 isolated exhibited carbamoylase activity towards *N*CβPhe. In literature, only the *N*-carbamoyl-β-alanine amidohydrolase of *A. tumefaciens* C58 is described to hydrolyze nonnatural *N*-carbamoyl-β-amino acids. Interestingly, this enzyme shows activity towards *N*-carbamoyl-β-homoalanine but not against *N*CβPhe (Martínez-Gómez *et al.*, 2009).

All of our newly isolated strains were also able to grow in screening medium without PheDU, therefore we assume that mainly nitrogen-fixing bacteria were isolated. This assumption is supported by the 16SrDNA sequencing of strain *Rhizobium* sp. DSM24917 which revealed that this isolate belongs to the typically nitrogen-fixing genus *Rhizobium* (see Table 5.3).

Towards a modified hydantoinase process

In this study, it was demonstrated that hydrolysis of aryl-substituted dihydropyrimidines by D-hydantoinases and strains exhibiting hydantoinase activity is possible. Furthermore, some of the tested biocatalyst showed a stereoselectivity towards the D- or L-PheDU enantiomer. Next steps will be to elucidate the gene sequences of the responsible hydantoinases. An appropriate carbamoylase able to catalyze the follow-up reaction to the corresponding β-amino acids was not detected. Therefore, further screenings will be conducted and also already described *N*-carbamoyl amino acid hydrolases will be tested for their activity towards the novel substrates described in this study. Finally racemization of dihydropyrimidines and *N*-carbamoyl-β-amino acids is to be studied to gain a modified hydantoinase process with a 100 % yield.

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6 Novel amidases of two *Aminobacter* sp. strains: Biotransformation experiments and elucidation of gene sequences

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AMB Express

The amidase activities of two *Aminobacter* sp. strains towards the aryl-substituted substrates phenylhydantoin, indolylmethyl hydantoin, D,L-6-phenyl-5,6-dihydrouracil and *para*-chloro-D,L-6-phenyl-5,6-dihydrouracil were compared. Both strains showed hydantoinase and dihydropyrimidinase activity by hydrolyzing all substrates to the corresponding *N*-carbamoyl-α- or *N*-carbamoyl-β-amino acids. However, carbamoylase activity and thus a further degradation of these products to α- and β-amino acids was not detected.

Additionally, the genes coding for a dihydropyrimidinase and a carbamoylase of *Aminobacter* sp. DSM24754 were elucidated. For *Aminobacter* sp. DSM24755 a dihydropyrimidinase gene flanked by two genes coding for putative ABC transporter proteins was detected. The deduced amino acid sequences of both dihydropyrimidinases are highly similar to the well-studied dihydropyrimidinase of *Sinorhizobium meliloti* CECT4114. The latter enzyme is reported to accept substituted hydantoins and dihydropyrimidines as substrates. The deduced amino acid sequence of the carbamoylase gene shows a high similarity to the very thermostable *N*-carbamoyl-D-amino acid amidohydrolase of *Pseudomonas* sp. KNK003A.

Keywords: beta-amino acid, hydantoinase, carbamoylase

Introduction

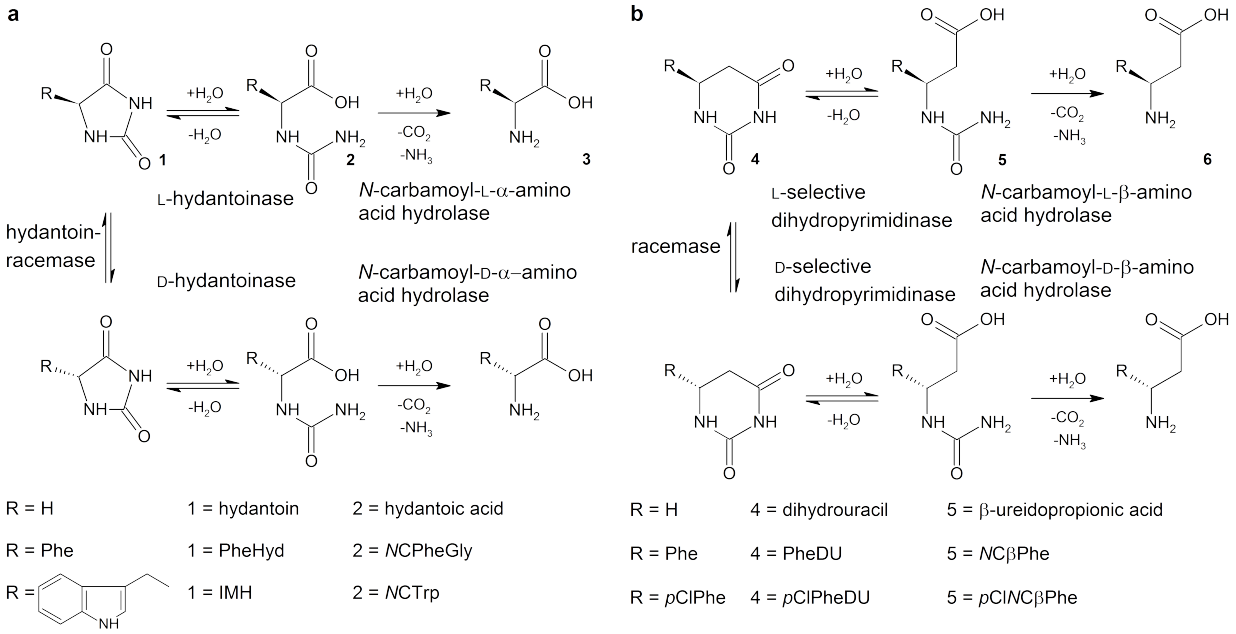

Figure 6.1 a) hydantoinase process for the synthesis of chiral D- or L-α-amino acids starting from racemic 5'-monosubstituted hydantoins via *N*-carbamoyl-α-amino acids applying a hydantoin racemase, a specific hydantoinase, and a specific *N*-carbamoyl-α-amino acid hydrolase; b) proposed modified hydantoinase process for the synthesis of chiral D- or L-β-amino acids starting from racemic 6'-monosubstituted dihydropyrimidines via *N*-carbamoyl-β-amino acids applying a racemase, a dihydropyrimidinase, and a *N*-carbamoyl-β-amino acid hydrolase; (1) 5'-monosubstituted hydantoin, (2) *N*-carbamoyl-α-amino acid, (3) α-amino acid, (4) 6'-monosubstituted dihydropyrimidine, (5) *N*-carbamoyl-β-amino acid, (6) β-amino acid; PheHyd: phenylhydantoin, *N*CPheGly: *N*-carbamoyl-α-phenylglycine, PheDU: phenyldihydrouracil, *N*CβPhe: *N*-carbamoyl-β-phenylalanine, IMH: indolymethyl hydantoin, *N*CTrp: *N*-carbamoyl-α-tryptophan, *p*ClPheDU: *para*-chloro-phenyldihydrouracil, *p*Cl*N*CβPhe: *para*-chloro-*N*-carbamoyl-β-phenylalanine

Hydantoinases (EC 3.5.2.2) were thought to be the microbial counterparts of eukaryotic dihydropyrimidinases. For this reason the terms hydantoinase and dihydropyrimidinase are used synonymously in EC nomenclature. The eukaryotic enzymes catalyze the second step in the reductive pyrimidine degradation pathway by hydrolyzing the dihydropyrimidines dihydrouracil and dihydrothymine to the corresponding *N*-carbamoyl-β-amino acids (Vogels & van der Drift, 1976). However, several bacterial hydantoinases are reported to lack the ability of hydrolyzing these natural substrates, e.g. D-hydantoinase from *Bacillus thermocatenulatus* GH-2, phenylhydantoinase from *Escherichia coli* and hydantoinase from *Agrobacterium* sp. IP 1-671 (Kim *et al.*, 2000; Park *et al.*, 1999; Runser & Meyer, 1993). Therefore, the natural function of hydantoinases is still unclear.

Apart from that, hydantoinases are of high interest as they are utilized for the biocatalytic production of unnatural enantiopure α-amino acids. In the so called hydantoinase process a

racemic hydantoin is converted to a D- or L-*N*-carbamoyl-α-amino acid and subsequently to a D- or L-α-amino acid applying a hydantoin racemase, a D- or L-specific hydantoinase and finally a D- or L-specific *N*-carbamoylase (see figure 6.1a). This industrially applied process has a theoretical yield of 100 %. Nowadays there is also a rising demand for optically pure β-amino acids. These compounds are promising building blocks for pharmaceuticals and fine chemicals (Seebach & Gardiner, 2008). However, the efficient production of chiral β-amino acids is still a challenging task (Weiner *et al.*, 2010).

In a previous study we tested the potential of using a modified hydantoinase process for the production of optically pure β-amino acids (see figure 6.1b). We demonstrated that three recombinant D-hydantoinases were able to convert aryl-substituted dihydropyrimidines to the corresponding *N*-carbamoyl-β-amino acids. Additionally, we detected several bacterial strains exhibiting activity towards aryl-substituted dihydropyrimidines. Some strains, among them *Aminobacter* sp. DSM24755, showed an enantioselective conversion of phenyldihydrouracil (Engel *et al.*, 2011).

Here we report the dihydropyrimidinase and hydantoinase activities of two *Aminobacter* strains. Moreover the genes coding for a carbamoylase and a dihydropyrimidinase in *Aminobacter* sp. DSM24754 and a gene coding for another dihydropyrimidinase in *Aminobacter* sp. DSM24755 are described.

Experimental

Chemicals

Chemicals were of reagent grade and obtained from commercial sources if not stated otherwise. D,L-5-Indolylmethyl-*N*-3-methyl hydantoin (CH_3-IMH) was supplied by former Degussa AG (now Evonik Industries AG). D-β-Phenylalanine (D-βPhe) and L-β-phenylalanine (L-βPhe) were obtained from Pep-Tech Corporation (Burlington, USA). *para*-Chloro-phenyldihydrouracil (*p*ClPheDU) was provided from Fraunhofer Institute for Chemical Technology (Pfinztal Germany). Phenylhydantoin (PheHyd), 5-indolylmethyl hydantoin (IMH) and *N*-carbamoyl-α-tryptophan (*N*CTrp) were synthesized according to (Stark & Smyth, 1963; Suzuki *et al.*, 1973). Phenyldihydrouracil (PheDU), *N*-rac-carbamoyl-β-phenylalanine (*N*CβPhe) and L-*N*CβPhe as standard for HPLC analysis were prepared according to (Dakin & Dudley, 1914; Dürr, 2007). The purity was proven by HPLC and ^{1}H-NMR. PheDU used for biotransformation experiments and *para*-chloro-*N*-carbamoyl-β-phenylalanine (*p*Cl*N*CβPhe) as HPLC standard were prepared as described elsewhere (Engel *et al.*, 2011).

Media

The following media were used: Lysogeny broth (LB) medium modified after Bertani, 1951 was used for cultivation of strains for biotransformation assays and growing cells for DNA extraction: 10 g/L bacto-tryptone, 5 g/L yeast extract and 5 g/L NaCl. The pH was adjusted to 7.2 with NaOH. For LBi medium 0.1 g/L CH_3-IMH was added to LB.

Bacterial strains

The strains *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 were kindly provided by Dr. A. Puñal and first described by Engel *et al.*, 2011.

Cultivation conditions

A bacterial colony was inoculated in 4 mL LB at 30 °C with 140 rpm overnight. The resulting preculture was added to 100 mL LB or LBi in a 1 L shaking flask and incubated at 30 °C and 140 rpm.

Assay of enzyme activity

Cells were harvested in the late exponential growth phase by centrifugation (8000 x g, 10 min, 12 °C). The supernatant was discarded and the cells were washed twice with potassium phosphate buffer (0.1 M, pH 8) followed by centrifugation. Finally resting cells were obtained by resuspending the cells in a small volume of the same buffer.

Preparation of substrate solutions: The assay substrates PheDU, PheHyd and IMH were dissolved in potassium phosphate buffer (0.1 M, pH 8) to a concentration of 4 mM assisted by heating to 70 °C for 30 min. *p*ClPheDU was dissolved in DMSO to a concentration of 400 mM due to its poor water solubility. *N*CβPhe and *N*CTrp were dissolved to a concentration of 4 mM in potassium phosphate buffer (0.1 M, pH 8).

Biotransformation reactions with PheDU, IMH, PheHyd, *N*CTrp or *N*CβPhe were started by the addition of 500 µL substrate solution to 500 µL suspension of resting cells. For biotransformation reactions with *p*ClPheDU 5 µL of this substrate solution were added to 495 µL of potassium phosphate buffer (0.1 M, pH 8) and subsequently biocatalysis was started by adding 500 µL suspension of resting cells. All assays were carried out in a thermomixer at 40 °C, 1400 rpm for 24 h. Reactions were stopped by centrifugation (13.000 x g, 1 min). Supernatants were harvested and stored at −28 °C until analysis.

Analysis

All substrate and product concentrations were analyzed by HPLC on an Agilent 1100 system (Agilent Technologies, Santa Clara, USA) using a Nucleodur 100-5 C18 ec column (Macherey-Nagel, Germany). The mobile phase for the analysis of PheDU, *N*CβPhe, βPhe, IMH, *N*CTrp, tryptophan (Trp), PheHyd, *N*-carbamoyl-α-phenylglycine (NCPheGly) and phenylglycine (PheGly) consisted of 20 % MeOH/80 % (0.1 % (v/v) H_3PO_4, pH 3.0 (NaOH)). The flow rate was 0.8 mL/min, the temperature 30 °C and the detection wavelength 210 nm. For the analysis of *p*ClPheDU and pCl*N*CβPhe the mobile phase was composed of 40 % MeOH/60 % (0.04 M potassium phosphate buffer pH 6.5). The flow rate was 0.8 mL/min, the temperature 30 °C and the detection wavelength 210 nm.

DNA preparation and sequencing of amidase genes

To extract genomic DNA from bacterial isolates the ZR Soil Microbe DNA Kit ™was applied according to the manufacturer's instructions. Quality and quantity of genomic DNA were controlled by agarose gel electrophoresis using 1 % agarose in TBE buffer. The gel was stained with ethidium bromide solution and analyzed under UV light.

Elucidation of amidase gene sequences is based on dihydropyrimidinase gene fragments amplified and provided by Dr. A. Puñal (Karlsruhe Institute of Technology (KIT) former University of Karlsruhe) with the primers dhyd-f (AAACGGTT) (5'-GCCGCAGCATGC-GGNGGNACNAC-3') and dhyd-r (DADIVIWDPNGE) (5'-CACCATTAGGGTCCCATAT-GACTADRTCNGCRT-3') for the strain *Aminobacter* sp. DSM24754 and the degenerate primers dhp-f (AAAFGG) (5'-GCSGCVTTYGGNGGNACNAC-3') and dhp-r (VHAENG) (5'-TCNCCRTTYTCNGCRTGNAC-3') for the strain *Aminobacter* sp. DSM24755 (Dürr, 2007; Lin *et al.*, 2005). The complete gene sequences were amplified by performing a modified TAIL-PCR. For the first TAIL-PCR the arbitrary degenerate primers AD1 and AD2 according to Liu & Whittier, 1995 and the specific primers 728Tf1 (5'-GCTGCATATGTGCGT-CAATGGCTGG-3') and 728Tf2 (5'-GAGCGTGGCGTCAACACCTTCAAG-3') for strain *Aminobacter* sp. DSM24754 and the specific primers 735Tf1 (5'-GTCGACAAGGGCATC-ACCTCGTTC-3') and 735Tf2 (5'-GGTGGACGACGACGAGATGTATTCG-3') for strain *Aminobacter* sp. DSM24755, synthesized by MWG-Biotech (Germany), were used. In contrast to the originally described method, PCR products were separated after the secondary PCR on a 1 % agarose gel. PCR products were purified with the MinElute Gel Extraction Kit (Qiagen, Hilden Germany), ligated into pDrive PCR cloning vector (Qiagen, Hilden Germany), and sequenced (GATC Biotech AG, Germany). Subsequently the sequences were assembled with the known fragments. With the newly identified sequences further specific primers were designed and TAIL-PCR was conducted as described above until the gene sequences were elucidated completely. Sequence fragments were aligned using BioEdit program, BLAST was applied for comparison with other sequences. ClustalW2 was used for global sequence alignments in order to compare the new *Aminobacter* sequences with sequences in the NCBI database (Altschul *et al.*, 1990, 1997; Chenna *et al.*, 2003). The aligned sequences were printed with ESPript (Gouet *et al.*, 1999). For comparison of the *Aminobacter* sp. DSM24754 gene cluster with other prokaryotic genomes the program Comparative Genome Cluster Viewer (CGCV, Revanna *et al.*, 2009) was applied.

Results

Biotransformation experiments

Due to previous results *Aminobacter* sp.DSM24754 was cultivated in LB and strain *Aminobacter* sp. DSM24755 in LBi for all experiments (Engel *et al.*, 2011). The dihydropyrimidinase, hydantoinase and carbamoylase activities of *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 were tested. The results are summarized in figure 6.2. Besides dihydropyrimidinase activity towards PheDU and *p*ClPheDU hydantoinase activity towards PheHyd and IMH was detected for both strains. Under the chosen conditions PheDU and PheHyd were the best substrates for *Aminobacter* sp. DSM24754 and PheHyd was the

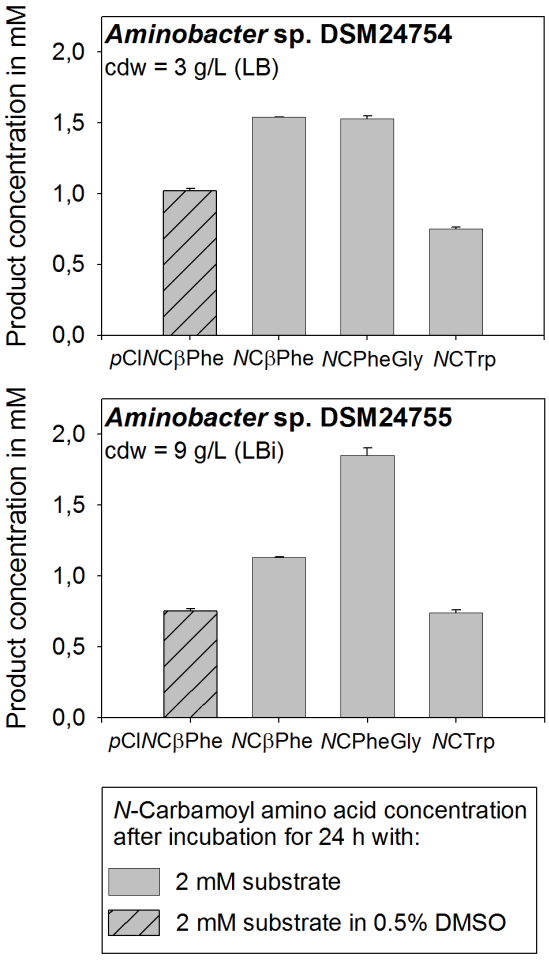

Figure 6.2 Product concentrations measured after 24 h of resting cell biotransformation experiments with *Aminobacter* sp. DSM24754 compared to the results determined for *Aminobacter* sp. DSM24755 with four different substrates: *para*-Chlorophenyldihydrouracil (*p*ClPheDU) is converted to *para*-chloro-*N*-carbamoyl-β-phenylalanine (*p*ClNCβPhe), phenyldihydrouracil (PheDU) is converted to *N*-carbamoyl-β-phenylalanine (*N*CβPhe); phenylhydantoin (PheHyd) is converted to *N*-carbamoyl-α-phenylglycine (*N*CPheGly) and indolymethyl hydantoin (IMH) is converted to *N*-carbamoyl-α-tryptophan (*N*CTrp). The substrate concentrations were 2 mM. cdw = cell dry weight

best substrate for *Aminobacter* sp. DSM24755. None of the *Aminobacter* strains exhibited carbamoylase activity towards the *N*-carbamoyl-β-amino acid *N*CβPhe or the *N*-carbamoyl-α-amino acid *N*CTrp.

Identification of amidase genes

A modified TAIL-PCR was conducted for both strains based on dihydropyrimidinase gene fragments. With this method a 3252 base pairs (bp) long DNA fragment of strain *Aminobacter* sp. DSM24754 and a 3127 bp long DNA fragment of *Aminobacter* sp. DSM24755

were amplified and subsequently sequenced.

Sequence analysis of the genomic DNA fragment of *Aminobacter* sp. DSM24754

The 3252 bp genomic DNA fragment of *Aminobacter* sp. DSM24754 has an overall GC content of 62 % and comprises two complete open reading frames (ORFs) and one incomplete ORF all starting with an ATG start codon (see table 6.1, figure 6.5). The first ORF consists of 942 bp coding for a protein of 313 amino acids. It has the highest amino acid identity of 85 % to the primary sequence of non-putative *N*-carbamoyl-D-amino acid amidohydrolase of *Pseudomonas* sp. KNK003A (BAD00008.1).

ORF2 is located downstream to ORF1 and points into the same direction. It is either 1485 bp or 1458 bp long, as there is a second ATG start codon 27 bp downstream to the first. The real start codon has to be determined by a heterologous expression of the longer and the shorter version of the gene and subsequent activity tests. However, most of the similar enzymes in the database are aligning beginning with the second start codon. A possible Shine-Dalgarno sequence (RBS) is only detected upstream to the second ATG codon (see table 6.1). For these reasons all following identity data are related to this shorter gene product coding for a protein of 485 amino acids. This protein shares highest amino acid identity (83 %) with a putative dihydropyrimidinase of *Mesorhizobium* sp. BNC1 (YP_675206).

The third ORF is 435 bp long, incomplete and points into the opposite direction. Its deduced amino acid sequence shows highest identity of 78 % to a hypothetical protein of *Agrobacterium radiobacter* K84 (YP_002542208.1). The possible function of the gene product of ORF3 remains unclear as it exhibits no clear similarity with non-putative proteins.

The sequence of the 3252 bp genomic DNA fragment of *Aminobacter* sp. DSM24754 was deposited at EMBL database (HE651322).

Sequence analysis of the genomic DNA fragment of *Aminobacter* sp. DSM24755

The 3127 bp DNA fragment has an overall GC content of 63 % and harbors two complete ORFs and one incomplete ORF (see table 6.1, figure 6.5). All ORFs point into the same direction and start with an ATG start codon. The first ORF is 1452 bp long and encodes a protein of 483 amino acids. It shows highest amino acid identity (90 %) to the putative dihydropyrimidinase of *Mesorhizobium loti* MAFF303099 (NP_103173.1).

A second ORF of 822 bp is located downstream to ORF1. Its deduced amino acid sequence displays highest identity (91 %) to a putative ATP-binding protein of an ABC transporter of *Mesorhizobium loti* MAFF303099 (NP_103169.1). The incomplete third open reading frame starts only 4 bp downstream to ORF2 and consists of 521 bp. The deduced partial amino acid sequence exhibits highest identity (88 %) to the transmembrane component of an ABC transporter of *Mesorhizobium amorphae* CCNWGS0123 (ZP_09089625.1).

The sequence of the 3127 bp genomic DNA fragment of *Aminobacter* sp. DSM24755 was deposited at EMBL database (HE651323).

The two genes coding for putative dihydropyrimidinases of the *Aminobacter* sp. strains DSM24754 and DSM24755 have an overall gene sequence identity of 74 %. The identity of the deduced amino acid sequences is 68 %.

Table 6.1 Location and properties of genes and predicted proteins of the sequenced fragments of *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 respectively. (-) no data available, due to an incomplete open reading frame. GC = guanine-cytosine content; aa = amino acids. (*) = values in brackets are for the 1485 bp long gene fragment of ORF2; underlined sequence parts = putative Shine-Dalgarno sequence (RBS), sequence in boldface = assumed start codon, sequence in italics = potential alternative start codon

	Gene	Nucleotide position	GC (%)	Putative RBS	Protein length (aa)	Calculated molecular mass (kDa)	Protein with highest identity in BLAST	Accession number	Organism	Identical aa (%)
DSM24754	ORF1	274-1215	62	TGAGGAGGTA-AGACC**ATG**	313	35	*N*-carbamoyl-D-amino acid amidohydrolase	BAD00008.1	*Pseudomonas* sp. KNK003A	85
	ORF2*	(1238)/ 1265-2722	(62.5)/ 62.7	*ATG*ACGGCAA-TCAATGGGAG-AACGAGC**ATG**	(494)/ 485	(53.8)/ 52.9	putative dihydropyrimidinase	YP_675206	*Mesorhizobium* sp. BNC1	83
	ORF3	3235-2818	59.8	-	-	-	hypothetical protein	YP_002542208.1	*Agrobacterium radiobacter* K84	78
DSM24755	ORF1	80-1531	63.6	TAGGGGAACT-GAAGAA**ATG**	483	53.2	putative phenylhydantoinase	NP_103173.1	*Mesorhizobium loti* MAFF303099	90
	ORF2	1780-2601	62.8	TGGGGTAAAA-CCGGGCA**ATG**	273	67.5	putative ATP-binding protein of ABC transporter	NP_103169.1	*Mesorhizobium loti* MAFF303099	91
	ORF3	2607-3127	62.8	-	-	-	putative permease protein of ABC transporter	ZP_09089625.1	*Mesorhizobium amorphae* CCNWGS0123	88

Discussion

Biotransformation results

In this study the dihydropyrimidine, hydantoin and carbamoyl amino acid hydrolyzing abilities of *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 were determined. Both *Aminobacter* strains showed dihydropyrimidinase and hydantoinase activity towards PheDU, *p*ClPheDU, PheHyd and IMH by converting these substrates to the corresponding *N*-carbamoyl-β- and *N*-carbamoyl-α-amino acids. *Aminobacter* sp. DSM24754 hydrolyzed the same amount of PheHyd and PheDU under the chosen conditions. *Aminobacter* sp. DSM24755 also showed a high conversion of PheHyd but in contrast to the results obtained for the other *Aminobacter* strain the dihydropyrimidine PheDU was not hydrolyzed to the same extend. This may be due to the fact that a D-selectivity for PheDU is described for *Aminobacter* sp. DSM24755 while *Aminobacter* sp. DSM24754 is reported to be unselective for this substrate (Engel *et al.*, 2011). Nothing is known about the stereoselectivity for PheHyd of both biocatalysts. But this substrate is known to spontaneously racemizes under alkaline conditions because of its keto-enol-tautomerism (Pietzsch & Syldatk, 1995). For this reason a potential stereoselectivity of the biocatalyst would probably not influence the biotransformation reaction. The dihydropyrimidine PheDU can not racemize spontaneously due to its different chemical structure. Thus a stereoselective biocatalyst would hydrolyze this substrate less effective.

When only the hydantoins PheHyd and IMH were compared PheHyd appeared to be the better substrate for both biocatalysts as a higher amount of this compound was degraded, respectively. This hydantoin is composed of a phenyl group directly bound to the hydantoin ring, which is highly similar to PheDU consisting of a phenyl group linked to a dihydropyrimidine ring. By contrast, the bulky aromatic indol ring of IMH is connected to the hydantoin ring via a methyl bridge. This difference in substrate structure may be a reason for the differences observed in the experiments. In general D-hydantoinases preferably hydrolyze phenyl-substituted hydantoins while L-hydantoinases show higher activities towards benzyl-substituted hydantoins such as IMH which is assumed to be a consequence of their different three-dimensional structures (Abendroth *et al.*, 2002a).

A further degradation of the resulting *N*-carbamoyl amino acids was not observed. Additionally, no carbamoylase activities were detectable in resting cell biotransformation experiments directly applying *N*CTrp and *N*CβPhe as substrates. We see two possible explanations for these results: Either (i) there are no genes coding for carbamoylases present in the genomes of the two tested *Aminobacter* strains or (ii) the experimental settings prevented the detection of carbamoylase activity. For example the expression of carbamoylases is reported to be inducible (Mei *et al.*, 2008). Consequently, the lack of an inducer during growth of *Aminobacter* sp. DSM24754 or the use of the wrong inducer (CH_3-IMH) during growth of *Aminobacter* sp. DSM24755 may account for not detectable carbamoylase activities. Otherwise it may be that carbamoylases were expressed but not active towards the substrates tested. Explanation (ii) seems to be more plausible as at least for strain *Aminobacter* sp. DSM24754 a gene product coding for a D-carbamoylase was detected (see above).

Comparison with other functionally related carbamoylases

Table 6.2 Comparison of the primary amino acid sequence of the putative carbamoylase found in *Aminobacter* sp. DSM24754 with the amino acid sequences of *Pseudomonas* sp. KNK003 (BAD00008.1), *Agrobacterium radiobacter* CCRC14924 (1FO6), *Agrobacterium* sp KNK712 (1ERZ) and *Agrobacterium tumefaciens* RU-OR HyuC2 (ABS11194.1) within a global alignment.

Amino acid sequence identities and similarities of carbamoylases		**DSM24754**	**BAD00008.1**	**1FO6**	**1ERZ**	**ABS11194.1**
		Identity %				
Aminobacter sp. **DSM24754**			85	58	57	49
Pseudomonas sp. KNK003 **BAD00008.1**	Similarity %	92		59	58	51
Agrobacterium radiobacter CCRC14924 **1FO6**		68	70		96	57
A. sp. KNK712 **1ERZ**		69	70	97		56
A. tumefaciens RU-OR HyuC2 **ABS11194.1**		56	57	64	93	

For strain *Aminobacter* sp. DSM24754 a gene coding for a carbamoylase was identified and the deduced amino acid sequence showed highest amino acid identity (85 %) to the D-carbamoylase of *Pseudomonas* sp. KNK003A (BAD00008.1, Ikenaka *et al.*, 1998). Furthermore it exhibited $49 - 58$ % identity to the three well studied D-carbamoylases of *Agrobacterium radiobacter* CCRC14924 (1FO6) *Agrobacterium* sp. KNK712 (1ERZ) and *Agrobacterium tumefaciens* RU-OR (HyuC2; ABS11194.1) within a global alignment (see table 6.2, figure 6.3,Wang *et al.*, 2001; Nakai *et al.*, 2000; Jiwaji *et al.*, 2009). In this alignement important residues were annotated and the residue numbers refer to the protein sequence of *Aminobacter* sp. DSM24754.

All of the alligned non-putative enzymes are D-carbamoylases able to catalyze the hydrolytic cleavage of *N*-carbamoyl-α-amino acids. For none of these enzymes activity towards *N*-carbamoyl-β-amino acids is decribed (Martínez-Rodríguez *et al.*, 2010b). The residues identified as the catalytic triad (Glu47, Lys127, Cys172), the residues proposed to be essential for enzyme conformation (His129, Glu136, His144, His215) and the residues assumed to be involved in the catalytic mechanism (Arg175, Arg176, Gly194, Thr198) are highly conserved for all sequences aligned (Nakai *et al.*, 2000; Wang *et al.*, 2001; Chen *et al.*, 2003; Han *et al.*, 2009). Due to its sequence characteristics the *Aminobacter* sp. DSM24754 enzyme can also be classified as D-carbamoylase.

Table 6.3 Comparison of the primary amino acid sequences of the putative hydantoinases detected in *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 with the amino acid sequences of *Pseudomonas* sp. KNK003A (BAE20330.1), *Ochrobactrum* sp. G21 (ABS84244.1), *Jannaschia* sp. CCS1 (YP_510647.1), *Sinorhizobium meliloti* CECT4114 3DC8 and *Bacillus stearothermophilus* SD1 (1K1D) within a global alignment.

Amino acid sequence identities and similarities of hydantoinases		**DSM24754**	**DSM24755**	**BAE20330.1**	**ABS84244.1**	**YP_510647.1**	**3DC8**	**1K1D**
		Identity %						
Aminobacter sp. **DSM24754**			69	82	73	73	70	42
Aminobacter sp. **DSM24755**		83		68	74	68	79	46
Pseudomonas sp. KNK003 **BAE20330.1**		91	82		73	71	69	42
Ochrobactrum sp. G21 **ABS84244.1**	Similarity %	86	87	86		69	71	42
Jannaschia sp. CCS1 **YP_510647.1**		85	80	83	81		68	42
Sinorhizobium meliloti CECT4114 **3DC8**		81	89	81	84	78		44
Bacillus stearothermophilus SD1 **1K1D**		59	64	61	60	59	61	

The putative carbamoylase of *Aminobacter* sp. DSM24754 exhibited highest identity to the *Pseudomonas* sp. KNK003A D-carbamoylase, which is described to be the most thermostable carbamoylase known today (Martínez-Rodríguez *et al.*, 2010b). However, the low stability of D-carbamoylases is one major drawback for their efficient use in an industrial hydantoinase process. Therefore various attempts have been made to engineer their thermal and oxidative stability. For the carbamoylase of *A. radiobacter* CCRC14924 it was reported that the residues Gln23, His58, Met184, Val237 and Thr262 influence the stability against temperature and oxidation, the residues Val40 and Gly75 affect the oxidative stability solely (Oh *et al.*, 2002; Chiang *et al.*, 2008), and that the residue Ala302 influences thermostability and catalytic activity (Chiu *et al.*, 2006). Thermal and pH stability of the carbamoylase of *Agrobacterium* sp. KNK712 was shown to be affected by the residues His58, Pro204 and Val237 (Ikenaka *et al.*, 1999). Except for Met184, all residues described to be involved in thermal stability of the two *Agrobacterium* enzymes differ from the residues in the carbamoylase sequences of *Aminobacter* sp. DSM24754 and *Pseudomonas* sp. KNK003A. Remarkably 5

of these 6 substitutions (Asp23, Gly204, Cys237, Ala262, Val302) are identical while one is similar (Val/Leu58) for the both latter enzymes. Furthermore in the D-carbamoylase of *A. radiobacter* CCRC14924 a single mutation of Thr262 to Ala led to a significant increase in oxidative and thermal stability (Oh *et al.*, 2002). The enzymes of *Pseudomonas* sp. KNK003A and *Aminobacter* sp. DSM24754 already possess an Ala residue in this position. Due to its sequences characteristics the *Aminobacter* sp. DSM24754 carbamoylase could have a similar high temperature stability like the*Pseudomonas* sp. KNK003A D-carbamoylase. This is to be studied in more detail within further experiments by its recombinant expression and biochemical characterization.

The recently discovered second carbamoylase of *A. tumefaciens* RU-OR (HyuC2) has as well substitutions in four positions (Arg24, Ile60, Met237, Ser262) compared to the above mentioned seven residues probably important for thermal stability (Jiwaji *et al.*, 2009). Thus the authors assumed that the thermal and oxidative stability of the enzyme may differ from those of the other well characterized *Agrobacterium* enzymes. However, the exchanged residues are neither identical with the residues of *Aminobacter* sp. DSM24754 carbamoylase nor with the corresponding residues of the *Pseudomonas* sp. KNK003A carbamoylase protein sequence.

Comparison with other functionally related hydantoinases

Within a BLAST search against the protein database the deduced amino acid sequence of *Aminobacter* sp. DSM24754 ORF2 exhibited highest identity (83 %) to a putative dihydropyrimidinase of *Mesorhizobium* sp. BNC1. Surprisingly, the second BLAST hit was with the non-putative hydantoinase of *Pseudomonas* sp. KNK003A (BAE20330.1) having an overall identity of 82 %. This is the same *Pseudomonas* strain whose carbamoylase showed highest identity to *Aminobacter* sp. DSM24754 carbamoylase (see section above). When compared to other non-putative enzymes (see table 6.3, figure 6.4) the identities were $70 - 73$ % to the *Ochrobactrum* sp. G21 D-hydantoinase (ABS84244.1), to the D-hydantoinase of *Jannaschia* sp. CCS1 (YP_510647.1) and to the dihydropyrimidinase of *Sinorhizobium meliloti* CECT4114 (3DC8) (Dürr *et al.*, 2008; Cai *et al.*, 2009; Martínez-Rodríguez *et al.*, 2010a). The identity to the well-studied D-hydantoinase of *Bacillus stearothermophilus* SD1 (1K1D) was 42 % (Cheon *et al.*, 2002). The overall identity to the deduced dihydropyrimidinase of *Aminobacter* sp. DSM24755 was 68 % only, although both gene products were detected in strains belonging to the same genus.

The deduced dihyropyrimidinase of *Aminobacter* sp. DSM24755 ORF1 showed highest identity of 91 % to the putative phenylhydantoinase of *Mesorhizobium loti* MAFF303099 in a BLAST search. The highest overall identity of 79 % to a non-putative protein was detected with the dihydropyrimidinase of *S. meliloti* CECT4114. A global alignment with further non-putative enzymes (see table 6.3, figure 6.4) showed that the amino acid identities to *Ochrobactrum* sp. G21 D-hydantoinase, to the D-hydantoinase of *Pseudomonas* sp. KNK003A and to the D-hydantoinase of *Jannaschia* sp. CCS1 were 74 % to 68 %. The primary amino acid identity to the D-hydantoinase of the *B. stearothermophilus* SD1 was only 46 %.

In the global alignment of the protein sequences the residues described to be important

for structure or function were annotated (see figure 6.4). The following residue numbers always refer to the deduced sequence of *Aminobacter* sp. DSM24755 dihydropyrimidinase. Like other dihydropyrimidinases the deduced *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 enzymes possess the highly conserved GxxDxHxH motif (residues 51-58) (May *et al.*, 1998). Together with the histidin residues of this motif the residues Lys146, His179, His235 and Asp312 are suggested to form the catalytic core and are as well completely conserved within the aligned proteins. Furthermore the lysine residue (Lys146) is post-translationally carboxylated in most known hydantoinases/dihydropyrimidinases (Abendroth *et al.*, 2002b; Cheon *et al.*, 2002). It is confirmed for several hydantoinases/dihydropyrimidinases that these active site residues form the binuclear center with divalent metal ions (Martínez-Rodríguez *et al.*, 2010a; Zhang *et al.*, 2010). Due to the fact that these residues are completely conserved in the protein sequences of *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 and due to the overall high sequence similarity with the well-studied dihydropyrimidinase of *S. meliloti* CECT4114 a metal dependence of these newly described enzymes is most likely.

The C-termini of hydantoinases are supposed to be involved in quaternary structure composition (Kim & Kim, 1998, 2002). This region was described to be non-homologous among microbial hydantoinases. However, it was recently reported that the C-termini of hydantoinases are highly conserved for α-*Proteobacteria* (Martínez-Rodríguez *et al.*, 2010a). The C-terminal regions of the deduced amino acid sequences of the α-*Proteobacteria* strains *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 support this assumption. They are highly homologous to the C-termini of *S. meliloti* CECT4114 dihydropyrimidinase, *Ochrobactrum* sp. G21 D-hydantoinase and *Jannaschia* sp. CCS1 hydantoinase (see figure 6.4).

For *S. meliloti* CECT4114 it is suggested that the substrate's hydantoin ring is recognized by residues Tyr152, Ser286 and Asn334, which are highly conserved among hydantoinases (Martínez-Rodríguez *et al.*, 2010a). The corresponding residues in *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 dihydropyrimidinases are identical. The exocylic side chain of the substrate is reported to be recognized by the so called stereochemistry gate loops (SGL), which are less conserved. It was suggested that these SGLs may be involved in determining the substrate specificity of these enzymes (Cheon *et al.*, 2002). For the two *Aminobacter* sp. dihydropyrimidinases the residues of SGL3 (see figure 6.4) are identical to each other and to *S. meliloti* CECT4114 dihydropyrimidinase, *Ochrobactrum* sp. G21 hydantoinase and *Pseudomonas* sp. KNK003A hydantoinase and are highly similar to the *Jannaschia* sp. CCS1 hydantoinase. There are bigger differences with regard to SGL1 and SGL2 among the two *Aminobacter* enzymes. The *Aminobacter* sp. DSM24754 dihydropyrimidinase SGL1 and SGL2 residues are nearly identical to residues of SGL1 and SGL2 in *Jannaschia* sp. CCS1 hydantoinase. In contrast the SGL1 and SGL2 residues of *Aminobacter* sp. DSM24755 dihydropyrimidinase almost completely match with the residues of *S. meliloti* CECT4114 dihydropyrimidinase. These results could indicate that the substrate specificity of the two *Aminobacter* sp. dihydropyrimidinases may be slightly different. This would correspond to the results obtained in the resting cell biotransformation experiments with the two strains.

In the biotransformation experiments *Aminobacter* sp. DSM24754 and *Aminobacter* sp.

DSM24755 exhibited hydantoinase and dihydropyrimidinase activity. This raises the question whether these dihydropyrimidinase and the hydantoinase activities originate from one enzyme or from two different enzymes, a hydantoinase and a dihydropyrimidinase in the respective strain. The *S. meliloti* CECT4114 dihydropyrimidinase is described to hydrolyze substituted five-membered and also six-membered ring substrates (Martínez-Rodríguez *et al.*, 2010a). In a previous study we reported that *Ochrobactrum* sp. G21 hydantoinase, *Delftia* sp. I24 hydantoinase and *Arthrobacter crystallopoietes* DSM20117 hydantoinase can act on substituted hydantoins and on aryl-substituted dihydropyrimidines (Engel *et al.*, 2011). The hydantoinase of *Jannaschia* sp. CCS1 is described to accept hydantoins and to show highest activity towards dihydrouracil (Cai *et al.*, 2009). However, nothing is reported concerning the substrate specificity of *Pseudomonas* sp. KNK003A hydantoinase. Due to the fact that most of the similar hydantoinases and dihydropyrimidinases have hydantoinase and dihydropyrimidinase activity we hypothesize that the detected deduced dihydropyrimidinases of *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 are responsible for both measured activities as well. The major difference was that strain *Aminobacter* sp. DSM24755 showed a D-stereoselectivity for phenyldihydrouracil while strain *Aminobacter* sp. DSM24754 did not (Engel *et al.*, 2011). Whether this is related to the differences in the primary structure of the dihydropyrimidinases has to be elucidated in further experiments with the pure enzymes.

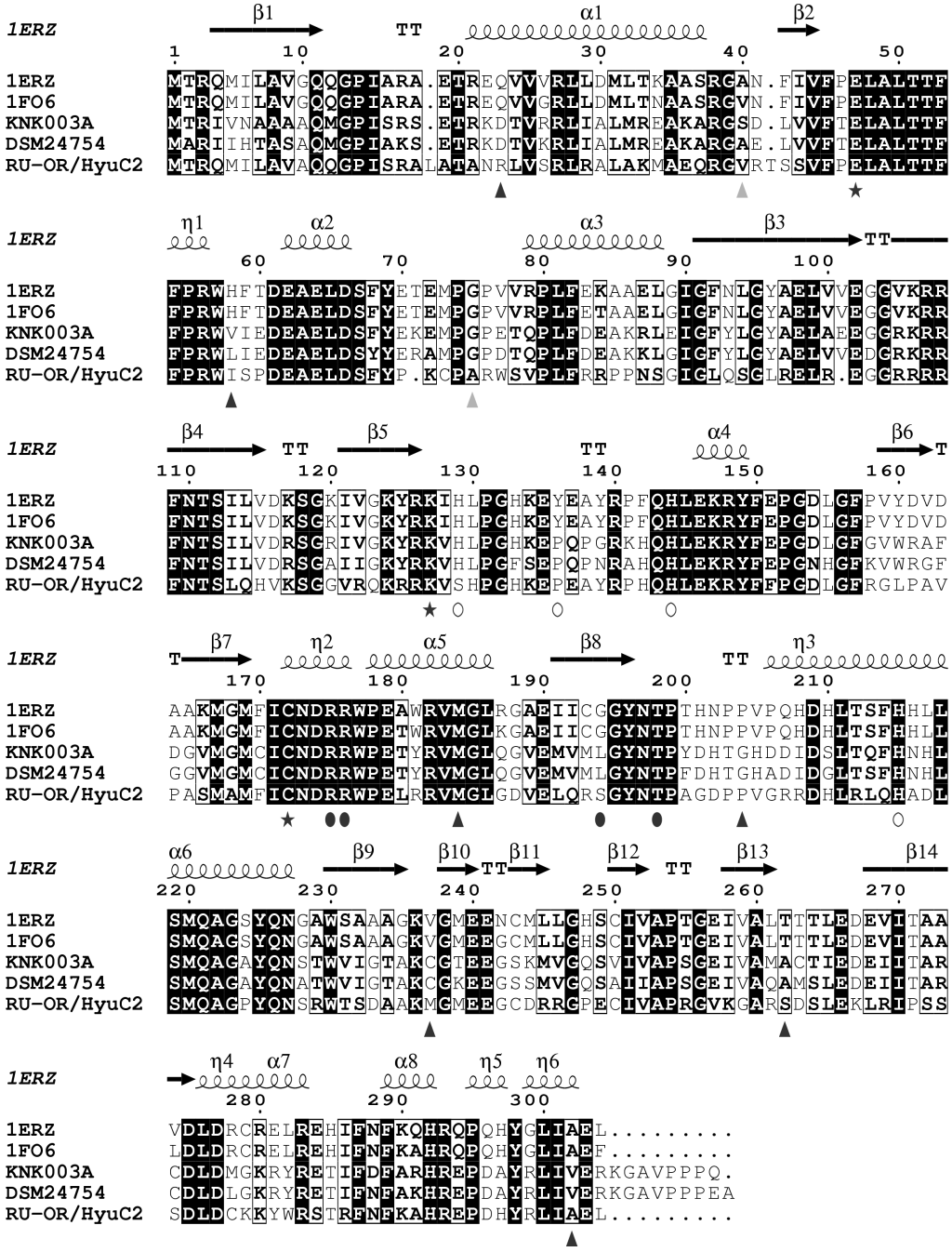

Figure 6.3 Alignment of primary amino acid sequences of the non-putative D-carbamoylases of *Agrobacterium tumefaciens* RU-OR (HyuC2; ABS11194.1), *Pseudomonas* sp. KNK003A (BAD00008.1), *Agrobacterium radiobacter* NRRL B11291 (1FO6), *Agrobacterium* sp. KNK712 (1ERZ) and the newly identified carbamoylase sequence of *Aminobacter* sp.DSM24754. The dark shading symbolizes identical residues while bold letters represents similar residues. (⋆) residues identified as catalytic triad (Chen *et al.*, 2003; Wang *et al.*, 2001; Nakai *et al.*, 2000), (•) residues assumed to play a key role in substrate recognition (Nakai *et al.*, 2000; Wang *et al.*, 2004; Chen *et al.*, 2003), (▲) residues supposed to influence thermal stability (Oh *et al.*, 2002; Ikenaka *et al.*, 1999; Chiu *et al.*, 2006; Chiang *et al.*, 2008), (▲) residues potentially influencing oxidative stability (Oh *et al.*, 2002).

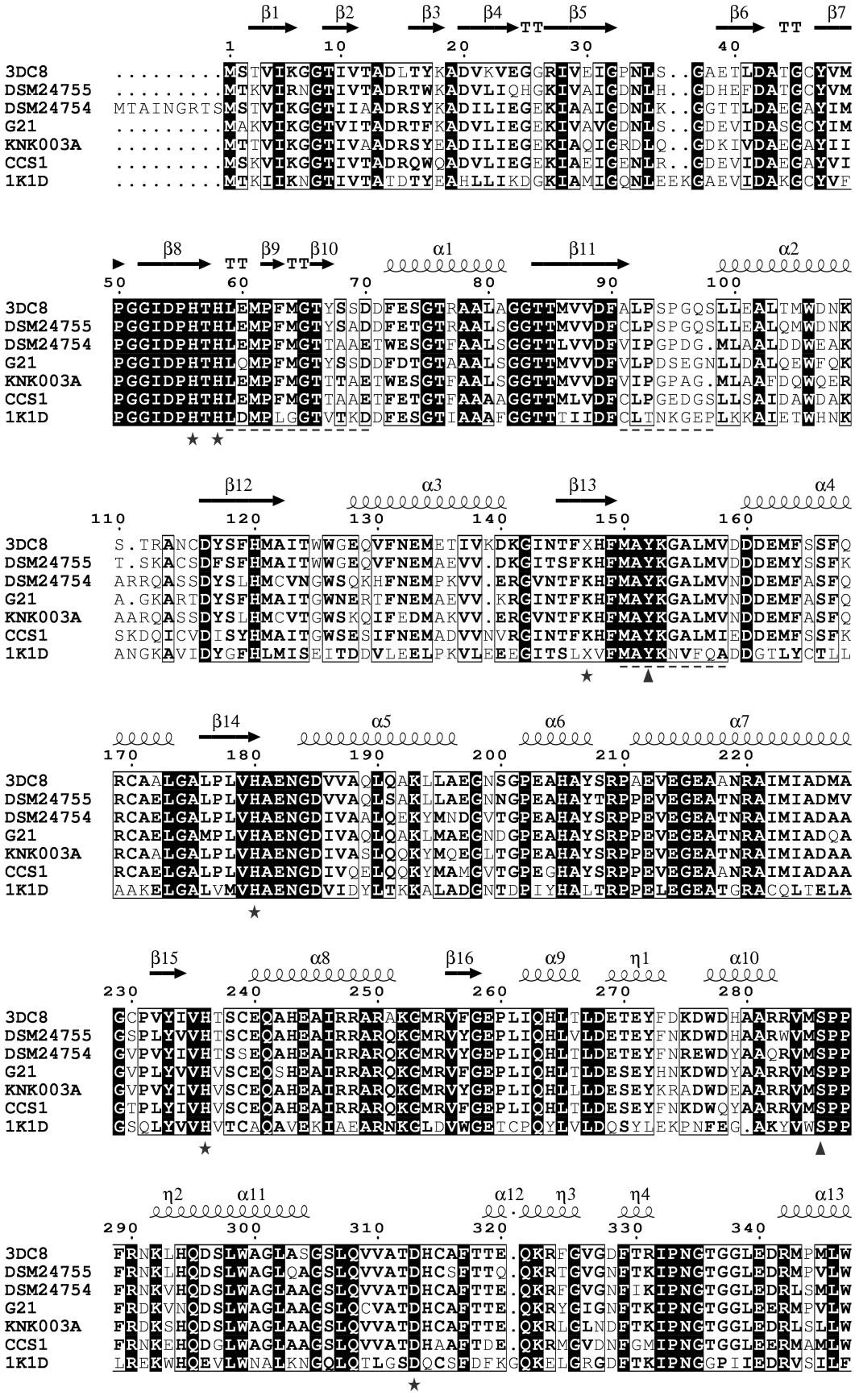

Figure 6.4 Continues on next page

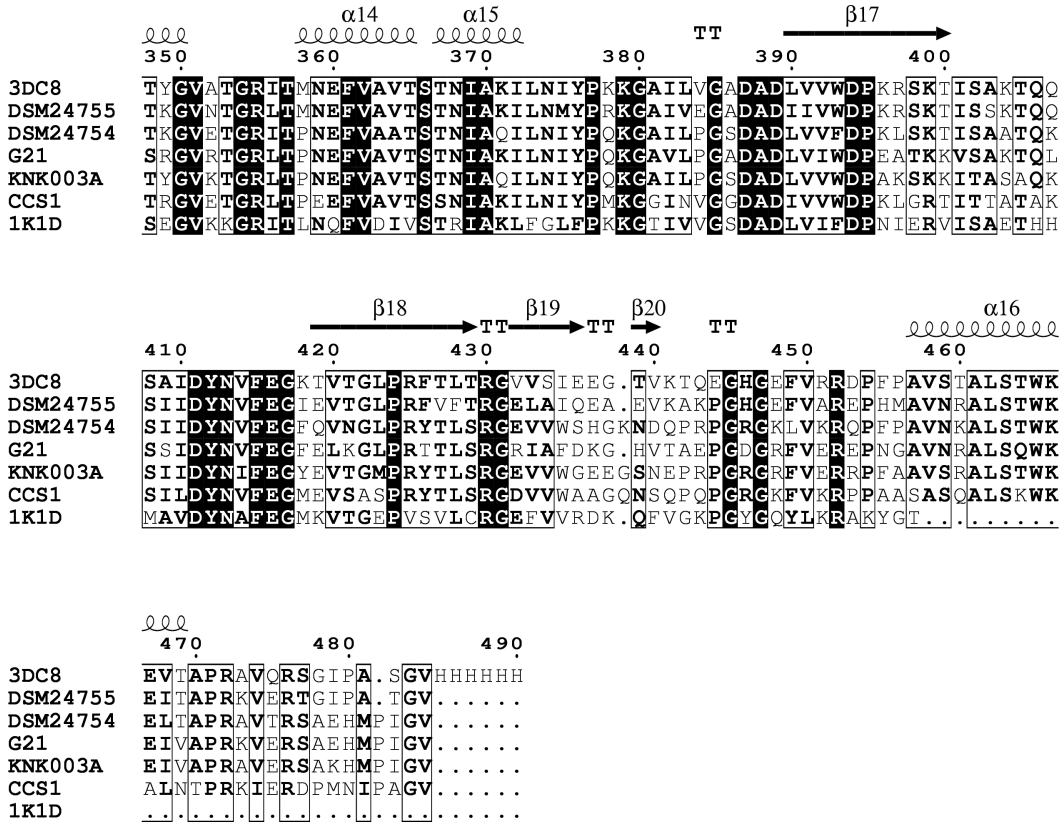

Figure 6.4 Alignment of primary amino acid sequences of the non-putative dihydropyrimidinase of *Sinorhizobium meliloti* CECT4114 (3DC8), D-hydantoinase of *Ochrobactrum* sp. G21 (ABS84244.1), D-hydantoinase of *Pseudomonas* sp. KNK003A (BAE20330.1), *Jannaschia* sp. CCS1 (YP_510647.1) and D-hydantoinase of *Bacillus stearothermophilus* SD1 (1K1D) and the newly identified dihydropyrimidinase sequences of *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755. The dark shading symbolizes identical residues while bold letters represents similar residues. (—) stereochemistry gate loops (SGL1: 59-70; SGL2: 91-98, SGL3: 150-158), (⋆) residues assumed to build the catalytic core, (▲) residues assumed to be involved in the recognition the hydantoin ring (Martínez-Rodríguez *et al.*, 2010a).

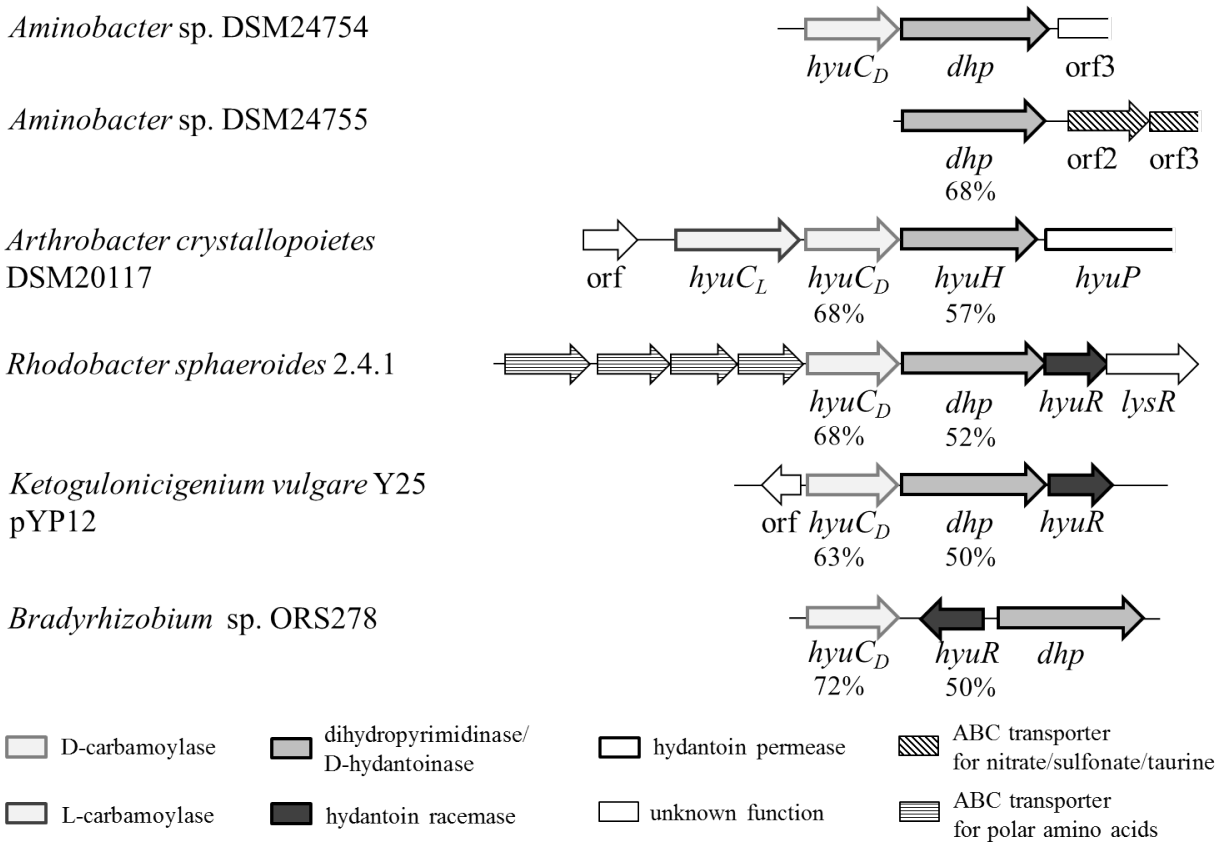

Figure 6.5 Comparison of the gene cluster of *Aminobacter* sp. DSM24754 to similar gene clusters. The putative gene clusters of *Rhodobacter sphaeroides* 2.4.1 (NC_007494.1), *Ketogulonicigenium vulgare* Y25 pYP12 (NC_014626), *Bradyrhizobium* sp. ORS278 (NC_009445) and the partial sequence of *Arthrobacter crystallopoietes* DSM20117 (AY185303.1) were obtained from NCBI. The values in % show the similarity of the deduced proteins to the deduced proteins of *Aminobacter* sp. DSM24754.

Comparison to other hydantoin/dihydropyrimidine cleaving gene clusters

Bacterial hydantoin utilizing (hyu) genes especially hydantoinases/dihydropyrimidinases and carbamoylases are often organized in gene clusters. Compared to other described hyu gene clusters the arrangement of the D-carbamoylase (hyuC) gene upstream to the D-hydantoinase (hyuH) gene in *Aminobacter* sp. DSM24754 appears to be unusual (Dürr *et al.*, 2008; Martínez-Rodríguez *et al.*, 2010b). To our knowledge *Arthrobacter crystallopoietes* DSM20117 is the only bacterial strain with a similar gene organization and approved hydantoinase and carbamoylase activities reported in literature (Werner *et al.*, 2004). The similarity of the two *Aminobacter* sp. dihydropyrimidinases to the D-hydantoinase protein of *A. crystallopoietes* DSM20117 is 57 % and the similarity of *Aminobacter* sp. DSM24754 carbamoylase to the D-carbamoylase protein of *A. crystallopoietes* DSM20117 is 68 % (see figure 6.3).

A database search against prokaryotic genomes resulted in only eight hits showing a comparable gene organization of putative hyuC and hyuH genes (see figure 6.5). In the chromosome of four *Rhodobacter* strains (*R. sphaeroides* ATCC 17025, *R. sphaeroides* ATCC 17029, *R. sphaeroides* 2.4.1, *R. sphaeroides* KD131) and on the plasmid pYP12 of *Ketogulonicigenium vulgare* Y25 the same arrangement of a putative D-carbamoylase gene and a putative D-hydantoinase gene as observed in *Aminobacter* sp. DSM24754 was detected. A similar gene organization was found in three *Bradyrhizobium* strains with the difference that a gene coding for a putative hydantoin racemase pointing in the opposite direction (for *Bradyrhizobium* sp. BTAi1, *Bradyrhizobium* sp. ORS278) or two hypothetical genes (for *B. japonicum* USDA 110) are located between hyuC and hyuH.

The *Rhodobacter sphaeroides* strains harbor two genes coding for putative hydantoinases respectively. This may be due to the fact that the *Rhodobacter* strains except for the *R. sphaeroides* ATCC 17025 possess two chromosomes. The second putative hydantoinase gene is always located on the other chromosome. Only *R. sphaeroides* ATCC 17025 harbors both genes on its single chromosome. One hydantoinase is always arranged downstream to a putative D-carbamoylase like in the hyu cluster of *Aminobacter* sp. DSM24754. The second hydantoinase forms a hyu gene cluster as well, but with a putative β-ureidopropionase located upstream to the hydantoinase gene, respectively. Interestingly the similarity of *Aminobacter* sp. DSM24754 and DSM24755 dihydropyrimidinases to the *Rhodobacter* hydantoinases clustering with the β-ureidopropionases is higher ($79 - 89$ %) than to the *Rhodobacter* hydantoinases clustering with the D-carbamoylases ($50 - 53$ %).

The same result is found for *Ketogulonicigenium vulgare* Y25, which harbors a hyu gene cluster similar to that of *Aminobacter* sp. DSM24754 on its plasmid pYP12. Additionally *K. vulgare* Y25 possess a putative hydantoinase located downstream to an putative L-carbamoylase on its chromosome. Again the latter hydantoinase has a higher similarity ($66 - 70$ %) to the deduced *Aminobacter* sp. proteins than to the hydantoinase clustering with the D-carbamoylase ($50 - 51$ %).

The *Bradyrhizobium* strains possess three genes coding for putative D-hydantoinases respectively. For each strain the sequence similarity of the deduced proteins to *Aminobacter* sp. dihydropyrimidinases is again higher ($49 - 78$ %) to putative hydantoinases not forming a cluster with a D-carbamoylase than to the hydantoinase clustering with the putative D-carbamoylase ($47 - 51$ %). Additionally, the *Bradyrhizobium* strains harbor three to four genes coding for putative D-carbamoylases, respectively. Like observed for the dihydropyrimidinases only one carbamoylase is organized in a cluster together with a hydantoinase in each strain whereas all the others are not. The similarities of *Bradyrhizobium* carbamoylases clustering with a hydantoinase to the *Aminobacter* sp. DSM24754 carbamoylase are $71 - 74$ %. The similarities of the carbamoylases not arranged together with a hydantoinase to the *Aminobacter* sp. carbamoylases are between 46 and 74 % respectively.

To our knowledge no biochemical data is available in literature for neither of the genes discussed above. This raises the questions whether or not the two *Aminobacter* sp. strains also possess several hydantoinases and carbamoylases and what role the hyu genes and their clustering play for these strains.

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Part III

Unpublished results

7 Putative pyrimidine degradation genes of *Alternaria brassicicola*

Background

Alternaria brassicicola is an ubiquitous ascomycete causing the black spot disease on most economically important *Brassica* species. During an *in silico* screening for methyltransferases of this fungus Dipl.-Biol K. Brzonkalik (Technical Biology, KIT) coincidentally detected a gene coding for a putative hydantoinase and a gene coding for a putative L-carbamoylase in the genome of *A. brassicicola*. In literature only a few eukaryotic genes involved in the pyrimidine degradation pathway are described (see sections 2.2.2 and 2.3). For some of these enzymes the hydrolysis of unnatural dihydropyrimidines like 5-bromo-dihydrouracil and 5-iodo-dihydrouracil is reported (Brooks *et al.*, 1983; Kikugawa *et al.*, 1994). As the intention of this thesis was to find novel dihydropyrimidine utilizing enzymes able to cleave unusual dihydropyrimidines via *N*-carbamoyl β-amino acids to β-amino acids the examination of the *A. brassicicola* gene sequences is of interest.

Material and Methods

Genome sequences from *A. brassicicola*

The genome sequences were obtained from http://genome.jgi-psf.org/Altbr1/Altbr1.home.html.

Sequence containing the putative hydantoinase/dihydropyrimidinase gene:

```
>contig 5:2346779-2345472
AAGATCGCGAACGTAGTTTCAAGAGGAGGTCTGAAGGACGCAGCGATGACGAGAACGGTA 2346720
GACGCTGAAGACGGCAATGTTATGACAGGAGGCGTGGACGCTCACGTCCATCTTCAGAAC 2346660
CTCCACTGTTCGGAAACGGCTCAACGGCAGATAATTATGAGACTGGCAGTCGATCAGCAG 2346600
CGTGTGGTGACACCACTACAATGGCGACCTTTACGCCACAAAAGAAGACCGAGGACATTC 2346540
TTCTCGATACCCTAGCGGCCACTCATGAACGAGCAAAGGATAACTGCTACATCGACTATT 2346480
CTTTCCGTATCATCGTCGGTAACCCCCTCGGAAAAGGCACTTTCAGAGTTCATGATCCTA 2346420
CGCGAGGAAGCGTATATCCTCACTGAAAATTTATATGACCTTCGAGGCCCTCCAGCTACA 2346360
CGGCGGGCAGATTCTAGACGTCCTCCTTGAGGCCCGAAGACAAGGCATAACTACCATGAT 2346300
CCACGCGGAGAACGGCCAAGTAATCGACTGGATGAGGACGCAACTCGAAAAGAAGAAGCT 2346240
TTTCGCGCCAAAATACCACGGCTCCTCTCACCCACCTATGGCAGAAACAGAAGTCACCTA 2346180
CCGCGCAATCAGCCTATCAGAATTCATGGACACACCCATCCTCATCGTCCACGTATCCAC 2346120
GCCCGCAGCAGCCGCTCATATAAAAGCAGCACAAGACCGCGGTCTACCCATCTACGCAGA 2346060
AACCTGCCCACAGTACCTCTTCCTCACAAAACAAAATCTCGACTAACCCGGTTTCAAAGG 2346000
CGCAAAATGCGTATGCTCGCCCCCACCACGGGAGAGCGAGGCCGACTGCGACATCATCTG 2345940
GGAAGGCCTTGCAAACAGCACTTTTACCATTCTCCCCTCGGACCATTGTCCCTTCCGCTA 2345880
```

```
CAACGACGTAGAGTCAGGCAAGAAATCTAGCATCAGCGCTGTATACCCCCTGGGCCACTT 2345820
CAAGTACATACCAGACGACTGTCCAGGCATCGAAACACGACTCTCGCTCACCCTCAGCGC 2345760
GAACCGTCTCGAACTCACTACATTCGTCCGAGTTACATCCGCCAACCCCGCCAAGCTATA 2345700
CGGCTTGTACCCAAAGAAAGGCGTGTAGGTCGAGGGCGAATCGGATGCTGATATCAATAT 2345640
CTGGTGTCCGGAGGGCGAAATGGACGAGTTTAAGCTGATGAATAGAGTGTCGCATCATGA 2345580
TGTTGATTATACACCTTTGAGGGGAGGACGCTGAAGCAGTGGCCGAGGTGGACGTTGCTA 2345520
AGGGGTAAGGTGGTTTGGGATAGGGAGAATGGGGGTTGGTAGGCTAGA 2345472
```

Table 7.1 First hits when *A. brassicicola* contig 5: 2346779-2345472 was compared with blastx to the SwissProt-Database (Altschul *et al.*, 1997; Boeckmann *et al.*, 2003).

Accession number	Enzyme	Organism	Query coverage	E-value
Q9P903	Dihydropyrimidinase	*Saccharomyces kluyveri*	80%	9e-86
Q8VTT5.1	D-hydantoinase	*Burkholderia pickettii*	80%	9e-48
Q44184.1	D-hydantoinase	*Agrobacterium tumefaciens*	76%	3e-47

Sequence containing the putative carbamoylase gene:

```
>contig 7:  684331-686282
CTATTAACCGACGGATAACTTGCATGTTAGAAAAGCGGGACAGTCATGCACTACTGTTAT 684390
CGGACATCGAACCCGCTCCTTCCCCGCGACACGACTGGGCGCCTGCATGTCATCTCACCT 684450
TTTGAGATGCCGACTTCCATAACCCGTACCGTCATGAACTTCATCTTTCTTCTTCCTCTG 684510
TTATTCCCACACAATGGCTCTGCTGGCTAATCGGTTCCCTGCTGCGATCCGCATTGCAAG 684570
CAGGTGGTACCCCGCACCAAGAGCTGTACCAACTGCGCAAGCAAGGATAGGGCATAACCA 684630
TGTCAGGTGTTTCTCCCATTCGCCACAATGTCAGATCAGAACAAAAGAGCTGGACGATGA 684690
TTTGATGAGGGATCTGAAAGTCAACCAGGCAAGGCTGATGGAAGATATACACCATACATG 684750
TCAATGGGGTACAGGCGAGCCGTGGGGCGAGTATGTAGTCCAAACACCCGATCATTATGT 684810
TGCTGACGATAGCAGGAAGTCAACGGAAACGGGTATGAGTCGACTGGCGCTCTCCGATGC 684870
CGACAAAGCTGCACGTGATTGGTTCGCAGATACTACAAAGTCGCTTGGCTGTGAGGTCAC 684930
TGTTGATGCCATGGGAAACCAATTCGCAGTTCGTGCTGGACTCAAAAATGACAAACCGCC 684990
TACATTTGTGGGAAGCCACTTAGACACACAGCCTACTGGTGGACGATACGATGGAATCCT 685050
GGGAGTCACTGCTGGTGTGGAAATGCTCAGGGTGCTCGCCGACAATTGGACGGAGACCGA 685110
GTATCCTGTCGGCGTAATCAACTGGACTAACGAAGAGGGCGCTCGCTTCCCCATGTCGAT 685170
GGTCTCTAGCGGAGTTTGGGCAGGTTCTATTGCTCTTGAGACAGCACACAACCTCCGTGA 685230
AGTTCATCCTGGAACAGCAACCATGAAGTCTGAACTTGAGCGAATTGGCTACCTGGGAAG 685290
TACACCAGCAAGCTACGAAGCCATGCCCATGGCGGCGCACTTTGAACTGCATATCGAGCA 685350
GGGCCCACTGTTGGAGATGGCAAACAAGAAGATCGGAGTTGTTACTGGAGTCCAGGCTTA 685410
CAAATGGATGACGGTCAAGGTCAAGGGACGAGACACGCATACTGGAACCACAGATCTCAA 685470
GTCACGAGCTGATGCGTTGCTAACAGCAAGCAAAATGATTCTACACTCTCATCGCCTTGC 685530
AACCGCCAACTCCGCGCTAGCATCCACGGGTATACTCAACTTAAAACCTGGTTCGACAAA 685590
CACAGTACCTGGCGAAGTGACATTCAGTCTCGACATCAGATCACCAAACGACGAGACAGT 685650
CGCAAAGATGAAAGAGTTAGTCTTGCGCGATTTCCCCAAGATCGCCGCAGGTGAGGACGT 685710
TGAAGGCTCGAACCACGGCTGCACGAGCGGTCTTCCTCTCACTGTCGAGATAGTCGAGGA 685770
CTTCGATAGCCCAGCCACCAAATTCCACGCAGACTGCATCACGTCCGTCAGCCAGTCTGC 685830
```

```
GCGCAGCATCCTCGGCCCAAACCAGTCTATGGAGATGACTTCTGGTGCCGGACACGACAG 685890
CGTATATGCGAGTAAACGCTGCCCGACGAGCATGATTTTCGTGCCATGTAGAGAAGGTGT 685950
GAGCCATAACCCACGAGAGTTTTGTAAAGAAGAAGACTGTGCATTAGGCGCACAGGTCCT 686010
GCTACAGAGTGTGGTGAGGTTTGATAGAATGAGAGACGAGAGAAGGGTTTCGTGGAAACA 686070
GGAGCCGTACACTCTATAAGAGGGGTCGTATGGGTGACAGTCTGGCATTCTACGGCGCAC 686130
GCCCAGAGAAGGGAGGCATAGCGGAAGCGCGCGTAGACATATCCAACAAATCTTCGATAC 686190
TTCCCTAAGATTGCTTGCCGTGCCATATTAGAATAAGCAGTATCTTTCCATTGACCCATC 686250
TGTTCGATGAAAGAATGTAATGGTCAAAACGA 686282
```

Table 7.2 First hits when *A. brassicicola* contig 7:684331-686282 was compared with blastx to the SwissProt-Database.

Accession number	Enzyme	Organism	Query coverage	E-value
Q57051.1	uncharacterized hydrolase	*Saccharomyces kluyveri*	58%	3e-49
Q53389.1	*N*-carbamoyl-L-amino acid hydrolase	*Geobacillus stearothermophilus*	61%	9e-44
P37113.2	*N*-carbamoyl-L-amino acid hydrolase	*Geobacillus stearothermophilus*	61%	6e-43

Polymerase chain reactions for the amplification of amidase genes

Genomic DNA from *A. brassicicola* was kindly provided by K. Brzonkalik (Technical Biology, KIT). RNA samples extracted after 1, 2, 3, 6, 7, and 8 days of cultivation were kindly provided by A. Stoppa (Technical Biology, KIT, Stoppa, 2009). The RNA was used to synthesize cDNA applying the oligo-dT primer and Omniscript™Reverse Transcriptase (Qiagen, Hilden Germany) following the manufacturer's instructions.

For the amplification of the putative hydantoinase gene the primers AbrasF (5'-ATGGAATACGACCTAATAATCGTCAACGGG-3') and AbrasR (5'-CTACAACGGCAGCACCCATCCGCC-3') and for the putative carbamoylase gene the primers AbrasCarbF (5'-ATGGCTCTGCTGGCTAATCG-3') and AbrasCarbR (5'-TTATAGAGTGTACGGCTCCT-3') were designed and orded at MWG-Biotech (Germany). All polymerase chain reactions were performed in a total volume of 25 µL containing the appropriate reaction buffer, reagents, primers and the Crimson Taq (NEB) following the manufacturer's instructions. A Mastercycler gradient (Eppendorf) was used and the conditions for the amplification of the appropriate genes from genomic DNA and cDNA were as follows: initial denaturation (30 s 95 °C), followed by 30 cycles of denaturation (20 s 95 °C), annealing (45 s 60 °C) and primer extension (1.5 min 68 °C) and final primer extension (5 min 68 °C). The PCR products were analyzed on a 1 % agarose gel stained with ethidium bromide. PCR products were purified with MinElute Gel Extraction Kit (Qiagen, Hilden Germany), ligated into pDrive PCR cloning vector (Qiagen, Hilden Germany), sequenced (GATC Biotech AG, Germany) and compared to *A. brassicicola* genome using the BLAST program (Altschul *et al.*, 1990).

Results

The amplification of the hydantoinase gene and the carbamoylase gene from genomic DNA seemed to be successful when analyzing the agarose gel picture (see figure 7.1). For cDNA generated from RNA harvested after 1, 2, 6, 7 and 8 days of cultivation no PCR products with none of the primer pairs were received (data not shown). A PCR product from cDNA was only obtained when using the hydantoinase primers AbrasF and AbrasR and cDNA generated from RNA harvested after 3 days of cultivation. However, the DNA sequencing of the PCR product revealed that the amplificated DNA showed highest identity to the 60S ribosomal protein L4-A of *Pyrenophora tritici-repentis* Pt-1C-BFP (XM_001930509) and was not the desired hydantoinase gene.

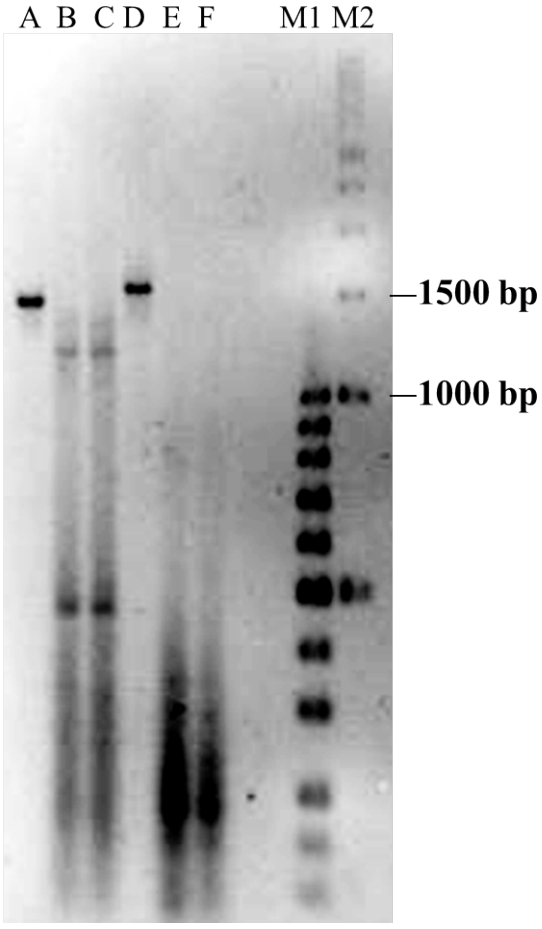

Figure 7.1 Agarose gel of PCR products amplified with the primers AbrasF and AbrasR (A-C) and the primers AbrasCarF and AbrasCarbR (D-E) from genomic DNA (A, D) and cDNA (B, C, E, F) of *Alternaria brassicicola*. The markers were 1 kb DNA ladder (M1) and 100 bp DNA ladder (M2) (Roth, Germany)

Discussion and outlook

Possible reasons for the achieved results are:

i Under the chosen cultivation conditions of *A. brassicicola* the prospected genes were not expressed. For this reason no appropriate mRNA was comprised in the RNA extracts. *A. brassicicola* mRNA samples were provided by A. Stoppa who cultivated the fungus on standard medium for molds (Stoppa, 2009). An adjustment of the medium e.g. with dihydropyrimidines as sole nitrogen source could possibly lead to the expression of the target genes and result in a successful amplification of the intron-free genes.

ii The primers mismatched the real start and the end of the gene sequences. The work with *A. brassicicola* was conducted in 2009 but the annotation of the *A. brassicicola* genome was improved in the last years. A recent comparison of the sequences with the database (http://genome.jgi-psf.org/Altbr1/Altbr1.home.html.) revealed that the primers sequences used for the amplification of the hydantoinase gene seem to be beyond the actual gene. For this reason it is impossible to amplify the hydantoinase gene with the primers AbrasF/AbrasR from the cDNA. In contrast to AbrasF/AbrasR the primer pair AbrasCarbF/AbrasCarbR exactly matched with start and the end of the putative carbamoylase gene.

iii The PCR conditions like the melting temperature were too unspecific.

For future experiments various cultivation conditions for *A. brassicicola* should be tested in order to force the expression of pyrimidine degradation genes. A novel primer pair for the amplification of the hydantoinase gene should be designed. Finally the PCR conditions should be adjusted by testing different melting temperatures, polymerases and variants of PCR.

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Part IV

Final remarks

8 Concluding remarks and outlook

8.1 Concluding remarks

The aim of this thesis was to examine the applicability of a modified hydantoinase process to the production of chiral β-amino acids.

Within this work it was demonstrated that the synthesis of two unnatural dihydropyrimidines PheDU and *p*ClPheDU from cheap bulk chemicals is accomplishable. The synthesis of the model substrate PheDU was upscaled to a achieve an amount of several hundred grams.

It was shown that several well-studied hydantoinases have dihydropyrimidinase activity towards PheDU and *p*ClPheDU. Additionally, a variety of microorganisms exhibited activity towards these substrates. Most of them displayed a D-stereoselective conversion of PheDU. However, for none of the biocatalysts tested carbamoylase activity towards *N*CβPhe was detected as well as no racemase activity was observed. In literature no racemases with activity towards dihydropyrimidines and only a few carbamoylases able to cleave unnatural *N*-carbamoyl-β-amino acids are described (Martínez-Gómez *et al.*, 2009). However, the decarbamoylation of carbamoyl amino acids can be achieved by a chemical reaction instead of the application of an appropriate enzyme (Keil *et al.*, 1995). The real challenge of a modified hydantoinase is the racemization of dihydropyrimidines. In contrast to hydantoins, 6'-monosubstituted dihydropyrimidines can not racemize via a keto-enol tautomerism. It has to be tested whether there are chemical methods or enzymes able to racemize 6'-monosubstituted aryl-substituted dihydropyrimidines or aryl-substituted *N*-carbamoyl-β-amino acids in order to develop a modified hydantoinase process with a potential yield of 100 %.

8.2 Outlook

Based on this thesis future studies could focus on the following tasks:

- ▷ Synthesis strategies in order to produce a larger variety of unnatural dihydropyrimidines should be developed e.g by the application of cinnamic acid derivatives instead of pure cinnamic acid (Vuano *et al.*, 2000) for the synthesis of 6'-substituted dihydropyrimidines. Subsequently, the hydrolysis of these novel substrates and their products by hydantoinases and carbamoylases should be examined.

- ▷ The novel dihydropyrimidinase genes of *Aminobacter* sp. DSM24754 and *Aminobacter* sp. DSM24755 and the novel carbamoylase gene of *Aminobacter* sp. DSM24755 (see chapter 6) should be recombinantly expressed and biochemically characterized. This work could prove that the dihydropyrimidinases are responsible for the activities towards hydantoins and dihydropyrimidines detected for the *Aminobacter* wild-type strains within this thesis. Additionally, a precise analysis and comparison of the crystal

structures of the dihydropyrimidinases could possibly explain why in biotransformation experiments one strain exhibited a enantioselective conversion of PheDU while for the other strain no enantiopreference for one of the PheDU enantiomers was detected. A characterization of the *Aminobacter* sp. DSM24755 carbamoylase could resolve the enzyme's substrate spectrum and also explore the enzyme's oxidative and thermal stability. The results achieved could be used to prove or disprove the hypothesis that the *Aminobacter* sp. DSM24755 carbamoylase may have a similar high thermostability like the *Pseudomonas* sp. KNK003A carbamoylase (Ikenaka *et al.*, 1998).

▷ Enzyme engineering of well studied D- and L-carbamoylases and β-ureidopropionases could be performed in order to enlarge or shift their substrate spectra towards *N*-carbamoyl-β-amino acids. Today, solely one carbamoylase is described to catalyze the hydrolysis of unnatural *N*-carbamoyl-β-amino acids to their corresponding β-amino acids (Martínez-Gómez *et al.*, 2009). Based on this study and on crystal structures available for L- and D-carbamoylases (Martínez-Rodríguez *et al.*, 2008; Wang *et al.*, 2001; Nakai *et al.*, 2000) strategies for a rational design of an appropriate carbamoylase could be developed and examined.

▷ A screening for enzymes able to racemize 6'-monosubstituted dihydropyrimidines or *N*-carbamoyl-β-amino acids should be accomplished. To the best of the author's knowledge there is no racemase with activity towards dihydropyrimidines described in literature. In contrast, several *N*-acylamino acid racemases (Tokuyama & Hatano, 1995; Bommarius *et al.*, 2002; Hsu *et al.*, 2007) and one *N*-succinylamino acid racemase (Pozo-Dengra *et al.*, 2009) are reported to accept *N*-carbamoyl-α-amino acids as substrates. These enzymes should be screened for their activity towards *N*-carbamoyl-β-amino acids. Additionally, it should to be tested if there are methods for a chemical racemization of 6'-monosubstituted dihydropyrimidines or *N*-carbamoyl-β-amino acids. An successful accomplishment of this task would be a further step towards a modified hydantoinase process.

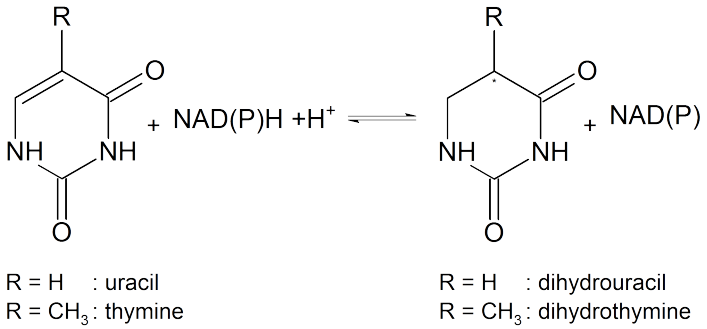

Figure 8.1 Enzymatic conversion of uracil or thymine to dihydrouracil or dihydrothymine by dihydropyrimidine dehydrogenase (West, 2001; Schnackerz *et al.*, 2004).

▷ The application of enzymes other than racemases in a modified hydantoinases process should be considered. For example, activity of dihydropyrimidine dehydrogenases (see figure 8.1) towards 6'-monosubstituted dihydropyrimidines like PheDU and *p*ClPheDU should be examined. The natural function of this enzyme is the reduction of uracil or thymine to dihydrouracil or dihydrothymine in the reductive pyrimidine degradation pathway (Vogels & van der Drift, 1976). Activity of dihydropyrimidine dehydrogenases towards unnatural pyrimidines is described for 5'-fluorouracil, an antitumor drug (for a review see van Kuilenburg, 2004) as well as for 5'-bromouracil, 5'-iodouracil, hydrouridylic acid, 5'-diazouracil, 5'-nitrouracil and 5'-aminouracil (Grisolia & Cardoso, 1957; Shiotani & Weber, 1981; Schmitt *et al.*, 1996). However, enzyme activity towards 6'-monosubstituted pyrimidines has not been described in literature so far. A dihydropyrimidine dehydrogenase also accepting 6'-monosubstituted dihydropyrimidines as substrates could be applied in a modified hydantoinase process for catalyzing the back reaction of the non-hydrolyzed dihydropyrimidine enantiomer e.g. D- or L-PheDU to achiral phenyluracil. The subsequent reversal of the dihydropyrimidine dehydrogenase reaction would yield the racemic dihydropyrimidine, e.g. PheDU, again. An appropriate dihydropyrimidine dehydrogenase together with an enantiospecific hydantoinase and a carbamoylase applied in a modified hydantoinase process would lead to a process with maximum possible yield of 100 %.

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