Biocatalytic Synthesis of Pharmaceutically Relevant Amides and Amino Acids

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SUMMARY AND FUTURE PERSPECTIVES

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Summary

The work described in this thesis focused on the application of enzymes and enzyme cascades for the synthesis of complex amino acids and amides that serve as versatile synthons for biologically active molecules. The target compounds included *N*-arylalkyl-substituted L-aspartic acid derivatives and vitamin B_5 derivatives that have proven to be difficult or tedious to synthesize. The established biocatalytic synthesis strategies are likely to advance our ability to prepare and search for new pharmaceutically active compounds, including those that target CoA biosynthesis and utilisation in a variety of pathogens.

In **Chapter 1** of this thesis, we reviewed the biochemical properties, structures, and catalytic mechanisms of two C-N lyases, MAL and EDDS lyase. We specifically discussed the recent application of these two enzymes in preparing a range of L-aspartic acid derivatives and complex bioactive molecules of pharmaceutical and nutraceutical importance via chemoenzymatic and multienzymatic cascades.

Subsequently, in the study described in **Chapter 2** we investigated the asymmetric synthesis of *N*-arylalkyl-substituted L-aspartic acids using EDDS lyase from *Chelativorans* sp. BNC1. Poelarends and coworkers previously reported the preparative usefulness of the enzyme for preparation of a series of aminocarboxylic acids (and derivatives) such as toxin A, AMA (aspergillomarasmine A), AMB (aspergillomarasmine B)[1], *N*-substituted L-aspartic acids (*N*-cycloalkyl, *N*-arylated, *N*-alkyl), heterocycles such as pyrazolidinones[2–4], as well as important precursors to food additives (neotame and advantame). Due to the broad amine substrate scope of this enzyme, we further continued to explore the synthetic potential of EDDS lyase for biocatalytic preparation of *N*-arylalkyl-substituted L-aspartic acids, including useful building blocks for known inhibitors of aspartate *N*-acetyl transferase. Commonly, these molecules are synthesized using metal-catalyzed reductive *N*-alkylation of L-aspartic acid. In this study, we demonstrated that EDDS lyase is an effective biocatalyst to synthesize *N*-arylalkyl-substituted L-aspartic acids through enantioselective hydroamination of fumaric acid. Good conversion of starting substrates into the wanted amino acid products was achieved (17-91%) within 24-48

h using 0.15 mol% catalyst loading. Seven enzymatically produced amino acid products were purified in good isolated yield (28-76%) and identified by ^{1}H NMR, ^{13}C NMR, and HRMS as the desired *N*-arylalkyl-substituted L-aspartic acids with high optical purity (>99% e.e.). Hence, EDDS lyase shows excellent enantioselectivity and a very broad substrate scope, including many structurally diverse non-native amines. This capability makes EDDS lyase a powerful tool for the biocatalytic asymmetric synthesis of versatile L-aspartic acid derivatives.

In **Chapter 3**, we describe the application of the C-N lyase MAL and its engineered variant MAL-H194A in multienzymatic cascade synthesis of (*R*)-pantothenic acid (vitamin B_5) and both diastereoisomers of α-methyl-substituted vitamin B_5 with high stereocontrol. These molecules are important synthetic precursors to antimicrobial pantothenamides, but have proven to be difficult or tedious to synthesize, hampering the investigation of their therapeutic potential. The established synthetic enzymatic cascade consists of three consecutive steps including amination, decarboxylation, and condensation. First, a one-pot, two-step enzymatic cascade was established with the help of MAL (or MAL-H194A) and an amino acid decarboxylase [aspartate α-decarboxylase (ADC), β-methylaspartate α-decarboxylase (CrpG), or glutamate decarboxylase (GAD)]. The respective reactions resulted in β-alanine and both enantiomers of α-methyl-β-alanine, building blocks of pantothenic acid and α-methyl-pantothenic acid, respectively, with high conversion (75-99%) and very good isolated product yield (63-85%) and enantiopurity (>99% e.e). Secondly, this two-step cascade was extended by including pantothenate synthetase (PS) to form a three-step enzymatic cascade, generating (*R*)–pantothenic acid and the desired diastereoisomers of α-methyl-pantothenic acid in a one-pot system (>75% conversion over three steps, 46-70% isolated yield). This study showed that the combination of these three different biocatalysts allowed the rapid synthesis of complex diastereopure synthons for biologically active amide compounds from inexpensive and simple starting materials.

In the work described in **Chapter 4**, we investigated the biocatalytic potential of the PS enzyme for the synthesis of pharmaceutically-relevant amides, including known vitamin B_5 antimetabolites. We show that besides the natural amine substrate β-alanine, the enzyme accepts a wide range of structurally diverse amines for coupling to the native carboxylic acid substrate (*R*)-pantoic acid to give amide products with up

to >99% conversion. Four interesting amide products, which may serve as synthons for promising antimicrobial pantothenamides, were prepared at mg-scale, purified and identified by ^{1}H NMR, ^{13}C NMR, and HRMS as the desired pantothenic acid derivatives, achieving >99% conversion and 71-89% isolated product yield. Of high interest are the fluorinated pantothenic acid derivatives, prepared by the enzymatic concensation of (*R*)-pantoate and α-fluoro-substituted β-alanine or α-CF_3-substituted β-alanine, which have proven to be extremely difficult to synthesize by conventional methods. Hence, the established enzymatic strategy provides an attractive alternative approach for the current chemical methods to prepare difficult pantothenic acid derivatives.

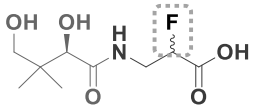
FUTURE PERSPECTIVES

In the work described in this thesis, we have exploited the synthetic potential of C-N lyases (MAL and EDDS lyase) for the biocatalytic asymmetric hydroamination of unsaturated carboxylic acids to produce versatile L-aspartic acid derivatives. In addition, pantothenate synthetase (PS) was exploited for the condensation reaction between structurally diverse amines and (*R*)-pantoate to generate substituted pantothenic acids, which are important synthons to prepare antimicrobial compounds, using single-step biotransformations as well as multienzymatic synthesis cascades. Both EDDS lyase and PS have a broad nucleophile substrate scope and display good catalytic activity, thus showing great potential for biocatalytic application.

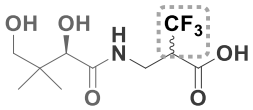
Next, there is a need to expand the electrophile substrate scope of EDDS lyase, which only accepts fumaric acid as substrate and shows no activity towards analogues like crotonic acid, mesaconic acid, itaconic acid, 2-pentenoic acid, and glutaconic acid. Structure-based protein engineering of EDDS lyase seems a good strategy to expand its electrophile scope, allowing the asymmetric synthesis of other amino acids including versatile β-amino acids. Aspartase from *Bacillus* sp. YM55-1 (AspB), an enzyme from the aspartate/fumarase superfamily, was successfully engineered aided by computational redesign to give promising activities towards several α,β-unsaturated monocarboxylic acids yielding β-amino acids[5]. Interestingly, EDDS lyase and AspB are highly conserved with regard to the amino acid residues that are responsible for α-carboxylate binding, providing support for using a similar engineering strategy to enlarge the electrophile scope of EDDS lyase[5,6]. If the enzyme engineering approach

would be successful in enlarging the electrophile scope of EDDS lyase, without harming its broad nucleophile scope, it would provide great opportunities for biocatalytic synthesis of an extremely large library of synthetically useful and/or biologically active noncanonical amino acids. Also the various decarboxylases (ADC, CrpG, and GAD) used in our studies are interesting targets for enzyme engineering to expand their substrate scope, enabling the conversion of a wide range of L-aspartic acid derivatives to produce valuable β-alanine derivatives.

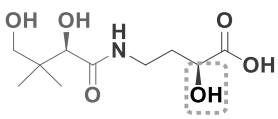
alpha-Fluoro (F)-Pantothenic acid [*rac*-**3g**]



alpha-Trifluoromethyl (CF_3)-Pantothenic acid [*rac*-**3h**]



(*S*)-(alpha)-hydroxy (OH)-Pantothenic acid [(*S*)-**3i**]



(*S*)-(beta)-hydroxy (OH)-Pantothenic acid [(*S*)-**3j**]

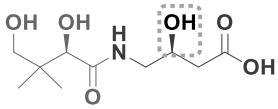


Figure 1. The chemical structure of pantothenic acid analogues synthesized by using the PS enzyme as biocatalyst.

Finally, in the work described in **Chapter 4**, we demonstrated the usefulness of the PS enzyme for the synthesis of substituted pantothenic acids (Figure 1), which are important synthons for preparing novel antimicrobial compounds (Figure 2). To synthesize these potential antimicrobials, the substituted pantothenic acid precursors need to be coupled with *N*-phenethylamine derivatives by amide bond formation selectively at the α-carboxylic acid group[7] (Figure 2). The potency of these novel pantothenamides needs to be tested, both in terms of stability and biological activity. Further studies into the therapeutic potential of novel biologically active pantothenamides are urgently needed, and their facile preparation via chemoenzymatic synthesis strategies may prove to be highly useful in further expanding and optimizing this interesting group of antimicrobials that target coenzyme A biosynthesis and utilisation.

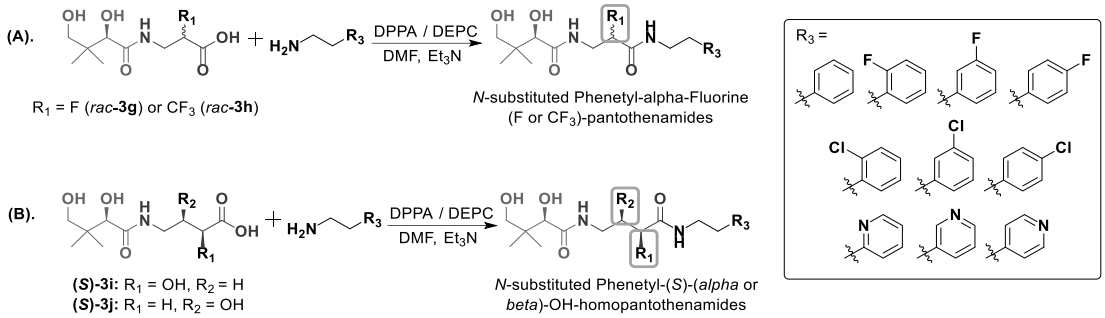


Figure 2. The proposed chemical synthesis of a novel pantothenamides as potential antimicrobial drugs. (**A**). Synthesis of *N*-substituted-phenetyl-α-F/CF_3-pantothenamides (**B**). Synthesis of *N*-substituted-phenetyl-(*S*)-(α/β)-OH-homopantothenamides.

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