

University of Groningen

Biocatalytic asymmetric hydroamination by native and engineered carbon-nitrogen lyases

Zhang, Jieli

DOI:
[10.33612/diss.93007154](https://doi.org/10.33612/diss.93007154)

IMPORTANT NOTE: You are advised to consult the publisher's version (publisher's PDF) if you wish to cite from it. Please check the document version below.

Document Version
Publisher's PDF, also known as Version of record

Publication date:
2019

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Zhang, J. (2019). *Biocatalytic asymmetric hydroamination by native and engineered carbon-nitrogen lyases: new enzymes to prepare amino acid precursors to pharmaceuticals and food additives*. [Thesis fully internal (DIV), University of Groningen]. University of Groningen. <https://doi.org/10.33612/diss.93007154>

Copyright

Other than for strictly personal use, it is not permitted to download or to forward/distribute the text or part of it without the consent of the author(s) and/or copyright holder(s), unless the work is under an open content license (like Creative Commons).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

Take-down policy

If you believe that this document breaches copyright please contact us providing details, and we will remove access to the work immediately and investigate your claim.

Downloaded from the University of Groningen/UMCG research database (Pure): <http://www.rug.nl/research/portal>. For technical reasons the number of authors shown on this cover page is limited to 10 maximum.

VII

Summary and Future Perspectives

In biocatalysis, enzymes or microorganisms are used as catalysts to accelerate chemical reactions. The use of enzymes as catalysts offers multiple benefits such as high catalytic efficiency, good chemo-, regio- and stereoselectivity, and adaptability via molecular biological manipulations. This has attracted strong interest from both academia and industry to apply biocatalysis for chemical synthesis. Recent years witnessed rapid advances in biocatalysis, which has now become a widely accepted strategy for sustainable and scalable production of important organic molecules, especially those of pharmaceutical significance. Developments in directed evolution, high throughput screening, bioinformatics and computational design accelerated enzyme discovery and engineering, rapidly generating biocatalysts with desired properties and reaction selectivity for a certain synthetic step.

Carbon-nitrogen lyases (C-N lyases) are enzymes catalyzing the cleavage of C-N bonds by an α,β -elimination reaction, yielding α,β -unsaturated molecules as products. C-N lyases can also work in reverse and thus be used as synthetic enzymes in C-N bond-forming reactions. These enzymes are found in a broad range of prokaryotic and eukaryotic organisms, with a vast diversity in structural and mechanistic characteristics. C-N lyases show great potential as biocatalytic tools for asymmetric synthesis of unnatural α - and β -amino acids through hydroamination of simple unsaturated carboxylic acids. Despite their great synthetic potential, only a few C-N bond-forming lyases have been identified and characterized for their substrate scope and synthetic usefulness. Developing new C-N lyases as effective C-N bond-forming biocatalysts by enzyme discovery and engineering, as well as expanding the synthetic applications of this fascinating group of enzymes, are promising areas to investigate.

Chapter 1 of this thesis provides a review on the biochemical properties, structures and catalytic mechanisms of two biotechnologically-relevant C-N lyases, MAL and EDDS lyase. Recent synthetic applications of these two enzymes in preparing a broad range of L-aspartic acid derivatives and building complex compounds of pharmaceutical and nutraceutical interest through chemoenzymatic and multienzymatic cascades are discussed.

In the work described in **Chapter 2**, an EDDS lyase from *Chelativorans* sp. BNC1 was investigated as biocatalyst for (chemo)enzymatic asymmetric synthesis of the important fungal natural products aspergillomarasmine A (AMA), aspergillomarasmine B (AMB) and toxin A, as well as related compounds. AMA is a potent inhibitor of *metallo*- β -lactamases such as NDM-1 and VIM-2, which are widely recognized as a major cause of bacterial resistance to β -lactam antibiotics, which is one of the biggest threats to global health. Given that AMA is difficult to synthesize chemically, EDDS lyase was used to catalyze the addition of different amino acids to fumaric acid to yield toxin A, the biosynthetic precursor of AMA, as well as seven analogues of Toxin A, with high conversion (91-98%), good isolated yield (34-82%) and excellent regio- and stereoselectivity (d.e. >98%, e.e. >99%). Based on this biocatalytic process, a one-pot chemoenzymatic synthetic strategy was developed for rapid derivatization of toxin A and related compounds by chemical *N*-alkylation using bromoacetic acid and bromopropanoic acid, generating AMB and two derivatives with only two synthetic

steps. Lastly, the biocatalytic synthesis of AMA, AMB or derivatives in one asymmetric step was performed by the regio- and stereoselective addition of retrosynthetically-designed amino acid substrates to fumaric acid by EDDS lyase. Reactions gave 65-80% conversion and enzymatic products were obtained with 20-47% isolated yield and in the desired absolute configuration (>98% d.e.). In summary, EDDS lyase is an important synthetic tool for the (chemo)enzymatic synthesis of the biologically active aminocarboxylic acids AMA, AMB, toxin A and their derivatives. EDDS lyase shows a broad amine scope, including structurally distinct amino acids, and excellent regio- and stereoselectivity. As such, this C-N lyase has great potential for practical synthesis of optically-pure (metal-chelating) aminocarboxylic acids.

To broaden our knowledge of EDDS lyase, an EDDS lyase from *Chelativorans* sp. BNC1 was cloned and characterized for its structural and mechanistic features (**Chapter 3**). The purified EDDS lyase reversibly catalyzed a sequential two-step amination of fumaric acid with ethylene diamine to give the natural metal chelator (*S,S*)-EDDS via the intermediate compound AEAA. Except for ethylene diamine, EDDS lyase showed broad substrate scope towards a variety of mono- and diamines. Crystal structures of EDDS lyase in native form and in complex with formate, succinate, fumarate, AEAA and (*S,S*)-EDDS were determined, revealing that the enzyme has a tertiary and quaternary structure that is characteristic for members of the aspartase/fumarase superfamily. A general base-catalyzed deamination mechanism was suggested, involving an enediolate intermediate that is highly stabilized by hydrogen bonding with active site residues. This work broadens our understanding of mechanistic diversity within the aspartase/fumarase superfamily and will aid in the optimization of EDDS lyase for asymmetric synthesis of valuable aminocarboxylic acids.

In **Chapter 4**, the substrate spectra of the MAL-Q73A mutant and EDDS lyase towards ring-substituted amines were investigated for biocatalytic asymmetric synthesis of *N*-cycloalkyl substituted L-aspartic acids. Cycles, especially heterocycles, are good modifiers of properties and biological activities of chemical molecules. A set of homo- and heterocycloalkyl amines substituted with four-, five-, and six-membered rings were evaluated for selective enzymatic addition to fumaric acid. The MAL-Q73A mutant displayed a relatively narrow scope for cycloalkyl amines, accepting only three tested amines as substrates, yet displaying perfect enantioselectivity (>99% e.e.). EDDS lyase accepted all ten amine substrates for addition to fumaric acid, giving the corresponding *N*-cycloalkyl L-aspartic acids with good conversions and in the desired L-configuration (>99% e.e.). EDDS lyase thus shows excellent enantioselectivity in the addition of various cycloalkylamines to fumaric acid, exhibiting great potential for biocatalytic asymmetric synthesis of difficult L-aspartic acid derivatives carrying homo- and heterocycles, which may be exploited for follow-up chemistry.

Chapter 5 describes the use of enzyme engineering to extend the synthetic application of EDDS lyase for biocatalytic preparation of *N*-(3,3-dimethylbutyl)-L-aspartic acid, an

important precursor to the artificial dipeptide sweetener neotame. Approved as a low-calorie artificial sweetener, neotame has wide-ranging applications in the food industry. First, results demonstrated that wild-type EDDS lyase has low-level hydroamination activity allowing the synthesis of *N*-(3,3-dimethylbutyl)-L-aspartic acid and five derivatives via enantioselective amine addition to fumaric acid (82-97% conversion in 7 days, 34-74% isolated yield, e.e >99%). The activity of EDDS lyase for synthesis of the neotame precursor was further enhanced by two rounds of site-saturation mutagenesis and activity screening, yielding double mutant D290M/Y320M that displays an 1140-fold increase in activity. This newly engineered C-N lyase enabled the efficient synthesis of the neotame precursor within 2.5 h (instead of 7 days) using low biocatalyst loading (0.05 mol%), and achieving 96% conversion and optically pure product (>99% e.e.) in 83% isolated yield. Five related aspartic acid derivatives were also synthesized with good conversions and enantioselectivity, including *N*-[3-(3-hydroxy-4-methoxyphenyl)propyl]-L-aspartic acid, an important precursor to the artificial sweetener advantame. This study provided the first biocatalyst to synthesize valuable amino acid precursors for neotame and advantame in a single asymmetric step, opening up new opportunities to develop practical multienzymatic processes for the more sustainable and step-economic synthesis of an important class of food additives.

In **Chapter 6**, an application of MAL and its H194A mutant was demonstrated in enzymatic cascade synthesis of (*R*)-pantothenic acid (vitamin B5) and its derivatives with high stereocontrol. (*R*)-Pantothenic acid and its α -methyl-substituted derivatives are synthetic precursors to pantothenamides, which are promising antimicrobial compounds. First, a one-pot two-step enzymatic cascade starting from unsaturated carboxylic acids was established containing a C-N lyase (MAL or MAL-H194A) and an amino acid decarboxylase [aspartate- α -decarboxylase (ADC), β -methylaspartate- α -decarboxylase (CrpG) or glutamate decarboxylase (GAD)], producing β -alanine and both enantiomers of α -methyl- β -alanine with good conversion (75-99%), isolated yield (63-85%) and optical purity (>99% e.e). Next, the synthetic pathway was extended by including pantothenate synthetase (PS) to form a one-pot three-step enzymatic cascade for production of (*R*)-pantothenic acid and both diastereoisomers of α -methyl-(*R*)-pantothenic acid (75-99% conversion, 46-70% isolated yield over three steps).

Conclusion and future perspectives

The work described in this thesis explored the synthetic application of C-N lyases for biocatalytic asymmetric hydroamination of unsaturated carboxylic acids to produce L-aspartic acid derivatives of pharmaceutical and nutraceutical importance. A new member of the aspartase/fumarase superfamily, EDDS lyase from *Chelativorans* sp. BNC1, was identified and characterized to elucidate its properties, structure and catalytic mechanism. EDDS lyase has a broad nucleophile scope and excellent regio- and stereoselectivity, showing great

potential for biocatalytic application. Successful structure-based engineering of EDDS lyase generated a tailor-made variant with significant improvement in catalytic efficiency, implicating its evolvability to an efficient biocatalyst for unnatural substrates. A collection of substituted L-aspartic acid derivatives with structural diversity were synthesized using EDDS lyase and MAL as biocatalysts, leading to novel enzymatic and chemoenzymatic routes to several valuable pharma- and nutraceutical compounds, including the metal chelators EDDS and AMA, low-calorie artificial sweeteners such as neotame and advantame, and vitamin B5 and its derivatives.

There is a need to further explore the substrate scope of EDDS lyase. Aromatic rings are versatile structures in natural products and pharmaceuticals, playing functions in numerous biological activities and in modifying compound properties. While the alkylamine scope of EDDS lyase has been elaborated, its ability to accept arylamines remains unknown. It is an interesting topic to explore the arylamine scope of EDDS lyase for addition to fumaric acid which would yield *N*-aryl substituted L-aspartic acids with extended applications. In addition, it would be interesting to investigate whether EDDS lyase can accept other nucleophiles such as thiols, alcohols, nitroalkanes, and others, which would result in non-native bond-forming reactions.

Similarly, expanding the electrophile scope of EDDS lyase is also an interesting yet challenging research topic to inspect. In contrast to the broad nucleophile scope, wild-type EDDS lyase shows a narrow electrophile scope. Except for fumaric acid, analogues like crotonic acid, mesaconic acid, itaconic acid, 2-pentenoic acid, and glutaconic acid are not processed by EDDS lyase. Structure-guided engineering of EDDS lyase, especially the redesign of its α -carboxylate-binding site (Figure 1), is a potential strategy to enlarge its electrophile scope with the aim to use EDDS lyase as catalyst for the asymmetric synthesis of β -amino acids. Based on the catalytic mechanism, the β -carboxyl group of the proximal aspartic acid moiety of the (*S,S*)-EDDS substrate is crucial for catalysis because it's involved in formation of the enediolate intermediate, while the α -carboxylate does not seem to be important for the catalytic mechanism as it mainly plays a role in substrate binding, forming hydrogen bond interactions with several active site residues. Modifying the α -carboxylate-binding site hypothetically could lead to binding and proper positioning of fumarate analogues in the modified active site, which might enable the attack of amines to produce β -amino acids.

A recent successful computational redesign of AspB (aspartase from *Bacillus* sp. YM55-1) into a β -amino acid lyase revealed mutations at four residue positions in the α -carboxylate-binding site (Thr187/Met321/Lys324/Asn326).¹ This engineered AspB enzyme catalyzes the enantioselective hydroamination of α,β -unsaturated monocarboxylic acids to yield several β -amino acids. As these four residues are highly conserved in members of the aspartase/fumarase superfamily, they are also found in EDDS lyase (Thr158/Met283/Lys286/Asn288), serving as good candidates to start with a focused

engineering approach. If engineering of EDDS lyase is successful and results in an enlarged electrophile scope, and given its broad nucleophile scope, it may be possible to use such an EDDS lyase variant to produce a large set of valuable β -amino acids.

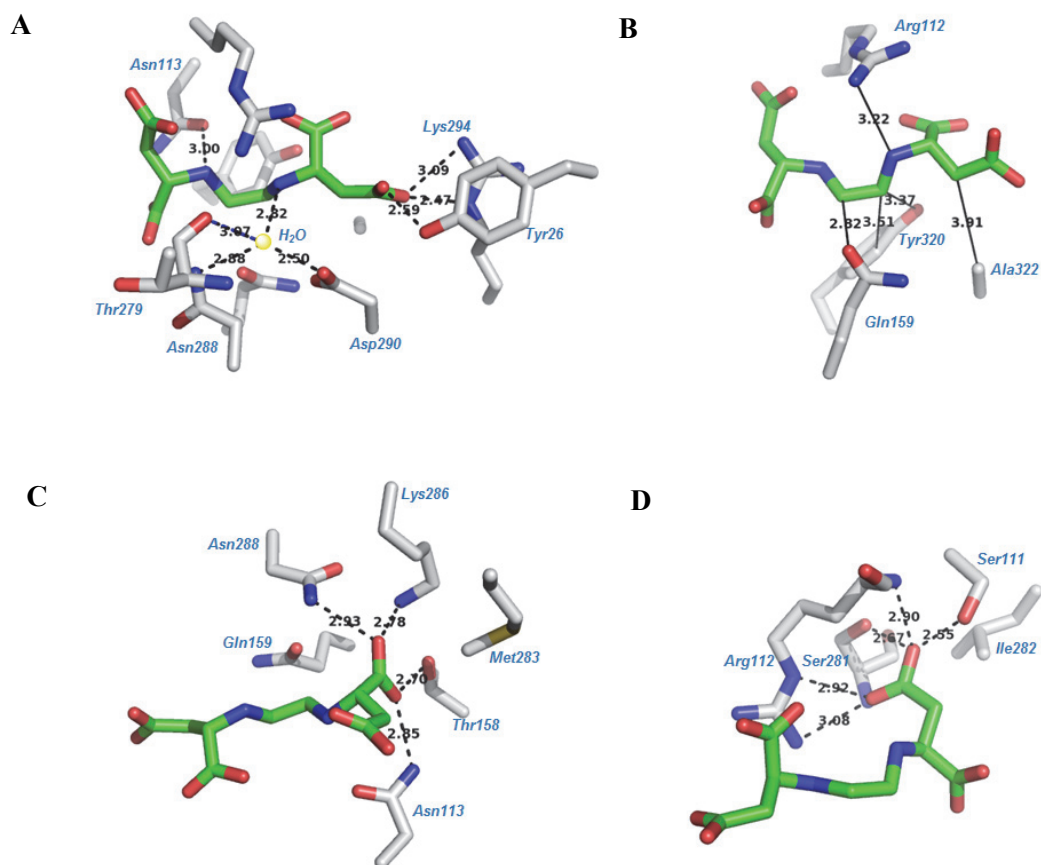


Figure 1. Crystal structure of EDDS lyase bound with (*S,S*)-EDDS. (A) Amine binding site showing hydrogen-bonding interactions; (B) Amine binding site showing distances between (*S,S*)-EDDS and residues that do not directly interact with the substrate; (C) α -Carboxylate binding site showing hydrogen-bonding interactions; (D) β -Carboxylate binding site showing hydrogen-bonding interactions. (*S,S*)-EDDS (green) and residues (grey) are shown in stick representation. The water molecule is shown as a yellow sphere. Hydrogen bonds and observed distances are shown as dash lines and solid lines, respectively.

Chapter 5 of this thesis described the application of an engineered EDDS lyase variant (D290M/Y320M) for efficient and enantioselective synthesis of complex *N*-substituted L-aspartic acids serving as neotame and advantame precursors. To fully synthesize the neotame and advantame dipeptides, these precursors would have to be coupled with L-phenylalanine methyl ester by amide bond formation via the α -carboxylic acid group. Current chemical coupling methods are relatively laborious because protecting/deprotecting the β -carboxylic acid group of the precursors and activating the α -carboxylic acid group are needed. An

