CHAPTER 1

Introduction and Scope of the Thesis
1. Introduction

1.1. Biocatalysis

Enzymes are nature’s catalysts that have fascinated chemists for centuries. The application of enzymes for chemical transformations is referred to as biocatalysis. In recent years, the field of biocatalysis has been rapidly expanding, and it has significant impact on the production of pharmaceuticals, fine chemicals, food ingredients and biofuels.\(^1\)–\(^4\) Biocatalysis is attractive to both academia and industry for many reasons, the most appealing feature of biocatalysts (enzymes) is their excellent chemo-, regio- and stereoselectivity that could be difficult or even impossible to achieve using their chemocatalyst counterparts.\(^5\)–\(^6\) Biocatalytic approaches are typically operated under mild conditions (e.g. moderate temperature and pressure, and near-neutral pH) and, therefore, innately have better safety profiles over chemocatalytic processes. Besides, enzymes themselves are nonhazardous and biodegradable catalysts, and are produced from natural renewable resources, fulfilling the sustainability requirements of tomorrow’s chemical industry.\(^7\) Additionally, enzymes can be immobilized which often provide advantages in terms of stability, recyclability as well as easy downstream processing.\(^8\)

The quantity and diversity of the biocatalytic toolbox for synthetic chemistry are continuously expanding due to the revolutionary developments in enzyme discovery (e.g. metagenome screening, genome mining, and computational design) and enzyme engineering (e.g. directed evolution, high-throughput screening, and rational design) in the past decades.\(^2\)–\(^4\),\(^9\)–\(^11\) Nowadays, organic chemists can design and refine synthetic strategies for target molecules by using appropriate biocatalysts for the key bond-forming steps in their retrosynthesis analysis (i.e. biocatalytic retrosynthesis), particularly in cases where enantioselectivity is critical.\(^12\)–\(^14\) Moreover, the combination of chemocatalysis (e.g. metal-, organo-, photo-, electro-, and heterocatalysis) and biocatalysis, albeit challenging, creates tremendous opportunities for the development of rapid and efficient routes to target molecules.\(^15\),\(^16\)

1.2. Biocatalytic synthesis of chiral unnatural amino acids using carbon-nitrogen lyases

Chiral unnatural amino acids are highly valuable as tools for biological research and as chiral building blocks for pharmaceuticals, nutraceuticals, agrochemicals, and more.\(^17\)–\(^19\) The impressive number of applications of enantiomerically pure unnatural amino acids have stimulated the development of various chemocatalytic asymmetric methodologies for their preparation.\(^20\),\(^21\) Despite the broad applicability of these conventional chemocatalytic
strategies, they usually suffer from limited availability of the starting materials, the use of hazardous reagents and expensive ligands, harsh reaction conditions with by-products, and generation of partially racemized products that need tedious purifications. The direct asymmetric chemical synthesis of chiral unnatural amino acids is still challenging due to the difficulty of setting a precise configuration as well as the necessity of protecting the reactive amine and carboxylic acid groups.

Biocatalysis provides a valuable alternative route to chiral unnatural amino acids. In recent years, various asymmetric enzymatic strategies have been developed for the synthesis of chiral unnatural amino acids, for example (i) reductive amination of keto acids with ammonia or amines using dehydrogenases; (ii) transferring the amino group of an amino acid to keto acids applying aminotransferases; and (iii) conjugate addition of ammonia or amines to α,β-unsaturated carboxylic acids catalyzed by carbon-nitrogen lyases. Among others, carbon-nitrogen lyase-catalyzed enantioselective addition represents a highly attractive strategy for the synthesis of chiral unnatural amino acids. This enzymatic strategy makes use of readily available α,β-unsaturated carboxylic acids as starting substrates without a requirement for cofactor recycling, circumvents steps of protecting or activating carboxylic groups, provides 100% theoretical yield, and normally gives high stereocontrol under mild and potentially green reaction conditions. Several synthetically useful carbon-nitrogen lyases, including aspartate ammonia lyases (DALs), methylaspartate ammonia lyases (MALs), ethylenediamine-\(N,N'\)-disuccinic acid lyase (EDDS lyase), phenylalanine ammonia lyases (PALs) and phenylalanine aminomutases (PAMs), have been successfully applied in the asymmetric synthesis of optically pure noncanonical α- or β-amino acids. In this thesis, our work mainly focuses on the biocatalytic applications of MALs and EDDS lyase for the asymmetric synthesis of functionalized aspartic acids, and these enzymes will be discussed in more detail in the following sections.

1.2.1. Methylaspartate ammonia lyase (MAL)

The enzyme MAL (EC 4.3.1.2) naturally catalyzes the reversible deamination of (L-threo)-3-methylaspartic acid to produce mesaconic acid (Figure 1). In 1959, MAL activity was firstly detected by Barker et al. in cell-free extracts of Clostridium tetanomorphum, where MAL catalyzes the second step in the glutamate catabolic pathway that converts \((S)\)-glutamic acid via (L-threo)-3-methylaspartic acid to give acetyl-coenzyme A. MAL is a homodimeric protein that belongs to the enolase superfamily, and exploits a deamination mechanism that involves general-base catalyzed formation of an enolate anion (\(\text{aci-carboxylate}\)) intermediate which is stabilized by coordination to the essential active site Mg\(^{2+}\) ion (Figure 1).
The wild-type MAL (MAL-WT) was first used in the asymmetric synthesis of aspartic acid (Asp) derivatives by Gani and co-workers. However, the substrate scope of wild-type MAL is very narrow, for which only a few small substituted amines and fumarates were accepted, yielding a limited number of substituted aspartic acids (Figure 2a). The detailed knowledge of the structure and catalytic mechanism of MAL served as a guide to expand the synthetic applicability of this enzyme by protein engineering. Two single-mutant variants of MAL were generated by Poelarends and co-workers, one having an enlarged nucleophile scope (MAL-Q73A) and the other having an enhanced electrophile scope (MAL-L384A). The variant MAL-Q73A exhibited a remarkably broad nucleophile spectrum, accepting a wide range of linear amines and cyclic alkylamines in the addition to mesaconic acid (Figure 2b). Later, this engineered variant MAL-Q37A was further used in the asymmetric synthesis of a large variety of valuable N-substituted L-aspartic acids with excellent stereoselectivity. The other variant MAL-L384A showed an exceptionally broad electrophile spectrum, including fumarate derivatives with various alkyl, aryl, alkoxy, aryloxy, alkylthio and arylthio substituents at the C-2 position (Figure 2b). Subsequently, a three-step chemoenzymatic approach that employed the engineered variant MAL-L384A as biocatalyst was developed for the rapid preparation of a series of C-3-aryloxy, -heteroaryloxy, -cycloalkyloxy and -alkyloxy substituted (L-threo)-aspartic acids as a class of potent excitatory amino acid transporters (EAATs) inhibitors (Figure 3, Chapter 3). Recently, an efficient and step-economic chemoenzymatic route that gives access to the most potent EAATs inhibitor (L-threo)-3-[3-4-(trifluoromethyl)benzoylamino]benzyloxyaspartate (L-TFB-TBOA, Figure 5) and its derivatives at multigram scale was achieved, using the mutant MAL-L384A as biocatalyst (Chapter 2).
CHAPTER 1. Introduction and Scope of the Thesis

1.2.2. Ethylenediamine-\(N,N'\)-disuccinic acid lyase (EDDS lyase)

The enzyme EDDS lyase naturally catalyzes a reversible sequential two-step deamination of (S, S)-EDDS, converting (S, S)-EDDS via the intermediate \(N\)-(2-aminoethyl)aspartic acid (AEAA) into ethylenediamine and two molecules of fumaric acid (Figure 4).\(^44,45\) The cloning of a gene (from Brevundimonas sp. TN3) encoding an EDDS lyase was first reported by Mizunashi in 2001.\(^46\) Recently, Poelarends and co-workers described the identification and cloning of the gene encoding EDDS lyase from the bacterium Chelativorans sp. BNC1, which was isolated from industrial sewage receiving EDTA-containing wastewater effluents.\(^45\) The crystal structure of EDDS lyase reveals that it is a homotetrameric protein that belongs to the aspartase/fumarase superfamily, and exploits a deamination mechanism that
involves general-base catalyzed formation of a carbanion stabilized as its aci-carboxylate (enediolate) form (Figure 4).\textsuperscript{45} Wild-type EDDS lyase has a large nucleophile scope (amine scope) and accepts various mono- and diamines as unnatural substrates in the amination of fumarate.\textsuperscript{45} However, EDDS lyase was found to be highly specific for fumarate, with other α,β-unsaturated carboxylic acids, including crotonic acid, mesaconic acid, itaconic acid, 2-pentenoic acid, or glutaric acid, not accepted as alternative electrophiles.\textsuperscript{45} The fruitful biocatalytic applications of EDDS lyase for asymmetric preparation of unnatural amino acids will be demonstrated in Chapters 5-7 of this thesis.

Figure 4. Natural reaction catalyzed by EDDS lyase and the proposed catalytic mechanism.
2. Scope of the thesis

Optically pure functionalized L-aspartic acids are widely found as core structures in pharmaceuticals, nutraceuticals, and agrochemicals, such as fibrinogen receptor antagonist Lotrafiban (2, Figure 5)\(^4\), artificial sweetener Neotame (3, Figure 5)\(^4\), excitatory amino acid transporters (EAATs) inhibitor L-TFB-TBOA (4, Figure 5)\(^4\), metallo-\(\beta\)-lactamase inhibitor Aspergillomarasmine A (AMA, 5, Figure 5)\(^5\), and biodegradable metal-chelator \((S, S)\)-EDDS (6, Figure 5)\(^6\). Despite their broad applications, the direct asymmetric synthesis of functionalized L-aspartic acids remains a challenge. The aim of the research presented in this thesis was therefore to develop novel and efficient biocatalytic methodologies for the direct asymmetric synthesis of functionalized L-aspartic acid derivatives. The scope of the thesis focuses attention on (i) biocatalytic asymmetric synthesis of C-3 substituted aspartic acids using MALs (Part 1, Chapters 2-4); and (ii) biocatalytic asymmetric synthesis of N-substituted aspartic acids applying EDDS lyase (Part 2, Chapters 5-7).

**Figure 5.** Representative biologically active compounds containing the structure of aspartic acid.

**Part 1: Chemoenzymatic synthesis of C-3 substituted aspartic acids using MAL-L384A**

In Chapter 2, we describe an efficient and step-economic chemoenzymatic route involving MAL-L384A as the biocatalyst that gives access to enantio- and diastereopure L-TFB-TBOA and its derivatives in 9 steps with 6% overall yield at multigram scale. These difficult aspartic acid derivatives are privileged compounds for studying the roles of EAATs in regulation of glutamatergic neurotransmission. Compared to the previously reported 20-step chemical synthesis of L-TFB-TBOA, our chemoenzymatic methodology gives a dramatic reduction in step count with fewer than half the steps.
In Chapter 3, we describe the chemoenzymatic asymmetric synthesis of a series of novel aspartic acid derivatives comprising (cyclo)alkyloxy and (hetero)aryloxy substituents at the C-3 position, using MAL-L384A as the biocatalyst in the stereoselective step. Remarkably, all these aspartic acid derivatives were found to be potent non-substrate pan inhibitors of EAAT1-4 with IC_{50} values ranging from 0.49 to 15 μM. In addition, two unique hybrid compounds were designed and synthesized, which displayed considerably lower IC50 values at EAAT1-4 (11-140 nM) than those shown by the respective parent molecules.

In Chapter 4, we demonstrate the reversible and temporal control over prokaryotic aspartate transporter GltTk using photo-controlled inhibitors and light. Based on the known inhibitor L-TFB-TBOA, seven novel inhibitors exhibiting a photoswitchable azobenzene moiety were synthesized using a key stereoselective enzymatic step. Of the seven azo-TBOAs, those with alkyloxy substituents on the para-position showed excellent photochemical properties and long half-lives of the cis isomer. The largest difference in inhibitory activity was observed for p-MeO-azo-TBOA, with the trans isomer (IC_{50} = 2.5 ± 0.4 μM) being 3.6-fold more active than the cis isomer (IC_{50} = 9.1 ± 1.5 μM), which was successfully used to reversibly control the transport rate by light in situ.

Part 2: Biocatalytic synthesis of N-substituted aspartic acids using EDDS lyase

The fungal natural product AMA was recently identified as a potent and selective inhibitor of metallo-β-lactamases and a promising co-drug candidate to fight antibiotic resistant bacteria. In Chapter 5, we report the first biocatalytic asymmetric synthesis of AMA as well as the related natural products Aspergillomarasmine B and Toxin A from retrosynthetically designed precursors. This synthetic route highlights a highly regio- and stereoselective carbon-nitrogen bond-forming step catalyzed by EDDS lyase. The enzyme shows broad substrate promiscuity, accepting a wide variety of amino acids with terminal amino groups for selective addition to fumarate. In addition, we also report a two-step chemoenzymatic cascade route for the rapid diversification of enzymatically prepared aminocarboxylic acids by N-alkylation in one pot. As such, our (chemo)enzymatic methodology provides a useful alternative route to complex aminocarboxylic acid products.

In Chapter 6, we report the asymmetric synthesis of various N-cycloalkyl-substituted L-aspartic acids using EDDS lyase and MAL-Q73A as biocatalysts. Particularly, EDDS lyase shows broad non-natural substrate promiscuity, allowing the selective addition of homo- and heterocycloalkyl amines (comprising four-, five- and six-membered rings) to fumarate, giving the corresponding N-cycloalkyl-substituted L-aspartic acids with excellent
enantiomeric excess (ee >99%). This biocatalytic methodology offers an alternative synthetic choice to prepare difficult \(N\)-cycloalkyl-substituted amino acids.

In Chapter 7, we report a biocatalytic methodology for the synthesis of optically pure (\(S\))-\(N\)-arylated aspartic acids using EDDS lyase as the biocatalyst. This enzyme shows a remarkably broad substrate scope, enabling the addition of a variety of arylamines to fumarate with high conversions, yielding the corresponding \(N\)-arylated aspartic acids in good isolated yields and with excellent optical purity (ee >99%). Furthermore, we developed a chemoenzymatic approach towards synthetically challenging chiral 2-aryl-5-carboxylpyrazolidin-3-ones, using arylhydrazines as bisnucleophilic donors in the EDDS lyase-catalyzed hydroamination of fumarate followed by an acid-catalyzed intramolecular amidation. In addition, we successfully combined the EDDS lyase-catalyzed hydroamination and acid-catalyzed cyclization steps in one pot, thus providing a simple chemoenzymatic cascade route for synthesis of enantiomerically pure pyrazolidin-3-ones. Hence, these newly developed biocatalytic methods provide convenient alternative routes to important chiral \(N\)-arylated aspartic acids and difficult 2-aryl-5-carboxylpyrazolidin-3-ones.

In Chapter 8, we summarize the work described in this thesis and suggest some perspectives for future research.

References


44. Witschel, M. & Egli, T. Purification and characterization of a lyase from the EDTA-degrading bacterial strain DSM 9103 that catalyzes the splitting of [S,S]-ethylenediaminedisuccinate, a structural isomer of EDTA. *Biodegradation* 8, 419–428 (1997).


PART 1

(CHAPTERS 2-4)

Chemoenzymatic Synthesis of C-3 Substituted Aspartic Acids Using MAL-L384A