Biocatalytic asymmetric hydroamination by native and engineered carbon-nitrogen lyases
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Introduction
Chapter 1

1. Carbon-Nitrogen Lyases and Biocatalytic Hydroamination of Unsaturated Carboxylic Acids

Lyases are a class of biocatalysts that cleave covalent bonds (C-C, C-O, C-N, C-S and others) through elimination reactions, yielding double bonds or ring structures in the resulting products. Carbon-nitrogen (C-N) lyases (EC 4.3.X.X.) selectively catalyze C-N bond cleavage.[1] They have been isolated and characterized from different prokaryotic and eukaryotic sources, playing roles in various physiological activities such as nitrogen metabolism, amino acid metabolism, biosynthesis of natural products, etc.[1–3] Based on the chemical nature of their products, C-N lyases are further divided into four subclasses, which are ammonia-lyases (4.3.1.X), amide/amidine-lyases (4.3.2.X), amine-lyases (4.3.3.X) and others (4.3.99.X), showing rich diversity in structural and mechanistic features. The well-studied biotechnologically relevant C-N lyases such as aspartate ammonia lyase (aspartase), 3-methylaspartate ammonia lyase (MAL), and histidine and phenylalanine ammonia lyase (HAL and PAL) belong to three different protein superfamilies, namely aspartase/fumarase superfamily, enolase superfamily, and 4-methylideneimidazole-5-one (MIO) cofactor dependent enzyme family, respectively. In recent years, several new C-N lyases were identified, like nitrosuccinate lyase[4] and choline trimethylamine-lyase[5], expanding our knowledge of C-N lyases regarding reactions, structures and catalytic mechanisms.

C-N lyases have shown great potential as biocatalysts for synthesis of optically pure (un)natural amino acids via asymmetric hydroamination of α,β-unsaturated mono- or dicarboxylic acids (Figure 1).[3,6] C-N lyase catalyzed reactions employ ubiquitous alkenes as starting materials, require no external cofactors and are atom-economic and highly selective, providing a complementary strategy to form chiral amino acids lining up with other biocatalytic approaches (Figure 1).[7] Early in the 1970s, ammonia-lyases, such as aspartase and phenylalanine ammonia lyase (PAL), were exploited in reverse to synthesize their natural substrates L-aspartic acid and L-phenylalanine starting from fumaric acid and cinnamic acid, respectively.[8] In recent years, C-N lyases with desired biocatalytic profiles (substrate scope, activity, selectivity) have been obtained by discovery or engineering. The rapidly expanding toolbox of C-N lyases enables enzymatic production of a broad range of unnatural amino acids, including substituted aspartic acids, β-substituted α- and β-alanines, with most reactions having no counterparts in organic chemistry (Table 1 and 2).[6] Besides, multienzymatic and chemoenzymatic cascades containing C-N lyases have been developed, giving access to complex bioactive molecules in a sustainable and step-economic manner. C-N lyases provide an alternative approach to synthesize valuable amino acids and offer more options for biocatalytic retrosynthesis.

L-aspartic acid derivatives are important bioactive molecules with wide applications in pharmaceutical and nutraceutical fields (Figure 2). Three types of C-N lyases, aspartase, MAL and ethylenediamine-\(N,N'\)-disuccinic acid lyase (EDDS lyase), have been used for biocatalytic preparation of L-aspartic acid derivatives. Here a review on EDDS lyase and
**Introduction**

**A** Representative biocatalytic synthesis of chiral amino acids

*Transamination*

\[
\text{R}_1^\text{COOH} + \text{R}_2^\text{NH}_2 \xrightarrow{\text{transaminase} \ PLP} \text{R}_1^\text{NH}_2\text{COOH} + \text{R}_2^\text{R}_3
\]

\( n = 0,1,2 \)

*Reductive amination*

\[
\text{R}^\text{COOH} + \text{NH}_3 \xrightarrow{\text{AADH} \ NAD(P)H \ NAD(P)^+} \text{R}^\text{NH}_2\text{COOH} + \text{H}_2\text{O}
\]

**Resolution**

\[
\text{R}^\text{COOH} \xrightarrow{\text{lipase/acylase/amidase}} \text{R}^\text{NH}_2\text{COOH}
\]

**B** Biocatalytic asymmetric hydroamination

\[
\text{R}_1^\text{COOH} + \text{H}_2\text{N}_\text{R}_2 \xrightarrow{\text{C-N lyase}} \text{R}_1^\text{NH}_2\text{R}_2
\]

**Biocatalytic asymmetric hydroamination**

- Atom economic
- Starting with versatile alkenes
- Enantioselective
- Regioselective

---

**Figure 1.** (A) Biocatalytic synthetic approaches for chiral (un)natural amino acids; (B) C-N lyase-catalyzed synthesis of (un)natural amino acids via asymmetric hydroamination of \( \alpha,\beta \)-unsaturated carboxylic acids.

**Table 1.** Synthesis of optically pure \( \alpha \)-amino acids using C-N lyases.

<table>
<thead>
<tr>
<th>( R_1 )</th>
<th>( R_2 )</th>
<th>( R_3 )</th>
<th>C-N lyase</th>
<th>Products</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>COOH</td>
<td>H</td>
<td>H, Me, OH, OMe, NH₂</td>
<td>AspB</td>
<td>Aspartate and its derivatives</td>
<td>[9]</td>
</tr>
<tr>
<td>COOH</td>
<td>H, Me, Et, Pr, iPr, Cl</td>
<td>H, Me, Et, OH, OMe, NH₂</td>
<td>CtMAL</td>
<td>Aspartate and its derivatives</td>
<td>[10,11]</td>
</tr>
<tr>
<td>COOH</td>
<td>H, alkyl, aryl, alkoxy, aryloxy, alkylthio, arylthio</td>
<td>H</td>
<td>CtMAL-L384A</td>
<td>3-substituted aspartates</td>
<td>[12–14]</td>
</tr>
<tr>
<td>COOH</td>
<td>H, Me</td>
<td>alkyl</td>
<td>CtMAL-Q73A</td>
<td>N-substituted aspartates</td>
<td>[12,15]</td>
</tr>
<tr>
<td>COOH</td>
<td>H</td>
<td>alkyl</td>
<td>EDDS lyase</td>
<td>N-substituted aspartates</td>
<td>[16–18]</td>
</tr>
<tr>
<td>Ph, aryl</td>
<td>H</td>
<td>H</td>
<td>RgPAL, AvPAL, PcPAL</td>
<td>Phenylalanine, ( \beta )-aryl ( \alpha )-alaniines</td>
<td>[6,19–23]</td>
</tr>
</tbody>
</table>
Table 2. Synthesis of optically pure β-amino acids using C-N lyases.

<table>
<thead>
<tr>
<th>R</th>
<th>Biocatalyst</th>
<th>Products</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>-Me,</td>
<td>AspB variants</td>
<td>(R)-β-Aminobutanoic acid, (R)-β-</td>
<td>[24]</td>
</tr>
<tr>
<td>-Et,</td>
<td></td>
<td>Aminopentanoic acid, (S)-β-</td>
<td></td>
</tr>
<tr>
<td>-CONH₂,</td>
<td></td>
<td>Asparagine,</td>
<td></td>
</tr>
<tr>
<td>-Ph,</td>
<td></td>
<td>(S)-β-Phenylalanine</td>
<td></td>
</tr>
<tr>
<td>-Ph, aryl</td>
<td>PAM</td>
<td>β-aryl β-alanines</td>
<td>[6,25]</td>
</tr>
</tbody>
</table>

MAL is provided, discussing their structural and mechanistic characteristics and recent applications in synthesis of important substituted L-aspartic acids.

2. Ethylenediamine-\(N,N'\)-Disuccinic Acid Lyase (EDDS lyase)

2.1 Properties, structure and catalytic mechanism

EDDS lyase is an amine-lyase, which naturally catalyzes two successive steps of reversible deamination of \((S,S)\)-EDDS to fumaric acid and ethylene diamine via the intermediate N-(2-aminoethyl)aspartic acid (AEAA) (Figure 3). \((S,S)\)-EDDS is a metal-chelating compound widely used in industry for a wide range of applications, such as soil remediation, paper manufacturing, waste water treatment and so on. In 1984, \((S,S)\)-EDDS was isolated as a secondary metabolite from the actinomycete Amycolatopsis japonicum, hypothetically serving as a scavenger of zinc (zincophore) and whose bioproduction was strictly repressed by zinc.[26,27] Recently, the biosynthetic gene cluster of \((S,S)\)-EDDS was uncovered using unique bioinformatics approaches by elucidating the zinc-responsive regulation mechanism.[28] The microbial biodegradation of \((S,S)\)-EDDS undergoes another set of reactions different from those in biosynthesis. The biodegradation of \((S,S)\)-EDDS was first observed in some microorganisms isolated from soil and sludge from different sources in the late 1990s, revealing a lyase that supposedly initiated the breakdown of \((S,S)\)-EDDS to fumarate and AEAA or ethylene diamine via a non-hydrolyzing cleavage.[29–31] These microorganisms were exploited for production of \((S,S)\)-EDDS and related aminopolycarboxylic acid chelators from fumarate and diamines at gram scale.[31–34] EDDS lyase was first isolated from Brevundimonas sp. TN3 in 2001, and was sequenced and recombinantly expressed, and used for \((S,S)\)-EDDS production.[35] Yet, detailed biochemical properties and mechanistic and structural features remained unexplored since then.

Our group recently identified an EDDS lyase from Chelativorans sp. BNC1 by using a BLAST search with the TN-3 EDDS lyase gene as the query (79% identity), and subsequently
Inhibitors of excitatory amino acid transporter (EAATi)

![Chemical structures of L-TBOA, L-TFB-TBOA, L-3-BA, and L-3-OH-Asp](image)

Aminopolycarboxylic acid metal chelators

![Chemical structures of (S,S)-EDDS, (S,S)-IDS, ASMA, and AM](image)

Inhibitor of metallo-β-lactamase

![Chemical structures of (S,S)-EDDS, (S,S)-IDS, ASMA, and AM](image)

Low-calorie artificial sweeteners

![Chemical structures of aspartame, neotame, and advantame](image)

**Figure 2.** Representative bioactive molecules and natural products comprising an L-aspartic acid moiety.

**Figure 3.** The EDDS-lyase-catalyzed reversible deamination of (S,S)-EDDS to fumaric acid and ethylene diamine via AEAA serves as the first step in the bacterial biodegradation of (S,S)-EDDS.

investigated the structure and catalytic mechanism of an EDDS lyase for the first time.\[18\] The EDDS lyase from strain BNC1 catalyzes the reversible deamination of (S,S)-EDDS with maximum activity at 60 °C and pH 8.0. Except for its natural substrates, it also shows activity towards ammonia and various mono- and diamines in addition to fumaric acid.

Crystal structures of EDDS lyase in unliganded form and those bound with fumarate, AEAA and (S,S)-EDDS were solved by X-ray diffraction analysis.\[18\] EDDS lyase is a homotetramer (56 kDa per subunit) sharing the characteristic tertiary and quaternary structural features of members of the aspartase/fumarase superfamily.\[2\] Interestingly, the catalytically essential SS-loop of EDDS lyase shows little movement upon substrate binding, which is different from the flexible SS-loop undergoing an open/closed conformational change upon substrate binding as observed in most aspartase/fumarase superfamily members. EDDS lyase
has a composite active site located at the interface of three subunits, with most residues forming the α- and β-carboxylate binding pockets being highly conserved.\[^{[18]}\] The amine binding site is less conserved, and rich in polar and charged side chains making it quite hydrophilic. Residues Asn288 and Asp290 form water-mediated hydrogen-bond interactions with the distal amino group of the bound (S,S)-EDDS, appearing to play important roles in binding and positioning of the natural substrate. Ser280 was proposed to serve as the crucial base catalyst, positioned close to the Cβ carbon with a proper orientation for proton abstraction to start the deamination reaction.

Based on the crystal structures and previous mechanistic studies of aspartase/fumarase superfamily members, a general base-catalyzed, sequential two-step deamination mechanism was proposed.\[^{[18]}\] The Ser280 oxyanion first abstracts the proton from Cβ of (S,S)-EDDS, leading to formation of an enediolate intermediate, which is stabilized by interactions with residues from the β-carboxylate binding pocket (Ser111, Arg112, Ser281). The collapse of the enediolate intermediate leads to cleavage of the C-N bond, releasing AEAA and fumarate. AEAA will re-bind in the active site, allowing another round of deamination to give ethylene diamine and fumarate as final products.

2.2 Synthetic applications

(S,S)-EDDS is a widely used metal-chelating compound in industry as well as a pharmaceutically active compound serving as inhibitor of Zn-dependent enzymes like phospholipase C (PL-C)\[^{[26]}\] and metallo-β-lactamase.\[^{[36]}\] Consequently, EDDS degrading microorganisms and the purified EDDS lyase have been exploited for the production of (S,S)-EDDS\[^{[34,35]}\] and related metal chelators (for example, phenylene-diamine,\[^{[33]}\], propanediamine-, and cyclohexylenediamine-N,N'-disuccinic acid\[^{[32]}\]) starting from fumarate and diamines. Suzuki and coworkers first reported the production of (S,S)-EDDS at multigram scale by incubating *Acidovorax* sp. TNT149 cells with fumarate and ethylene diamine at optimum pH (7.5) and temperature (35°C).\[^{[34]}\]

Recently, EDDS lyase from *Chelativorans* sp. BNC1 was used for chemoenzymatic synthesis of the natural products aspergillomarasmine A (AMA), aspergillomarasmine B (AMB) and toxin A, as well as related aminocarboxylic acids with excellent regio- and stereoselective control.\[^{[16]}\] AMA is a potent inhibitor of metallo-β-lactamas (including the notorious NDM-1 and VIM-2) with a low IC\(_{50}\) value at micromolar concentration. It received extensive academic attention yet remained difficult to synthesize with correct (2S,2′S,2″S)-configuration. Toxin A is the direct biosynthetic precursor to AMA and its analogue AMB. EDDS lyase was incubated with fumaric acid and a series of retrosynthetically designed amine substrates in aqueous solution at pH 8.5-9 and 25 or 37 °C. AMA, AMB, toxin A and their derivatives were obtained with excellent stereoselectivity (e.e >99%, d.e. >98%) and in moderate to good yields. Based on the biocatalytic synthesis of toxin A, a one-pot two-step chemoenzymatic approach was developed for rapid and efficient synthesis of AMB and its
derivatives. Compared to previous chemocatalytic methods, this chemoenzymatic approach significantly reduced the total synthetic steps for AMA, AMB, and toxin A, showing great potential for efficient practical synthesis of complex amino(poly)carboxylic acids.

EDDS lyase from *Chelativorans* sp. BNC1 was also employed for enantioselective synthesis of complex *N*-cycloalkyl substituted L-aspartic acids which are structurally distinct from its natural substrates. Reactions were performed with fumaric acid, various homo- and heterocycloalkyl amines (comprising four-, five- and six-membered rings) and EDDS lyase at pH 8.5 and room temperature. EDDS lyase exhibited broad substrate promiscuity, accepting a variety of cycloalkylamines in hydroamination of fumarate to give the corresponding *N*-cycloalkyl-L-aspartic acids with excellent enantioselectivity (e.e. >99%).

Another synthetic application of EDDS lyase is described in Chapter 5 of this thesis, demonstrating the use of an engineered variant of EDDS lyase for asymmetric biocatalytic synthesis of an important precursor of neotame. Neotame is a commercial low-calorie artificial sweetener used as sugar substitute in a broad range of food products. In fact, it is a *N*-substituted derivative of the widely used dipeptide sweetener aspartame (Figure 2). The chemical synthesis of neotame involves an intermediate *N*(3,3-dimethylbutyl)-L-aspartic acid, which is prepared by metal-catalyzed reductive alkylation of L-aspartic acid. Wild-type EDDS lyase (0.15 mol%) was incubated with fumaric acid and various alkylamines in sodium phosphate buffer at pH 8.5 and room temperature. The reactions yielded the neotame precursor *N*(3,3-dimethylbutyl)-L-aspartic acid and five related compounds with 82-97% conversion (after 7 days) and >99% e.e. To further improve the synthetic usefulness of EDDS lyase, two rounds of site-saturation mutagenesis and activity screening were performed at two positions selected on basis of the crystal structure. A highly efficient EDDS lyase variant (D290M/Y320M) was obtained with a 1140-fold activity improvement over the wild-type enzyme, allowing the selective synthesis of the neotame precursor and related compounds (including the precursor to advantame, Figure 2) with high enantioselectivity and conversion, requiring only a few hours reaction time instead of 7 days, while using low biocatalyst loadings (0.05 mol%).

### 3. 3-Methylaspartate Ammonia Lyase (MAL)

#### 3.1 Properties, structure and catalytic mechanism

Identified from several facultative anaerobic bacteria, the MAL enzyme forms part of the glutamate catabolic pathway and reversibly deaminates *L*-threo- and *L*-erythro-3-methylaspartate to give mesaconate. Two best studied MAL enzymes were isolated from *C. tetanomorphum* (CtMAL) and *C. amalonaticus* (CaMAL), with the first crystal structures reported in 2002. The overall structure of the homodimeric MAL (45 kDa per subunit) resembles that of members of the enolase superfamily, sharing the characteristic TIM barrel
fold (8-fold α/β barrel). MAL requires K\(^+\) and Mg\(^{2+}\) for optimal activity, and the catalytic
mechanism of MAL is proposed to involve general base-catalyzed proton abstraction
resulting in the formation of an enolate anion intermediate, which is stabilized by interactions
with the active site Mg\(^{2+}\) ion and side chains of amino acid residues.\(^{[9,38]}\) Based on structural
work and mutagenesis studies, residues Lys331 and His194 have been identified as the \(S\)- and
\(R\)-specific base catalysts, respectively.\(^{[40]}\)

### 3.2 Synthetic applications

Based on its natural substrate promiscuity, CtMAL has been used for asymmetric synthesis of
L-aspartic acid derivatives with small \(N\)- and \(\beta\)-substitutions.\(^{[6,9]}\) The scope of biocatalytic
applications was greatly enlarged by structure-guided engineering of CtMAL, which led to an
engineered variant (MAL-Q73A) with broad nucleophile scope, accepting linear and
cycloalkylamines, and an engineered variant (MAL-L384A) with wide electrophile scope,
accepting non-native fumarate derivatives with alkyl, aryl, alkoxy, aryloxy, alkylthio, and
aryltio substitutions at the C-2 position.\(^{[12]}\) Using these engineered MAL enzymes, a diverse
collection of \(N\)- and \(\beta\)-substituted L-aspartic acids were synthesized by asymmetric
hydroaminations with moderate to good stereoselectivity.\(^{[12,15]}\) In another study, a variant of
CtMAL (MAL-H194A) with strongly enhanced diastereoselectivity, which was obtained by
mechanism-based engineering, has been exploited for asymmetric synthesis and kinetic
resolution of various 3-substituted aspartic acids.\(^{[40,41]}\)

Inspired by the expanded scope towards 2-substituted fumarate derivatives, MAL variants
have been exploited in chemoenzymatic synthesis of excitatory amino acid transporter
(EAAT) inhibitors that are extremely difficult to synthesize chemically. Located in the
membranes of mammalian neurons and surrounding glia cells, EAATs are responsible for
regulating the concentration of the excitatory neurotransmitter glutamate in the synaptic cleft.
Inhibitors of EAATs are therefore useful tools to study the precise functions of these
transporters in glutamatergic neurotransmission and in neurological disorders related with
extracellular glutamate accumulation. For example, L-\(\text{threo}\)-3-benzyloxyaspartate (L-TBOA,
Figure 2) is a widely-used nonselective inhibitor of EAATs, the chemical synthesis of which
is highly challenging (11 steps in total with an overall yield of <1%).

By using an engineered variant of MAL as biocatalyst, de Villiers and coworkers
developed an elegant three-step chemoenzymatic synthesis route to L-TBOA and several
ring-substituted derivatives.\(^{[13]}\) 2-Benzyloxyfumarate and a series of derivatives with F, CH\(_3\)
and CF\(_3\) groups at the ortho, meta and para position of the aromatic ring, which were
obtained via two-step chemical synthesis from dimethyl acetylenedicarboxylate, were reacted
with ammonia in the presence of the MAL-L384A or MAL-L384G variant at room
temperature and pH 9.0. Reactions achieved >89% conversion within 24 h, with products L-
TBOA and seven analogues purified with good yield (57-78%) and identified as the desired
L-\(\text{threo}\) isomers with excellent stereoselectivity (e.e. >99%; d.e. >95%).
The synthetic usefulness of MAL variants for preparation of EAAT blockers was further extended by synthesizing a series of L-TBOA derivatives whose pharmacological properties were evaluated.[14] Catalyzed by MAL-L384A, aspartate derivatives with (cyclo)alkyloxy and (hetero)aryloxy substituents at the C-3 position were prepared by hydroamination of the corresponding fumarate derivatives (prepared from dimethyl acetylenedicarboxylate) with excellent stereoselectivity. These derivatives showed potent inhibitory activities towards EAAT1-4 subtypes with IC₅₀ values ranging from micro- to nanomolar concentrations.

Based on the synthesis of L-TBOA, the chemoenzymatic preparation of another potent and widely used nontransportable EAAT inhibitor, (L-threo)-3-[3-[4-(trifluoromethyl)benzoylamino]benzyl氧]aspartate (L-TFB-TBOA, Figure 2) was achieved with excellent stereochemical control and a dramatically reduced number of synthetic steps.[42] L-TBOA was first synthesized by the MAL-L384A catalyzed hydroamination of 2-benzyloxyfumarate, and subsequently used as starting material for debenzylation and protection (3 steps), yielding the key intermediate dimethyl (L-threo)-N-Boc-3-hydroxyaspartate (71-73% yield). This chiral intermediate was subsequently subjected to O-alkylation and global deprotection, which gave rise to L-TFB-TBOA at multigram scale with 6% overall yield in 9 steps (for comparison, chemical synthesis takes 20 steps). This method provides a convenient strategy to produce L-aspartic acid derivatives with large aryloxy substituents at the C3 position, enabling stereoselective preparation of four L-TFB-TBOA derivatives with strong inhibitory activities for EAAT1-4 subtypes (IC₅₀ values range from 5 to 530 nM).[14] This strategy was later exploited for the design and preparation of novel photo-controlled glutamate transporter inhibitors by functionalization of L-TBOA with a photoswitchable azobenzene moiety (azo-TBOAs).[43] Remarkably, (L-threo)-trans-3-(3-((4-(methoxy)phenyl)diazenyl)benzyl氧) aspartate (p-MeO-azo-TBOA) showed good photochemical properties, reversibly switched from the trans to cis isomer in response to irradiation, with the isomers showing a 3.6-fold difference in inhibitory activity towards the prokaryotic transporter GltrN.

Lastly, application of MAL and its H194A variant in multienzymatic cascade synthesis was demonstrated for the production of vitamin B₅ [(R)-pantothenic acid] and its derivatives.[44] Vitamin B₅ is the biosynthetic precursor of coenzyme A, and its derivatives serve as important synthetic precursors to antimicrobial pantothiamides. A one-pot, three-step enzymatic cascade was developed, consisting of asymmetric hydroamination (catalyzed by MAL or MAL-H194A), α-decarboxylation (catalyzed by an appropriate decarboxylase) and condensation (catalyzed by pantothenate synthase) reactions. Starting from achiral fumarate or mesaconate, ammonia and (R)-pantoate, vitamin B₅ and both diastereoisomers of α-methyl-substituted vitamin B₅ were produced in good isolated yield and with excellent stereoselectivity (>99% e.e.).
4. Concluding Remarks

Asymmetric hydroamination of unsaturated carboxylic acids by C-N lyases is an attractive biocatalytic strategy to synthesize optically pure amino acids. C-N lyases have been applied in multienzymatic and chemoenzymatic cascade syntheses of complex molecules with pharmaceutical significance. EDDS lyase has been discovered more than ten years ago, yet its biochemical properties and structural and mechanistic features have been elucidated recently. EDDS lyase from *Chelativorans* sp. BNC1 has broad substrate promiscuity and displays excellent selectivity and evolvability, providing great potential for practical synthesis of important L-aspartic acid derivatives as tools for neurobiological research and synthetic precursors to pharmaceuticals and food additives.

References


Introduction


