Biocatalytic Enantioselective Hydroaminations for Production of N-Cycloalkyl-Substituted L-Aspartic Acids Using Two C–N Lyases

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Manuscript received: November 22, 2018; Revised manuscript received: February 12, 2019;
Version of record online: March 5, 2019

Supporting information for this article is available on the WWW under https://doi.org/10.1002/adsc.201801569

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Abstract: N-cycloalkyl-substituted amino acids have wide-ranging applications in pharma- and nutraceutical fields. Here we report the asymmetric synthesis of various N-cycloalkyl-substituted L-aspartic acids using ethylenediamine-N,N'-disuccinic acid lyase (EDDS lyase) and a previously engineered variant of methylaspartate ammonia lyase (MAL-Q73A) as biocatalysts. Particularly, EDDS lyase shows broad non-natural substrate promiscuity and excellent enantioselectivity, 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 >99% e.e. This biocatalytic methodology offers an alternative synthetic choice to prepare difficult N-cycloalkyl-substituted amino acids. Given its very broad amine scope, EDDS lyase is an exceptionally powerful synthetic tool that nicely complements the rapidly expanding toolbox of biocatalysts for asymmetric synthesis of noncanonical amino acids.

Keywords: Biocatalysis; Hydroamination; EDDS lyase; Noncanonical amino acids

N-substituted L-aspartic acids are noncanonical amino acids that have wide applications in pharma- and nutraceutical fields, serving as drug candidates and chiral building blocks for pharmaceutically active molecules, artificial sweeteners and peptidomimetics. [1–7] Therefore, the development of methodologies for the efficient synthesis of N-substituted aspartic acids in enantioenriched form is of high academic and industrial interest. The most common chemocatalytic synthetic strategy is the Michael addition of suitable amines to maleic acid, fumaric acid, their ester or amide derivatives, or monoalkalaki salts [7–9] However, in these chemocatalytic reactions, racemic product mixtures are obtained. To achieve the desired single L-enantiomer, purification or resolution is needed, leading to unsatisfactory product yields lower than 50%.

Asymmetric hydroamination of alkenes is a desirable atom-economic route to introduce nitrogen-based functionalities into organic molecules [10–12] Enzymatic addition of ammonia or amines to appropriate α,β-unsaturated mono- or dicarboxylic acids using C–N lyases as biocatalysts has become an attractive methodology to synthesize chiral α-amino acids, such as phenylalanine and aspartic acid, and their derivatives (Scheme 1) [10,13–15] This enzymatic strategy employs readily available α,β-unsaturated acids as starting materials, escaping steps of protecting/activating carboxylic groups by derivatization as the corresponding esters or amides, and normally gives high stereoccontrol under mild and potentially green reaction conditions. Using this concept, a range of N-substituted L-aspartic acids has previously been prepared [16–18] For instance, aspartate ammonia lyase (AspB) from Bacillus sp.
YM55-1 and methylaspartate ammonia lyase (MAL) from *Clostridium tetanomorphum* were found to accept several small substituted amines, like hydroxylamine, methoxyamine and methylamine, as substrates for hydroamination of fumarate or mesaconate, yielding the corresponding *N*-substituted L-aspartic acid derivatives.\(^{[16,17]}\) 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 (acyl-carboxylate) intermediate that is stabilized by coordination to the essential active site Mg\(^{2+}\) ion.\(^{[14]}\) The detailed knowledge of the structure and catalytic mechanism of MAL served as a guide to expand the synthetic usefulness of this enzyme by protein engineering.\(^{[19]}\)

Two variants of MAL were generated, one having an enlarged nucleophile scope (MAL-Q73A) and the other having an enlarged electrophile scope (MAL-L384A).\(^{[19]}\) Using MAL-Q73A, a large variety of *N*-substituted L-aspartic acids were synthesized with high enantioselectivity (>99% e.e.).\(^{[19]}\) Structural analysis of MAL-Q73A showed that this mutant enzyme has an enlarged amine binding pocket, without changes in the orientation of active site residues, thus rationalizing its ability to convert the new amine substrates.\(^{[19]}\)

Recently, we reported another C–N lyase, ethylenediamine-\(N,\text{ }N\text{-disuccinic acid (EDDS)}\) lyase from *Chelativorans* sp. BNC1, that can catalyze the reversible addition of ethylene diamine to two molecules of fumarate to produce (S,S)-EDDS, which is an attractive biodegradable metal-chelator.\(^{[21]}\) Wild-type EDDS lyase has a large amine scope, including linear mono- and diamines, and its preparative usefulness was recently demonstrated in the chemoenzymatic synthesis of aspergillosammin A (AMA), an important metallo-\(\beta\)-lactamase inhibitor, as well as various related aminoacarboxylic acids.\(^{[22]}\)

Cycles are versatile and important structural moieties present in organic molecules, which act as good modifiers of properties and biological activities.\(^{[23–26]}\) Functionalization of amino acids with cycles is a subject of great interest, leading to a diversity of useful noncanonical amino acids with broad applications.\(^{[27–29]}\)

Here we report the asymmetric synthesis of various *N*-cycloalkyl-substituted L-aspartic acids using MAL-Q73A and EDDS lyase as biocatalysts. This biocatalytic methodology provides an alternative synthetic choice to prepare difficult *N*-cycloalkyl-substituted amino acids.

Previous work from our group demonstrated that the Q73A mutant of MAL exhibits an expanded amine scope, accepting various structurally distinct amines in hydroamination reactions.\(^{[19,20]}\) This prompted us to first test the potential of MAL-Q73A for the asymmetric synthesis of *N*-cycloalkyl-substituted L-aspartic acids. Out of ten amines tested, MAL-Q73A only accepted amines 2b, 2e and 2f as substrates (Table 1). However, the observed conversions for the reactions with cycloalkyl amines 2b, 2e and 2f were quite low (20–25%). The enzymatic products 3b, 3e, and 3f were purified and identified as the corresponding *N*-substituted aspartic acid derivatives by \(^1\)H NMR, \(^{13}\)C NMR and HRMS (see Supporting Information).

As MAL-Q73A showed a narrow cycloalkyl amine scope, we investigated the amine scope of EDDS lyase. Remarkably, EDDS lyase accepted all ten amines as substrates for addition to fumarate, giving high conversions (83–99%) for most reactions (Table 1). Relatively low conversions were observed for reactions with homocycloalkyl amines 2b and 2e (25% and 10%, respectively) as well as with heterocycloalkyl amines 2h and 2i (14% and 46%, respectively). The enzymatic products were isolated and identified as the anticipated *N*-substituted aspartic acids by \(^1\)H NMR, \(^{13}\)C NMR and HRMS (see Supporting Information). Hence, EDDS lyase shows a broad amine scope, accepting structurally distinct homo- and heterocycloalkyl amines in the hydroamination of fumarate.

The absolute configuration and optical purity of the enzymatic products was determined by HPLC using a chiral stationary phase. For this, *N*-substituted L-aspartic acids and *N*-substituted D-aspartic acids were prepared by chemical synthesis and used as authentic standards (for detailed procedures, see Supporting Information). The three products from the MAL-Q73A-catalyzed hydroamination reactions (3b, 3e and 3f) were identified as the desired L-configured enantiomers, with >99% enantiomeric excess (e.e.) (Table 1, Figures S31, S34, S40). Analysis of eight selected products from the EDDS-lyase-catalyzed hydroamination reactions (3b–d, 3f–j) showed that the absolute configuration of the newly formed stereogenic center was L in all cases (>99% e.e., Table 1, Figures S31–S39), while no D-configured enantiomers were observed. With regard to amino acid products 3c and 3d, pairs of diastereoisomers (S,S- and S,R-configured) were formed from addition of racemic mixtures of 2c and 2d to fumarate, and the diastereomeric ratio (d.r.) values were determined to be 50:50 (Figures S32 and S33). This revealed that EDDS lyase accepts both enantiomers of the starting racemic substrates 2c or 2d in the hydroamination reactions.

**Scheme 1.** Direct hydroamination of \(\alpha,\beta\)-unsaturated carboxylic acids catalyzed by a C–N lyase with enantiocontrol to synthesize optically pure α-amino acids.
Table 1. Enantioselective synthesis of \( N \)-cycloalkyl-substituted \( \text{L} \)-aspartic acids via addition of amines \( 2a-2j \) to fumarate catalyzed by MAL-Q73A or EDDS lyase.

\[
\begin{align*}
-\text{O}_2\text{C} &-\text{C} &-\text{CO}_2^- &+ &\text{RNH}_2 &\xrightarrow{\text{MAL-Q73A or EDDS lyase}} &-\text{O}_2\text{C} &-\text{C} &-\text{NH} &\text{CO}_2^- \\
1 &\text{2a-2j} &\text{3a-3j} \\
\end{align*}
\]

<table>
<thead>
<tr>
<th>Entry</th>
<th>Amine Substrate</th>
<th>Amino Acid Product</th>
<th>Conv. [%]</th>
<th>e.e. [%]</th>
<th>Conv. [%]</th>
<th>e.e./d.r. [%]</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>( \text{2a} )</td>
<td>( \text{3a} )</td>
<td>0</td>
<td>–</td>
<td>99</td>
<td>n.d.[e]</td>
</tr>
<tr>
<td>2</td>
<td>( \text{2b} )</td>
<td>( \text{3b} )</td>
<td>20</td>
<td>&gt;99</td>
<td>25</td>
<td>&gt;99</td>
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<tr>
<td>3[a]</td>
<td>( \text{2c} )</td>
<td>( \text{3c} )</td>
<td>0</td>
<td>–</td>
<td>89</td>
<td>&gt;99/50:50(f)</td>
</tr>
<tr>
<td>4[a]</td>
<td>( \text{2d} )</td>
<td>( \text{3d} )</td>
<td>0</td>
<td>–</td>
<td>83</td>
<td>&gt;99/50:50(f)</td>
</tr>
<tr>
<td>5</td>
<td>( \text{2e} )</td>
<td>( \text{3e} )</td>
<td>20</td>
<td>&gt;99</td>
<td>10</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>( \text{2f} )</td>
<td>( \text{3f} )</td>
<td>25</td>
<td>&gt;99</td>
<td>84</td>
<td>&gt;99</td>
</tr>
<tr>
<td>7</td>
<td>( \text{2g} )</td>
<td>( \text{3g} )</td>
<td>0</td>
<td>–</td>
<td>91</td>
<td>&gt;99</td>
</tr>
<tr>
<td>8</td>
<td>( \text{2h} )</td>
<td>( \text{3h} )</td>
<td>0</td>
<td>–</td>
<td>14</td>
<td>&gt;99</td>
</tr>
<tr>
<td>9</td>
<td>( \text{2i} )</td>
<td>( \text{3i} )</td>
<td>0</td>
<td>–</td>
<td>46</td>
<td>&gt;99</td>
</tr>
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</table>
Thus, both MAL-Q73A and EDDS lyase exhibit excellent enantioselectivity in the addition of substituted amines to fumarate, yielding the desired optically pure L-aspartic acid derivatives.

To further demonstrate the synthetic usefulness of EDDS lyase, preparative-scale synthesis of amino acid was performed. Accordingly, substrates 1 (10 mM) and 2f (100 mM) were incubated with EDDS lyase (0.15 mol% based on fumaric acid) in H2O at pH 9 and room temperature. Reactions were allowed to proceed for 5 d. Conversions were determined using 1H NMR spectroscopy.

The e.e. and d.r. values were determined by chiral HPLC analysis using chemically synthesized reference compounds with known configuration.

The isolated amino acid product could be tentatively assigned the L configuration on the basis of analogy.

Products 3c and 3d were mixtures of (S,S)- and (S,R)-isomers (Figures S32 and S33).

The absolute configuration of product 3j is assigned to be L-trans; Figure S39).

**Table 1.** continued

<table>
<thead>
<tr>
<th>Entry</th>
<th>Amine Substrate</th>
<th>Amino Acid Product</th>
<th>Conv. [%]</th>
<th>e.e. [%]</th>
<th>Conv. [%]</th>
<th>e.e./d.r. [%]</th>
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<tr>
<td>10</td>
<td>2j</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2a-2j</td>
<td>3j</td>
<td>0</td>
<td>92</td>
<td>&gt;99/99:1</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

[a] Substrates 2c and 2d were used as racemic mixtures.
[b] Reaction conditions: fumaric acid (1, 10 mM), amine 2a-j (100 mM), MgCl2 (20 mM), and MAL-Q73A (0.1 mol% based on fumaric acid) in H2O at pH 9 and room temperature. Reactions were allowed to proceed for 5 d. Conversions were determined using 1H NMR spectroscopy.
[c] The e.e. and d.r. values were determined by chiral HPLC analysis using chemically synthesized reference compounds with known configuration.
[d] Reaction conditions: fumaric acid (1, 10 mM), amines 2a-j (100 mM) and EDDS lyase (0.15 mol% based on fumaric acid) in buffer (20 mM NaH2PO4/NaOH, pH 8.5) at room temperature. Reactions were allowed to proceed for 7 d. Conversions were determined using 1H NMR spectroscopy.
[e] The isolated amino acid product could be tentatively assigned the L configuration on the basis of analogy.
[f] Products 3c and 3d were mixtures of (S,S)- and (S,R)-isomers (Figures S32 and S33).
[g] The absolute configuration of product 3j is assigned to be L-trans; Figure S39).

**Experimental Section**

**General procedure for enzymatic synthesis of N-cycloalkyl-substituted aspartic acids.** For a typical MAL-Q73A reaction, an initial reaction mixture (15 ml) consisting of fumaric acid (0.2 mmol, 200 ul of 1 M stock solution), an amine (2a-2j; 2 mmol), and MgCl2 (0.4 mmol, 400 ul of 1 M stock solution) was prepared in demineralized (demi) water and the pH was adjusted to 9.0. MAL-Q73A (0.1 mol% based on fumaric acid) was added to start the reaction, and the volume of the reaction mixture was immediately adjusted to 20 ml with demi water.
The reaction was allowed to proceed for 5 d, and was stopped by heating at 70°C for 10 min. Reaction progress was monitored by 1H NMR spectroscopy. The conversions were determined by comparing the signals corresponding to fumaric acid (6.5 ppm) and amino acid product.

For a typical EDDS lyase reaction, an initial reaction mixture (15 ml) containing fumaric acid (0.2 mmol, 200 ul of 1 M stock solution) and an amine (2a–2j; 2 mmol) in NaH2PO4–NaOH buffer (20 mM, pH 8.5) was prepared. The pH was adjusted to 8.5 with hydrochloric acid solution. To start the reaction, EDDS lyase (0.15 mol% based on fumaric acid) was added, and the final volume of the reaction mixture was immediately adjusted to 20 ml with the same buffer. The reaction was allowed to proceed for 7 d, and stopped by heating at 70°C for 10 min. The reaction progress was monitored using 1H NMR spectroscopy by comparing signals corresponding to fumaric acid (6.5 ppm) and amino acid product.

Enzymatic products were purified by two steps of ion-exchange chromatography. The purified products were lyophilized and their identity was determined by using 1H NMR, 13C NMR and HRMS. The enantiomeric excess and absolute configuration of the product was determined by HPLC analysis on a chiral stationary phase.

Further experimental details and product characterization are given in the Supporting Information.

Acknowledgements

Jielin Zhang and Haigen Fu acknowledge funding from the China Scholarship Council. The authors thank Dr. Hans Raj, Dr. Thangavelu Saravanan, and Dr. Sabry H. H. Younes for insightful discussions, and Dr. Robert H. Cool for assistance with enzyme purification.

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