Biocatalytic asymmetric hydroamination by native and engineered carbon-nitrogen lyases
Zhang, Jielin

DOI:
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):

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).

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.

Download date: 06-03-2020
Biocatalytic Enantioselective Hydroaminations for Production of N-Cycloalkyl-Substituted L-Aspartic Acids Using Two C-N Lyases

Jielin Zhang, Haigen Fu, Pieter G. Tepper and Gerrit J. Poelarends

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 methylaaspartate 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.
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 peptido-mimetics.[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 monoalkali 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 stereocontrol 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, methoxylamine 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 (aci-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.).[20] 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,N'-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 aspergillomarasmine A (AMA), an important metallo-β-lactamase inhibitor, as well as various related aminocarboxylic acids.[22]

Scheme 1. Direct hydroamination of α,β-unsaturated carboxylic acids catalyzed by a C-N lyase with enantiocontrol to synthesize optically pure α-amino acids.

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 1H NMR, 13C 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 1H NMR, 13C 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.
Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

Table 1. Enantioselective synthesis of N-cycloalkyl-substituted L-aspartic acids via addition of amines 2a-2j to fumarate catalyzed by MAL-Q73A or EDDS lyase.

\[
\begin{array}{cccccc}
\text{Entry} & \text{Amine Substrate} & \text{Substrate} & \text{Amino Acid Product} & \text{Conv. [%]} & \text{e.e. [%]} & \text{Conv. [%]} & \text{e.e./d.r. [%]} \\
\hline
1 & & 2a & 3a & 0 & - & 99 & n.d. \\
2 & & 2b & 3b & 20 & >99 & 25 & >99 \\
3 & & 2c & 3c & 0 & - & 89 & >99:50:50 \\
4 & & 2d & 3d & 0 & - & 83 & >99:50:50 \\
5 & & 2e & 3e & 20 & >99 & 10 & n.d. \\
6 & & 2f & 3f & 25 & >99 & 84 & >99 \\
7 & & 2g & 3g & 0 & - & 91 & >99 \\
8 & & 2h & 3h & 0 & - & 14 & >99 \\
9 & & 2i & 3i & 0 & - & 46 & >99 \\
10 & & 2j & 3j & 0 & - & 92 & >99:99:1 \\
\end{array}
\]

[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 (Figure S2 and S3). [g] The absolute configuration of product 3j is assigned to be L-trans; Figure S9).

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, Figure S1, S4, S10). 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, Figure S1-S9), 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 (Figure S2 and S3). This revealed that EDDS lyase accepts both enantiomers of the starting racemic substrates 2c or 2d in the hydroamination reactions. 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 3f was performed. Accordingly, substrates 1 (10 mM) and 2f (100 mM) were incubated with EDDS lyase (0.15 mol%) in 20 mM NaH2PO4-NaOH at pH 8.5 and room temperature. Under these conditions, excellent conversion (85%) and good isolated yield (54%, 117 mg) of optically pure (>99% e.e.) product 3f were achieved.

In conclusion, we explored the substrate scope of two C-N lyases, a previously engineered variant of MAL (mutant Q73A) [14,19] and wild-type EDDS lyase, [21,22] towards a series of homo- and heterocycloalkyl amines. Pleasingly, EDDS lyase was found to possess broad non-natural substrate promiscuity accepting various cycloalkyl amines in the hydroamination of fumarate. A set of N-cycloalkyl-substituted L-aspartic acids was synthesized with excellent stereoselectivity (>99% e.e. for all amino acid products), including those with interesting heterocyclic substituents that might allow ring opening and further derivatization for various applications. [30-33] Previous studies on EDDS lyase revealed that this C-N lyase, when working in reverse, accepts a wide variety of amino acids and diamines as substrates in the hydroamination of fumarate, giving rise to a large number of useful aminocarboxylic acid products, including biodegradable metal chelators and potent metallo-β-lactamase.
Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

inhibitors.\cite{21,22} Hence, EDDS lyase is a powerful synthetic tool that nicely complements the rapidly expanding toolbox of biocatalysts for asymmetric synthesis of unnatural amino acids. In contrast to its broad amine scope, EDDS lyase was found to be specific for fumarate, and not capable to accept fumaric acid monomethyl ester, crotonic acid, mesaconic acid, itaconic acid, 2-pentenoic acid or glutaric acid as alternative substrate for hydroamination.\cite{21} Work is in progress to expand the electrophile scope of EDDS lyase by structure-based protein engineering.

Acknowledgements

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

References


Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

Supporting Information

Table of content

1. Materials and Instrumentation
2. General Procedure for Enzymatic Synthesis of N-Cycloalkyl-Substituted Aspartic Acids
3. General Procedure for Chemical Synthesis of N-Substituted L- or D-Aspartic Acids
4. Chiral HPLC Chromatograms

References

1. Materials and Instrumentation

All reagents that were used to prepare media and buffers were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO) or Merck (Darmstadt, Germany) and were used without further purification. Fumaric acid and the starting amines, 3-aminooxetane, cyclopentylamine, 3-aminotetrahydrofuran, cyclohexylamine, 4-aminotetrahydropyran, 4-aminopiperidine, tetra-2H-thiopyran-3-amine, 1-methyl-4-piperidinamine, and trans-1,4-diaminocyclohexane were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO). Tetrahydrothiophen-3-amine and tetrahydro-2H-thiopyran-4-amine were synthesized according to protocols reported previously.\textsuperscript{[1,2]} Dowex 50W X8 resin (100-200 mesh) and Dowex 1X8 resin (chloride form, 100-200 mesh) were purchased from Sigma-Aldrich Chemical Co. Ni sepharose 6 fast flow resin and a HiLoad 16/600 Superdex 200 pg column were purchased from GE Healthcare Bio-Sciences AB (Uppsala, Sweden).
NMR analysis was performed on a Brucker 500 MHz machine at the Drug Design laboratory of the University of Groningen. High resolution mass spectrometry (HRMS) was performed by the Mass Spectrometry core facility of the University of Groningen. HPLC analysis was performed on a Shimadzu VP HPLC system. Enzyme purification on a HiLoad 16/600 Superdex 200 pg column was conducted using an AKTAexplorer 10S protein purification system.

2. General Procedure for Enzymatic Synthesis of N-Cycloalkyl-Substituted Aspartic Acids

The MAL-Q73A and EDDS lyase enzymes were overproduced and purified to homogeneity by following previously described protocols.\textsuperscript{[3,4]} 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 (\textit{2a-2j}; 2 mmol), and MgCl\textsubscript{2} (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 (9.6 mg, 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 \textit{\textsuperscript{1}H} 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 (\textit{2a-2j}; 2 mmol) in NaH\textsubscript{2}PO\textsubscript{4}-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 (16.5 mg, 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 \textit{\textsuperscript{1}H} 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, as described previously.\textsuperscript{[3]} The purified products were lyophilized and their identity was determined by using \textit{\textsuperscript{1}H} NMR, \textit{\textsuperscript{13}C} NMR and HRMS. The enantiomeric excess and absolute configuration of the product was determined by HPLC analysis on a chiral stationary phase.
Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

*N-(3-oxetanyl)aspartic acid (3a)*

Synthesis of 3a using EDDS lyase as biocatalyst was performed, and the product was purified according to a slightly modified protocol. The reaction (20 ml) was performed with fumaric acid (0.2 mmol, 200 ul of 1 M stock solution), 2a (2 mmol, 146 mg) and EDDS lyase (16.5 mg, 0.15 mol% of fumaric acid) in demi water. The product was purified by flash silica chromatography (CH2Cl2/MeOH: 50/50, v/v).

7 mg (18% yield); ¹H NMR (500 MHz, D2O) δ 4.97 – 4.92 (m, 2H), 4.85 – 4.79 (m, 2H), 4.56 – 4.50 (m, 1H), 3.77 (dd, J = 8.0, 3.8 Hz, 1H), 2.78 (dd, J = 17.8, 3.8 Hz, 1H), 2.70 (dd, J = 17.8, 8.1 Hz, 1H); ¹³C NMR (126 MHz, D2O) δ 176.74, 172.75, 73.82, 73.75, 58.25, 50.90, 35.68; HRMS (ESI⁺): calcd. for C7H12O5N, 190.0710 [M+H]⁺, found, 190.0710.

*N-cyclopentyl-L-aspartic acid (3b)*

MAL-Q73A: 5 mg (12% yield); EDDS lyase: 10 mg (25% yield); ¹H NMR (500 MHz, D2O) δ 3.83 (dd, J = 9.3, 3.8 Hz, 1H), 3.66 – 3.60 (m, 1H), 2.79 (dd, J = 17.5, 3.7 Hz, 1H), 2.64 (dd, J = 17.5, 9.3 Hz, 1H), 2.08 – 2.01 (m, 2H), 1.79 – 1.61 (m, 6H); ¹³C NMR (126 MHz, D2O) δ 176.95, 173.40, 58.97, 58.57, 35.87, 29.64, 28.95, 23.23 (2C); HRMS (ESI⁺): calcd. for C9H16O4N, 202.1074 [M+H]⁺, found, 202.1073. The enantiopurity was determined by chiral HPLC analysis on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO₄ at 1 ml/min at 60°C, detected at 240 nm. Retention time: 13.7 min.

*N-[(S/R)-3-tetrahydrofuranyl]-L-aspartic acid (3c)*

7 mg (17% yield); ¹H NMR (500 MHz, D2O) δ 4.10 – 3.95 (m, 3H), 3.92 – 3.80 (m, 3H), 2.83 – 2.75 (m, 1H), 2.64 – 2.56 (m, 1H), 2.45 – 2.32 (m, 1H), 2.22 – 2.08 (m, 1H); ¹³C NMR (126 MHz, D2O) δ 177.75 (2C), 174.88(2C), 70.23, 69.62, 66.72(2C), 59.24, 58.84, 57.44(2C), 37.17, 37.11, 29.85, 29.02; HRMS (ESI⁺): calcd. for C₈H₁₄O₅N, 204.0867 [M+H]⁺, found, 204.0867. The stereochemistry was determined by chiral HPLC analysis on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO₄ solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 45.4 min and 55.0 min.

*N-[(S/R)-3-tetrahydrothiophenyl]-L-aspartic acid (3d)*

12 mg (27% yield); ¹H NMR (500 MHz, D2O) δ 4.08 – 4.00 (m, 1H), 3.96 – 3.90 (m, 1H), 3.20 – 3.12 (m, 1H), 3.08 – 2.89 (m, 3H), 2.87 – 2.81 (m, 1H), 2.68 – 2.61 (m, 1H), 2.38 – 2.22 (m, 2H); ¹³C NMR (126 MHz, D2O) δ 177.64, 177.54, 173.80, 173.68, 61.21, 60.69, 59.26, 58.55, 36.31, 36.13,
34.11, 33.82, 32.96, 31.64, 27.24, 27.08; HRMS (ESI\(^{+}\)): calcd. for C\(_8\)H\(_{14}\)O\(_4\)NS, 220.0638 [M+H]\(^{+}\), found, 220.0638. The stereochemistry was determined by chiral HPLC analysis on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO\(_4\) solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 13.2 min and 20.0 min.

\textbf{N-cyclohexyl-\textit{l}-aspartic acid (3e)}

\begin{align*}
\text{MAL-Q73A: 5 mg (12\% yield); } & \text{\textsuperscript{1}H NMR (500 MHz, D\(_2\)O) } \delta 3.95 \text{ (dd, } J = 8.9, 3.9 \text{ Hz, 1H}), 3.17 – 3.11 \text{ (m, 1H), 2.84 (dd, } J = 17.6, 3.8 \text{ Hz, 1H), 2.71 (dd, } J = 17.6, 8.9 \text{ Hz, 1H), 2.10 – 2.02 \text{ (m, 2H), 1.82 – 1.79 \text{ (m, 2H), 1.63 (dt, } J = 12.4, 3.5 \text{ Hz, 1H), 1.45 – 1.27 \text{ (m, 4H), 1.25 – 1.15 \text{ (m, 1H),}} \text{[5] 13C NMR (126 MHz, D\(_2\)O) } \delta 176.75, 175.36, 57.03, 56.87, 35.75, 29.53, 28.81, 24.43, 23.81, 23.74; \text{ HRMS (ESI\(^{+}\)): calcd. for C\(_{10}\)H\(_{18}\)O\(_4\)N, 216.1230 [M+H]\(^{+}\), found, 216.1229. The enantiopurity was determined by chiral HPLC analysis on a Nucleosil 5\(\mu\) chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO\(_4\) at 1 ml/min at 60°C, detected at 240 nm. Retention time: 13.7 min.}
\end{align*}

\textbf{N-(4-tetrahydropyranyl)-\textit{l}-aspartic acid (3f)}

\begin{align*}
\text{MAL-Q73A: 7 mg (16\% yield), EDDS lyase: 18 mg (41\% yield); } & \text{\textsuperscript{1}H NMR (500 MHz, D\(_2\)O) } \delta 4.07 – 4.03 \text{ (m, 2H), 3.98 (dd, } J = 9.0, 3.8 \text{ Hz, 1H), 3.53 – 3.45 \text{ (m, 3H), 2.85 (dd, } J = 17.6, 3.8 \text{ Hz, 1H), 2.71 (dd, } J = 17.7, 9.0 \text{ Hz, 1H), 2.12 – 2.04 \text{ (m, 2H), 1.82 – 1.70 \text{ (m, 2H); 13C NMR (126 MHz, D\(_2\)O) } \delta 177.34, 173.81, 65.72 \text{ (2C), 57.08, 53.35, 36.27, 29.47, 29.00; HRMS (ESI\(^{+}\)): calcd. for C\(_{9}\)H\(_{16}\)O\(_5\)N, 218.1023 [M+H]\(^{+}\), found, 218.1023. The enantiopurity was determined by chiral HPLC analysis on a Nucleosil 5\(\mu\) chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO\(_4\) at 1 ml/min at 60°C, detected at 240 nm. Retention time: 6.7 min.}
\end{align*}

\textbf{N-(4-piperidinyl)-\textit{l}-aspartic acid (3g)}

\begin{align*}
\text{29 mg (67\% yield); } & \text{\textsuperscript{1}H NMR (500 MHz, D\(_2\)O) } \delta 3.94 \text{ (dd, } J = 10.1, 3.4 \text{ Hz, 1H), 3.58 – 3.49 \text{ (m, 3H), 3.13 – 3.07 \text{ (m, 2H), 2.80 (dd, } J = 17.4, 3.5 \text{ Hz, 1H), 2.60 (dd, } J = 17.4, 10.0 \text{ Hz, 1H), 2.43 – 2.33 \text{ (m, 2H), 1.96 – 1.85 \text{ (m, 2H); 13C NMR (126 MHz, D\(_2\)O) } \delta 177.25, 173.53, 57.70, 51.91, 42.28 \text{ (2C), 36.24, 25.91, 25.40; HRMS (ESI\(^{+}\)): calcd. for C\(_{9}\)H\(_{17}\)O\(_4\)N\(_2\), 217.1183 [M+H]\(^{+}\), found, 217.1181. The enantiopurity was determined by chiral HPLC analysis on a Nucleosil 5\(\mu\) chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO\(_4\) at 1 ml/min at 60°C, detected at 240 nm. Retention time: 6.4 min.}
\end{align*}
Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

**N-(tetrahydro-2H-thiopyran-4-yl)-L-aspartic acid (3h)**

5 mg (11 % yield); $^1$H NMR (500 MHz, D$_2$O) $\delta$ 3.94 (dd, $J = 9.1, 3.7$ Hz, 1H), 3.25 – 3.14 (m, 1H), 2.80 (dd, $J = 17.6, 3.7$ Hz, 1H), 2.76 – 2.71 (m, 1H), 2.66 (dd, $J = 17.6, 9.1$ Hz, 1H), 2.45 – 2.33 (m, 2H), 1.86 – 1.69 (m, 2H); $^{13}$C NMR (126 MHz, D$_2$O) $\delta$ 176.64, 173.16, 56.96, 56.28, 35.65, 30.74, 30.16, 26.32, 26.29. The enantiopurity was determined by chiral HPLC analysis on a Nucleosil 5µ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO$_4$ at 0.6 ml/min at 60°C, detected at 240 nm. Retention time: 6.9 min.

**N-(1-methylpiperidin-4-yl)-L-aspartic acid (3i)**

12 mg (26% yield); $^1$H NMR (500 MHz, D$_2$O) $\delta$ 3.89 (dd, $J = 9.9, 3.4$ Hz, 1H), 3.55 – 3.50 (m, 2H), 3.41 – 3.39 (m, 1H), 3.06 – 3.02 (m, 2H), 2.81 (s, 3H), 2.77 (dd, $J = 17.2, 3.6$ Hz, 1H), 2.57 (dd, $J = 17.2, 9.9$ Hz, 1H), 2.40 – 2.30 (m, 2H), 1.97 – 1.85 (m, 2H); $^{13}$C NMR (126 MHz, D$_2$O) $\delta$ 177.18, 173.11, 57.89, 52.45 (2C), 51.48, 42.90, 36.01, 26.54 (2C); HRMS (ESI$^+$): calcd. for C$_{10}$H$_{19}$O$_4$N$_2$, 231.1339 [M+H]$^+$, found, 231.1338. The enantiopurity was determined by chiral HPLC analysis on a Nucleosil 5µ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO$_4$ at 1 ml/min at 60°C, detected at 240 nm. Retention time: 11.2 min.

**N-(trans-4-aminocyclohexyl)-L-aspartic acid (3j)**

4 mg (9% yield); $^1$H NMR (500 MHz, D$_2$O) $\delta$ 3.93 (dd, $J = 9.8, 3.6$ Hz, 1H), 3.27 - 3.16 (m, 2H), 2.79 (dd, $J = 17.5, 3.5$ Hz, 1H), 2.61 (dd, $J = 17.5, 9.8$ Hz, 1H), 2.31 – 2.16 (m, 4H), 1.64 – 1.46 (m, 4H); $^{13}$C NMR (126 MHz, D$_2$O) $\delta$ 177.26, 173.48, 57.61, 55.00, 48.47, 36.08, 28.00, 27.94, 27.24, 26.65; HRMS (ESI$^+$): calcd. for C$_{10}$H$_{19}$O$_4$N$_2$, 231.1339 [M+H]$^+$, found, 231.1337. The stereochemistry was determined by chiral HPLC analysis on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO$_4$ solution:isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 15.2 min.

### 3. General Procedure for Chemical Synthesis of N-Substituted L- or D-Aspartic Acids

Chemical synthesis of N-substituted L- or D-aspartic acids was performed using a previously described method with a slight modification.$^{[3][6]}$ In general, a solution (1 ml) of sodium cyanoborohydride (72.5 mg, 1.15 mmol, 1.5 eq) in dry methanol was added to a mixture of L- or D-aspartic acid (100 mg, 0.75 mmol, 1 eq) and an appropriate ketone/aldehyde (0.90 mmol, 1.2 eq) in dry methanol (2 ml). The mixture was stirred for 24 - 48 hours at room temperature.
The reaction mixture was coated first on silica and then purified by flash silica chromatography [dichloromethane/methanol (50-60%)] without workup. The purified fractions were combined and dried under vacuum.

**N-cyclopentyl-L-aspartic acid (L-3b)**

The synthesis of compound L-3b was conducted with a slightly modified protocol. Compound L-3b was obtained by reacting cyclopentanone (101 mg, 1.20 mmol) with L-aspartic acid (147 mg, 1.10 mmol) and sodium cyanoborohydride (95.0 mg, 1.50 mmol). The reaction mixture was filtered. The filtrate was evaporated, washed with ethyl acetate (3 x 6 ml), and dried under vacuum. 40 mg product was further purified by silica chromatography (DCM/MeOH: 50:50, v/v).

23 mg (10% yield); 1H NMR (500 MHz, D2O) δ 3.83 (dd, J = 9.5, 3.7 Hz, 1H), 3.66 – 3.60 (m, 1H), 2.79 (dd, J = 17.5, 3.7 Hz, 1H), 2.63 (dd, J = 17.5, 9.5 Hz, 1H), 2.08 – 2.01 (m, 2H), 1.77 – 1.61 (m, 6H); 13C NMR (126 MHz, D2O) δ 177.13, 173.46, 58.87, 58.64, 35.95, 29.69, 28.96, 23.25(2C); HRMS (ESI+): calcd. for C9H16O4N, 202.1074 [M+H]+, found, 202.1073.

The chiral HPLC analysis was conducted on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO4 at 1 ml/min at 60°C, detected at 240 nm. Retention time: 13.8 min.

**N-cyclopentyl-D-aspartic acid (D-3b)**

The synthesis of compound D-3b was conducted with a slightly modified protocol. Compound D-3b was obtained by reacting cyclopentanone (101 mg, 1.20 mmol) with D-aspartic acid (147 mg, 1.10 mmol) and sodium cyanoborohydride (95.0 mg, 1.50 mmol). The reaction mixture was filtered. The filtrate was evaporated, washed with ethyl acetate (3 x 6 ml), and dried under vacuum. 57 mg product was further purified by silica chromatography (DCM/MeOH: 50:50, v/v).

14 mg (6% yield); 1H NMR (500 MHz, D2O) δ 3.83 (dd, J = 9.5, 3.7 Hz, 1H), 3.66 – 3.60 (m, 1H), 2.79 (dd, J = 17.4, 3.7 Hz, 1H), 2.62 (dd, J = 17.4, 9.5 Hz, 1H), 2.08 – 2.01 (m, 2H), 1.76 – 1.65 (m, 6H); 13C NMR (126 MHz, D2O) δ 177.23, 173.49, 58.91, 58.69, 35.99, 29.71, 28.93, 23.18(2C); HRMS (ESI+): calcd. for C9H16O4N, 202.1074 [M+H]+, found, 202.1075. The chiral HPLC analysis was conducted on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO4 at 1 ml/min at 60°C, detected at 240 nm. Retention time: 6.0 min.
Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

\[\text{N-[(S/R)-3-tetrahydrofuranyl]-L-aspartic acid [L, (S/R)]-3c}\]

Compound \(\text{L-3c}\) was obtained by reacting dihydrofuran-3(H)-one (73 mg, 0.85 mmol) with L-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 65 mg (43% yield); \(^1\)H NMR (500 MHz, D₂O) \(\delta 4.18 - 3.99\) (m, 3H), \(3.94 - 3.76\) (m, 3H), \(2.88 - 2.77\) (m, 1H), \(2.71 - 2.58\) (m, 1H), \(2.49 - 2.31\) (m, 1H), \(2.28 - 2.10\) (m, 1H); \(^{13}\)C NMR (126 MHz, D₂O) \(\delta 176.96, 176.89, 172.89, 172.82, 69.64, 68.90, 66.70, 66.65, 58.81, 58.50, 57.89, 57.79, 35.77\) (2C), 29.19, 28.41; HRMS (ESI\(^+\)): calcd. for \(\text{C}_8\text{H}_{14}\text{O}_5\text{N}\), 204.0867 [M+H]\(^+\), found, 204.0866. The chiral HPLC analysis was performed on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO\(_4\) solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 45.7 min and 55.4 min.

\[\text{N-[(S/R)-3-tetrahydrofuranyl]-D-aspartic acid [D, (S/R)]-3c}\]

Compound \(\text{D-3c}\) was obtained by reacting dihydrofuran-3(H)-one (73 mg, 0.85 mmol) with D-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 51 mg (33% yield); \(^1\)H NMR (500 MHz, D₂O) \(\delta 4.18 - 3.99\) (m, 3H), \(3.94 - 3.76\) (m, 3H), \(2.88 - 2.77\) (m, 1H), \(2.71 - 2.59\) (m, 1H), \(2.50 - 2.32\) (m, 1H), \(2.28 - 2.08\) (m, 1H); HRMS (ESI\(^+\)): calcd. for \(\text{C}_8\text{H}_{14}\text{O}_4\text{N}\), 204.0867 [M+H]\(^+\), found, 204.0867. The chiral HPLC analysis was performed on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO\(_4\) solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 21.1 min and 40.9 min.

\[\text{N-[(S/R)-3-tetrahydrothiophen]-L-aspartic acid [L, (S/R)]-3d}\]

Compound \(\text{L-3d}\) was obtained by reacting 4.5-dihydro-3(2H)-thiophenone (92 mg, 0.90 mmol) with L-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 56 mg (40% yield); \(^1\)H NMR (500 MHz, D₂O) \(\delta 4.09 - 4.00\) (m, 1H), \(3.96 - 3.87\) (m, 3H), \(3.21 - 3.10\) (m, 1H), \(3.09 - 2.88\) (m, 3H), \(2.87 - 2.80\) (m, 1H), \(2.68 - 2.60\) (m, 1H), \(2.40 - 2.21\) (m, 2H); \(^{13}\)C NMR (126 MHz, D₂O) \(\delta 177.30, 177.18, 172.95, 172.87, 61.38, 60.81, 59.06, 58.33, 35.71, 35.54, 33.94, 33.18, 32.77, 31.39, 27.16, 26.99\); HRMS (ESI\(^+\)): calcd. for \(\text{C}_8\text{H}_{14}\text{O}_4\text{NS}\), 220.0638 [M+H]\(^+\), found, 220.0638. The chiral HPLC analysis was performed on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO\(_4\) solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 13.3 min and 20.3 min.
Chapter 4

N-[(S/R)-3-tetrahydrothiophen]-d-aspartic acid [D, (S/R)-3d]

Compound **D-3d** was obtained by reacting 4,5-dihydro-3(2H)-thiophenone (92 mg, 0.90 mmol) with D-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 56 mg (40% yield); $^1$H NMR (500 MHz, D$_2$O) $\delta$ 4.13 – 3.99 (m, 1H), 3.98 – 3.87 (m, 1H), 3.20 – 3.11 (m, 1H), 3.09 – 2.91 (m, 3H), 2.87 – 2.78 (m, 1H), 2.68 – 2.56 (m, 1H), 2.42 – 2.18 (m, 2H); $^{13}$C NMR (126 MHz, D$_2$O) $\delta$ 177.31, 177.19, 172.95, 172.87, 61.11, 60.79, 59.12, 58.39, 35.72, 35.55, 33.95, 33.18, 32.78, 31.40, 27.17, 27.00; HRMS (ESI$^+$): calcd. for C$_8$H$_{14}$O$_4$NS, 220.0638 [M+H]$^+$, found, 220.0638. The chiral HPLC analysis was on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO$_4$ solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 11.5 min and 52.9 min.

N-cyclohexyl-L-aspartic acid (L-3e)

Compound **L-3e** was obtained by reacting cyclohexanone (118 mg, 1.20 mmol) with L-aspartic acid (147 mg, 1.10 mmol) and sodium cyanoborohydride (95.0 mg, 1.50 mmol). The reaction mixture was filtered. The filtrate was evaporated, washed with ethylacetate (3 x 6 ml), and dried under vacuum. 40 mg product was further purified by silica chromatography (DCM/MeOH: 50:50, v/v).

25 mg (11% yield); $^1$H NMR (500 MHz, D$_2$O) $\delta$ 3.89 (dd, $J = 9.4, 3.7$ Hz, 1H), 3.12 – 3.06 (m, 1H), 2.76 (dd, $J = 17.2, 3.7$ Hz, 1H), 2.60 (dd, $J = 17.3, 9.5$ Hz, 1H), 2.08 – 2.00 (m, 2H), 1.81 – 1.77 (m, 2H), 1.64 – 1.61 (m, 1H), 1.46 – 1.16 (m, 5H); $^{13}$C NMR (126 MHz, D$_2$O) $\delta$ 177.07, 173.57, 56.94, 56.90, 35.87, 29.52, 28.82, 24.43, 23.79, 23.72; HRMS (ESI$^+$): calcd. for C$_{10}$H$_{18}$O$_4$N, 216.1230 [M+H]$^+$, found, 216.1229. The chiral HPLC analysis was conducted on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO$_4$ at 1 ml/min at 60°C, detected at 240 nm. Retention time: 13.5 min.

N-cyclohexyl-D-aspartic acid (D-3e)

Compound **D-3e** was obtained by reacting cyclohexanone (118 mg, 1.20 mmol) with D-aspartic acid (147 mg, 1.10 mmol) and sodium cyanoborohydride (95.0 mg, 1.50 mmol). The reaction mixture was filtered. The filtrate was evaporated, washed with ethylacetate (3 x 6 ml), and dried under vacuum. 50 mg product was further purified by silica chromatography (DCM/MeOH: 50:50, v/v).

24 mg (10% yield); $^1$H NMR (500 MHz, D$_2$O) $\delta$ 3.92 (dd, $J = 9.3, 3.7$ Hz, 1H), 3.16 – 3.10 (m, 1H), 2.78 (dd, $J = 17.4, 3.7$ Hz, 1H), 2.64 (dd, $J = 17.4, 9.3$ Hz, 1H), 2.09 – 2.02 (m, 2H), 1.82 – 1.78 (m, 2H), 1.65 – 1.61 (m, 1H), 1.48 – 1.16 (m, 5H); $^{13}$C NMR (126 MHz, D$_2$O) $\delta$
177.24, 173.62, 57.04, 56.87, 35.94, 29.54, 28.83, 24.43, 23.79, 23.71; HRMS (ESI\(^+\)): calcd. for C\(_{10}\)H\(_{18}\)O\(_4\)N, 216.1230 [M+H]\(^+\), found, 216.1230. The chiral HPLC analysis was conducted on a Nucleosil 5\(\mu\) chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO\(_4\) at 1 ml/min at 60°C, detected at 240 nm. Retention time: 6.4 min.

**N-(4-tetrahydro-2H-pyranyl)-L-aspartic acid (L-3f)**

Compound **L-3f** was obtained by reacting tetrahydro-4H-pyran-4-one (90 mg, 0.90 mmol) with L-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 75 mg (46% yield); \(^1\)H NMR (500 MHz, D\(_2\)O) \(\delta\) 4.07 – 4.03 (m, 2H), 3.95 (dd, \(J = 9.3, 3.7\) Hz, 1H), 3.53 – 3.43 (m, 3H), 2.80 (dd, \(J = 17.5, 3.7\) Hz, 1H), 2.65 (dd, \(J = 17.5, 9.3\) Hz, 1H), 2.12 – 2.04 (m, 2H), 1.82 – 1.71 (m, 2H); \(^1\)C NMR (126 MHz, D\(_2\)O) \(\delta\) 177.11, 173.28, 65.66 (2C), 57.03, 53.48, 35.90, 29.33, 28.88. HRMS (ESI\(^+\)): calcd. for C\(_9\)H\(_{16}\)O\(_5\)N, 218.1023 [M+H]\(^+\), found, 218.1023. The chiral HPLC analysis was performed on a Nucleosil 5\(\mu\) chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO\(_4\) at 1 ml/min at 60°C, detected at 240 nm. Retention time: 6.8 min.

**N-(4-tetrahydro-2H-pyranyl)-D-aspartic acid (D-3f)**

Compound **D-3f** was obtained by reacting with tetrahydro-4H-pyran-4-one (90 mg, 0.90 mmol) with D-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 73 mg (45% yield); \(^1\)H NMR (500 MHz, D\(_2\)O) \(\delta\) 4.08 – 4.02 (m, 2H), 3.95 (dd, \(J = 9.3, 3.8\) Hz, 1H), 3.52 – 3.43 (m, 3H), 2.81 (dd, \(J = 17.5, 3.8\) Hz, 1H), 2.66 (dd, \(J = 17.5, 9.2\) Hz, 1H), 2.13 – 2.03 (m, 2H), 1.82 – 1.71 (m, 2H); \(^1\)C NMR (126 MHz, D\(_2\)O) \(\delta\) 177.00, 173.24, 65.66 (2C), 56.80, 53.52, 35.85, 29.29, 28.86. HRMS (ESI\(^+\)): calcd. for C\(_9\)H\(_{16}\)O\(_5\)N, 218.1023 [M+H]\(^+\), found, 218.1023. The chiral HPLC analysis was performed on a Nucleosil 5\(\mu\) chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO\(_4\) at 1 ml/min at 60°C, detected at 240 nm. Retention time: 4.3 min.

**N-(4-piperidinyl)-L-aspartic acid (L-3g)**

Tert-butyl 4-oxo-1-piperidinecarboxylate (179 mg, 0.90 mmol), L-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol) were reacted to yield (1- (tert-butoxycarbonyl)piperidin-4-yl)-L-aspartic acid (81 mg, 34 % yield). Next, (1-(tert-butoxycarbonyl)piperidin-4-yl)-L-aspartic acid (38 mg, 0.12 mmol) was mixed with water in a closed vial of 4 ml and heated for 20 min at 100 °C. After heating, extra dry ice was added at room temperature, and the heating was continued for 1 h. The compound was purified by flash silica chromatography (gradient elution with dichloromethane/methanol from 50/50 to 10/90) and lyophilized.
26 mg (99.8% yield); H NMR (500 MHz, D$_2$O) $\delta$ 3.58 (dd, $J = 9.3, 4.4$ Hz, 1H), 3.45 – 3.33 (m, 2H), 3.01 – 2.91 (m, 2H), 2.85 – 2.80 (m, 1H), 2.54 (dd, $J = 15.4, 4.5$ Hz, 1H), 2.30 (dd, $J = 15.3, 9.3$ Hz, 1H), 2.19 – 1.97 (m, 2H), 1.61 – 1.47 (m, 2H); C NMR (126 MHz, D$_2$O) $\delta$ 179.04 (2C), 58.23, 50.89, 42.65, 42.59, 40.51, 28.47, 27.38; HRMS (ESI$^+$): calcd. for C$_9$H$_{17}$O$_4$N$_2$, 217.1183 [M+H]$^+$, found, 217.1182.

In order to facilitate the comparison between H NMR of L-3g and enzymatic 3g, L-3g was loaded onto a cation-exchange column (Dowex 50WX8, 100-200 mesh), and eluted in the same way as in the last-step purification of enzymatic 3g (described in previous section). H NMR (500 MHz, D$_2$O) $\delta$ 3.95 (dd, $J = 10.0, 3.3$ Hz, 1H), 3.59 – 3.49 (m, 3H), 3.14 – 3.07 (m, 2H), 2.81 (dd, $J = 17.4, 3.3$ Hz, 1H), 2.61 (dd, $J = 17.6, 10.1$ Hz, 1H), 2.45 – 2.34 (m, 2H), 2.00 – 1.85 (m, 2H). Chiral HPLC analysis was performed on a Nucleosil 5$\mu$ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO$_4$ at 1 ml/min at 60°C, detected at 240 nm. Retention time: 6.4 min.

N-(4-piperidinyl)-D-aspartic acid (D-3g)

Tert-butyl 4-oxo-1-piperidinecarboxylate (179 mg, 0.90 mmol), D-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol) were reacted to yield (1-(tert-butoxycarbonyl)piperidin-4-yl)-D-aspartic acid (85 mg, 36 % yield). Next, (1-(tert-butoxycarbonyl)piperidin-4-yl)-D-aspartic acid (39 mg, 0.12 mmol) was mixed with water in a closed vial of 4 ml and heated for 20 min at 100 °C. After heating, extra dry ice was added at room temperature, and the heating was continued for 1 h. The compound was purified by flash silica chromatography (gradient elution with dichloromethane/methanol from 50/50 to 10/90) and lyophilized.

25 mg (96% yield); H NMR (500 MHz, D$_2$O) $\delta$ 3.58 (dd, $J = 9.2, 4.4$ Hz, 1H), 3.45 – 3.35 (m, 2H), 3.04 – 2.88 (m, 2H), 2.87 – 2.80 (m, 1H), 2.54 (dd, $J = 15.3, 4.4$ Hz, 1H), 2.30 (dd, $J = 15.3, 9.3$ Hz, 1H), 2.15 – 2.03 (m, 2H), 1.61 – 1.47 (m, 2H); C NMR (126 MHz, D$_2$O) $\delta$ 179.00 (2C), 58.26, 50.92, 42.65, 42.58, 40.46, 28.44, 27.41; HRMS (ESI$^+$): calcd. for C$_9$H$_{17}$O$_4$N$_2$, 217.1183 [M+H]$^+$, found, 217.1182. Chiral HPLC analysis was performed on a Nucleosil 5$\mu$ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO$_4$ at 1 ml/min at 60°C, detected at 240 nm. Retention time: 5.4 min.

N-(tetrahydro-2H-thiopyran-4-yl)-L-aspartic acid (L-3h)

Compound L-3h was obtained by reacting tetrahydro-4H-thiopyran-4-one (105 mg, 0.90 mmol) with L-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 26 mg (15 % yield); H NMR (500 MHz, D$_2$O) $\delta$ 3.94 (dd, $J = 9.5, 3.6$ Hz, 1H), 3.27 – 3.16 (m, 1H), 2.82 – 2.70 (m, 5H), 2.62 (dd, $J = 17.5, 9.6$ Hz, 1H), 2.49 – 2.34 (m, 2H), 1.89 – 1.72 (m, 2H); C NMR (126 MHz, D$_2$O) $\delta$ 177.27, 173.45, 57.34, 56.34, 36.02, 31.00, 30.35,
Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

N-(tetrahydro-2H-thiopyran-4-yl)-D-aspartic acid (D-3h)

Compound D-3h was obtained by reacting tetrahydro-4H-thiopyran-4-one (105 mg, 0.90 mmol) with d-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 9 mg (5 % yield); 1H NMR (500 MHz, D2O) δ 3.95 (dd, J = 9.3, 3.6 Hz, 1H), 3.26 – 3.13 (m, 1H), 2.86 – 2.69 (m, 5H), 2.64 (dd, J = 17.5, 9.4 Hz, 1H), 2.49 – 2.33 (m, 2H), 1.90 – 1.69 (m, 2H); 13C NMR (126 MHz, D2O) δ 177.23, 173.44, 57.36, 56.39, 35.97, 31.07, 30.35, 26.52, 26.50. HRMS (ESI\(^+\)): calcd. for C9H16O4NS, 234.07946 [M+H]\(^+\), found, 234.07942. The chiral HPLC analysis was performed on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO\(_4\) at 0.6 ml/min at 60°C, detected at 240 nm. Retention time: 7.1 min.

N-(1-methylpiperidin-4-yl)-L-aspartic acid (L-3i)

Compound L-3i was obtained by reacting N-methyl-piperidone (102 mg, 0.90 mmol) with L-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 156 mg (90% yield); 1H NMR (500 MHz, D2O) δ 3.67 (dd, J = 9.2, 4.2 Hz, 1H), 3.22 – 3.20 (m, 2H), 2.94 – 2.89 (m, 1H), 2.68 – 2.59 (m, 3H), 2.55 (s, 3H), 2.39 (dd, J = 15.9, 9.3 Hz, 1H), 2.14 – 2.03 (m, 2H), 1.68 – 1.59 (m, 2H); 13C NMR (126 MHz, D2O) δ 178.66, 178.44, 58.16, 58.03, 52.65, 51.32, 43.25, 39.61, 29.00, 28.02. HRMS (ESI\(^+\)): calcd. for C10H19O4N2, 231.1339 [M+H]\(^+\), found, 231.1339.

In order to facilitate the comparison between 1H NMR of L-3g and enzymatic 3g, L-3g was loaded onto a cation-exchange column (Dowex 50WX8, 100-200 mesh), and eluted in the same way as in the last-step purification of enzymatic 3g (described in previous section).[3] 1H NMR (500 MHz, D2O) δ 3.95 (dd, J = 10.0, 3.5 Hz, 1H), 3.66 – 3.50 (m, 3H), 3.15 – 3.09 (m, 2H), 2.88 (s, 3H), 2.81 (dd, J = 17.5, 3.5 Hz, 1H), 2.62 (dd, J = 17.5, 10.0 Hz, 1H), 2.47 – 2.37 (m, 2H), 2.04 – 1.91 (m, 2H). Chiral HPLC analysis was performed on Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO\(_4\) at 1 ml/min at 60°C, detected at 240 nm. Retention time: 11.3 min.

N-(1-methylpiperidin-4-yl)-D-aspartic acid (D-3i)

Compound D-3i was obtained by reacting N-methyl-piperidone (102 mg, 0.90 mmol) with d-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol). 39 mg (36% yield); 1H NMR
(500 MHz, D$_2$O) δ 3.68 (dd, $J = 9.0, 3.9$ Hz, 1H), 3.24 – 3.19 (m, 2H), 2.94 (t, $J = 10.5$ Hz, 1H), 2.74 – 2.60 (m, 3H), 2.57 (s, 3H), 2.40 (dd, $J = 16.0, 9.4$ Hz, 1H), 2.16 – 2.05 (m, 2H), 1.71 – 1.59 (m, 2H); $^{13}$C NMR (126 MHz, D$_2$O) δ 178.52, 178.08, 58.06 (2C), 52.54, 51.42, 43.27, 39.38, 28.96, 28.00; HRMS (ESI$^+$): calcd. for C$_{10}$H$_{19}$O$_4$N$_2$, 231.1339 [M+H]$^+$, found, 231.1338. Chiral HPLC analysis was performed on a Nucleosil 5µ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO$_4$ at 1 ml/min at 60°C, detected at 240 nm. Retention time: 8.6 min.

$N$-(cis/trans-4-aminocyclohexyl)-L-aspartic acid [L, (cis/trans)]-3j

Compound L-3j was obtained with a slightly modified protocol. The reaction was started with tert-butyl(4-oxocyclohexyl)carbamate (192 mg, 0.90 mmol), L-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol) in 3 ml methanol to yield (4-((tert-butoxycarbonyl)amino) cyclohexyl)-L-aspartic acid (90 mg, 36% yield). 40 mg of (4-((tert-butoxycarbonyl)amino) cyclohexyl)-L-aspartic acid was mixed with water in a closed vial of 4 ml and heated for 20 min at 100 °C. After heating, extra dry ice was added at room temperature, and the heating was continued for 1 h. The compound was purified by flash silica chromatography (gradient elution with dichloromethane/methanol from 50/50 to 10/90) and lyophilized. 15 mg (54% yield); HRMS (ESI$^+$): calcd. for C$_{10}$H$_{19}$O$_4$N$_2$, 231.1339 [M+H]$^+$, found, 231.1339. The stereochemistry was determined by chiral HPLC analysis on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO$_4$ solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 10.3 min, 15.2 min; d.r. (L, trans / L, cis) = 40: 60.

$N$-(cis/trans-4-aminocyclohexyl)-D-aspartic acid [D, (cis/trans)]-3j

Compound D-3j was obtained with a slightly modified protocol. The reaction was started with tert-butyl(4-oxocyclohexyl)carbamate (192 mg, 0.90 mmol), D-aspartic acid (100 mg, 0.75 mmol) and sodium cyanoborohydride (72.5 mg, 1.15 mmol) in 3 ml methanol to yield (4-((tert-butoxycarbonyl)amino) cyclohexyl)-D-aspartic acid (100 mg, 40%). 40 mg of (4-((tert-butoxycarbonyl)amino) cyclohexyl)-D-aspartic acid was mixed with water in a closed vial of 4 ml and heated for 20 min at 100 °C. After heating, extra dry ice was added at room temperature, and the heating was continued for 1 h. The compound was purified by flash silica chromatography (gradient elution with dichloromethane/methanol from 50/50 to 10/90) and lyophilized. 24 mg (80% yield); $^1$H NMR (500 MHz, D$_2$O) δ 3.72 – 3.65 (m, 2H), 3.29 – 3.20 (m, 1H), 3.15 – 3.02 (m, 1H), 3.02 – 2.92 (m, 1H), 2.78 – 2.69 (m, 1H), 2.65 – 2.54 (m, 2H), 2.47 – 2.33 (m, 2H), 2.17 – 2.10 (m, 1H), 2.09 – 1.93 (m, 3H), 1.88 – 1.60 (m, 9H), 1.41 – 1.28 (m, 3H). HRMS (ESI$^+$): calcd. for C$_{10}$H$_{19}$O$_4$N$_2$, 231.1339 [M+H]$^+$, found, 231.1339. The stereochemistry was determined by chiral HPLC analysis on Chirex
3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO₄ solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm. Retention time: 6.0 min, 7.1 min; d.r. = 47: 53.

4. Chiral HPLC Chromatograms

**Figure S1.** Chiral HPLC analysis of N-cyclopentylaspartic acid (3b). (a) Chromatogram of a racemic mixture of 3b, which was prepared by mixing chemically synthesized L-3b and D-3b in a molar ratio of 1:1; (b) Chromatogram of 3b obtained with MAL-Q73A as biocatalyst; (c) Chromatogram of 3b obtained with EDDS lyase as biocatalyst. Chiral HPLC analysis was performed on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO₄ at 1 ml/min at 60°C, detected at 240 nm.
Figure S2. Chiral HPLC analysis of \(N\)-([S/R]-3-tetrahydrofuranyl]-aspartic acid (3c). (a) Chromatogram of [L, (S/R)]-3c synthesized chemically; (b) Chromatogram of [D, (S/R)]-3c synthesized chemically; (c) Chromatogram of a mixture of 4 stereoisomers of 3c, which was prepared by mixing [L, (S/R)]-3c and [D, (S/R)]-3c in a molar ratio of 1:1; (d) Chromatogram of 3c obtained with EDDS lyase as biocatalyst. Chiral HPLC analysis was performed on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO\(_4\) solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm.
Figure S3. Chiral HPLC analysis of N-[(S/R)-3-tetrahydrothiophenyl]-aspartic acid (3d). (a) Chromatogram of [L, (S/R)]-3d synthesized chemically; (b) Chromatogram of [D, (S/R)]-3d synthesized chemically; (c) Chromatogram of a mixture of 4 stereoisomers of 3d, which was prepared by mixing [L, (S/R)]-3d and [D, (S/R)]-3d in a molar ratio of 1:1; (d) Chromatogram of 3d obtained with EDDS lyase as biocatalyst. Chiral HPLC analysis was performed on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO₄ solution: isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm.
Figure S4. Chiral HPLC analysis of N-cyclohexyl-aspartic acid (3e). (a) Chromatogram of a racemic mixture of 3e, which was prepared by mixing L-3e and D-3e synthesized chemically in a molar ratio of 1:1; (b) Chromatogram of 3e obtained with MAL-Q73A as biocatalyst. Chiral HPLC analysis was performed on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO4 at 1 ml/min at 60°C, detected at 240 nm.

Figure S5. Chiral HPLC analysis of N-(4-tetrahydropyranyl)-aspartic acid (L-3f). (a) Chromatogram of a racemic mixture of 3f, which was prepared by mixing L-3f and D-3f synthesized chemically in a molar ratio of 1:1; (b) Chromatogram of 3f obtained with EDDS lyase as biocatalyst. Chiral HPLC analysis was performed on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO4 at 1 ml/min at 60°C, detected at 240 nm.
Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

Figure S6. Chiral HPLC analysis of N-(4-piperidinyl)-aspartic acid (3g). (a) Chromatogram of a mixture of 3g, which was prepared by mixing L-3g and D-3g synthesized chemically; (b) Chromatogram of 3g obtained with EDDS lyase as biocatalyst. Chiral HPLC analysis was performed on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO₄ at 1 ml/min at 60°C, detected at 240 nm.
Figure S7. Chiral HPLC analysis of N-(tetrahydro-2H-thiopyran-4-yl)-aspartic acid (3h). (a) Chromatogram of L-3h synthesized chemically; (b) Chromatogram of (rac)-3h prepared by mixing L-3h with D-3h (1:1); (c) Chromatogram of 3h obtained with EDDS lyase as biocatalyst. Chiral HPLC analysis was performed on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO₄ at 0.6 ml/min at 60°C, detected at 240 nm.
Figure S8. Chiral HPLC analysis of N-(1-methylpiperidin-4-yl)-aspartic acid (3i). (a) Chromatogram of a racemic mixture of 3i, which was prepared by mixing L-3i and D-3i synthesized chemically in a molar ratio of 1:1; (b) Chromatogram of 3i obtained with EDDS lyase as biocatalyst. Chiral HPLC analysis was performed on a Nucleosil 5μ chiral-1 120A (250*4.6 mm) column, using isocratic 0.5 mM CuSO₄ at 1 ml/min at 60°C, detected at 240 nm.
Figure S9. Chiral HPLC analysis of N-(trans-4-aminocyclohexyl)-aspartic acid (3j). (a) Chromatogram of [L, (cis/trans)]-3j synthesized chemically; (b) Chromatogram of [D, (cis/trans)]-3j synthesized chemically; (c) Chromatogram of a mixture of 4 isomers of 3j, which was prepared by mixing [L, (cis/trans)]-3j and [D, (cis/trans)]-3j in a molar ratio of 1:1; (d) Chromatogram of 3j obtained with EDDS lyase as biocatalyst. Chiral HPLC analysis was performed on a Chirex 3126-D-penicillamine column (250 mm x 4.6 mm, Phenomenex, USA), using isocratic 2 mM CuSO₄ solution:isopropanol (95:5, v/v) at 1 ml/min at 25°C, detected at 254 nm.
Biocatalytic Synthesis of N-Cycloalkyl-Substituted L-Aspartic Acids

Figure S10. Chiral HPLC analysis of N-(4-tetrahydropyranyl)-aspartic acid (3f). (a) Chromatogram of L-3f synthesized chemically; (b) Chromatogram of D-3f synthesized chemically; (c) Chromatogram of 3f obtained with MAL-Q73A as biocatalyst. Chiral HPLC conditions: Nucleosil 5μ chiral-1 120A (250*4.6 mm) column. Mobile phase A: 0.5 mM CuSO₄, mobile phase B: acetonitrile; A/B = 80:20 (v/v). Flow rate 1.2 mL/min, 60 °C, UV detection at 270 nm.
References


