Engineered C–N Lyase: Enantioselective Synthesis of Chiral Synthons for Artificial Dipeptide Sweeteners

Jielin Zhang, Eleonora Grandi, Haigen Fu, Thangavelu Saravanan, Laura Bothof, Pieter G. Tepper, Andy-Mark W. H. Thunnissen, and Gerrit J. Poelarends*

Abstract: Aspartic acid derivatives with branched N-alkyl or N-arylalkyl substituents are valuable precursors to artificial dipeptide sweeteners such as neotame and advantame. The development of a biocatalyst to synthesize these compounds in a single asymmetric step is an as-yet-unmet challenge. Reported here is an enantioselective biocatalytic synthesis of various difficult N-substituted aspartic acids, including N-(3,3-dimethylbutyl)-l-aspartic acid and N-[3-(3-hydroxy-4-methoxyphenyl)propyl]-l-aspartic acid precursors to neotame and advantame, respectively, using an engineered variant of ethylenediamine-N,N’-disuccinic acid (EDDS) lyase from Chelatiorans sp. BNC1. This engineered C–N lyase (mutant D290M/L59/S38 and two extra methyl group(s) at C3 compared with that of the wild-type enzyme. These results present new opportunities to develop practical multienzymatic processes for the more sustainable and step-economic synthesis of an important class of food additives.

Introduction

Artificial low-calorie sweeteners are used as sugar replacements in the food industry, with the benefits of controlling energy intake and blood glucose levels, improving dental health, and other health concerns related to sugar overconsumption.[1–4] The dipeptide aspartame, which is about 200-fold sweeter than sucrose (Scheme 1A), is one of the most widely used artificial sweeteners with a substantial production volume each year.[5] The derivatization of aspartame with branched N-alkyl- or N-arylalkyl groups generates even sweeter compounds, such as the more recently approved food additives neotame and advantame (Scheme 1A).[6–10] Notably, neotame is 7000–13000 times sweeter than sucrose, while advantame is about 20000 times sweeter than sucrose. A common synthetic method for neotame and advantame production is the reductive N-alkylation of aspartame with the corresponding aldehyde in the presence of hydrogen using a palladium (Pd/C) or platinum (Pt/C) hydrogenation catalyst (Scheme 1B, Method 1).[10–14] An alternative strategy for neotame production involves N-(3,3-dimethylbutyl)-l-aspartic acid [l-3a] as a precursor, which is linked to l-phenylalanine methyl ester by amide-bond coupling (Scheme 1B, Method 2).[15–17] This precursor is chemically synthesized by reductive N-alkylation of l-aspartic acid (or its ester derivative) using transition-metal catalysts (Pd/C or Pt/C). Similarly, N-[3-(3-hydroxy-4-methoxyphenyl)propyl]-l-aspartic acid [l-3f] could be chemically prepared by reductive N-alkylation of l-aspartic acid and serve as precursor to advantame. However, the development of a biocatalyst for enantioselective synthesis of these difficult N-substituted aspartic acids l-3a and l-3f, in a single asymmetric step, is to date an unmet challenge.

Here we report the engineering of an effective C–N lyase, based on ethylenediamine-N,N’-disuccinic acid (EDDS) lyase from Chelatiorans sp. BNC1[18–20] for the enantioselective syntheses of l-3a and l-3f, precursors to neotame and advantame, respectively, as well as related chiral synthons for aspartame-based sweeteners starting from the simple non-chiral compound fumaric acid (1, Scheme 1C). This newly engineered C–N lyase shows a 1140-fold increase in activity for the selective hydroamination of fumarate compared to that of the wild-type enzyme, opening up new opportunities to design practical multienzymatic processes for the more sustainable and step-economic synthesis of an important class of food additives.

Results and Discussion

Our group has previously reported that an engineered variant of 3-methylaspartate ammonia lyase (MAL-Q73A) accepts various amines, including butylamine (2e, Table 1), for enantioselective hydroamination of fumarate (1).[21,22] Structurally, the amines 2b and 2a have, respectively, one and two extra methyl group(s) at C3 compared with 2c. This difference prompted us to start our investigations by testing the branched amines 2a and 2b as unnatural substrates in the
MAL-Q73A-catalyzed hydroamination of 1. Although 2b was accepted by MAL-Q73A for slow hydroamination of 1 (see Figure S1 in the Supporting Information), yielding optically pure l-3b (ee > 99%), 2a was unfortunately not accepted as a substrate by MAL-Q73A. This observation suggests that the bulky tert-butyl group of 2a prevents productive binding in the enzyme active site, making 2a a challenging substrate for C–N lyases.

We continued our investigations by testing whether EDDS lyase, which has previously been shown to possess an exceptionally broad amine scope,[18–20] can accept 2a as an unnatural substrate in the hydroamination of 1. Pleasingly, EDDS lyase accepted 2a for addition to 1, giving rise to 3a. Under optimized reaction conditions, excellent conversion (92% after 7 days) and good yield (67%) of isolated 3a were achieved using a 0.15 mol% biocatalyst loading (Table 1; see Figure S2). The enzymatic product 3a was identified as the desired l enantiomer with greater than 99% ee. The amines 2b and 2c were also readily converted by EDDS lyase to afford the respective optically pure products l-3b and l-3c (> 99% ee) with 93–95% conversion and in 66–74% yield upon isolation. Interestingly, the bulky arylalkylamines 2d–f were also accepted as substrates by EDDS lyase, yielding the respective products l-3d–f. High conversions (82–97% after 7 days) and excellent enantioselectivities (> 99% ee) were observed.

Although EDDS lyase is the first identified biocatalyst to synthesize l-3a in a single asymmetric step, its catalytic activity for this transformation is quite low, resulting in a rather long reaction time of 7 days when using a 0.15 mol% biocatalyst loading. Therefore, a structure-based protein engineering strategy was applied to enhance this hydroamination activity of EDDS lyase. On the basis of the structure of EDDS lyase in complex with its natural substrate
two residues (Asp290 and Tyr320) were chosen for site-saturation mutagenesis (SSM) because of their presumed roles in positioning of the amine substrate for addition to fumarate. Specifically, residue Asp290 forms a water-mediated hydrogen bond with the internal amino group connected to the distal succinyl moiety of (S,S)-EDDS, which appears to be an important interaction for binding and positioning of ethylenediamine and other diamine substrates (but not for monoamines such as 2a) for addition to fumarate. The bulky aromatic ring of Tyr320 may further preclude optimal positioning of 2a.

Accordingly, two focused libraries were constructed by randomizing the positions Asp290 and Tyr320, yielding libraries D290X and Y320X. The libraries were transformed into Escherichia coli cells and screened by evaluating about 100 transformants of each library. Initially, we evaluated mutants in the D290X library by monitoring the depletion of 1 in an aspE photometric kinetic assay in multiwell plates using cell-free extracts (CFEs). However, this screening was unsuccessful because 1 was converted at a similar rate by all CFEs, including a CFE prepared from E. coli cells not producing EDDS lyase (see Figure S3). We assumed that this relatively high background consumption of 1 was caused by indigenous fumarase (FumC) activity present in the E. coli CFE, resulting in the undesired hydration of 1 to give L-malic acid, which outcompeted the slower EDDS lyase mediated hydroamination of 1.

Considering that the removal of fumarase by enzyme purification from CFEs is quite laborious and not suitable for library screening, we tested whether the addition of fumarase inhibitors (d-malate, citrate, and glycerol) could suppress FumC-catalyzed hydration of 1. While d-malate and citrate did not show sufficient inhibition (data not shown), the addition of glycerol (45%, v/v) to the screening assay effectively inhibited FumC-catalyzed hydration of 1 (see Figures S4A and S5). It has been reported that glycerol inhibits FumC by affecting a conformational change, which appears to be the rate-limiting step, based on its viscosity effect. Importantly, control experiments demonstrated that the activity of EDDS lyase, measured by the addition of ethylene diamine to 1, was not inhibited by glycerol (see Figure S4B). Based on these optimizations, 45% (v/v) glycerol was included in the screening assay as additive.

Table 1: Enantioselective synthesis of neotame and advantame precursors, as well as related compounds, using EDDS lyase or its engineered variant D290M/Y320M as a biocatalyst.

| Entry | Amin​e | Product | Conv. (yield)% | t [days] | Conv. (yield)% | t [h] | ee% |
|-------|--------|---------|----------------|----------|----------------|------|-----|
| 1     | 2a     | 3a      | 92 (67)        | 7        | 96 (83)        | 2.5  | >99 |
| 2     | 2b     | 3b      | 93 (74)        | 7        | 93 (81)        | 2.5  | >99 |
| 3     | 2c     | 3c      | 95 (66)        | 7        | 92 (68)        | 2.5  | >99 |
| 4     | 2d     | 3d      | 97 (45)        | 7        | 96 (49)        | 6    | >99 |
| 5     | 2e     | 3e      | 93 (40)        | 7        | 90 (40)        | 6    | >99 |
| 6     | 2f     | 3f      | 82 (34)        | 7        | 82 (34)        | 6    | >99 |

[a] Reaction conditions: fumaric acid (1, 10 mM), amine (2a–f, 50 or 100 mM), and EDDS lyase WT (0.05 or 0.15 mol% based on fumaric acid) in NaH₂PO₄/NaOH buffer (pH 8.5) at room temperature. [b] Reaction conditions: fumaric acid (1, 10 mM), amine (2a–f, 50 or 100 mM), glycerol (45%, v/v), and EDDS lyase D290M/Y320M (0.05 mol% based on fumaric acid) in NaH₂PO₄/NaOH buffer (pH 8.5) at room temperature. [c] Conversion was determined by 1H NMR spectroscopy. Yield of isolated product determined after ion-exchange chromatography. [d] The ee value was determined by high-performance liquid chromatography on a chiral stationary phase using chemically synthesized authentic standards. [e] The apparent kcat/Km values (using 5 mM 1) were estimated to be 5.1 (2c), 12.8 (2b), and 14.5 M⁻¹s⁻¹ (2a).
to suppress the FumC-catalyzed hydration of 1, enabling
hydroamination activity screening of mutant libraries using
CFEs instead of purified proteins.

Using this optimized assay, screening of the D290X and
Y320X libraries resulted in the identification of five mutants
(D290L, D290V, Y320M, Y320V, and Y320L) with signifi-
cantly improved activity. These mutant enzymes were purified
to homogeneity and assayed for their ability to catalyze the
addition of 2a to 1 to yield 3a. The best mutant from the
D290X library (D290L) showed a 55-fold enhanced activity,
while the best mutant from the Y320X library (Y320M)
displayed a remarkable 620-fold increase in activity compared
to that of the wild-type enzyme (Figure 2; see Table S2).

To further improve the catalytic activity of EDDS lyase,
we used an iterative saturation mutagenesis (ISM) strategy,
using the best four hits from the single-site libraries as
templates and randomizing the other respective position.
Accordingly, the libraries D290L/Y320X, Y320M/D290X,
Y320V/D290X, and Y320L/D290X were constructed. The
screening of these libraries resulted in the identification of
four double mutants, D290M/Y320M, D290H/Y320M,
D290L/Y320M, and D290M/Y320V, which showed activity
improvement over the best single mutant Y320M. Based on
assays using purified enzymes, the mutant D290M/Y320M
was shown to be the best mutant enzyme, with a striking 1140-
fold increase in activity compared to that of the wild-type
enzyme (Figure 2; see Table S2). Notably, the mutant enzyme
D290L/Y320M, in which the two best single mutations at each
position are combined, displayed a lower activity compared
to that of mutant D290M/Y320M (Figure 2), illustrating the
importance of using an ISM approach to identify the best
mutant.

To understand how the mutations cause the large increase
in activity we determined crystal structures of the EDDS
lyase variant D290M/Y320M. Crystal structures were ob-
tained with either fumarate or formate occupying the active
site (see Table S3 and Figure S6), like previously shown for
the wild-type enzyme.[18] Similar as in the wild-type structure,
fumarate is bound tightly in the active site of the mutant
through interactions with its two carboxylate groups, while its
Cβ atom faces the hydroxy oxygen atom of Ser280, the
catalytic base, at a distance of 3 Å. A second fumarate is
bound somewhat away from the active site, in a region which
in the wild-type structure is responsible for binding the distal
succinate group of (S,S)-EDDS. The formate-bound mutant
structure contains three formate ions, two of which occupy
positions in the active site where the carboxylate groups of
fumarate bind, further denoting the integrity of the active site.
A detailed comparison of the mutant and wild-type structures
revealed no significant differences in overall protein struc-
ture, nor in the conformations of the active site residues
responsible for binding and activating fumarate prior to the
amine addition. Mutations D290M and Y320M are located at
opposite sides of the presumed amine binding pocket, and the
main structural consequences of the amino-acid substitutions
are a slight reshaping of the pocket and a significant increase
of its hydrophobicity (Figure 3A,B). Further insights were
obtained from docking 3a in the EDDS lyase wild-type and
mutant crystal structures (efforts to obtain crystal structures
with bound 3a were unsuccessful). The modeling results show
that while residues Asp290 and Tyr320 in the wild-type
structure form unfavorable polar–apolar contacts with the
3,3-dimethylbutyl moiety of 3a, residues Met290 and Met320
in the mutant structure are able to form favorable apolar–
apolar contacts (Figure 3C,D). The increased hydrophobicity
of the pocket resulting from the D290M and Y320M
mutations thus leads to improved binding interactions with
the apolar 3,3-dimethylbutyl moiety of 3a, suggesting that the

Figure 1. A) Structures of natural substrate (S,S)-EDDS and target compound (S)-3a. B) A close-up of the active site of EDDS lyase with bound (S,S)-EDDS (PDB: 6G3H). The bound (S,S)-EDDS (green) and side chains of residues forming the amine binding pocket are shown using stick representation. The two target residues for mutagenesis, Asp290 and Tyr320, are shown in yellow.
large increase in activity of mutant enzyme D290M/Y320M is a consequence of much stronger, and possibly more productive, binding of 2a.

Importantly, we observed that the activity of the mutant enzyme D290M/Y320M for the addition of 2a (100 mM) to 1 (5 mM) was affected by glycerol. The enzymatic activity decreased significantly (ca. 2.5-fold) when the glycerol concentration in the reaction mixture was reduced from 45% to 30% (v/v) and became almost undetectable when the concentration in the reaction mixture was reduced from 45% to 30% (v/v) and became almost undetectable when the glycerol concentration was lowered to less than or equal to 20% (see Figure S7). This decrease in enzyme activity upon lowering the glycerol concentration was accompanied by slight protein precipitation. Since the D290M/Y320M mutant was observed to be stable and fully active after several hours of incubation in buffer (without amine substrate) at room temperature, it appears that in the presence of high concentrations of 2a (100 mM), the D290M/Y320M mutant is not stable and loses activity. In the reaction mixture with 100 mM 2a, the D290M/Y320M mutant was stabilized by glycerol (45%, v/v), which is a routinely used stabilizing agent for proteins.[26,27] Note that the addition of 45% (v/v) glycerol did not effect the hydroamination activity of the wild-type enzyme under the same reaction conditions (see Figure S8).

Interestingly, this implies that glycerol played dual roles in mutant library screening. It served both as fumarase inhibitor and as protein stabilizer. The presence of 45% (v/v) glycerol during library screening was thus essential for the identification of the D290M/Y320M mutant, suggesting that the incorporation of cosolvents in screening assays is an appealing strategy to identify mutants with the desired activity, but having reduced stability, in enzyme evolution. Our results provide support for the notion that protein stability is a major constraint in enzyme evolution, and buffering mechanisms such as the inclusion of stabilizing cosolvents are key in relieving this constraint.[28]

Having generated an EDDS lyase variant with strongly improved catalytic activity (mutant D290M/Y320M), we tested its performance as biocatalyst for the synthesis of our target 3a. With a 0.05 mol% biocatalyst loading, starting substrates 1 and 2a were readily converted to afford the optically pure l-3a (> 99% ee) with 96% conversion after only 2.5 hours (instead of 7 days as observed for the same transformation with the wild-type enzyme) and in 83% yield upon isolation (entry 1 in Table 1; see Figure S9). To further demonstrate the synthetic usefulness of this newly engineered C–N lyase, 2b–f were tested as substrates in the hydroamination of 1. The enzymatic reactions proceeded smoothly to afford enantiomerically pure products l-3b–f (> 99% ee) with 82–96% conversion (after a few hours rather than 7 days) and in 34–81% yield (Table 1, entries 2–6; see Figure S10). These amino-acid products (except l-3d) are key building blocks for N-functionalized aspartame derivatives that were reported to be much sweeter than sucrose.[8] Notably, l-3f is a chiral precursor for the synthesis of advantame (Scheme 1), which, like neotame, has already been approved for application in food products.

**Conclusion**

In conclusion, we have successfully engineered a C–N lyase for efficient asymmetric addition of challenging amines to fumarate to yield optically pure N-(3,3-dimethylbutyl)-l-aspartic acid and N-[3-(3-hydroxy-4-methoxyphenyl)propyl]-l-aspartic acid, which are important precursors to neotame and advantame, respectively. Interestingly, the presence of glycerol during laboratory evolution was essential for the identification of this improved C–N lyase, suggesting that the inclusion of stabilizing cosolvents is an appealing strategy to reduce the constraining effects of protein stability during enzyme evolution. The newly developed biocatalytic methodology offers a useful alternative route to important chiral synthons for artificial dipeptide sweeteners. The engineered C–N lyase nicely supplements the toolbox of biocatalysts for production of unnatural amino acids, and opens up new opportunities to develop an entirely enzymatic route for the straightforward synthesis of valuable aspartame-based sweet-
eners, starting from the simple nonchiral dicarboxylic acid 1 (Scheme 1 C). Although the engineered EDDS lyase variant D290M/Y320M exhibits a respectable specific activity of 1.74 U mg\(^{-1}\) for the addition of 2a to 1, ongoing enzyme engineering work in our group is focused on further improving its stability (in the absence of glycerol) and catalytic efficiency (in terms of \(k_{\text{cat}}/K_m\)) for this reaction. As such, this work sets the stage for further development of practical multienzymatic processes for the more sustainable and step-economic synthesis of an important class of food additives.

**Experimental Section**

**Experimental Details**: All experimental details can be found in the Supporting Information.

**Acknowledgements**

We acknowledge funding from The Netherlands Organization of Scientific Research (VICI grant 724.016.002) and the European Research Council (PoC grant 713483). Jielin Zhang and Haigen Fu acknowledge funding from the China Scholarship Council. The authors thank Harshwardhan Poddar and Lieuwe Biewenga for insightful discussions, and Gea Cruiming for assistance with synthesis and Dr. Robbert H. Cool for assistance with enzyme purification. Dr. Daniele de Sanctis from the European Synchrotron Radiation Facility (Grenoble) is acknowledged for assistance with X-ray data collection at beamline ID29.

**Conflict of interest**

The authors declare no conflict of interest.

**Keywords**: enzymes · biocatalysis · hydroamination · protein engineering · synthetic methods

How to cite: Angew. Chem. Int. Ed. 2020, 59, 429–435
Angew. Chem. 2020, 132, 437–443

[1] R. K. Johnson, A. H. Lichtenstein, C. A. M. Anderson, J. A. Carson, J. P. Després, F. B. Hu, P. M. Kris-Etherton, J. J. Otten, A. Towfighi, J. Wylie-Rosett, Circulation 2018, 138, 126–140.
