University of Groningen

Chemoenzymatic asymmetric synthesis of the metallo- β -lactamase inhibitor aspergillomarasmine A and related aminocarboxylic acids

Fu, Haigen; Zhang, Jieli; Saifuddin, Mohammad; Cruiming, Ge; Tepper, Pieter G.; Poelarends, Gerrit J.

Published in:
Nature Catalysis

DOI:
[10.1038/s41929-018-0029-1](https://doi.org/10.1038/s41929-018-0029-1)

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:
2018

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Fu, H., Zhang, J., Saifuddin, M., Cruiming, G., Tepper, P. G., & Poelarends, G. J. (2018). Chemoenzymatic asymmetric synthesis of the metallo- β -lactamase inhibitor aspergillomarasmine A and related aminocarboxylic acids. *Nature Catalysis*, 1(3), 186-191. <https://doi.org/10.1038/s41929-018-0029-1>

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).

The publication may also be distributed here under the terms of Article 25fa of the Dutch Copyright Act, indicated by the "Taverne" license. More information can be found on the University of Groningen website: <https://www.rug.nl/library/open-access/self-archiving-pure/taverne-amendment>.

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.

Chemoenzymatic asymmetric synthesis of the metallo- β -lactamase inhibitor aspergillomarasmine A and related aminocarboxylic acids

Haigen Fu^{1,2}, Jieli Zhang^{1,2}, Mohammad Saifuddin^{1,2}, Gea Cruiming¹, Pieter G. Tepper¹ and Gerrit J. Poelarends^{1*}

Metal-chelating aminocarboxylic acids are being used in a broad range of domestic products and industrial applications. With the recent identification of the fungal natural product aspergillomarasmine A as a potent and selective inhibitor of metallo- β -lactamases and a promising co-drug candidate to fight antibiotic-resistant bacteria, the academic and industrial interest in metal-chelating chiral aminocarboxylic acids further increased. Here, we report a biocatalytic route for the asymmetric synthesis of aspergillomarasmine A and various related aminocarboxylic acids from retrosynthetically designed substrates. This synthetic route highlights a highly regio- and stereoselective carbon-nitrogen bond-forming step catalysed by ethylenediamine-*N,N'*-disuccinic acid lyase. The enzyme shows broad substrate promiscuity, accepting a wide variety of amino acids with terminal amino groups for selective addition to fumarate. We also report a two-step chemoenzymatic cascade route for the rapid diversification of enzymatically prepared aminocarboxylic acids by *N*-alkylation in one pot. This biocatalytic methodology offers a useful alternative route to difficult aminocarboxylic acid products.

Aminocarboxylic acids that contain several carboxylate groups bound to one or more nitrogen atoms form an important group of chelating agents¹. Their ability to form stable complexes with metal ions is the basis for their use in a broad range of domestic products, as well as industrial and medical applications^{1–4}. An important aminocarboxylic acid that recently received considerable attention is the fungal natural product aspergillomarasmine A (AMA) (**1a**, Fig. **1a**)^{5–7}. It was originally discovered and characterized for its plant-wilting and necrotic activity in 1965 (ref. ⁸). Later, in the 1980s, the molecule was evaluated as an inhibitor against human angiotensin-converting enzyme⁹. Recently, AMA has been highlighted as a potent and selective inhibitor of New Delhi metallo- β -lactamase-1 via a zinc-chelation mechanism, with an IC₅₀ value at low-micromolar concentration⁵. Moreover, AMA could efficiently restore the activity of meropenem in a mouse model infected with a lethal dose of New Delhi metallo- β -lactamase-1-expressing *Klebsiella pneumonia*, demonstrating the potential of AMA as a promising co-drug candidate to rescue or potentiate β -lactam antibiotics in combination therapies⁵.

Retrosynthetic analysis of AMA (**1a**, Fig. **1a**) suggests the compound can be deconstructed into three amino acid moieties— aspartic acid (Asp) and two 2-aminopropionic acids (Apa1 and Apa2)—where the amino acid moieties are connected through C–N bonds rather than conventional peptide bonds. The stereochemical assignment (configuration at C2, C2' and C2'') of natural AMA was reassigned from the original incorrectly proposed (2*S*, 2'*R*, 2''*R*) to the correct configuration (2*S*, 2'*S*, 2''*S*) through total synthesis^{10,11}. Replacing the terminal Apa2 moiety of AMA with glycine provides another related natural product, aspergillomarasmine B (AMB) (**2a**, Fig. **1a**), with (2*S*, 2'*S*) absolute configuration¹². Toxin A (**3a**, Fig. **1a**),

a biosynthetic precursor of AMA, is built from two amino acid moieties (Asp and Apa) through a C–N bond. The absolute configuration of **3a** was first incorrectly assigned as (2*S*, 2'*R*) in 1979 (ref. ¹³), but later corrected to (2*S*, 2'*S*) through chemical synthesis and feeding experiments in 1991 (ref. ¹⁴).

The intriguing structural features and important biological activities of AMA have attracted attention from synthetic chemists, culminating in the elegant total synthesis of AMA and related compounds (Fig. **1a**)^{10–12,15}. A modular approach towards AMA via a late-stage oxidation strategy (14 steps, 4% yield) was first reported¹⁰. Another study employed *o*-nosyl aziridine as a key intermediate in the total synthesis of AMA (9 steps, 1% yield)¹¹. Since then, an improved route utilizing a sulfamidate approach towards AMA has been achieved (6 steps, 19% yield)¹². However, creating a biocatalytic methodology as an alternative route to AMA, AMB and other difficult aminocarboxylic acids is an as yet unmet challenge.

The enzyme ethylenediamine-*N,N'*-disuccinic acid (EDDS) lyase catalyses an unusual two-step sequential addition of ethylenediamine (**4**) to two molecules of fumaric acid (**5**) providing (*S*)-*N*-(2-aminoethyl)aspartic acid (AEAA, **6**) as an intermediate and (*S*, *S*)-EDDS (**7**) as the final product (Fig. **1b**); it also catalyses the reverse reaction, converting **7** into **4** and two molecules of **5** (refs ^{16–18}). Interestingly, structural comparisons showed that toxin A (**3a**), AMA (**1a**) and AMB (**2a**) show striking similarities to the natural EDDS lyase substrates AEAA (**6**) and EDDS (**7**). This prompted us to explore EDDS lyase as a biocatalyst for the asymmetric synthesis of toxin A, AMA and AMB.

Here, we report a biocatalytic methodology for the asymmetric synthesis of toxin A, AMA, AMB and related aminocarboxylic acids

¹Department of Chemical and Pharmaceutical Biology, Groningen Research Institute of Pharmacy, University of Groningen, Groningen, The Netherlands.

²These authors contributed equally: Haigen Fu, Jieli Zhang, Mohammad Saifuddin. *e-mail: g.j.poelarends@rug.nl

Fig. 1 | Natural aminocarboxylic acid products. Structural similarities between EDDS lyase substrates and the natural products AMA, AMB and toxin A. **a**, Previous total synthesis strategies and our biocatalysis strategy towards AMA. **b**, Natural two-step sequential addition reaction catalysed by EDDS lyase.

Table 1 | Enzymatic synthesis of toxin A and related aminocarboxylic acids

$\text{HO}_2\text{C}-\text{CH}=\text{CH}-\text{CO}_2\text{H} + \text{H}_2\text{N}-\text{R} \xrightarrow[\text{conditions}^a]{\text{EDDS lyase}} \text{HO}_2\text{C}-\text{CH}(\text{CO}_2\text{H})-\text{CH}_2-\text{NH}-\text{R}$					
5		8a-h	3a-h		
Entry	Substrate	Product	Conversion ^b (yield ^c , %)	d.e./e.e. ^d (%)	Absolute configuration
1	8a 	3a 	98 (52)	>98	(2S, 2'S) ^e
2	8b 	3b 	97 (82)	>98	(2S, 2'R) ^e
3	8c 	3c 	96 (75)	>98	(2S, 3'S) ^f
4	8d 	3d 	94 (72)	>98	(2S, 4'S) ^g
5	8e 	3e 	95 (55)	>98	(2S, 5'S) ^g
6	8f 	3f 	91 (34)	>99	(S) ^h
7	8g 	3g 	92 (37)	>99	(S) ^h
8	8h 	3h 	92 (53)	>99	(S) ⁱ

^aConditions and reagents: fumaric acid (**5**, 10 mM), substrates **8a-h** (100 mM) and EDDS lyase (0.05 mol% based on fumaric acid) in buffer (20 mM NaH₂PO₄/NaOH, pH 8.5) at room temperature for 24 h (**8f**, 48 h). The amount of applied purified enzyme was chosen such that reactions were completed within 24–48 h. ^bConversions were determined by comparing ¹H NMR signals of substrates and corresponding products. ^cIsolated yield after ion-exchange chromatography. ^dThe d.e. for **3a-e** was determined by ¹H NMR (Supplementary Figs. 6 and 32–37), whereas the e.e. for **3f-h** was determined by high-performance liquid chromatography on a chiral stationary phase using authentic standards (Supplementary Figs. 38–42 and 57–59). ^eDetermined by referring to the literature¹⁴. ^fAssigned by ¹H NMR spectroscopy using an authentic standard and diastereomeric mixture with known (2S, 3'S) and (2S/R, 3'S) configurations, respectively (Supplementary Figs. 30–33). ^gTentatively assigned the (S, S) configuration on the basis of analogy. ^hDetermined by chiral high-performance liquid chromatography using authentic standards with known R or S configuration. ⁱTentatively assigned by optical rotation on the basis of comparison with the closely related product **3g**.

from retrosynthetically designed substrates. This biocatalytic strategy highlights a highly regio- and stereoselective carbon–nitrogen bond-forming step catalysed by EDDS lyase, and offers an alternative synthetic choice for preparing difficult aminocarboxylic acid products.

Results

Biocatalytic synthesis of toxin A and related compounds. Inspired by previous studies showing that toxin A (**3a**, Asp–Apa) is a precursor in the biosynthesis of AMA (**1a**, Asp–Apa1–Apa2)¹⁴, we envisioned that **3a** could serve as a precious building block for the synthesis of more complex aminocarboxylic acids, such as AMA, AMB and their derivatives. Structurally, toxin A has only one extra carboxylate group at the C2' position compared with AEAA (**6**), which is the intermediate product formed in the natural EDDS-lyase-catalysed two-step addition reaction (Fig. 1). This prompted us to start our investigations by testing (S)-2,3-diaminopropionic acid (**8a**, Table 1) as an unnatural substrate in the EDDS-lyase-catalysed amination of fumarate (**5**). Remarkably, the enzymatic amination reaction occurred exclusively at the less sterically hindered terminal 3-amino group of **8a** while the 2-amino group of **8a** remained untouched, providing **3a** as a single product. Notably, no further addition of the 2'-amino group of product **3a** to fumarate was observed. Under optimized conditions, excellent conversion (98%) and good isolated yield (52%) of **3a** were achieved using only 0.05 mol% biocatalyst loading (Table 1). Product **3a** was identified as the desired (2S, 2'S)-diastereomer (diastereomeric excess (d.e.) > 98%, Table 1), with the (2S)-stereogenic centre being set by EDDS lyase and the (2'S)-stereogenic centre derived from starting substrate **8a**. Interestingly, the (R)-enantiomer of 2,3-diaminopropionic acid (**8b**) was also accepted as substrate by EDDS lyase, giving (2S, 2'R)-**3b** as the anticipated diastereomeric product with excellent conversion (97%) and stereoselectivity (d.e. > 98%), and a high isolated yield (82%, Table 1).

Substrates with longer chains, including (S)-2,4-diaminobutyric acid (**8c**), (S)-2,5-diaminopentanoic acid (ornithine, **8d**) and (S)-2,6-diaminohexanoic acid (lysine, **8e**), were also well accepted by EDDS lyase, yielding the respective products **3c–e**. High conversions (94–96%), good isolated product yields (55–75%) and excellent regio- and stereoselectivity (d.e. > 98%) were observed (Table 1). As anticipated, only the less sterically hindered terminal amino groups of **8c–e** functioned as the nucleophile in the enzymatic additions to fumarate. In comparison with the previously reported four-step

chemical synthesis of (2S, 2'S)-toxin A (**3a**)¹² and the four-step chemical synthesis of (2S, 3'S)-**3c** (Supplementary Section 7), our biocatalytic methodology highlights a high-yielding one-step enzymatic synthesis of toxin A (**3a**) and its homologues **3c–e** with excellent regio- and stereoselectivities.

Substrates with only one terminal NH₂ group, such as glycine (**8f**), β-alanine (**8g**) and γ-aminobutyric acid (**8h**) were also well accepted by EDDS lyase, giving excellent conversions (91–92%) and yielding the corresponding aminocarboxylic acids **3f–h** as the (S)-configured enantiomers with >99% enantiomeric excess (e.e.) (Table 1). Notably, while the α-amino acid glycine (**8f**) was accepted as substrate by EDDS lyase, other tested α-amino acids with substituents at the Cα-position failed to give corresponding products (Supplementary Section 3), indicating that the steric environment of the nucleophilic amino group is essential for substrate conversion by EDDS lyase. It is also noteworthy that product **3f** (aspartic-N-monoacetic acid (ASMA)) was shown to be an efficient tricarbonyl ligand in the formation of ^{99m}Tc(CO)₃(ASMA) and Re(CO)₃(ASMA) complexes as renal (radio)tracers^{19,20}. Hence, our biocatalytic approach provides a convenient method to prepare such tricarbonyl ligands in optically pure form, which could be used for further development of (radio)tracers.

Two-step chemoenzymatic synthesis of AMB and its homologues.

Previous studies^{11,12} have confirmed that the primary amino group (2'-NH₂) in the Apa moiety of toxin A (**3a**) is much more reactive than the secondary amino group (2-NH) in the Asp moiety, providing an opportunity for regioselective N-alkylation at the 2'-NH₂ position of **3a**. Encouraged by the exquisite EDDS-lyase-catalysed biotransformation providing **3a** and its derivatives with excellent conversions and stereoselectivities, we set out to combine the biotransformation and chemical N-alkylation in one pot to prepare more complex molecules, including the important metallo-β-lactamase inhibitor AMB (**2a**) and its homologues (Fig. 2). To effect the second N-alkylation step in the one-pot synthesis of **2a**, the first requirement was the complete consumption of the starting amine substrate **8a** in the first enzymatic step to prevent it from reacting (and lowering the desired product yield) during the subsequent chemical N-alkylation step. Therefore, the enzymatic step was modified using a threefold excess of fumarate (rather than an excess of amine) to drive **8a** to completion. Remarkably, excellent conversion (98% after 24 h) of substrate **8a** was achieved providing (2S, 2'S)-**3a** as a single enzymatic product. Without any purification, (2S, 2'S)-**3a** was subjected to N-alkylation using bromoacetic acid in the same pot, of which the pH was adjusted and maintained at 11 for 6 h at 70 °C,

Fig. 2 | One-pot, two-step chemoenzymatic synthesis of AMB and its homologues. Reagents and reaction conditions: ^asubstrate (**8a** or **8c**, 1 eq.), fumaric acid (3 eq.) and EDDS lyase (0.05 mol% based on **8a** or **8c**) in buffer (20 mM NaH₂PO₄/NaOH, pH 8.5) at room temperature for 24 h; ^bbromoacetic acid, pH 11 at 70 °C for 6 h; ^c3-bromopropanoic acid, pH 11 at 70 °C for 6 h. Conversions are shown as percentage values.

Fig. 3 | Retrosynthesis of AMA and AMB. Biocatalytic retrosynthesis suggests that AMA and AMB can be prepared via EDDS lyase catalysed enantioselective amine addition to fumarate.

providing high conversion (84%) for the second step. Comparison of proton nuclear magnetic resonance (^1H NMR) and two-dimensional ^1H – ^1H correlation spectroscopy NMR spectra of product **2a** (23% isolated yield) with those of (2S, 2'S)-**3a** showed that the *N*-alkylation had unambiguously occurred at the 2'- NH_2 position of **3a**, demonstrating that product **2a** has the desired Asp–Apa–Gly structure (Supplementary Figs. 13 and 43–46). With both stereogenic centres being derived from intermediate (2S, 2'S)-**3a**, product **2a** has the correct (2S, 2'S) configuration.

To further demonstrate the synthetic usefulness of this one-pot strategy, we successfully modified the third Gly moiety or the second Apa moiety of AMB by either replacing the alkylation agent (3-bromopropanoic acid) or the starting substrate (**8c**) in the chemoenzymatic cascade, yielding AMB homologues (2S, 2'S)-**2b** or (2S, 3'S)-**2c**, respectively (Fig. 2 and Supplementary Figs. 14 and 15). Note that the stereogenic centres of **2b** and **2c** have been derived from the respective intermediates **3a** and **3c**, of which the absolute configurations have been unambiguously established (Table 1).

Enzymatic synthesis of AMA, AMB and related compounds.

The one-pot chemoenzymatic cascade described above provides a rapid route to synthesize (2S, 2'S)-AMB (**2a**) and its homologues. With the aim of developing a convenient synthetic methodology for (2S, 2'S, 2'')-AMA (**1a**), we envisioned a retrosynthesis of **1a** by incorporating an EDDS-lyase-catalysed amination step (Fig. 3). This stereoselective disconnection of the C–N bond would result in (2S, 2'S)-**9d** as a challenging starting substrate for the EDDS-lyase-catalysed amination reaction. Similar retrosynthesis could be applied to **2a**, leading to (S)-**9a** as a starting substrate for the enzyme-catalysed amination step (Fig. 3).

Considering that compound (S)-**9a** is structurally less bulky than (2S, 2'S)-**9d**, which may indicate a better chance of acceptance by the enzyme, we first prepared and tested (S)-**9a** (Supplementary Fig. 16) as a retrosynthetically designed substrate for EDDS lyase. Remarkably, using a fourfold excess of fumarate over (S)-**9a**, and only 0.05 mol% biocatalyst loading in 20 mM sodium phosphate buffer (pH 8.5) at room temperature, (S)-**9a** was readily converted by EDDS lyase to afford a single product **2a** with 80% conversion and 47% isolated yield (Table 2 and Supplementary Figs. 23 and 51). The enzymatic product **2a** was identified as the desired (2S, 2'S)-diastereomer of AMB (d.e. > 98%, Table 2), with the (2S)-stereogenic centre being set by EDDS lyase and the (2'S)-stereogenic centre derived from starting substrate (S)-**9a**.

To tackle our original target AMA (**1a**), retrosynthetically designed substrate (2S, 2'S)-**9d** was prepared via a β -lactone approach (Supplementary Figs. 20 and 21). To our delight, EDDS lyase accepted (2S, 2'S)-**9d** as substrate in the amination of fumarate to give product **1a** under the same reaction conditions that were used for the conversion of substrate (S)-**9a**, although low conversion (30%) was observed. We optimized the reaction conditions and found that 0.15 mol% of biocatalyst loading in 50 mM $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$ buffer at pH 9.0 and 37 °C

Table 2 | Enzymatic synthesis of AMA, AMB and their homologues

Entry	Substrate	Product	Conversion ^d (yield ^e , %)	d.e. ^f (%)	Absolute configuration
1 ^a	9a	2a (AMB)	80 (47)	>98	(2S, 2'S) ^g
2 ^b	9b	2b	71 (46)	>98	(2S, 2'S) ^g
3 ^b	9c	2c	65 (31)	>98	(2S, 3'S) ^g
4 ^c	9d	1a (AMA)	79 (26)	>98	(2S, 2'S, 2'') ^h
5 ^b	9e	1b	65 (20)	>98	(2S, 3'S, 2'') ⁱ

^aSubstrate **9a** (1 eq.), fumaric acid (4 eq.) and EDDS lyase (0.05 mol% based on **9a**) in buffer (20 mM $\text{NaH}_2\text{PO}_4/\text{NaOH}$, pH 8.5) at room temperature for 24 h. ^bSubstrate **9b**, **9c** and **9e** (1 eq.), fumaric acid (4 eq.) and EDDS lyase (0.15 mol% based on **9b**, **9c** and **9e**) in buffer (50 mM Tris/HCl , pH 9.0) at 37 °C for 48 h. ^cSubstrate **9d** (1 eq.), fumaric acid (4 eq.) and EDDS lyase (0.15 mol% based on **9d**) in buffer (50 mM $\text{NaHCO}_3/\text{Na}_2\text{CO}_3$, pH 9.0) at 37 °C for 48 h. ^dConversions were determined by ^1H NMR. ^eIsolated yield after ion-exchange chromatography. No formation of side products was observed and isolated yields of the desired products may be further improved by optimizing the purification protocols. ^fThe d.e. was determined by ^1H NMR (Supplementary Figs. 23–26 and 29). ^gAssigned by ^1H NMR spectroscopy using chemoenzymatically synthesized authentic standards **2a** with known (S, S) configuration. ^hDetermined by referring to the literature (Supplementary Fig. 27 and Supplementary Table 1). ⁱTentatively assigned the (2S, 3'S, 2'') configuration on the basis of analogy.

afforded product **1a** with good conversion (79% in 48 h) and in 26% isolated yield (Supplementary Figs. 26 and 54). A comparison of the ^1H NMR spectrum of enzymatic product **1a** with those reported for (2*S*, 2'*S*, 2''*S*)-AMA (Supplementary Table 1) and (2*R*, 2'*S*, 2''*S*)-AMA confirmed that product **1a** had the desired (2*S*, 2'*S*, 2''*S*) configuration (d.e. > 98%, Table 2), with two stereogenic centres derived from the starting substrate (2*S*, 2'*S*)-**9d** and the other one set by the enzyme. In addition, small amounts of the cyclic anhydro-AMA were obtained during purification (Supplementary Figs. 28 and 56).

With the aim of further exploring the substrate promiscuity and synthetic capability of EDDS lyase, we prepared and analysed compounds **9b**, **9c** and **9e** (Supplementary Figs. 17–19 and 22) as substrates in the enzymatic amination of fumarate. The corresponding AMA homologue **1b** and AMB homologues **2b** and **2c** were obtained with good conversions (65–71%), excellent stereoselectivities (d.e. > 98%) and in 20–46% isolated yields (Table 2 and Supplementary Figs. 24, 25, 29, 47, 48, 50, 52, 53 and 55).

Discussion

Using a biocatalytic retrosynthesis approach^{21–23}, we have successfully demonstrated that EDDS lyase can be applied as a biocatalyst for the asymmetric synthesis of the natural products toxin A, AMA and AMB, as well as related chiral aminocarboxylic acids. This enzyme shows remarkably broad substrate promiscuity, and excellent regio- and stereoselectivity, allowing the selective addition of a wide variety of amino acids to fumarate. Only less sterically hindered terminal amino groups of the starting substrates functioned as the nucleophile in the enzymatic additions, providing insight into the regioselectivity of this enzyme. We also developed a two-step chemoenzymatic cascade route for the rapid diversification of enzymatically prepared aminocarboxylic acids by *N*-alkylation in one pot. Our (chemo)enzymatic methodology offers a useful alternative route to difficult aminocarboxylic acid products, as exemplified by the synthesis of the natural products toxin A (1 step, 52% yield), AMA (4 steps, 10% yield) and AMB (one-pot, 2 steps, 22% yield). The isolated product yields are comparable to those of previously reported chemical synthesis strategies for toxin A (4 steps, 46% yield), AMA (6–14 steps, 1–19% yield) and AMB (5–6 steps, 8–10% yield)^{10–12}.

In addition to EDDS lyase, several other enzymes have previously been characterized that catalyse the asymmetric addition of substituted amines to fumarate or its derivatives. These include aspartate ammonia-lyases, methylaspartate ammonia-lyases, argininosuccinate lyases and adenylosuccinate lyases^{17,18,24,25}. With the notable exception of an engineered variant of methylaspartate ammonia-lyase, which accepts various alkylamines as non-natural substrates²⁶, these enzymes have a rather narrow nucleophile scope. In contrast, EDDS lyase has a very broad nucleophile scope, accepting a wide variety of structurally diverse amino acids, allowing the biocatalytic preparation of numerous valuable aminocarboxylic acids. As such, EDDS lyase nicely complements the rapidly expanding toolbox of enzymes for asymmetric synthesis of unnatural amino acids²⁴.

We have initiated studies aimed at further exploring novel substrates (for example, unsaturated mono- and dicarboxylic acids, including fumarate derivatives) for EDDS lyase to find new promiscuous reactions. In addition, work is in progress to determine crystal structures of EDDS lyase, both uncomplexed and in complex with substrates and products, and the results will be reported in due course. Such structures could guide the engineering of EDDS lyase to enhance its catalytic activity (allowing lower catalyst loadings) and further expand its unnatural substrate scope.

Methods

Enzyme expression and purification. *Escherichia coli* TOP10 cells containing the pBADN (EDDS lyase-His) plasmid were collected from an Luria-Bertani (LB) agar plate, containing $100\ \mu\text{g ml}^{-1}$ ampicillin (Ap) and used to inoculate LB/Ap medium (10 ml). After overnight incubation at 37°C, the culture was used to inoculate fresh LB/Ap medium (1 l). The cells were grown at 37°C for about 4 h, until the OD₆₀₀ reached 0.8–1.0. Arabinose (0.05%, w/v) was added to induce enzyme expression. Cultures were incubated at 20°C for 18 h with vigorous shaking. Cells were harvested by centrifugation and stored at –20°C until further use.

In a typical purification experiment, 4 g of wet cells (from 1 l culture) were suspended in lysis buffer (15 ml, 50 mM Tris-HCl, 300 mM NaCl, 20 mM imidazole, pH 8.0). Cells were disrupted by sonication for 4 × 40 s (with a 5 min interval between each cycle) at a 60 W output. The unbroken cells and debris were removed by centrifugation. The supernatant was filtered through a filter (pore diameter 0.45 μm) and incubated with Ni sepharose resin (1 ml slurry in a small column at 4°C for 18 h), which had previously been equilibrated with lysis buffer. The unbound proteins were eluted from the column by gravity flow. The column was first washed with lysis buffer (15 ml) and then with buffer A (30 ml, 50 mM Tris-HCl, 300 mM NaCl, 30 mM imidazole pH 8.0). Retained proteins were eluted with buffer B (5 ml, 50 mM Tris-HCl, 300 mM NaCl, 500 mM imidazole pH 8.0). Fractions were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis on gels containing acrylamide (4–12%). Fractions containing EDDS lyase were combined and loaded onto a HiLoad 16/600 Superdex 200 pg column, which was previously equilibrated with buffer C (180 ml, 20 mM NaH₂PO₄-NaOH buffer, pH 8.5). The column was eluted with buffer C at 1 ml min^{–1} for 1.2 column volumes. Fractions were collected and analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis on gels containing acrylamide (4–12%). The purified enzyme was stored at –20°C until further use (Supplementary Fig. 1).

Biotransformations and purification of toxin A and related compounds.

Reaction mixtures (15 ml) consisted of fumaric acid (0.2 mmol) and amines **8a–h** (2 mmol) prepared in 20 mM NaH₂PO₄-NaOH buffer, and the pH of the reaction mixture was adjusted to 8.5. The enzymatic reaction was started by the addition of freshly purified EDDS lyase (0.05 mol%) and the final volume of the reaction mixture was adjusted immediately to 20 ml with the same buffer. The reaction mixture was then incubated at room temperature for 24 h (48 h for **8f**). After completion of the reaction, it was stopped by heating to 70°C for 10 min. The reaction progress was monitored by ^1H NMR spectroscopy by comparing the signals of the substrates and corresponding products.

The enzymatic products were purified by two steps of ion-exchange chromatography. For a typical purification procedure, the precipitated enzyme was removed by filtration (diameter 0.45 μm). The filtrate was loaded slowly onto an anion-exchange column (5 g of AG 1-X8 resin, acetate form, 100–200 mesh), which was pre-treated with 5 column volumes of 1 M acetic acid (aqueous solution) and then water (until the pH was near neutral). The column was washed with water (3 column volumes) and then 0.1 M acetic acid (3 column volumes) until all the excess substrates **8a–h** were removed. The products were eluted with 2 M acetic acid for **3a–e** or 1 M HCl for **3f–h**. The ninhydrin-positive fractions were collected and loaded onto a cation-exchange column (5 g of Dowex 50WX8 resin, 100–200 mesh), which was pre-treated with 2 M aqueous ammonia (5 column volumes), 1 M HCl (3 column volumes) and water (5 column volumes). After product loading, the column was washed with water (3 column volumes) to remove the remaining fumaric acid and subsequently eluted with 2 M aqueous ammonia until the desired product was collected. The ninhydrin-positive fractions were collected, concentrated under vacuum and lyophilized to provide the desired products as ammonium salts, which were characterized by ^1H NMR, ^{13}C NMR and high-resolution mass spectrometry (Supplementary Figs. 2–12).

Detailed experimental procedures, chemical synthesis and analysis. Detailed experimental procedures and characterizations of compounds are provided in the Supplementary Information.

Data availability. All data are available from the corresponding author upon reasonable request.

Received: 12 September 2017; Accepted: 11 January 2018;
Published online: 8 March 2018

References

1. Bucheli-Witschel, M. & Egli, T. Environmental fate and microbial degradation of aminopolycarboxylic acids. *FEMS Microbiol. Rev.* **25**, 69–106 (2001).
2. Kolodnynska, D. in *Expanding Issues in Desalination* 339–370 (InTech, London, 2011).
3. Almubarak, T., Ng, J. H. & Nasr-El-Din, H. Oilfield scale removal by chelating agents: an aminopolycarboxylic acids review. In *SPE Western Regional Meeting* (Society of Petroleum Engineers, Richardson, 2017).

4. Repo, E., Warchol, J. K., Bhatnagar, A., Mudhoo, A. & Sillanpää, M. Aminopolycarboxylic acid functionalized adsorbents for heavy metals removal from water. *Water Res.* **47**, 4812–4832 (2013).
5. King, A. M. et al. Aspergillomarasmine A overcomes metallo- β -lactamase antibiotic resistance. *Nature* **510**, 503–506 (2014).
6. Von Nussbaum, F. & Schiffer, G. Aspergillomarasmine A, an inhibitor of bacterial metallo- β -lactamases conferring *bla*_{NDM} and *bla*_{VIM} resistance. *Angew. Chem. Int. Ed. Engl.* **53**, 11696–11698 (2014).
7. Meziane-Cherif, D. & Courvalin, P. Antibiotic resistance: to the rescue of old drugs. *Nature* **510**, 477–478 (2014).
8. Haenni, A. et al. Structure chimique des aspergillomarasmies A et B. *Helv. Chim. Acta* **48**, 729–750 (1965).
9. Mikami, Y. & Suzuki, T. Novel microbial inhibitors of angiotensin-converting enzyme, aspergillomarasmies A and B. *Agric. Biol. Chem.* **47**, 2693–2695 (1983).
10. Liao, D. et al. Total synthesis and structural reassignment of aspergillomarasmine A. *Angew. Chem. Int. Ed. Engl.* **55**, 4291–4295 (2016).
11. Koteva, K., King, A. M., Capretta, A. & Wright, G. D. Total synthesis and activity of the metallo- β -lactamase inhibitor aspergillomarasmine A. *Angew. Chem. Int. Ed. Engl.* **128**, 2210–2212 (2016).
12. Albu, S. A. et al. Total synthesis of aspergillomarasmine A and related compounds: a sulfamidate approach enables exploration of structure–activity relationships. *Angew. Chem. Int. Ed. Engl.* **128**, 13259–13262 (2016).
13. Bach, E. et al. Structures, properties and relationship to the aspergillomarasmies of toxins produced by *Pyrenophora teres*. *Physiol. Plant Pathol.* **14**, 41–46 (1979).
14. Friis, P., Olsen, C. & Møller, B. Toxin production in *Pyrenophora teres*, the ascomycete causing the net-spot blotch disease of barley (*Hordeum vulgare* L.). *J. Biol. Chem.* **266**, 13329–13335 (1991).
15. Zhang, J. et al. Synthesis and biological evaluation of aspergillomarasmine A derivatives as novel NDM-1 inhibitor to overcome antibiotics resistance. *Bioorg. Med. Chem.* **25**, 5133–5141 (2017).
16. Witschel, M. & Egli, T. Purification and characterization of a lyase from the EDTA-degrading bacterial strain DSM 9103 that catalyzes the splitting of [S,S]-ethylenediaminedisuccinate, a structural isomer of EDTA. *Biodegradation* **8**, 419–428 (1997).
17. Puthan Veetil, V., Fibriansah, G., Raj, H., Thunnissen, A.-M. W. & Poelarends, G. J. Aspartase/fumarase superfamily: a common catalytic strategy involving general base-catalyzed formation of a highly stabilized aci-carboxylate intermediate. *Biochemistry* **51**, 4237–4243 (2012).
18. Wu, B., Szymanski, W., Crismaru, C. G., Feringa, B. L. & Janssen, D. B. in *Enzyme Catalysis in Organic Synthesis* 3rd edn 749–778 (Wiley, Weinheim, 2012).
19. Lipowska, M., Klenc, J., Marzilli, L. G. & Taylor, A. T. Preclinical evaluation of ^{99m}Tc(CO)₃-aspartic-N-monoacetic acid, a renal radiotracer with pharmacokinetic properties comparable to ¹³¹I-o-iodohippurate. *J. Nucl. Med.* **53**, 1277–1283 (2012).
20. Klenc, J., Lipowska, M., Taylor, A. T. & Marzilli, L. G. Synthesis and characterization of *fac*-Re(CO)₃-aspartic-N-monoacetic acid: structural analogue of a potential renal tracer, *fac*-^{99m}Tc(CO)₃(ASMA). *Eur. J. Inorg. Chem.* **2012**, 4334–4341 (2012).
21. Hönig, M., Sondermann, P., Turner, N. J. & Carreira, E. M. Enantioselective chemo- and biocatalysis: partners in retrosynthesis. *Angew. Chem. Int. Ed. Engl.* **56**, 8942–8973 (2017).
22. De Souza, R. O. M. A., Miranda, L. S. M. & Bornscheuer, U. T. A retrosynthesis approach for biocatalysis in organic synthesis. *Chem. Eur. J.* **23**, 12040–12063 (2017).
23. Turner, N. J. & O'Reilly, E. Biocatalytic retrosynthesis. *Nat. Chem. Biol.* **9**, 285–288 (2013).
24. Parmeggiani, F., Weise, N. J., Ahmed, S. T. & Turner, N. J. Synthetic and therapeutic applications of ammonia-lyases and aminomutases. *Chem. Rev.* **118**, 73–118 (2018).
25. De Villiers, M., Puthan Veetil, V., Raj, H., de Villiers, J. & Poelarends, G. J. Catalytic mechanisms and biocatalytic applications of aspartate and methylaspartate ammonia lyases. *ACS Chem. Biol.* **7**, 1618–1628 (2012).
26. Raj, H. et al. Engineering methylaspartate ammonia lyase for the asymmetric synthesis of unnatural amino acids. *Nat. Chem.* **4**, 478–484 (2012).

Acknowledgements

H.F. and J.Z. acknowledge funding from the China Scholarship Council. The authors thank A. Boltjes and W. Szymanski for insightful discussions, and R. H. Cool for assistance with enzyme purification.

Author contributions

H.F., J.Z., M.S. and G.C. performed the preparative biotransformations and product analysis. H.F. and M.S. synthesized the starting substrates and reference compounds, and developed the one-pot chemoenzymatic cascade. J.Z. and P.G.T. performed the chiral high-performance liquid chromatography experiments. G.J.P. supervised the scientific work. All authors contributed to writing the paper.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary information is available for this paper at <https://doi.org/10.1038/s41929-018-0029-1>.

Reprints and permissions information is available at www.nature.com/reprints.

Correspondence and requests for materials should be addressed to G.J.P.

Publisher's note: Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.