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Modular Enzymatic Cascade Synthesis of Vitamin B5 and its Derivatives

Mohammad Z. Abidin, Thangavelu Saravanan, Jielin Zhang, Pieter G. Tepper, Erick Strauss and Gerrit J. Poelarends

Abstract

Access to vitamin B₅ [(R)-pantothenic acid] and both diastereoisomers of α-methyl-substituted vitamin B₅ [(R)- and (S)-3-((R)-2,4-dihydroxy-3,3-dimethylbutanamido)-2-methylpropanoic acid] has been achieved using a modular three-step biocatalytic cascade involving 3-methylaspartate ammonia lyase (MAL), aspartate-α-decarboxylase (ADC), β-methylaspartate-α-decarboxylase (CrpG) or glutamate decarboxylase (GAD), and pantoyl synthetase (PS) enzymes. Starting from simple non-chiral dicarboxylic acids (either fumaric acid or mesaconic acid), vitamin B₅ and both diastereoisomers of α-methyl-substituted vitamin B₅, which are valuable precursors for promising antimicrobials against Plasmodium falciparum and multidrug-resistant Staphylococcus aureus, can be generated in good yields (up to 70%) and excellent enantiopurity (>99% ee). This newly developed cascade process might be tailored and used for the biocatalytic production of various vitamin B₅ derivatives by modifying the pantoyl or β-alanine moiety.
Coenzyme A (CoA) is an essential enzyme cofactor in all organisms, and its biosynthetic pathway enzymes have been identified as attractive targets for new antimicrobial drugs.\textsuperscript{[1,2]} An interesting class of new antimicrobials that target CoA biosynthesis is the pantothenamides (PanAms), which are secondary or tertiary amides of pantothenic acid (vitamin B\textsubscript{5}, 5\texttextsuperscript{a}, Figure 1), the biosynthetic precursor of CoA. Various PanAms have been shown to possess potent antimicrobial activity against several organisms, including the pathogenic bacterium \textit{Staphylococcus aureus}\textsuperscript{[3]} as well as the malaria parasite \textit{Plasmodium falciparum}.\textsuperscript{[4]} However, pantetheinase enzymes that normally hydrolyse pantetheine in human serum also act on the PanAms, thereby reducing their efficacy.\textsuperscript{[5,6]} Interestingly, pantetheinase-mediated hydrolysis of PanAms could be prevented by modifying the β-alanine moiety of the compounds.\textsuperscript{[7,8]} Indeed, a PanAm with an added α-methyl group was shown to have superior antiplasmodial activity compared to its parent molecule.\textsuperscript{[9]} However, such modifications introduce stereochemical complexity to the molecules that recent results have indicated is highly relevant to the antimalarial activity of PanAm analogues.\textsuperscript{[10]} However, the more challenging chemical syntheses of these compounds poses a significant barrier to the discovery of their clinical potential. Therefore, the development of an asymmetric biocatalytic synthesis strategy that provides efficient and step-economic access to pantothenic acid (5\texttextsuperscript{a}) and both diastereoisomers of its α-methyl substituted derivative (5\texttextsuperscript{b}, Figure 1), avoiding (de-)
protecting steps and intermediate purifications, is of high interest. The desired PanAms can be easily prepared from the corresponding pantothenic acids by transforming the carboxylic acid group to an amide.\textsuperscript{[11]}

![Figure 1. Structures of vitamin B\textsubscript{5} and its α-methyl-substituted derivative.](image)

We envisioned that pantothenic acid (5\texttextsuperscript{a}) and its α-methyl substituted derivative (5\texttextsuperscript{b}) could be prepared from fumaric acid (1\texttextsuperscript{a}) and mesaconic acid (1\texttextsuperscript{b}), respectively, via a modular three-step enzymatic cascade involving 3-methylaspartate ammonia lyase (MAL), an appropriate decarboxylase such as aspartate-α-decarboxylase (ADC), β-methylaspartate-α-decarboxylase (CrpG) or glutamate decarboxylase (GAD), and pantothetate synthetase (PS) (Scheme 1). The expected chemoselectivity of each biocatalyst could allow for a one-pot reaction sequence due to the orthogonal reactivity of each enzyme. In this process, the new stereogenic center in product 5\texttextsuperscript{b} can be established by either regio- and diastereoselective
amination (as catalyzed by MAL), or diastereospecific decarboxylation by one of the decarboxylase enzymes.

MAL of *Clostridium tetanomorphum* is part of a catabolic pathway for L-glutamate, where it catalyzes the conversion of L-threo-3-methylaspartate to ammonia and mesaconate.\[^{12}\] Using a large molar excess of ammonia, the enzyme also efficiently catalyzes the amination of mesaconate (1b) to give both (2S,3S)-3-methylaspartate (L-threo-2b) and (2S,3R)-3-methylaspartate (L-erythro-2b) as products (Scheme 1). It was found that L-threo-2b is formed at a rate much faster than L-erythro-2b, but at equilibrium (using a 65-fold molar excess of ammonia over 1b at pH 9) the molar ratio of these diastereoisomers is approximately 1.\[^{13}\] In addition, MAL accepts fumarate (1a) as substrate, which is converted to L-aspartate (2a). The mechanism-inspired engineering of a MAL mutant (H194A) with strongly enhanced diastereoselectivity in the amination of 1b, giving exclusively L-threo-2b, has been reported previously.\[^{13}\] Moreover, the substrate scope of MAL has been expanded by structure-guided site-saturation mutagenesis, allowing for the biocatalytic production of a broad range of valuable 3-substituted L-aspartic acid derivatives.\[^{14}\]

ADC of *Escherichia coli* is part of the biosynthesis pathway for pantothenate, where it catalyzes the decarboxylation of 2a to give β-alanine (3a).\[^{15}\] This enzyme has a limited substrate scope and showed no decarboxylation activity towards 2b.\[^{16}\] The enzyme β-methylaspartate-α-decarboxylase (CrpG) of *Nostoc* sp. ATCC53789 is involved in a biosynthetic pathway for cryptophycin, where it catalyzes the decarboxylation of L-erythro-2b to yield (R)-3-amino-2-methylpropanoic acid (3b).\[^{17}\] CrpG is the only enzyme known that can catalyze the decarboxylation of 2b, with significant activity towards the L-erythro isomer only. GAD of the hyperthermophilic archaeon *Thermococcus kodakarensis* has been reported to function as an ADC and is most likely responsible for the production of β-alanine necessary for pantothenate biosynthesis.\[^{18}\] In this study, we demonstrate that this enzyme exhibits decarboxylase activity towards 2b, but with highest activity towards the L-threo isomer. Note that ADC and CrpG have to undergo self-processing leading to formation of the
catalytic pyruvoly group, whereas GAD is a PLP-dependent decarboxylase that does not require autocatalytic self-processing.

Pantothenate synthetase (PS) of *E. coli* is involved in the last step of pantothenate biosynthesis and catalyzes the ATP-dependent condensation of \((R)\)-pantoate \((4)\) and β-alanine \((3a)\) to form \((R)\)-pantothenate (vitamin B₅, 5a). PS enzymes typically accept a variety of β-alanine analogues in the condensation reaction, albeit with reduced catalytic efficiency compared to that with the natural substrate.\(^{[15,19]}\)

Initially, we set out to combine MAL and ADC in one-pot to prepare product 3a. Accordingly, substrate 1a and NH₄Cl were incubated with MAL and ADC, and the reaction was monitored by TLC (Figure S3). After 24 h, 1a was completely converted into product 3a, as confirmed by \(^1\)H NMR spectroscopy. These initial results showed that the two enzymes MAL and ADC are compatible for cascade synthesis in one pot. To further demonstrate the preparative usefulness of this two-step cascade system, a 100 mg-scale synthesis was performed. Accordingly, substrate 1a (25 mM) and NH₄Cl (500 mM) were incubated with MAL (0.02 mol%) and ADC (0.6 mol%) in one pot (25 mL of buffer, pH 8). Under these conditions, excellent conversion (>99% after 24 h) and good isolated yield of product 3a (85%) were achieved (Table 1).

Unfortunately, ADC showed no decarboxylase activity towards either L-threo-2b or L-erythro-2b. Therefore, we cloned, expressed and purified the decarboxylase CrpG, and incubated it with a 1:1 mixture of L-threo-2b and L-erythro-2b. Under these conditions the L-erythro isomer was fully decarboxylated, whereas the L-threo isomer was not converted, not even after prolonged incubation for 7 d (Figure S11). This indicates that CrpG is highly diastereoselective, with detectable activity only towards the L-erythro isomer.

**Table 1.** Two step enzymatic cascade synthesis of β-alanine (3a) and both enantiomers of 3-amino-2-methylpropanoic acid (3b)\(^{[a]}\)

<table>
<thead>
<tr>
<th>Product</th>
<th>Enzymes</th>
<th>Conversion (%(^{[b]}))</th>
<th>Isolated yield (%)(^{[c]})</th>
<th>ee (%(^{[d]}))</th>
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<tbody>
<tr>
<td>3a</td>
<td>MAL and ADC</td>
<td>&gt; 99</td>
<td>85</td>
<td>-</td>
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<tr>
<td>((R))-3b</td>
<td>MAL and CrpG</td>
<td>&gt; 99</td>
<td>78</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>((S))-3b</td>
<td>MAL-H194A and GAD</td>
<td>75</td>
<td>63</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

\^[a\] For synthesis of 3a, the reaction mixture contained MAL (0.02 mol%), ADC (0.6 mol%), 1a (25 mM), NH₄Cl (500 mM), and MgCl₂ (25 mM) in 25 mL Tris-HCl buffer (pH 8, 100 mM). For synthesis of \((R)\)-3b, the reaction mixture contained MAL (0.02 mol%), CrpG (0.47 mol%), 1b (30 mM), NH₄Cl (500 mM), and MgCl₂ (25 mM) in 25 mL potassium phosphate buffer (pH 8, 100 mM). For the synthesis of \((S)\)-3b, the reaction mixture contained MAL-H194A (0.04 mol%), TkGAD (0.6 mol%), 1b (10 mM), NH₄Cl (500 mM), PLP (1 mM), and MgCl₂ (25 mM) in 25 mL potassium phosphate buffer (pH 8, 100 mM); \^[b\] Conversion was analysed by \(^1\)H NMR spectroscopy; \^[c\] Products were purified by cation exchange chromatography; \^[d\] Enantiomeric excess (ee) was analysed by chiral HPLC.
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Having established the preference of CrpG for L-erythro-2b, a two-step enzymatic cascade reaction was performed at analytical scale by incubation of 1b and NH₄Cl with MAL and CrpG in one pot. Interestingly, full conversion of starting substrate 1b was observed (Figure S4), yielding solely the (R)-enantiomer of product 3b, as confirmed by ¹H NMR spectroscopy and chiral HPLC analysis. This is explained by a dynamic kinetic asymmetric transformation⁴²⁰,²¹ of the diastereoisomeric mixture of 2b (Scheme 2). In the first cascade step, MAL produces both L-erythro- and L-threo-2b as intermediate products. Subsequently, in the second step CrpG only decarboxylates L-erythro-2b to give exclusively (R)-3b. The remaining L-threo-2b is also converted into L-erythro-2b by MAL, leading to the full conversion of the starting material (1b) into the desired product (R)-3b.

Several control experiments were also performed. First, when 1b and NH₄Cl were incubated with MAL only, a ~1:1 mixture of L-threo-2b and L-erythro-2b was obtained (Figure S12A). After removal of MAL from the reaction mixture by heat inactivation and filtration, CrpG was added. After prolonged incubation (7 d), a mixture of unreacted L-threo-2b and product 3b was obtained (Figure S12B). Second, incubation of 1b and NH₄Cl with the diastereospecific mutant of MAL (MAL-H194A) and CrpG in one pot resulted in the formation of L-threo-2b but did not yield product 3b, consistent with the inability of CrpG to decarboxylate L-threo-2b (Figure S9). These results confirm that MAL is responsible for both the synthesis and epimerisation of L-threo- and L-erythro-2b, and that CrpG displays activity towards L-erythro-2b only, allowing for the selective synthesis of (R)-3b starting from the simple non-chiral dicarboxylic acid 1b.

**Scheme 2.** One pot two-step enzymatic cascade reaction involving MAL and CrpG that fully converts mesaconate (1b) to only (R)-3b. This is due to CrpG only acting on L-erythro-2b, and the MAL-mediated dynamic kinetic asymmetric transformation of the L-threo-2b into the desired diastereomer.
To demonstrate the synthetic usefulness of this two-step enzymatic cascade, a 100 mg-scale synthesis was performed. Accordingly, substrate 1b (30.8 mM) and NH₄Cl (500 mM) were incubated with MAL (0.02 mol%) and CrpG (0.47 mol%) in one pot (25 mL of buffer, pH 8). High conversion (>99% after 7 d), good isolated yield (78%), and excellent enantiopurity of product (R)-3b (>99% ee) were achieved (Table 1, Figure S16).

CrpG displays activity towards L-erythro-2b, enabling the enzymatic synthesis of (R)-3b. In order to synthesize the opposite enantiomer of 3b, a decarboxylase with activity towards L-threo-2b was required. Our attempts to obtain CrpG variants by directed evolution through screening of single-site saturation mutagenesis libraries did not yield any mutants with detectable activity towards L-threo-2b. We therefore cloned and produced two PLP-dependent GAD enzymes and tested their ability to decarboxylate L-threo-2b. Initially, we worked on the GAD from Pyrococcus furiosus (PfGAD), which was reported to accept L-aspartate, L-glutamate and L-tyrosine as substrates. [22] Although many different expression conditions were tested, we were not able to produce PfGAD in a soluble form in an Escherichia coli host. In an attempt to produce soluble protein, different constructs were made as fusions with three solubility enhancers: maltose-binding protein (MBP), small ubiquitin-like modifier protein (SUMO) and Fh8, a small protein secreted by the parasite Fasciola hepatica. However, inefficient solubilization of PfGAD limited the effectiveness of this approach. Therefore, we selected the GAD from the hyperthermophilic archaeon Thermococcus kodakarensis (TkGAD), which has 71% sequence similarity with PfGAD (Figure S2). TkGAD reportedly catalyzes the decarboxylation of L-glutamate and L-aspartate. [23] The gene encoding TkGAD was cloned and expressed, and the corresponding enzyme purified, yielding soluble and active protein (Figure S1). Initially, TkGAD activity was tested towards L-aspartate (2a) and L-erythro- and L-threo-2b (Figure S10). The enzyme completely converted 2a to 3a, whereas the reaction with L-erythro-2b showed <10% conversion. To our delight, L-threo-2b was also accepted as substrate by TkGAD, yielding the desired (S)-3b with more than 70% conversion.

Because TkGAD displays activity towards both diastereoisomers of 2b, a dynamic kinetic asymmetric transformation approach in which MAL and TkGAD are combined in one pot would not yield enantiopure (S)-3b product. Hence, for the one-pot, two-step enzymatic cascade synthesis of (S)-3b, TkGAD was used in combination with the diastereospecific MAL-H194A mutant, which produces exclusively L-threo-2b upon amination of 1b (Scheme 3 and Figure S5). Accordingly, substrate 1b (10 mM) and NH₄Cl (500 mM) were incubated with MAL-H194A (0.02 mol%) and TkGAD (0.3 mol%) in one pot (25 mL of buffer, pH 8). Because some protein precipitation occurred (due to instability of TkGAD), the same amount of each enzyme was added again after 24 h of incubation. Using these conditions, good conversion (75% after 48 h), good isolated yield (63%), and excellent enantiopurity of product (S)-3b (>99% ee) were achieved (Table 1, Figure S16).

Having developed one-pot, two-step enzymatic cascade reactions for the production of 3a,
Scheme 3. The one pot two-step enzymatic cascade reaction that converts mesaconate (1b) to only (S)-3b relies on a diastereospecific mutant MAL (MAL-H194A) and the newly discovered stereoselectivity of TkGAD towards L-threo-2b.

(R)-3b and (S)-3b, we next verified whether the PS enzyme is able to accept these compounds as substrates in condensation with (R)-pantoate (4) using small-scale (1 mL) reactions. We were pleased to find that PS accepted 3a, (R)-3b and (S)-3b as substrates in the condensation reaction, yielding the corresponding pantothenic acid products 5a and 5b, as confirmed by 1H NMR spectroscopy (Figure S13).

Having established that PS can be used to set up a three-step enzymatic cascade reaction, the enzymes MAL, ADC, and PS were combined in one pot. Initially, the reaction was tested by adding the three enzymes simultaneously and using an equimolar ratio of 1a, 4 and ATP. Although full conversion of the starting substrate 1a was observed after 24 h, the desired product (R)-5a was obtained in a crude yield of only 50% along with accumulation of 3a. This indicated that further optimization of the reaction conditions was necessary to improve the yield of product (R)-5a. After testing different ratios of 4 and ATP, a molar ratio of 2:3 was found to be best, which resulted in excellent conversion of the starting substrate into the desired product 5a (crude yield >99%; Figure S6 and S14). Hence, under these conditions, no significant accumulation of intermediate product 3a was observed.

To assess the performance of this one-pot multi-enzymatic cascade, a 32 mg-scale synthesis was performed. The three-step cascade reaction was performed by addition of all components simultaneously including the three enzymes. After 24 h, the starting material 1a was completely consumed (conversion >99%, Figure S15A) and the desired product (R)-5a was obtained in good isolated yield (70%) and with excellent ee (>99%) (Table 2). Importantly, the modularity of this enzymatic cascade approach also allows for the facile synthesis of both diastereoisomers of α-methyl-substituted vitamin Bs, that is (2R,2'R)-5b and (2S,2'R)-5b (Figure S7 and S8), with one stereogenic center being set by the selected combination of enzymes and the other by the substrate (R)-pantoate (4). Under suitable reaction conditions (for details, see section 8.2 in SI), and using the appropriate combination
of enzymes in one-pot, the desired products \((2R,2'R)-5b\) and \((2S,2'R)-5b\) were obtained with excellent de and ee values (>99%) and in 46-49% isolated yield (Table 2, Figure S15 and S17).

**Table 2.** Three-step enzymatic cascade synthesis of pantothenic acid \(5a\) and both diastereoisomers of its \(\alpha\)-methyl-substituted derivative \(5b\)\[^{[a]}\]

<table>
<thead>
<tr>
<th>Product</th>
<th>Enzymes</th>
<th>Conv (%)[[b]]</th>
<th>Isolated yield (%)</th>
<th>de and ee (%)[[c]]</th>
</tr>
</thead>
<tbody>
<tr>
<td>((R)-5a)</td>
<td>MAL, ADC and PS</td>
<td>&gt; 99</td>
<td>70[c]</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>((2R,2'R)-5b)</td>
<td>MAL, CrpG and PS</td>
<td>&gt; 99</td>
<td>49[c]</td>
<td>&gt; 99</td>
</tr>
<tr>
<td>((2S,2'R)-5b)</td>
<td>MAL-H194A, GAD and PS</td>
<td>75</td>
<td>46[d]</td>
<td>&gt;99</td>
</tr>
</tbody>
</table>

[a] The enzymes were found to be compatible for cascade synthesis at pH 9 (MAL, MAL-H194A and PS, optimum pH: 9.0-10.0; ADC, CrpG and GAD, optimum pH: 7.5-8.0). The amounts of applied enzymes were adjusted such that high conversions were achieved. For synthesis of \(5a\), the reaction mixture contained MAL (0.01 mol%), ADC (0.3 mol%), PS (0.07 mol%), \(1a\) (10 mM), \(4\) (20 mM), ATP (30 mM), NH\(_4\)Cl (500 mM) and MgCl\(_2\) (10 mM) in 20 mL Tris-HCl buffer (100 mM, pH 9). For synthesis of \((2R,2'R)-5b\), the reaction mixture contained MAL (0.01 mol%), CrpG (0.7 mol%), PS (0.07 mol%), \(1b\) (10 mM), \(4\) (20 mM), ATP (30 mM), NH\(_4\)Cl (500 mM) and MgCl\(_2\) (10 mM) in 20 mL Tris-HCl buffer (100 mM, pH 9). For synthesis of \((2S,2'R)-5b\), the reaction mixture contained MAL-H194A (0.04 mol%), \(7\) GAD (0.6 mol%), PS (0.07 mol%), \(1b\) (10 mM), \(4\) (20 mM), ATP (30 mM), PLP (1 mM), NH\(_4\)Cl (500 mM) and MgCl\(_2\) (10 mM) in 20 mL Tris-HCl buffer (100 mM, pH 9). [b] conversion was analysed by \(^1\)H NMR spectroscopy; [c] product was purified by preparative HPLC; [d] product was purified by silica gel column chromatography; [e] de and ee were analysed by \(^1\)H NMR spectroscopy and HPLC analysis.

In conclusion, we have successfully developed a one-pot cascade process for the synthesis of enantiomerically pure vitamin B\(_5\) starting from fumarate and utilizing MAL, ADC, and PS enzymes. Starting from mesaconic acid, the stereoselective synthesis of both diastereoisomers of \(\alpha\)-methyl-substituted vitamin B\(_5\), an important antibiotic precursor, was achieved by using either the CrpG or GAD enzyme instead of ADC, with one stereogenic center being set by the selected combination of MAL/CrpG or MAL-H194A/GAD and the other derived from one of the starting substrates. Given the availability of engineered MAL mutants and natural PS enzymes with a broad substrate scope, work is in progress to expand the substrate scope of CrpG and GAD by protein engineering.

Although the decarboxylation step actually is stereochemically deconstructive, with the loss of one chiral centre, the cascade approach strongly benefits from the use of stereo-divergent decarboxylases. These enzymes not only allow the synthesis of both diastereoisomers of \(\alpha\)-methyl-substituted vitamin B\(_5\), but they also provide a strong driving force to pull the equilibrium of the MAL-catalyzed reaction towards product formation. The use of an irreversible decarboxylation step, with stereochemical kinetic distinction, is an
important strategy in biocatalytic cascade synthesis to overcome thermodynamic limitations and maximize product yield. A possible constraint on the use of the developed cascade for large-scale transformations would be the dependence of the PS enzyme on the expensive cofactor ATP. This could be addressed by the incorporation of an auxiliary enzyme-catalyzed step for efficient ATP recycling. Several ATP recycling enzyme systems are available and a few have already been successfully implemented in preparative biocatalysis. However, as the starting materials (fumarate and mesaconate) can be efficiently produced in high yields by large-scale fermentation using metabolically engineered *E. coli* strains, we envision to co-express the enzymes employed for the cascade in such a fermentation host in order to directly obtain vitamin B₅ and its α-methyl-substituted derivatives from cheap carbon and nitrogen sources. Indeed, such a cell-based approach would eliminate the need for ATP recycling.

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Enzymatic Cascade Synthesis of Vitamin B5


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9. Spectral data
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11. References
1. Materials

Mesaconic acid, sodium fumarate, β-alanine, \((R/S)\)-3-aminoisobutyric acid, L-aspartic acid, sodium \((R)\)-pantoate, adenosine triphosphate (ATP), calcium \((R)\)-pantothenate and pyridoxal phosphate (PLP) were purchased from Sigma-Aldrich Chemical Co. A diastereomeric mixture of L-\(\text{threo}\)- and L-\(\text{erythro}\)-3-methylaspartic acid (2b) was obtained using the MAL enzyme by following a previously published procedure.\([1]\) Buffers and media were obtained from Duchefa Biochemie or Merck. Molecular biology reagents, including restriction enzymes, PCR reagents, T4 DNA ligase and DNA ladders, agarose, SDS-PAGE gels and protein markers were obtained from Fermentas. PCR purification kits and Miniprep kits were purchased from Macherey-Nagel. Ni Sepharose High Performance (HP) affinity resin and pre-packed Sephadex G-25 columns were purchased from GE Healthcare Life Sciences AB. Amylose resin was purchased from New England Biolabs Inc. Oligonucleotides for DNA amplification and synthetic genes were obtained from Operon Biotechnologies. Column chromatography was performed on Merck 60 silica gel (0.063 – 0.200 mesh; Milipore). Analytical thin layer chromatography (TLC) was performed on Merck silica plates 60 GF254 with ninhydrin staining for detection.

2. General methods

Techniques for transformation and other standard molecular biology manipulations were based on methods described elsewhere.\([2]\) Proteins were analyzed by polyacrylamide gel electrophoresis (PAGE) using sodium dodecyl sulfate (SDS) gels containing polyacrylamide (12%). The gels were stained with Instant Blue. Protein concentrations were determined by the Waddell method.\([3]\) NMR spectra were recorded on a Bruker DRX-500 (500 MHz) spectrometer. Chemical shifts for protons are reported in parts per million and are referenced to the residual water in D\(_2\)O. To monitor the reaction progress by \(^1\)H NMR, the reaction mixture was lyophilized and dissolved in D\(_2\)O. Analytical HPLC analysis was carried out using an in-house HPLC equipped with either a chiral CROWNPAK® CR-I(+) (150 × 3mm, 5µm) column or a Kinetex® 5µm EVO C-18 (100 Å; 150 × 4.6 mm; Phenomenex) column. Preparative HPLC analysis was performed using a Kinetex® 5µm C18, AXIA (100 Å; 100 x 21.2 mm, Phenomenex) column. The HPLC chromatographic data were analyzed by data processing software (LC Solutions) obtained from Shimadzu. Thin Layer Chromatography (TLC) analysis was performed in 2-butanol:acetic acid:water (8:2:2) as the mobile phase.
3. Molecular cloning, expression and purification of enzymes

Table S1. Bacterial strains, plasmids and growth conditions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Source</th>
<th>Plasmid</th>
<th>Restriction sites</th>
<th>E. coli strain</th>
<th>Induction</th>
<th>Expression Temp (°C) &amp; rpm</th>
<th>His-tag</th>
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</thead>
<tbody>
<tr>
<td>MAL</td>
<td>Clostridium tetanomorphum</td>
<td>pBADN/Myc -His A</td>
<td>NdeI/HindIII</td>
<td>TOP10</td>
<td>TOP10</td>
<td>Arabinose (0.05%)</td>
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<td>ADC</td>
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<td>pET28a</td>
<td>NdeI/XhoI</td>
<td>DH5α</td>
<td>BL21(DE3)</td>
<td>IPTG (1 mM)</td>
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</table>
3.1. Expression and purification of methylaspartate ammonia lyase (MAL) and the mutant enzyme MAL-H194A

The wildtype MAL and mutant MAL-H194A enzymes were overexpressed and purified by following a previously reported protocol.[1]

3.2. Expression and purification of aspartate-α-decarboxylase (ADC)

The ADC enzyme was expressed and purified according to previously reported procedures.[4] A preculture was prepared by inoculating 10 mL of LB-medium containing kanamycin (100 mg/L). The preculture was incubated overnight at 200 rpm and 37°C. The complete overnight culture was used to inoculate fresh LB medium (1 L) containing kanamycin (100 mg/L) in a 5 L Erlenmeyer flask. The culture was incubated at 200 rpm and 37°C to an initial OD600 of 0.4 – 0.6. The protein expression was induced by adding IPTG (1 mM, final concentration) and the incubation was continued for 18 h at 37°C and 200 rpm. Cells were harvested by centrifugation, the supernatant was removed and the cells stored at -20°C until further use.

Cell pellets were thawed and suspended in lysis buffer (30 mL, 20 mM Tris-HCl, 500 mM NaCl, 5 mM imidazole, pH 7.9). Then, cells were disrupted by sonication for 5 x 40 sec (with 5 min intervals between each cycle) at a 60 W output. The unbroken cells and debris were removed by centrifugation. The supernatant (25 mL) was filtered (0.45 µm filter), and incubated with Ni sepharose resin (1 mL slurry) in a column for 18 h at 4°C. 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 (5 mL, 20 mM Tris-HCl, 500 mM NaCl, 100 mM imidazole, pH 7.9). Retained proteins were eluted with buffer B (5 mL, 20 mM Tris-HCl, 500 mM NaCl, 250 mM imidazole, pH 7.9). Fractions were analyzed by SDS-PAGE and those containing pure enzyme were combined, and the buffer was exchanged against Tris-HCl (3.5 mL, 25 mM, pH 8.0), containing 5 mM MgCl2, using a pre-packed Sephadex G-25 column (GE-Healthcare). The purified ADC enzyme was activated at 50°C for 48 h (autocatalytic self-processing). Subsequently, the activated enzyme was aliquoted, snap-frozen in liquid nitrogen and stored in aliquots at −80 °C until further use.

3.3. Cloning, expression and purification of β-methylaspartate-α-decarboxylase (CrpG)

The sequence of the gene encoding CrpG from Nostoc sp. ATCC 53789 was obtained from the NCBI nucleotide database under GenBank accession no. EF159954. The DNA sequence was codon-optimized for E. coli and synthesized by Operon Biotechnologies. The synthetic gene was first cloned into the pGEM-T Easy Vector, using standard TA cloning. Subsequently, the gene was obtained from this vector using the restriction enzymes NdeI and XhoI, and subcloned into the pET-28 expression vector to produce N-terminally hexahistidine-tagged CrpG. Expression and purification of CrpG were performed as described
previously.[5] The buffers used for Ni-sepharose column chromatography and desalting of CrpG by using a pre-packed Sephadex G-25 column (GE-Healthcare) are given in Table S2. The purified enzyme was activated at 50°C for 48 h (autocatalytic self-processing). The activated enzyme was aliquoted, snap-frozen in liquid nitrogen and stored in aliquots at −80°C until further use.

3.4. Cloning and expression of glutamate decarboxylase from *Pyrococcus furiosus* (*PfGAD*)

The sequence of the gene encoding glutamate decarboxylase (GAD) from *Pyrococcus furiosus* was obtained from the NCBI nucleotide database under GenBank accession no. NC_003413. The DNA sequence was codon-optimized for *E. coli* and synthesized by Operon Biotechnologies. After initial cloning of the synthetic gene into the pGEM-T Easy Vector, it was finally subcloned into the pET-20b expression vector. For expression of *PfGAD*, a procedure was followed as described in an earlier study.[6] After lysis of the cells and subsequent centrifugation, the cell-free extract (supernatant) and pellet were analysed by SDS-PAGE, showing that the enzyme was produced in a insoluble form.

3.5. Cloning, expression and purification of *PfGAD* with fusion partner (MBP, SUMO, or Fh8)

The pCOBO-MBP vector was made by following our earlier reported protocol.[7] The pCOBO-MBP-*PfGAD* fusion construct was generated by the AQUA cloning technique. The first DNA fragment, containing the MBP gene, was mutated using PCR and an appropriate mutagenesis primer to remove an internal *Nco*I restriction site. For this, a silent mutation (C to G) was introduced at position 975 of the MBP ORF by the QuickChange method. The second fragment, containing the *PfGAD* gene, was amplified using pET-20b-*PfGAD* as the template. The *PfGAD* gene was amplified using the following primers: *PfGAD-Fw*: 5’-TG CTT TTT CAA GGC CCT GGA GGC GCC ATG GTG AAG TTC CCA AGA AAA GGT ATC-3’ (*Nco*I site in bold) and *PfGAD-Rev*: 5’-GCC GGA TCG TCA TTA CCC GGG TTT CTC GAG TTA TTA ATC ATG GCC TCC GTC ACA TGA-3’ (*Xho*I site in bold). After *NcoI*/*XhoI* digestion of both PCR products, the two fragments were ligated and fused into pCOBO-MBP using the AQUA cloning technique. The newly constructed expression vector was denoted pCOBO-MBP-*PfGAD* and its fidelity was confirmed by DNA sequencing.

The pCOBO-MBP-*PfGAD* plasmid was transformed into *E. coli* BL21(DE3). For protein production, a preculture was prepared by inoculating 10 mL of LB-medium containing ampicillin (100 µg/mL). The preculture was incubated overnight at 200 rpm and 37°C. The complete overnight culture was used to inoculate fresh LB medium (1 L), containing ampicillin (100 µg/mL) in a 5 L Erlenmeyer flask. Then, the culture was incubated at 200 rpm and 37°C to an initial OD600 of 0.4 – 0.6. The protein expression was induced by adding IPTG
(0.1 mM, final concentration) and the incubation was continued for 18 h at 20°C and 200 rpm. Cells were harvested by centrifugation and stored at -20°C until further use.

Cell pellets were thawed and suspended in lysis buffer (30 mL, 50 mM Tris-HCl, 10% glycerol, pH 7.4) supplemented with protease inhibitor (Roche, The Netherlands). Then, cells were disrupted by sonication for 5 x 40 sec (with 5 min interval between each cycle) at a 60 W output. The unbroken cells and debris were removed by centrifugation. The supernatant (25 mL) was filtered (0.45 µm filter) and incubated overnight at 4 °C with 5 mL MBPTrap resin, which was pre-equilibrated with buffer (50 mM Tris-HCl, 10% glycerol, pH 7.4), in a conical centrifuge tube. Then the resin was transferred into a column and the unbound proteins were eluted from the column by gravity flow. Bound protein was eluted with 3.5 mL elution buffer (50 mM Tris-HCl, 10 mM maltose, 10% glycerol, pH 7.4). Fractions were analyzed by SDS PAGE, those containing the fusion protein were pooled, aliquoted, snap-frozen in liquid nitrogen, and stored in aliquots at −80 °C. Unfortunately, the purified MBP-PfGAD fusion protein was found to be inactive.

The pCOBO-SUMO-PfGAD and pCOBO-Fh8-PfGAD expression constructs were made using a similar cloning strategy as that mentioned above for pCOBO-MBP-PfGAD. The pCOBO-SUMO and pCOBO-Fh8 vectors have been reported before. [7] The conditions for production of the fusion proteins SUMO-PfGAD and Fh8-PfGAD were the same as that followed for MBP-PfGAD. After lysis of the cells and subsequent centrifugation, the cell free extract (supernatant) and pellet were analyzed by SDS-PAGE, showing that the fusion proteins were produced in an insoluble form.

3.6. Cloning, expression and purification of glutamate decarboxylase from Thermococcus kodakarensis (TkGAD)

The sequence of the gene encoding GAD from Thermococcus kodakarensis KOD1 was obtained from the NCBI nucleotide database under GenBank accession no. AP006878.1. The DNA sequence was codon-optimized for E. coli and synthesized by Operon Biotechnologies. After initial cloning of the synthetic gene into the pGEM-T Easy Vector, it was finally subcloned into the pET-20b expression vector. Expression and purification of TkGAD were performed as described earlier. [8] The buffers used for Ni-sepharose column chromatography and desalting of the purified enzyme by using a pre-packed Sephadex G-25 column (GE-Healthcare) are given in Table S2. The purified enzyme was aliquoted, snap-frozen in liquid nitrogen, and stored in aliquots at −80 °C until further use.

3.7. Cloning, expression and purification of pantothenate synthetase (PS)

The sequence of the panC gene, encoding pantothenate synthetase of E. coli, was obtained from the NCBI nucleotide database under GenBank accession no. NP_414675. The DNA sequence was codon-optimized for E. coli and synthesized by Operon Biotechnologies. After
initial cloning of the synthetic gene into the pGEM-T Easy Vector, the PS gene was mutated using PCR and an appropriate mutagenesis primer to remove an internal NdeI restriction site. For this, a silent mutation (T to C) was introduced at position 171 of the PS ORF by single overlap extension PCR. The amplified gene encoding PS was cloned into the pET-28a vector. Expression and purification were performed as described earlier. The buffers used for Ni-sepharose column chromatography and desalting of the purified PS enzyme by using a pre-packed Sephadex G-25 column (GE-Healthcare) are given in Table S2. The purified enzyme was aliquoted, snap-frozen in liquid nitrogen, and stored in aliquots at −80 °C until further use.

Table S2: Protein purification conditions

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Step1 (Washing)</th>
<th>Step2 (Elution)</th>
<th>Step 3 (Desalting)</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAL/ MAL-H194A</td>
<td>i) 50 mM Tris-HCl, 300 mM NaCl, 10 mM Imidazole, pH 8.0 (15 mL)</td>
<td>50 mM Tris-HCl, 300 mM NaCl, 500 mM Imidazole, pH 8 (5 mL)</td>
<td>50 mM Tris buffer, 2 mM MgCl2, 0.1 mM KCl, pH 8 (3.5 mL)</td>
</tr>
<tr>
<td>ADC</td>
<td>i) 20 mM Tris-HCl, 500 mM NaCl, 5 mM Imidazole, pH 8 (15 mL)</td>
<td>20 mM Tris-HCl, 500 mM NaCl, 250 mM Imidazole, pH 8 (5 mL)</td>
<td>25 mM Tris-HCl, 5 mM MgCl2, pH 8 (3.5 mL)</td>
</tr>
<tr>
<td>CrpG</td>
<td>50 mM potassium phosphate, 300 mM NaCl, 20 mM Imidazole, pH 8 (15 mL)</td>
<td>50 mM potassium phosphate, 300 mM NaCl, 250 mM Imidazole, pH 8 (5 mL)</td>
<td>50 mM Potassium phosphate, pH 8 or 20 mM Tris-HCl, 500 mM NaCl, pH 8 (3.5 mL)</td>
</tr>
<tr>
<td>TkGAD</td>
<td>20 mM Na-phosphate buffer, 500 mM NaCl, 25 mM Imidazole, pH 7.4 (15 mL)</td>
<td>20 mM Na-phosphate buffer, 500 mM NaCl, 250 mM Imidazole, pH 7.4 (5 mL)</td>
<td>20 mM Na-phosphate buffer, 150 mM NaCl, 0.25 mM PLP, pH 7.4 (3.5 mL)</td>
</tr>
<tr>
<td>PS</td>
<td>20 mM Na-phosphate buffer, 500 mM NaCl, 30 mM Imidazole, pH 8 (15 mL)</td>
<td>20 mM Na-phosphate buffer, 500 mM NaCl, 250 mM Imidazole, pH 8 (5 mL)</td>
<td>20 mM Tris-HCl, 500 mM NaCl, pH 7.9 (3.5 mL)</td>
</tr>
</tbody>
</table>
Figure S1. SDS-PAGE analysis of purified proteins. (M) protein ladder (PageRuler Plus Prestained, Thermo Fisher Scientific); (1) MAL; (2) MAL-H194A; (3) ADC (after activation; \( \pi \) – unprocessed proenzyme form; \( \alpha \) – enzyme subunit which contains a C-terminal acid; \( \beta \) – enzyme subunit which contains the pyruvoyl group at its N terminus); (4) CrpG (after activation; \( \pi \) – unprocessed proenzyme form; \( \alpha \) – enzyme subunit which contains a C-terminal acid; \( \beta \) – enzyme subunit which contains the pyruvoyl group at its N terminus); (5) TkGAD; (6) PS.
4. Sequence alignment between *PfGAD* and *TkGAD*

![Figure S2](image)

**Figure S2.** Sequence alignment between *PfGAD* and *TkGAD*. The amino acid sequences were aligned with the DNAMAN program using default parameters. (-) in the *TkGAD* sequence denotes that its residues at that position are identical to those of *PfGAD*. 
5. TLC analysis

5.1. Monitoring the progress of two-step enzymatic cascade synthesis

- \(1a\) = fumarate (standard)
- \(2a\) = L-aspartic acid (standard)
- \(3a\) = \(\beta\)-alanine (standard)
- \(\text{RM}\) = sample of enzymatic reaction mixture

**Figure S3.** Two-step enzymatic cascade synthesis of \(3a\). The reaction mixture (1 mL) contained 0.01 mol% of MAL, 0.6 mol% of ADC, fumarate (\(1a\), 25 mM), NH\(_4\)Cl (500 mM) and MgCl\(_2\) (10 mM) in Tris-HCl buffer (100 mM, pH = 8).

- \(1b\) = mesaconic acid (standard)
- \(\text{ery-2b}\) = L-erythro-3-methylaspartic acid (standard)
- \((R\text{-})3b\) = \((R\text{-})\)-3-amino-2-methylpropanoic acid (standard)
- \(C\) = control of enzymatic reaction mixture (without enzyme)
- \(\text{RM}\) = sample of enzymatic reaction mixture

**Figure S4.** Two-step enzymatic cascade synthesis of \((R\text{-})3b\). The reaction mixture (1 mL) contained 0.01 mol% of MAL, 0.25 mol% of CrpG, mesaconic acid (\(1b\), 25 mM) and NH\(_4\)Cl (500 mM) in potassium phosphate buffer (100 mM, pH = 8).

- \(1b\) = mesaconic acid (standard)
- \(\text{thr-2b}\) = L-threo-3-methylaspartic acid (standard)
- \((S\text{-})3b\) = \((S\text{-})\)-3-amino-2-methylpropanoic acid (standard)
- \(\text{RM}\) = sample of enzymatic reaction mixture

**Figure S5.** Two-step enzymatic cascade synthesis of \((S\text{-})3b\); The reaction mixture (1 mL) contained 0.04 mol% of MAL-H194A, 0.6 mol% of \(Tk\text{GAD}\), mesaconic acid (\(1b\), 10 mM), NH\(_4\)Cl (500 mM) and PLP (1 mM) in potassium phosphate buffer (100 mM, pH = 8).
5.2. Monitoring the progress of three-step enzymatic cascade synthesis

\[
\begin{align*}
1a & = \text{fumarate (standard)} \\
2a & = \text{L-aspartic acid (standard)} \\
3a & = \beta\text{-alanine (standard)} \\
(R)-5a & = (R)\text{-pantothenic acid (standard)} \\
RM & = \text{sample of enzymatic reaction mixture}
\end{align*}
\]

Note: All three enzymes (MAL, ADC and PS) were added simultaneously and the reaction progress was monitored by TLC. Reaction completion was also confirmed by \(^1\text{H}\) NMR (Figure S15A).

**Figure S6.** Three-step enzymatic cascade synthesis of \((R)-5a\). The reaction mixture (1 mL) contained 0.02 mol\% of MAL, 0.3 mol\% of ADC, 0.07 mol\% of PS, fumarate \((1a, 10 \text{ mM})\), NH\(_4\)Cl (500 mM), \((R)\)-pantoate \((4, 20 \text{ mM})\), ATP (30 mM) and MgCl\(_2\) (10 mM) in Tris-HCl buffer (100 mM, pH = 9).

\[
\begin{align*}
1b & = \text{mesaconic acid (standard)} \\
\text{ ery-2b} & = \text{L-erythro-3-methylaspartic acid (standard)} \\
(R)-3b & = (R)\text{-3-amino-2-methylpropanoic acid (standard)} \\
RM & = \text{sample of enzymatic reaction mixture (before addition of the PS enzyme)}
\end{align*}
\]

Note: The first two steps of the cascade reaction were monitored by TLC. After addition of the PS enzyme, the completion of the third step (condensation reaction) was confirmed by \(^1\text{H}\) NMR (Figure S15B).

**Figure S7.** Three-step enzymatic cascade synthesis of \((2R,2^\prime R)-5b\). The reaction mixture (1 mL) contained 0.02 mol\% of MAL, 0.25 mol\% of CrpG, 0.07 mol\% of PS, mesaconic acid \((1b, 10 \text{ mM})\), NH\(_4\)Cl (500 mM), \((R)\)-pantoate \((4, 20 \text{ mM})\), ATP (30 mM) and MgCl\(_2\) (10 mM) in Tris-HCl buffer (100 mM, pH = 9).

\[
\begin{align*}
1b & = \text{mesaconic acid (standard)} \\
\text{ thr-2b} & = \text{L-threo-3-methylaspartic acid (standard)} \\
(S)-3b & = (S)\text{-3-amino-2-methylpropanoic acid (standard)} \\
\text{RM (24 h)} & = \text{sample of enzymatic reaction mixture after 24 h} \\
\text{RM (48 h)} & = \text{sample of enzymatic reaction mixture after 48 h (after addition of the second batch of enzyme)}
\end{align*}
\]

Note: The first two steps of the cascade reaction were monitored by TLC. After addition of the PS enzyme, the completion of the third step (condensation reaction) was confirmed by \(^1\text{H}\) NMR (Figure S15C).

**Figure S8.** Three-step enzymatic cascade synthesis of \((2S,2^\prime R)-5b\). The reaction mixture (1 mL) contained 0.04 mol\% of MAL-H194A, 0.6 mol\% of TkGAD, 0.07 mol\% of PS, mesaconic acid \((1b, 10 \text{ mM})\), NH\(_4\)Cl (500 mM), MgCl\(_2\) (10 mM), \((R)\)-pantoate \((4, 20 \text{ mM})\), and ATP (30 mM) in Tris-HCl buffer (100 mM, pH = 9).
5.3. Control experiment to test the diastereoselectivity of CrpG

![Figure S9](image)

**Figure S9.** The reaction mixture (1 mL) contained 0.01 mol% of MAL-H194A, 0.25 mol% of CrpG, mesaconic acid (1b, 25 mM) and NH₄Cl (500 mM) in potassium phosphate buffer (100 mM, pH = 8).

5.4. TkGAD activity test

![Figure S10](image)

**Figure S10.** The reaction mixtures contained 0.3 mol% of TkGAD, 10 mM of substrate (2a, L-threo-2b, or L-erythro-2b), and 1 mM PLP in 50 mM HEPES buffer (100 mM, pH = 8).
6. NMR Analysis

6.1. CrpG catalyzed diastereoselective decarboxylation of 2b

Figure S11. CrpG catalyzed diastereoselective decarboxylation of 2b. (A) $^1$H NMR spectrum of a 1:1 mixture of L-threo- and L-erythro-3-methylaspartic acid (2b). (B) $^1$H NMR analysis of the diastereoselective decarboxylation of a 1:1 mixture of L-threo-2b and L-erythro-2b by CrpG. The spectrum was recorded after 7 d of incubation, showing unreacted L-threo-2b and product (R)-3-amino-2-methylpropanoic acid [(R)-3b], which results from the CrpG-catalyzed decarboxylation of L-erythro-2b.
6.2. Control experiment to test the diastereoselectivity of CrpG

Figure S12. (A) $^1$H NMR spectrum monitoring the MAL-catalyzed amination of mesaconic acid (1b), yielding a ~1:1 mixture of L-threo- and L-erythro-3-methylaspartic acid (2b); the spectrum was recorded 24 h after the start of the amination reaction. (B) $^1$H NMR spectrum monitoring the CrpG catalyzed diastereoselective decarboxylation of L-erythro-3-methylaspartic acid yielding (R)-3-amino-2-methylpropanoic acid [(R)-3b]. After removal of MAL from the reaction mixture shown in Figure S12A by heat inactivation and filtration, CrpG was added. The spectrum was recorded after 7 d of incubation, showing complete decarboxylation of L-erythro-2b into (R)-3b, as well as unreacted L-threo-2b.
6.3. Testing the substrate scope of PS by $^1$H NMR analysis

Figure S13. (A) $^1$H NMR spectrum monitoring the PS-catalyzed condensation of $3\text{a}$ and $(R)$-pantoate (4) yielding $(R)$-$5\text{a}$; (B) $^1$H NMR spectrum monitoring the PS-catalyzed condensation of $(R)$-$3\text{b}$ and 4 yielding $(2R,2'R)$-$5\text{b}$; (C) $^1$H NMR spectrum monitoring the PS-catalyzed condensation of $(S)$-$3\text{b}$ and 4 yielding $(2S,2'R)$-$5\text{b}$. Reaction mixtures contained 0.07 mol% of PS, 10 mM of $3\text{a}$ or $(R)$-$3\text{b}$ or $(S)$-$3\text{b}$, 20 mM of 4, 20 mM of ATP, and 10 mM of MgCl$_2$ in 1 mL Tris-HCl buffer (pH 9, 100 mM); NMR spectra were recorded 24 h after the start of the condensation reactions. Characteristic signals of the products are labeled.
6.4. Reaction conditions optimization for three-step cascade synthesis of (R)-5a

**Figure S14.** $^1$H NMR spectra monitoring the progress of the cascade synthesis of (R)-5a using different conditions. (A) $^1$H NMR spectrum of reaction mixture which contained 1:1:1 ratio of fumarate (1a), (R)-pantoate (4), and ATP; (B) $^1$H NMR spectrum of reaction mixture which contained 1:2:2 ratio of 3a, 4, and ATP; (C) $^1$H NMR spectrum of reaction mixture which contained 1:2:3 ratio of 1a, 4, and ATP. The reaction mixtures contained 0.01 mol% of MAL, 0.03 mol% of ADC and 0.07 mol% of PS, 1a (10 mM), 4 (10-20 mM), ATP (10-30 mM), and MgCl$_2$ (10 mM) in 20 mL Tris-HCl buffer (pH 9, 100 mM); NMR spectra were recorded after 24 h. Characteristic signals of the products are labeled in Panels A-C.
6.5. Monitoring the progress of three-step cascade synthesis

Figure S15. $^1$H NMR spectra monitoring the progress of the multi-enzymatic synthesis of pantothenic acid (5a) and both diastereoisomers of α-methyl-pantothenic acid (5b). (A) $^1$H NMR spectrum of reaction mixture which contains (R)-5a; spectrum was recorded after 24 h; (B) $^1$H NMR spectrum of reaction mixture which contains (2R,2'R)-5b; spectrum was recorded after 8 d; (C) $^1$H NMR spectrum of reaction mixture which contains (2S,2'R)-5b; spectrum was recorded after 3 d. Characteristic signals of the products are labeled in Panels A-C.
7. Experimental procedure for analytical scale synthesis

7.1. One-pot two-step enzymatic cascade synthesis

7.1.1. 3-Aminopropanoic acid (3a): The reaction was performed in Tris-HCl buffer (100 mM, pH 8). The reaction mixture (1 mL) contained MAL (0.01 mol%), ADC (0.6 mol%), fumarate (1a, 25 mM), NH₄Cl (500 mM) and MgCl₂ (10 mM). The reaction mixture was incubated at 25 °C. Reaction progress was monitored by TLC analysis. After 24 h the reaction mixture was heated at 100°C for 5 min and the precipitated proteins were removed by centrifugation. The crude reaction mixture was analyzed by ¹H NMR and showed >99% conversion of 1a into 3a.

7.1.2. (R)-3-amino-2-methylpropanoic acid [(R)-3b]: The reaction was performed in potassium phosphate buffer (100 mM, pH 8). The reaction mixture (1 mL) contained MAL (0.01 mol%), CrpG (0.25 mol%), mesaconic acid (1b, 25 mM) and NH₄Cl (500 mM). The reaction mixture was incubated at 25 °C. Reaction progress was monitored by TLC analysis. After 7 d the reaction mixture was heated at 100°C for 5 min and the precipitated proteins were removed by centrifugation. The crude reaction mixture was analyzed by ¹H NMR and showed >99% conversion of 1b into the desired product (R)-3b.

7.1.3. (S)-3-amino-2-methylpropanoic acid [(S)-3b]: The reaction was performed in potassium phosphate buffer (100 mM, pH 8). The reaction mixture (1 mL) contained MAL-H194A (0.02 mol%), TkGAD (0.3 mol%), mesaconic acid (1b, 10 mM), NH₄Cl (500 mM), and PLP (1 mM). The reaction mixture was incubated at 37 °C for 24 h. The reaction progress was monitored by TLC analysis and after 24 h the reaction showed ~48% conversion. Then, one more equivalent of MAL-H194A (0.02 mol%) and TkGAD (0.3 mol%) were added and the reaction was allowed to proceed further for 24 h. After a total incubation time of 2 d, maximum conversion of 1b (75%) into the desired product (S)-3b was obtained, and further incubation did not show any improvement.

7.2. One-pot three-step enzymatic cascade synthesis

7.2.1. (R)-pantothenic acid [(R)-5a]: The reaction was performed in Tris-HCl buffer (100 mM, pH 9). All three enzymes, MAL (0.02 mol%), ADC (0.3 mol%) and PS (0.07 mol%) were added simultaneously into Tris-HCl buffer. To this 1a (10 mM), NH₄Cl (500 mM), ATP (30 mM) and MgCl₂ (10 mM) were added. The reaction mixture (1 mL) was incubated at 25 °C. After 24 h the reaction mixture was heated at 100 °C for 5 min and the precipitated proteins were removed by centrifugation. The crude reaction mixture was analyzed by ¹H NMR and showed >99% conversion of 1a into (R)-5a.

7.2.2. (2R,2'R)-α-methyl-pantothenic acid [(2R,2'R)-5b]: The reaction was performed in Tris-HCl buffer (100 mM, pH 9). Initially, the first two enzymes MAL (0.02 mol%) and CrpG (0.25 mol%) were added simultaneously into the buffer. To this 1b (10 mM), NH₄Cl
(500 mM), and MgCl₂ (10 mM) were added. The reaction mixture (1 mL) was incubated at 25 °C for 7 d. After complete conversion of 1b into (R)-3b, the third enzyme PS (0.07 mol%), 4 (20 mM) and ATP (30 mM) were added into the reaction mixture, and incubation was continued further for 24 h. Then, the reaction mixture was heated at 100 °C for 5 min and the precipitated proteins were removed by centrifugation. The crude reaction mixture was analyzed by ¹H NMR and showed >99% conversion of 1b into (2R,2′R)-5b.

7.2.3. (2S,2′R)-α-methyl-pantothenic acid [(2S,2′R)-5b]: The reaction was performed in Tris-HCl buffer (100 mM, pH 9). Initially, the first two enzymes MAL-H194A (0.04 mol%) and TkGAD (0.6 mol%) were added simultaneously into the buffer. To this 1b (10 mM), NH₄Cl (500 mM), MgCl₂ (10 mM), and PLP (1 mM) were added and the reaction mixture was incubated at 37 °C for 24 h to produce (S)-3b as mentioned earlier (section 7.1.3). Then, the third enzyme PS (0.07 mol%), 4 (20 mM) and ATP (30 mM) were added into the reaction mixture, and incubation of the reaction mixture was continued further at 25 °C for 24 h. Then, the reaction mixture was heated at 100 °C for 5 min and the precipitated proteins were removed by centrifugation. The crude reaction mixture was analyzed by ¹H NMR and showed 75% conversion of 1b into (2S,2′R)-5b.

8. Experimental procedure for preparative scale synthesis

8.1. One-pot two-step enzymatic cascade synthesis

8.1.1. 3-aminopropanoic acid (3a):

The reaction mixture consisted of 1a (100 mg, 25 mM), NH₄Cl (500 mM) and MgCl₂ (25 mM) in 25 mL of Tris-HCl buffer (100 mM, pH 8), and the pH of the reaction mixture was adjusted to pH 8 by addition of aqueous HCl or NaOH. The enzymatic cascade reaction was started by addition of MAL (0.02 mol%) and ADC (0.6 mol%), and the reaction mixture was incubated at 25 °C. The reaction progress was monitored by TLC. After 24 h, the reaction mixture was heated at 100 °C for 5 min and precipitated protein was removed by centrifugation. The reaction mixture was concentrated and then acidified with 1N HCl (5 mL). The desired product was purified using a cation exchange column (Dowex® 50W X8, 5 mL), which was pretreated with aqueous NH₃ (2 M, 4 column volumes) followed by HCl (1N, 2 column volumes) and finally washed with distilled water (4 column volumes). The crude reaction sample was loaded onto the cation exchange column and washed with water (2 column volume), after which the product was eluted with aqueous NH₃ (2 M, 3 column volumes). The collected fractions (analysed by TLC using ninhydrin staining) that contained the product were combined and concentrated, followed by lyophilization. Product 3a was isolated in 85% yield (48 mg).
8.1.2. (R)-3-amino-2-methylpropanoic acid [(R)-3b]:

The reaction mixture consisted of 1b (100 mg, 30.8 mM), NH₄Cl (500 mM) and MgCl₂ (25 mM) in 25 mL of pottasium phosphate buffer (100 mM, pH 8), and the pH of the reaction mixture was adjusted to pH 8 by addition of aqueous HCl or NaOH. The enzymatic cascade reaction was started by addition of MAL (0.02 mol%) and CrpG (0.47 mol%) and the reaction mixture was incubated at 25 °C. The reaction progress was monitored by TLC. After 7 d, the reaction mixture was heated at 100 °C and precipitated protein was removed by centrifugation. The reaction mixture was concentrated and then acidified with 1 N HCl (5 mL). The crude product was purified as mentioned above (section 8.1.1). Product (R)-3b was isolated in 78% yield (62 mg).

8.1.3. (S)-3-amino-2-methylpropanoic acid [(S)-3b]:

The reaction mixture consisted of 1b (32.5 mg, 10 mM), NH₄Cl (500 mM) and MgCl₂ (25 mM) in 25 mL of pottasium phosphate buffer (100 mM, pH 8), and the pH of the reaction mixture was adjusted to pH 8 by addition of aqueous HCl or NaOH. The enzymatic cascade reaction was started by addition of MAL-H194A (0.02 mol%) and TkGAD (0.3 mol%) and incubated at 37 °C for 24 h. After 24 h of incubation, the reaction showed only ~48% conversion of 1b into the final product (S)-3b. To achieve a better yield of 3b, MAL-H194A (0.02 mol%) and TkGAD (0.3 mol%) were added again and the reaction mixture was incubated further for 24 h. The reaction progress was monitored by TLC. After a total incubation time of 2 d, the reaction mixture was heated at 100 °C and precipitated protein was removed by centrifugation. The reaction mixture was concentrated and then acidified with 1 N HCl (5 mL). The crude product was purified as mentioned above (section 8.1.1). Product (S)-3b was isolated in 63% yield (16 mg).

8.2. One-pot three-step enzymatic cascade synthesis

8.2.1. (R)-pantothenic acid [(R)-5a]:

The reaction mixture consisted of 1a (32 mg, 10 mM), NH₄Cl (500 mM), 4 (20 mM), ATP (30 mM) and MgCl₂ (10 mM) in 20 mL of Tris-HCl buffer (100 mM, pH 9), and the pH of the reaction mixture was adjusted to pH 9 by addition of aqueous HCl or NaOH. All three enzymes MAL (0.01 mol%), ADC (0.3 mol%) and PS (0.07 mol%) were added simultaneously into the reaction mixture, and the reaction mixture was incubated at 25 °C. After 24 h, the reaction mixture was heated at 100°C for 5 min and the precipitated proteins were removed by centrifugation. The reaction mixture was concentrated and dissolved in 2 mL of NH₄HCO₃ (20 mM). The crude product was purified by preparative HPLC (20 mM ammonium bicarbonate, flow rate 15 mL/min). The eluted fractions (analysed by TLC using ninhydrin staining) that contained the product were combined and concentrated, followed by lyophilization. Product (R)-5a was isolated in 70% yield (31 mg).
8.2.2. \((2R,2'R)-\alpha\text{-methyl-pantothenic acid}\) [(2R,2’R)-5b]:

The reaction mixture consisted of \(1b\) (26 mg, 10 mM), NH\(_4\)Cl (500 mM) and MgCl\(_2\) (10 mM) in 20 mL of Tris-HCl (100 mM, pH 9), and the pH of the reaction mixture was adjusted to pH 9 by addition of aqueous HCl or NaOH. Initially the first two enzymes MAL (0.01 mol%) and CrpG (0.7 mol%) were added into the reaction mixture. The reaction mixture was incubated at 25 °C and reaction progress was monitored by TLC and \(^1\)H NMR analysis. After complete conversion of \(1b\) (7 d), the third enzyme PS (0.07 mol%), \(4\) (20 mM), and ATP (30 mM) were added, and incubation of the reaction mixture was continued at 25 °C. After 24 h the crude product was purified by preparative HPLC as mentioned above (section 8.2.1). Product \((2R,2'R)-5b\) was isolated in 49% yield (23 mg).

8.2.3. \((2S,2'R)-\alpha\text{-methyl-pantothenic acid}\) [(2S,2’R)-5b]:

The reaction mixture consisted of \(1b\) (26 mg, 10 mM), PLP (1 mM), NH\(_4\)Cl (500 mM) and MgCl\(_2\) (10 mM) in 20 mL of Tris-HCl (100 mM, pH 9), and the pH of the reaction mixture was adjusted to pH 9 by addition of aqueous HCl or NaOH. The first two steps of the three-step cascade reaction were carried out as mentioned above (section 8.1.3). The reaction progress was monitored by TLC and \(^1\)H NMR analysis. After 2 d, maximum conversion of \(1b\) into the intermediate product \((S)-3b\) was obtained (75%). Then, the third enzyme PS (0.07 mol%), \(4\) (20 mM), and ATP (30 mM) were added, and incubation of the reaction mixture was continued at 25 °C. After 24 h, the crude product was purified by silica gel column chromatography (ethyl acetate and methanol; 9:1). Product \((2S,2'R)-5b\) was isolated in 46% yield (23 mg).

9. Spectral data

9.1. 3-Aminopropanoic acid \((3a)\): Conversion >99%; Yield 85%; White solid. \(^1\)H NMR (500 MHz, Deuterium Oxide): \(\delta\) 3.10 (t, \(J = 6.6\) Hz, 1H), 2.49 (t, \(J = 6.6\) Hz, 1H); HRMS: m/z calc. for C\(_3\)H\(_8\)NO\(_2\), 90.0477 [M+H]\(^+\), found: 90.0549.

9.2. \((R)-3\text{-amino-2-methylpropanoic acid}\) [(R)-3b]: Conversion >99%; Yield 78%; White solid. \(^1\)H NMR (500 MHz, Deuterium Oxide): \(\delta\) 3.08 (dd, \(J = 12.8, 8.5\) Hz, 1H), 3.01 (dd, \(J = 12.8, 5.3\) Hz, 1H), 2.64 – 2.53 (m, 1H), 1.18 (d, \(J = 7.3\) Hz, 3H); HRMS: m/z calc. for C\(_4\)H\(_{10}\)NO\(_2\), 104.0633 [M+H]\(^+\), found: 104.0708.

9.3. \((S)-3\text{-amino-2-methylpropanoic acid}\) [(S)-3b]: Conversion 75%; Yield 63%; Yellow solid. \(^1\)H NMR (500 MHz, Deuterium Oxide): \(\delta\) 3.08 (dd, \(J = 12.8, 8.5\) Hz, 1H), 3.00 (dd, \(J = 12.8, 5.3\) Hz, 1H), 2.65 – 2.52 (m, 1H), 1.17 (d, \(J = 7.3\) Hz, 3H); HRMS: m/z calc. for C\(_4\)H\(_{10}\)NO\(_2\), 104.0633 [M+H]\(^+\), found: 104.0708.
9.4. \((R)-\text{pantothenic acid}\) \((R)-5a\): Conversion >99%; Yield 70%; White solid. \(^1\)H NMR (500 MHz, Deuterium Oxide): \(\delta 3.97\) (s, 1H), 3.50 (d, \(J = 11.2\) Hz, 1H), 3.45 (t, \(J = 6.7\) Hz, 2H), 3.39 (d, \(J = 11.2\) Hz, 1H), 2.46 (t, \(J = 6.7\) Hz, 2H), 0.90 (d, \(J = 18.3\) Hz, 6H); \(^1\)C NMR (126 MHz, Deuterium Oxide): \(\delta 174.93, 75.74, 75.64, 68.32, 38.51, 35.87, 35.76, 20.46, 19.04\); HRMS: m/z calc. for \(\text{C}_9\text{H}_{18}\text{O}_5\text{N}\), 220.1107 [M+H]^+, found: 220.1180.

9.5. \((2R,2'R)-\alpha\text{-methyl-pantothenic acid}\) \((2R,2'R)-5b\): Conversion >99%; Yield 49%; White solid. \(^1\)H NMR (500 MHz, Deuterium Oxide): \(\delta 3.98\) (s, 1H), 3.51 (d, \(J = 11.2\) Hz, 1H), 3.39 (d, \(J = 11.2\) Hz, 1H), 3.30 (d, \(J = 7.2\) Hz, 2H), 2.53 (h, \(J = 7.0\) Hz, 1H), 1.10 (d, \(J = 7.1\) Hz, 3H), 0.90 (d, \(J = 19.6\) Hz, 6H); \(^1\)C NMR (126 MHz, Deuterium Oxide): \(\delta 183.68, 175.03, 75.70, 68.36, 42.45, 42.33, 38.50, 20.45, 19.07, 15.28\); HRMS: m/z calc. for \(\text{C}_{10}\text{H}_{20}\text{O}_5\text{N}\), 234.1263 [M+H]^+, found 234.1334.

9.6. \((2S,2'R)-\alpha\text{-methyl-pantothenic acid}\) \((2S,2'R)-5b\): Conversion 75%; Yield 46%; White solid. \(^1\)H NMR (500 MHz, Deuterium Oxide): \(\delta 3.99\) (s, 1H), 3.53 – 3.43 (m, 2H), 3.41 – 3.33 (m, 2H), 2.75 (h, \(J = 7.1\) Hz, 1H), 1.16 (d, \(J = 7.1\) Hz, 3H), 0.90 (d, \(J = 17.1\) Hz, 6H); \(^1\)C NMR (126 MHz, Deuterium Oxide): \(\delta 179.86, 175.18, 75.84, 68.45, 41.61, 39.71, 38.59, 20.48, 19.11, 14.33\); HRMS: m/z calc. for \(\text{C}_{10}\text{H}_{20}\text{O}_5\text{N}\), 234.1263 [M+H]^+, found 234.1337.
Chapter 6

10. HPLC analysis of isolated target compounds
10.1. Chiral HPLC analysis of \((R)-3\text{-amino-2-methylpropanoic acid} [(R)-3b]\) and \((S)-3\text{-amino-2-methylpropanoic acid} [(S)-3b]\).

Figure S16. (A) HPLC chromatogram of rac-3b; (B) HPLC chromatogram of enzymatically obtained \((R)\)-3b; (C) HPLC chromatogram of enzymatically obtained \((S)\)-3b. **HPLC conditions:** CROWNPAK® CR-(+) column (150 x 3 mm, Daicel); HClO₄ (pH = 1.0) / ACN (80:20); 0.2 mL/min flow rate; 20°C; 210 nm; Retention times: \((S)\)-3b = 8.1 min, \((R)\)-3b = 9.3 min.
10.2 HPLC analysis of diastereoisomers of α-methyl substituted pantothenic acid (5b)

Figure S17. (A) HPLC chromatogram of a diastereomeric mixture of 5b; (B) HPLC chromatogram of enzymatically obtained (2R,2'R)-5b; (C) HPLC chromatogram of enzymatically obtained (2S,2'R)-5b. HPLC conditions: Kinetex® 5µm EVO C18 column (100 Å; 150 x 4.6 mm; Phenomenex); KH₂PO₄ / MeOH (90:10); 0.4 mL/min; 30°C; 210 nm; Retention times: (2R,2'R)-5b = 6.8 min, (2S,2'R)-5b = 7.4 min.
11. References


