Video Article

Enzymatic Cascade Reactions for the Synthesis of Chiral Amino Alcohols from L-lysine

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Abstract

Amino alcohols are versatile compounds with a wide range of applications. For instance, they have been used as chiral scaffolds in organic synthesis. Their synthesis by conventional organic chemistry often requires tedious multi-step synthesis processes, with difficult control of the stereochemical outcome. We present a protocol to enzymatically synthetize amino alcohols starting from the readily available L-lysine in 48 h. This protocol combines two chemical reactions that are very difficult to conduct by conventional organic synthesis. In the first step, the regio- and diastereoselective oxidation of an unactivated C-H bond of the lysine side-chain is catalyzed by a dioxygenase; a second regio- and diastereoselective oxidation catalyzed by a regiodivergent dioxygenase can lead to the formation of the 1,2-diols. In the last step, the carboxylic group of the alpha amino acid is cleaved by a pyridoxal-phosphate (PLP) decarboxylase (DC). This decarboxylative step only affects the alpha carbon of the amino acid, retaining the hydroxy-substituted stereogenic center in a beta/gamma position. The resulting amino alcohols are therefore optically enriched. The protocol was successfully applied to the semipreparative-scale synthesis of four amino alcohols. Monitoring of the reactions was conducted by high performance liquid chromatography (HPLC) after derivatization by 1-fluoro-2,4-dinitrobenzene. Straightforward purification by solid-phase extraction (SPE) afforded the amino alcohols with excellent yields (93% to >95%).

Video Link

The video component of this article can be found at https://www.jove.com/video/56926/

Introduction

Despite the benefits offered by biocatalysis, the integration of biocatalytic steps in synthetic pathways or total biocatalytic routes remains mostly limited to enzymatic kinetic resolutions. These routes have been widely used as a first step in asymmetric chemo-enzymatic synthesis, but biocatalysis offers many more possibilities in functional group interconversions with high stereoselectivity[1,2,3]. Moreover, as biocatalytic reactions are conducted in similar conditions, it is therefore feasible to perform cascade reactions in a one-pot fashion[4,5].

Chiral amino alcohols are versatile molecules for use as auxiliaries or scaffolds in organic synthesis[6]. The amino alcohol moiety is frequently found in secondary metabolites and in active pharmaceutical ingredients (API). Primary β-amino alcohols are readily available from the corresponding α-amino acids by conventional chemical synthesis, but access to chiral γ-amino alcohols or secondary amino alcohols often requires tedious synthetic pathways together with sensitive control of the stereochemistry[7,8,9,10]. Due to its high stereoselectivity, biocatalysis may provide a superior synthetic route to these chiral building blocks[11,12,13,14].

We previously reported the synthesis of mono- and di-hydroxy-L-lysines by diastereoselective enzymatic hydroxylation catalyzed by dioxygenases of the iron(II)/α-ketoacid-dependent oxygenase family (αKAO) (**Figure 1**)[15]. In particular, starting from L-lysine, the KDO1 dioxygenase catalyzes the formation of the (3*S*)-hydroxy derivative (**1**), while the (4*R*)-derivative (**2**) is formed by the reaction with KDO2 dioxygenase. Successive regiodivergent hydroxylations by KDO1 and KDO2 lead to the formation of the (3*R*,4*R*)-dihydroxy-L-lysine (**3**) in optically pure form. However, the limited substrate range of these enzymes impedes their large utilization in chemical synthesis, especially in the hydroxylation of simple amines, as a carboxylic acid moiety in the α-position of the amino group is essential for activity[16].

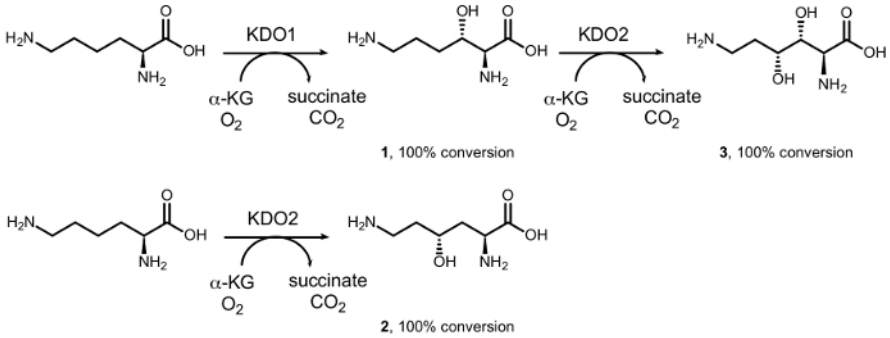

Figure 1: Biocatalytic conversions of L-lysine. Conversion into (3*S*)-hydroxy-L-lysine (**1**) catalyzed by KDO1 dioxygenase; (4*R*)-hydroxy-L-lysine (**2**) catalyzed by KDO2 dioxygenase; and (3*R*,4*R*)-dihydroxy-L-lysine (**3**) by cascade reaction catalyzed successively by KDO1 and KDO2 dioxygenases. Please click here to view a larger version of this figure.

Decarboxylation is a common reaction in metabolism[17]. In particular, amino acid DCs (EC 4.1.1) are cofactor-free (pyruvoyl-dependent) or PLP-dependent enzymes, and catalyze the decarboxylation of amino acids into the corresponding polyamines in bacteria and higher organisms[18,19,20,21,22]. The mono- and dihydroxy compounds (**Figure 3**) **4-7, 10-11** correspond to hydroxylated cadaverine, the diamine obtained by decarboxylation of L-lysine. Cadaverine is a key building block for the chemical industry, specifically it is a component of polyamide and polyurethane polymers. Therefore, bio-based production of this diamine from renewable resources has attracted attention as an alternative to the petroleum-based route, and various microorganisms have been engineered for this purpose. In these metabolic pathways, lysine DC (LDC) is the key enzyme. LDC is a PLP-dependent enzyme belonging to the alanine racemase (AR) structural family[23]. The PLP-dependent DCs (PLP-DCs) are known to be highly substrate-specific. However, a few enzymes own the capability of slight promiscuity, being active towards both L-ornithine and L-lysine amino acids, as for example the LDC from *Selenomonas rumirantium* (LDC_{Srum}), which has similar kinetic constants for lysine and ornithine decarboxylation[24,25]. This extended substrate specificity makes this enzyme a good candidate for the decarboxylation of mono- and di-hydroxy-L-lysine. In addition, to find DCs active towards the hydroxyl derivatives of lysine, we examined the genomic context of the genes encoding the αKAO enzymes. Indeed, in prokaryotic genomes the genes encoding enzymes involved in the same biosynthetic pathway are generally co-localized in gene clusters. The KDO2 (from *Chitinophaga pinensis*) gene was found co-localized with a gene encoding putative PLP-DC (**Figure 2**). In contrast, no gene encoding for DC has been found when analyzing the genomic context of the KDO1 dioxygenase. The PLP-DC protein from *C. pinensis* (DC_{Cpin}) was therefore selected as a promising candidate to catalyze the decarboxylation step of the cascade reaction.

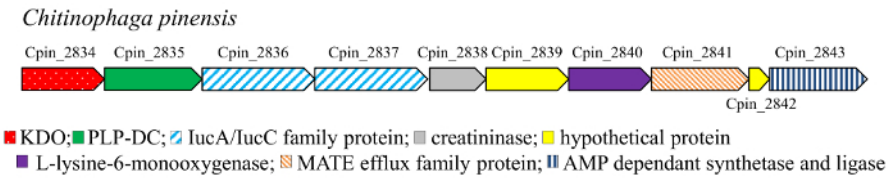

Figure 2: Genomic context of KDO2 gene in *C. pinensis*. Please click here to view a larger version of this figure.

Consequently, we designed enzymatic cascade reactions involving dioxygenases and DCs to achieve the synthesis of aliphatic chiral β- and γ-amino alcohols from amino acids (**Figure 3**). As previously reported, the C-H oxidation catalyzed by the αKAO introduces the hydroxy-substituted stereogenic center with total diastereoselectivity; the Cβ/γ chirality will be preserved in the decarboxylative step, which only affects the Cα carbon of the amino acid moiety[16].

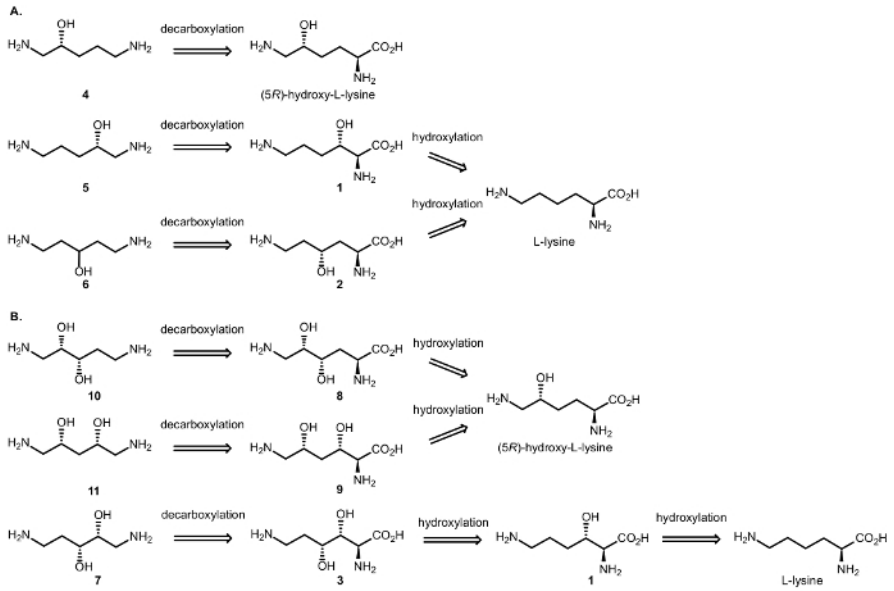

Figure 3: Retrosynthetic analysis. (**A**) Retrosynthesis of β- and γ-amino alcohols (*R*)-1,5- diaminopentan-2-ol (**4**) from (5*R*)-hydroxy-L-Lysine, and (*S*)-1,5-diaminopentan-2-ol (**5**) and 1,5-diaminopentan-3-ol (**6**) from L-lysine. (**B**) Retrosynthesis of β,γ- and β,δ-amino diols (2*S*,3*S*)-1,5-diaminopentane-2,3-diol (**10**) and (2*R*,4*S*)-1,5-diaminopentane-2,4-diol (**11**) starting from (5*R*)-hydroxy-L-lysine, and (2*R*,3*R*)-1,5-diaminopentane-2,3-diol (**7**) starting from L-lysine. Please click here to view a larger version of this figure.

Starting from L-lysine and its (5*R*)-hydroxy derivative, we herein report a two/three step, one pot, enzymatic procedure combining dioxygenases and PLP-DCs to obtain the target amino alcohols. Prior the synthesis at the laboratory scale of the target molecules, the method was developed at the analytical scale to adjust the reaction conditions, *e.g.*, the enzyme concentrations, required to allow full conversion of the starting materials; we present this procedure as well.

Protocol

1. Enzyme Preparation

1. Express and purify proteins as previously described[26].
   NOTE: Recombinant proteins were obtained with the following final concentrations: αKAO from *Catenulispora acidiphila*, UniProtKB ID: C7QJ42 (KDO1), 1.35 mg/mL; αKAO from *C. pinensis*, UniProtKB ID: C7PLM6 (KDO2), 2.29 mg/mL; PLP-DCs from *S. rumirantium*, UniProtKB ID: O50657 (LDC_{Srum}), cell free extract with total enzyme at 12.44 mg/mL; PLP-DC from *C. pinensis*, UniProtKB ID: C7PLM7 (DC_{Cpin}), 2.62 mg/mL.

2. Preparation of Solutions

NOTE: All the solutions below are prepared in deionized water.

1. Prepare 1 M of HEPES buffer, pH 7.5; adjust with 5 M of NaOH.
   NOTE: During the pH adjustment step, wear protective gloves, protective clothing, eye protection, and face protection (P280). In case of contact with eyes (P305, P351, P338), rinse cautiously with water for several min. Remove contact lenses if possible. Continue rinsing and immediately call a poison center or doctor (P310). The P code refers to the precautionary statement (hazard class and category; see, *e.g.*, pubchem.ncbi.nlm.nih.gov/ghs/).
2. Prepare 100 mM L-lysine, 100 mM (5*S*)-hydroxy-L-lysine, 150 mM α-ketoglutaric acid, 100 mM sodium ascorbate, 10 mM ammonium iron(II) sulfate hexahydrate (Mohr salt), 100 mM pyridoxal 5-phosphate (PLP), and 100 mM dithiothreitol (DTT).
3. Prepare 1 M, 2M, and 6M of hydrochloric acid (HCl) from a 37% HCl solution (approximatively 12 M solution), following the same safety instructions as those described in 2.1.
   NOTE: The solutions of α-ketoglutaric acid, sodium ascorbate, and ammonium iron(II) sulfate hexahydrate must be made fresh on the day of use. The lysine solution can be stored for several months at room temperature. The PLP solution can be stored for one month at 4 °C. The DTT solution can be stored for one month at -20 °C.

3. Analytical-scale Reactions

1. Method development for the decarboxylation reaction of (5*R*)-hydroxy-L-lysine
   1. Add 11 µL of 1 M solution of HEPES buffer pH 7.5 (final concentration 50 mM) to a 2 mL microtube. Then, add 22 µL of 100 mM (5*S*)-hydroxy-L-lysine (final concentration 10 mM), 2.2 µL of 100 mM PLP (final concentration 1 mM), and 2.2 µL of 100 mM DTT (final concentration 1 mM).

NOTE: In the case of reaction with LDC_{Srum}, the addition of DTT is not necessary.
2. Add the purified PLP-DC (final concentration 0.1 mg/mL). Complete with H_2O for a final volume of 220 µL.
3. Shake the reaction mixture at room temperature, with an open lid in open air, at 300 rpm for 3 h.
NOTE: Shaking the reaction mixture with an open lid in open air ensures a good oxygenation of the reaction media. It is not therefore necessary to bubble oxygen in the reaction media.
4. Collect 10 µL of the reaction mixture to be analyzed according to the reaction monitoring procedure described in step 6. The samples are preferably derivatized and analyzed directly after the sampling.

2. Method development for the enzymatic cascade reaction combining one hydroxylation step catalyzed by αKAO with decarboxylation catalyzed by PLP-DC
1. Add 11 µL of 1 M of HEPES buffer pH 7.5 (final concentration 50 mM) to a 2 mL microtube. Then, add 22 µL of 150 mM of α-ketoglutaric acid (final concentration 15 mM), 5.5 µL of 100 mM of sodium ascorbate (final concentration 2.5 mM), 22 µL of 10 mM of Mohr's salt (final concentration 1 mM), and 22 µL of 100 mM of L-lysine (final concentration 10 mM).
2. Complete with H_2O for a final volume of 220 µL, taking into account the volume of the enzyme solution to be added in step 3.2.3.
3. Add the required purified αKAO enzyme according to the targeted regioselectivity: KDO1 for hydroxylation in position C-3 or KDO2 for position C-4 of L-lysine. The final concentration of the purified enzyme (0.05-0.5 mg/mL) was determined to enable a complete conversion in approximatively 3 h.
4. Shake the reaction mixture at room temperature, with an open lid in open air, at 300 rpm for 3 h.
5. Add 2.2 µL of 100 mM of PLP (final concentration ~ 1 mM) and 2.2 µL of 100 mM of DTT (final concentration ~ 1 mM).
NOTE: In the case of reaction with LDC_{Srum}, the addition of DTT is not necessary.
6. Add the purified PLP-DC at a concentration enabling complete conversion in 18 h: LDC_{Srum} at an approximate final concentration of 0.1 mg/mL for the cascade reaction with KDO1, and DC_{Cpin} at an approximate final concentration of 0.5 mg/mL for the cascade reaction with KDO2.
7. Shake the reaction mixture at room temperature, with an open lid in open air at 300 rpm for 18 h.
8. Run a monitoring procedure as in step 3.1.4.

3. Method development for the enzymatic cascade reaction combining two hydroxylation steps catalyzed by αKAOs with decarboxylation catalyzed by PLP-DC
1. Run steps 3.2.1-3.2.2.
2. Add KDO1 at a final concentration of 0.05 mg/mL. Shake the reaction mixture at room temperature, with an open lid in open air, at 300 rpm for 3h.
3. Add KDO2 at an approximate final concentration of 0.5 mg/mL, calculated using the initial reaction volume. Shake the reaction mixture at room temperature, with an open lid in open air, at 300 rpm for 18 h.
4. Run step 3.2.5.
5. Add the purified PLP-DC DC_{Cpin} at an approximate final concentration of 0.5 mg/mL, calculated using the initial reaction volume. Shake the reaction mixture at room temperature, with an open lid in open air, at 300 rpm for 18 h.
6. Run the monitoring procedure as in step 3.1.4.

4. Semi-preparative One-pot Biocatalytic Reaction

NOTE: Enzymatic reactions are carried out on 0.1 mmol of L-lysine in an open-air 250 mL glass Erlenmeyer flask for a total volume of 10 mL.

1. Synthesis of the monohydroxylated derivatives
1. Add 0.5 mL of 1 M HEPES buffer, pH 7.5 (final concentration 50 mM) to the flask. Then, add 1 mL of 100 mM L-lysine (final concentration 10 mM), 1 mL of 150 mM α-ketoglutaric acid (final concentration 15 mM), 0.25 mL of 100 mM sodium ascorbate (final concentration 2.5 mM), and 1 mL of 10 mM Mohr's salt (final concentration 1 mM).
2. Complete with H_2O for a final volume of 10 mL, taking into account the volume of the enzyme solution to be added in step 4.1.3.
3. Add the required purified αKAO enzyme according to the targeted regioselectivity: KDO1 at a final concentration of 0.075 mg/mL for hydroxylation in position C-3 or KDO2 at a final concentration of 0.5 mg/mL for position C-4 of L-lysine. The final concentration of the purified enzyme was determined to enable a complete conversion in approximatively 3 h.
4. Shake the reaction mixture at room temperature at 300 rpm for the appropriate duration. When the reaction monitoring indicates a completed hydroxylation reaction (run all the steps detailed in step 6 for the reaction monitoring protocol), proceed to step 4.1.5. A typical reaction time is 3 h.
5. Add 100 µL of 100 mM PLP to the αKAO reaction mixture (final concentration ~ 1 mM). Then, add 100 µL of 100 mM DTT (final concentration ~ 1 mM), except when using LDC_{Srum}.
6. Add purified PLP-DC: LDC_{Srum} at an approximate final concentration of 0.05 mg/mL for the cascade reaction with KDO1 or DC_{Cpin} at an approximate final concentration of 0.5 mg/mL for the cascade reaction with KDO2.
7. Shake the reaction mixture at room temperature, 300 rpm, for 18 h and continue directly to the steps detailed in step 4.3.

2. Synthesis of the dihydroxylated derivative
1. Add 0.5 mL of 1 M HEPES buffer, pH 7.5 (final concentration 50 mM), 1 mL of 100 mM L-lysine (final concentration 10 mM), 1 mL of 150 mM α-ketoglutaric acid (final concentration 15 mM), 0.25 mL of 100 mM sodium ascorbate (final concentration 2.5 mM), and 1 mL of 10 mM Mohr's salt (final concentration 1 mM). Complete with H_2O for a final volume of 10 mL, taking into account the volume of the enzyme solution to be added in step 4.2.2.
2. Add KDO1 to a final concentration of 0.075 mg/mL. Shake the reaction mixture at room temperature, at 300 rpm for the appropriate duration.
3. When the reaction monitoring indicates a completed hydroxylation reaction (see step 6 for the reaction monitoring protocol), proceed to step 4.2.5. A typical reaction time is 3 h.

4. Add 1 mL of 150 mM α-ketoglutaric acid (final concentration 15 mM), 0.25 mL of 100 mM sodium ascorbate (final concentration 2.5 mM), and 1 mL of 10 mM Mohr's salt (final concentration 1 mM).
5. Add KDO2 to an approximate final concentration of 0.5 mg/mL, calculated using the initial reaction volume. Shake the reaction mixture at room temperature at 300 rpm for 18 h.
6. When the reaction monitoring indicates a completed dihydroxylation reaction (see step 6 for the reaction monitoring protocol), proceed to step 4.2.7. A typical reaction time is 18 h.
7. Add 100 µL of 100 mM of DTT (final concentration ~ 1 mM), except when using the LDC_{Srum}.
8. Add the purified DC_{Cpin} at an approximate final concentration of 0.5 mg/mL, calculated using the initial reaction volume.
9. Shake the reaction mixture at room temperature at 300 rpm for 18 h.

3. Quenching
    1. Cool down the reaction mixture by placing the 250 mL glass Erlenmeyer flask in an ice bath.
    2. Add carefully, over approximatively 1 min, 0.25 mL of 6 M HCl by manually gently shaking the cooled reaction mixture. Follow the same safety instructions as those described in step 2.1.
    3. Transfer the acidic mixture into a 50 mL conical-bottom centrifuge tube using a glass Pasteur pipette equipped with a bulb, then centrifuge at 1,680 x g and 4 °C for 15 min.
    4. Withdraw the supernatant and keep aside in a 250 mL round-bottom flask.
    5. Add 10 mL of deionized water to the conical-bottom centrifuge tube containing the pellet. Vortex to re-suspend the pellet.
    6. Centrifuge at 1,680 x g, 4 °C, for 15 min.
    7. Withdraw the supernatant and add it to the 250 mL round-bottom flask containing the first supernatant (step 4.3.4).
    8. Freeze the collected supernatants by immersion of the flask into liquid nitrogen with constant hand swirling, and transfer the flask immediately to a benchtop manifold freeze-dryer to prevent the material from thawing.
    9. After the freeze-drying process is complete (overnight), remove the flask from the freeze-dryer.

5. Purification of Amino Alcohols from Crude Enzymatic Reaction Mixture

1. Ion exchange resin
    1. Dissolve the freeze-dried product in the minimum amount of aqueous 0.1 M of HCl (approximatively 4 mL) until solid particles are no longer visible to the naked-eye.
    2. Condition a sulfonic acid functional group cation exchange resin, 200-400 mesh, in a glass column (20 x 250 mm) by eluting at atmospheric pressure 1M HCl (4 column volumes) followed by 0.1 M HCl (4 column volumes; column volume = 10 mL).
    3. Load the sample carefully at the top of the resin on the walls of the glass column using a 1,000 µL micropipette. Then, rinse the conical-bottom centrifuge tube that contained the crude product three times with 1 mL 0.1 M of HCl aqueous solution per wash, and load the resulting rinse volumes onto the column in the same way.
    4. Elute with a non-linear gradient: 4 column volumes of 0.1 M HCl, 4 column volumes of water, 4 column volumes of 5% NH_4OH, 4 column volumes of 10% NH_4OH, 4 column volumes of 15% NH_4OH, 4 column volumes of 20% NH_4OH, 4 column volumes of 25% NH_4OH and lastly 4 column volumes of 28% NH_4OH.
    5. Monitor the purification by HPLC (see step 6).
    6. Pool the fractions containing the compound (NH_4OH 20-28%) and freeze-dry as described in steps 4.3.8-4.3.9.
    NOTE: The ion exchange column can be reused after elution with deionized water to neutralization of elution water (approximatively 50 mL) followed by reconditioning by eluting with 50 mL of 1 M of HCl.

2. SPE
    1. Solubilize the freeze-dried compound in approximatively 2 mL of H_3PO_4/water (20 µL/mL).
    2. Place a mixed mode cation-exchange SPE 6 mL-cartridge on an extraction manifold connected to a water pump. Condition the cartridge by drawing through 4 mL of methanol under reduced pressure provided by the manifold. Equilibrate the cartridge by drawing through 4 mL of H_3PO_4/water (20 µL/mL).
    3. Load the sample onto the cartridge using a 1,000 µL micropipette at the top of the resin on the walls of the glass column, and rinse the vial containing the product three times with 0.75 mL of H_3PO_4/water (20 µL/mL) per wash. Load the resulting rinse volume onto the column in the same way.
    4. Wash the cartridge by eluting under reduced pressure, provided by the manifold, 4 mL of 2% HCOOH in water, then with 4 mL of water. Elute with a gradient of NH_4OH in water (4 mL for each elution, starting from 4% NH_4OH: 10%, 15%, 15%, 20%, and 28%).
    5. Monitor the purification by HPLC by analyzing each fraction according to the reaction monitoring protocol described in step 6. Keep all the fractions containing pure desired derivative according to ultraviolet (UV) chromatogram and proceed to step 5.2.6.
    6. Pool the fractions containing the desired compound into a 100 mL round bottom flask, rinse the tubes containing the fractions with water, and add the rinse volume to the 100 mL round bottom flask.
    7. Remove the solvent under reduced pressure using a rotary evaporator equipped with a thermostat water bath kettle at 40 °C and connected to a vacuum pump set at 10 mbar.
    8. Solubilize the solid in a minimum volume of water and transfer the solution into a weighed 25 mL round bottom flask. Rinse the flask with a minimum volume of water, add the rinse volume to the 25 mL flask, and proceed to step 5.2.9.
    9. Weigh the purified product and calculate the yield.
    10. Dissolve the purified product in the minimum volume of 2M of HCl until solid particles are no longer visible to the naked-eye and freeze-dry as described in steps 4.3.8-4.3.9.
    NOTE: The amino alcohols are sensitive compounds and are kept as their hydrochloride salts.

6. Reaction Monitoring and Product Analysis

1. Derivatization of substrates and products prior to HPLC analysis
   NOTE: The substrates and products need to be derivatized as aromatic derivatives to increase their UV chromatographic detectivity.We used 1-fluoro-2,4-dinitrobenzene (DNFB). Each sample was injected into the HPLC immediately after derivatization.
   1. Take 10 µL of the enzymatic reaction and transfer to a 1.5 mL microtube. Add 10 µL of 0.25 M of $NaHCO_3$ aqueous solution, 100 µL of ethanol, and 30 µL of 2.5 mg/mL of DNFB solution in ethanol.
   2. Close the microtube and shake the resulting solution for 1 h at 65 °C, at 1,000 rpm. Quench with 10 µL 1 M of HCl.
   3. Filter over a 4 mm diameter non-sterile syringe filter with a 0.22 µm pore size hydrophilic polyvinylidene fluoride (PVDF) membrane using a 1 mL Luer syringe.

2. Perform monitoring of the dioxygenase reaction using any system capable of separating derivatized amino acids. This protocol involves UV detection of amino acids derivatized by the dinitrobenzene (DNB) chromophore group[27].
   1. Fit the HPLC with C18 with a trimethyl silyl endcapping reversed phase column (5 µm, 3 x 150 mm).
   2. Equilibrate the C18 column with (30:70) eluent B (MeCN + 0.1% TFA) to eluent A (H_2O + 0.1% TFA) at a flow rate of 1 mL/min and 35 °C for 15 min (corresponding to approximatively 20 column volumes).
   3. Inject 10 µL of the derivatized reaction mixture (see step 6.1) onto the HPLC C18 column. Use a mixture of MeCN, 0.1% TFA/H_2O and 0.1% TFA as eluent with a linear gradient (ratio 30:70 to 70:30 in 9 min, temperature 35 °C, flow 1 mL/min).
   4. Use UV detection set at 400 nm to analyze the products eluted on the chromatographic column. DNB-lysine typically elutes around 6.5 min, hydroxylated DNB-lysine around 5.5 min, dihydroxylated DNB-lysine around 4.2 min, and DNB-aminodiol around 5.3 min.

7. NMR Analysis of Purified Amino Alcohols

1. Dissolve purified amino alcohols in D_2O (700 µL) and transfer the solution to a Nuclear Magnetic Resonance (NMR) tube.
2. Acquire ^{1}H and ^{13}C NMR spectra according to appropriate NMR facility protocols[28].

Representative Results

We have previously reported the synthesis of mono- and di-hydroxy-L-lysines by diastereoselective enzymatic hydroxylation catalyzed by dioxygenases of the iron(II)/αKAO family (**Figure 1**)[16]. To optimize the protocol of the entire cascades presented here, which combine one or two hydroxylation steps catalyzed by an αKAO followed by a decarboxylation step catalyzed by a PLP-DC, the reaction conditions were adjusted to satisfy the requirements of both enzymatic reactions. We started by investigating the activities of the two DCs, LDC_{Srum} and DC_{Cpin}, towards the commercially available (5*R*)-hydroxy-L-lysine. Then we assayed the DC activities towards the mono derivatives, 3-hydroxy-L-lysine (**1**) and 4-hydroxy-L-lysine (**2**), in cascade with the oxidation step catalyzed by the appropriate αKAO. **Table 1** presents the results of the biocatalytic decarboxylations of the mono-hydroxy-L-lysines. Conversions were measured by HPLC after derivatization of the reaction mixture with DNFB to give the corresponding DNB derivatized substrates and products.

Entry	Substrate	PLP-DC	Product	Conversion
1	**(5*R*)-hydroxy-L-lysine**	LDC_{Srum} [b)]	**4**	100%
2	**(5*R*)-hydroxy-L-lysine**	DC_{Cpin} [b)]	**4**	n.d.
3	**1** [a)]	LDC_{Srum} [b)]	**5**	100%
4	**1** [a)]	DC_{Cpin} [b)]	**5**	29%
5	**2** [a)]	LDC_{Srum} [c)]	**6**	60%
6	**2** [a)]	DC_{Cpin} [c)]	**6**	100%

Table 1: Biocatalytic decarboxylation of monohydroxy-L-lysines. Reaction conditions: substrate (10 mM), PLP-DC (0.1 to 0.15 mg mL^{-1}), HEPES buffer (50 mM, pH 7.5), PLP (1 mM), DTT (1 mM; not used with LDC_{Srum}), overnight, RT, 300 rpm. (**a**) Synthesized enzymatically *in situ*. (**b**) 0.1 mg/mL. (**c**) 0.15 mg/mL; n.d.: not detected

The DC from *S. rumirantium* (LDC_{Srum}) exhibited activity towards all the mono hydroxy lysines with best conversion of 3- and 5- derivatives to the corresponding chiral hydroxy diamines (entries 1, 3, and 5). As expected, DC_{Cpin}, the DC of the αKAO genomic context, turned out to be the most suitable for the decarboxylation of (4*R*)-hydroxy-L-lysine (**2**) (entry 6). Under standard reaction conditions the conversion of (3*S*)-hydroxy-L-lysine (**1**) into its decarboxylated counterpart (**5**) with DC_{Cpin} was low (entry 4), and no activity was observed towards (5*R*)-hydroxy-L-lysine (entry 2).

Lastly, we examined the activities of the two DCs towards the di-hydroxy-L-lysine derivatives **3**, **8**, and **9**, synthesized *in situ* by one or two hydroxylation steps catalyzed by KDO1, KDO2, or a combination of the two (**Figure 2**). The results of the biocatalytic decarboxylation of the di-hydroxy-L-lysines are summarized in **Table 2**.

Entry	Substrate	PLP-DC	Product	Conversion
1	**3**	LDC_{Srum} [b)]	**7**	n.d.
2	**3**	DC_{Cpin} [b)]	**7**	100%
3	**8** [a)]	LDC_{Srum} [b)]	**10**	19%
4	**8** [a)]	DC_{Cpin} [b)]	**10**	12%
5	**9** [a)]	LDC_{Srum} [c)]	**11**	n.d.
6	**9** [a)]	DC_{Cpin} [c)]	**11**	n.d.

Table 2: Biocatalytic decarboxylation of dihydroxy-L-lysines. Reaction conditions: substrate (10 mM), PLP-DC (0.1 to 0.15 mg/mL), HEPES buffer (50 mM, pH 7.5), PLP (1 mM), DTT (1 mM; not used with LDC_{Srum}), overnight, RT, 300 rpm. (**a**) Synthesized enzymatically *in situ*. (**b**) 0.1 mg/mL. (**c**) 0.15 mg/mL; n.d.: not detected.

Regarding the decarboxylation of the dihydroxy-L-lysines **3**, **8**, and **9**, the results were not fully satisfactory. In the standard reaction conditions, only (3*R*,4*R*)-dihydroxy-L-lysine (**3**) was quantitatively converted into the corresponding dihydroxy diamine **7** (entries 1-2). The conversion of 4,5-dihydroxy-L-lysine (**8**) was moderate (entries 3-4) but it is worth noting that it could be improved by greatly increasing the enzyme loading. None of the two tested PLP-DCs were found active towards 3,5-dihydroxy-L-lysine (**9**) (entries 5-6).

The enzymatic cascade reactions exhibiting quantitative conversion as determined by HPLC monitoring were successfully scaled up (**Figure 4**).

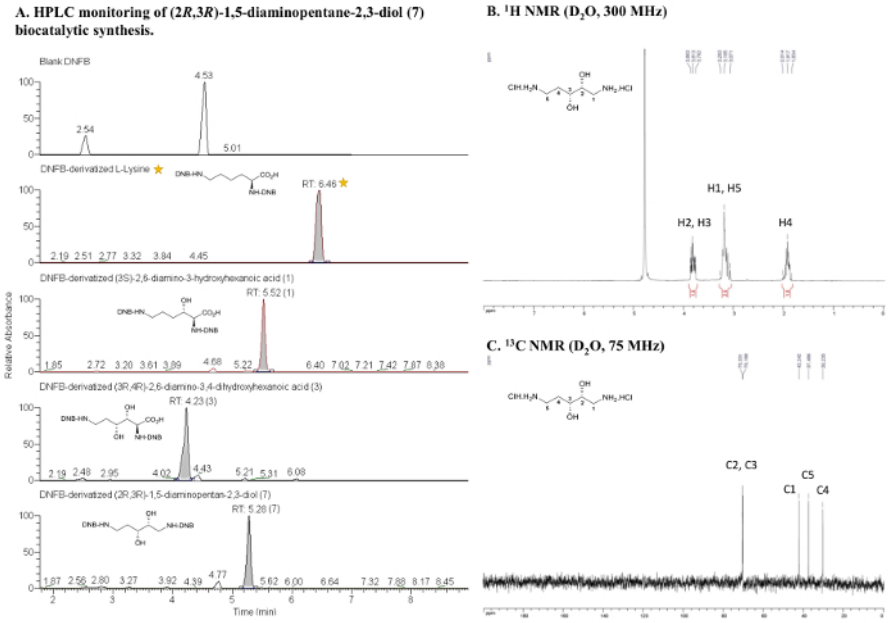

Figure 4: Representative analytical data for the three-step enzymatic cascade. (**A**) HPLC monitoring of biocatalytic conversion of L-lysine into (2*R*,3*R*)-1,5-diaminopentan-2,3-diol (**7**). RT = retention time. (**B**) ^{1}H NMR and (**C**) ^{13}C NMR of amino alcohol (**7**) after purification. Please click here to view a larger version of this figure.

The purification protocol presented herein allows efficient extraction of the amino alcohols from the complex enzymatic reaction mixture. The amino alcohols **4**, **5**, **6**, and **7** were obtained in excellent yields (**Figure 5**).

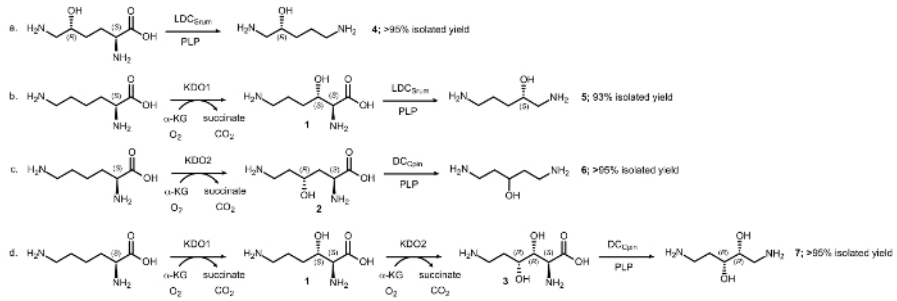

Figure 5: Enzymatic cascades for the synthesis of amino alcohols. Synthesis of (*R*)-1,5- diaminopentan-2-ol (**4**), (*S*)-1,5-diaminopentan-2-ol (**5**), 1,5-diaminopentan-3-ol (**6**), and (2*R*,3*R*)-1,5-diaminopentane-2,3-diol (**7**). Please click here to view a larger version of this figure.

Discussion

Chiral amino alcohols and derivatives have a wide range of applications, from chiral auxiliaries for organic synthesis to pharmaceutical therapy. Multistep synthesis for producing amino alcohols by conventional organic synthesis are numerous, but may not always be efficient because of tedious protection/deprotection steps together with a sensitive control of the stereochemistry[16]. A biocatalytic approach that dispenses with the protection/deprotection steps and is usually highly stereoselective represents a good alternative.

Previous work reported the synthesis of various β-amino alcohols with a 2-phenylethan-1-amine backbone by dynamic kinetic asymmetric transformation (DYKAT). In this route, the hydroxy-substituted stereogenic center was introduced by aldolisation on benzaldehyde and derivatives, catalyzed by a threonine aldolase. The low diastereoselectivity and the moderate yield of this first step were overcome by the subsequent stereospecific decarboxylation catalyzed by L-tyrosine DC, leading to enantioenriched aromatic β-amino alcohols[11,12].

We reported herein a straightforward protocol for the synthesis of various amino alcohols starting from the readily available L-lysine. Although very efficient, this protocol suffers from drawbacks due to limited substrate ranges of the αKAO and PLP-DC enzymes. Nevertheless, biocatalytic synthesis of various amino alcohols can be considered by using different combinations of other αKAO and amino acid DCs[29,30,31,32]. It is worth noting that the two-step order is critical in the cascade as the PLP-DCs are also active towards the L-lysine. Care must be taken to ensure that all the L-lysine is consumed in the first oxidative step before running the decarboxylation reaction catalyzed by the PLP-DC. This is ensured through a careful monitoring of the reaction by HPLC after derivatization of the reaction media by a chromophore agent. The low tolerance of the enzyme to high substrate concentration is a limitation for further development. To address this issue, enzyme evolution strategies might be used for optimizing the enzyme properties, such as substrate concentration tolerance[33]. Also, enzyme immobilization can be considered to ensure reuse of the enzyme.

This protocol is easy to carry out, and one of its most attractive features is the efficiency of the purification steps. In our preceding work, the direct extraction of polar molecules from the complex enzymatic reaction mixture was an issue, and derivatization of the compounds by hydrophobic groups was necessary[16]. The purification of the amino alcohols directly from the reaction without derivatization required extensive work. In this protocol, the two-step purification procedure combining ion exchange resin and solid phase extraction removes glycerol (contained in the enzyme solution) and HEPES buffer, two polar molecules with physical-chemical properties close to the ones of the targeted amino alcohols and therefore difficult to separate from these compounds. This purification protocol can be adapted for the extraction of various polar molecules from complex reaction mixtures such as those from enzyme reactions.

Disclosures

The authors have nothing to disclose.

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