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Enantioselective biocatalytic formal α-amination of hexanoic acid to L-norleucine†

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A three-step one-pot biocatalytic cascade was designed for the enantioselective formal α-amination of hexanoic acid to L-norleucine. Regioselective hydroxylation by $P450_{CLA}$ peroxygenase to 2-hydroxyhexanoic acid was followed by oxidation to the ketoacid by two stereocomplementary dehydrogenases. Combination with final stereoselective reductive amination by amino acid dehydrogenase furnished L-norleucine in >97% ee.

Upgrading of bio-based chemicals using biological methods presents the advantage of combining the use of natural resources as starting material and nature-derived catalysts for performing the reaction(s) of choice. This can translate into a major asset for commercialization of corresponding end-products, which do not suffer from a strong chemical footprint and in turn benefit from a better acceptance from end-consumers attentive to global change. In order to render such bio-synthetic schemes efficient and environmentally sound, a few strategies may be implemented that should result in the maximization of reaction yield and minimization of reagents and waste. In this regard, (artificial) linear cascade reactions appear ideal[1] as the combination of several biocatalytic steps in sequence without the need for intermediate purification steps significantly alleviates consumption of resources (including solvents) and reduces operating time.[2]

Saturated fatty acids are receiving increasing consideration as a source of raw materials, which upon targeted functionalization, grant access to a broad variety of synthons.[3] We recently developed an atom-efficient biocatalytic oxidative cascade for the transformation of fatty acids to corresponding α-ketoacids *via* internal H_2O_2 recycling. The two-step one-pot cascade relies on catalytic amounts of hydrogen peroxide, air (O_2) and three enzymes for full conversion to final products, liberating water as the only by-product.[4] The handle provided by the oxo-functionality renders these compounds ideal for subsequent transformation to *e.g.*, amino acids, aldehydes, amines, or alkanes.[5] Non-canonical amino acids (ncAAs) in particular may be ideally obtained through amination of 2-oxo-acids.

Inspired by pioneering work from Wandrey *et al.* on the development of hydrogen borrowing bioprocesses to access amino acids from corresponding α-hydroxy acids,[6] and following up our recent work on fatty acid oxyfunctionalization,[4] we envisaged a biocatalytic cascade for the formal enantioselective α-amination of hexanoic acid (**1a**) to ncAA L-norleucine (**4a**, Scheme 1). The concept of hydrogen borrowing by Wandrey and Kula was initially applied to amination of DL-lactate to L-alanine through formation of pyruvate as the intermediate, using two stereocomplementary lactate dehydrogenases, in combination with alanine dehydrogenase for final reductive amination.[6] The protocol was then adapted to L-leucine production by employing L- and D-hydroxyisocaproate dehydrogenases (Hic-DHs; L-Hic-DH from *Lactobacillus confusus* and D-Hic-DH from *Lactobacillus casei*) along with L-leucine dehydrogenase (L-Leu-DH from *Bacillus cereus*).[7] A similar design was also applied to *e.g.*, mandelic acid amination.[8] Complementary approaches for enzymatic asymmetric synthesis of ncAAs are numerous.[9,10]

Enantioselective formal amination of saturated fatty acids to enantiopure α-amino acids in an enzymatic cascade may proceed through (i) regioselective α-hydroxylation to 2-hydroxy acids catalyzed by P450 peroxygenase,[11] followed by (ii) dehydrogenation catalyzed by Hic-DHs to 2-oxo-acids and (iii) final stereoselective reductive amination by L-Leu-DH (Scheme 1).[12] Since the last two steps share complementary redox states of the nicotinamide cofactor,[7] we opted for the H_2O_2-driven hydroxylation catalyzed by P450 enzyme (*via* the so-called peroxide shunt[13]) in the first step. The reaction can also be driven by O_2 in conjunction with NADH and a suitable electron

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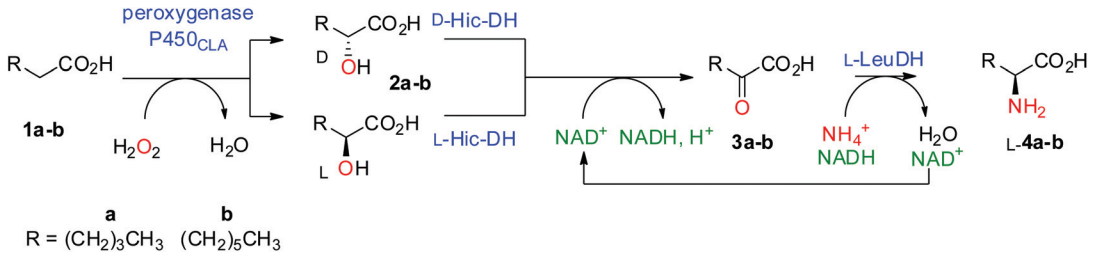


Scheme 1 Linear biocatalytic cascade for the enantioselective formal α-amination of fatty acids to amino acids. $P450_{CLA}$: P450 peroxygenase from *Clostridium acetobutylicum*; Hic-DH: 2-hydroxyisocaproate dehydrogenase from *Lactobacillus confusus* (L-Hic-DH) and *Lactobacillus casei* (D-Hic-DH); L-Leu-DH: leucine dehydrogenase from *Bacillus cereus*.

transport chain.[14] However, the peroxide shunt offers the advantage of simplified reaction design by not impacting the redox neutrality of the two combined last steps.‡

Since P450 from *Clostridium acetobutylicum* ($P450_{CLA}$) efficiently functions as peroxygenase, it was selected as the catalyst for the first step and could be obtained through recombinant expression in *E. coli* in a soluble pure form from high yielding preparation.[4] In contrast to the previously developed internal H_2O_2-regenerating system,[4] which allowed the use of catalytic amounts of hydrogen peroxide in a closed loop, the oxidant should be provided in this isolated hydroxylation step in (at least) stoichiometric amounts. Concomitant heme-dependent enzyme inactivation at high peroxide concentration[11a] can be practically avoided by fed-batch supplementation of H_2O_2. To identify a suitable amount of peroxide for the stepwise addition, a preliminary test was conducted; one portion (0.8 mM or 1.6 mM) was added to the buffer solution containing the substrate (10 mM of **1a–b**) and catalyst, and conversion of saturated fatty acids to corresponding hydroxy acids **2a–b** was monitored (Table 1). Data indicated that percentage yield is reduced at higher peroxide concentration (compare 0.8 mM to 1.6 mM, Table 1). The reaction appears to proceed quickly, with 87% of maximum product formation observed with **1a** after 5 min reaction time (entry 1, Table 1). Stepwise addition of 1.6 mM every 60 min was chosen to maximize product concentration. Long-term pulsing (12 h) in a concentration range usually troublesome for many heme-dependent proteins[15] was generally well-tolerated by $P450_{CLA}$ (Table 2). Full conversion of fatty acids (**1a–b**) could be obtained (10 mM) using max. 2 mol. equivalents of H_2O_2 (Table 2), forming corresponding 2-hydroxy acids **2a–b** with high regioselectivity[4] (>95%). The moderate coupling efficiency of ~50% ($mol_{product}$ $mol_{peroxide}^{-1}$) justifies the excess of supplied peroxide and is likely connected to depletion of H_2O_2 through spontaneous disproportionation.[16] Under such reaction design, fed-batch supplementation proved already superior to batch addition of the oxidant, which could not afford full conversion at 10 mM substrate.[4] Notably, a TON of ~3300 could be reached in the H_2O_2-driven hydroxylation of **1b** by $P450_{CLA}$ (entry 2, Table 2).

Table 2 α-Hydroxylation of fatty acids **1a–b** by $P450_{CLA}$ [a]

Entry	**1a–b** (10 mM)	Conv. (%)	[**2a–b**][b] (mM)	TON
1	**1a**	98	9.8	3267
2	**1b**	>99	>9.9	>3300

[a] Reactions performed in Tris-HCl (pH 7.5, 100 mM) using 3 μM $P450_{CLA}$ and 2.5–5% (v/v) EtOH (co-solvent) at RT and 170 rpm shaking in closed glass vials. 1.6 mM H_2O_2 was supplemented every hour (total 19.2 mM). [b] No β-hydroxylation product detected.

Since regioselective α-hydroxylation by $P450_{CLA}$ is poorly enantioselective (max. 36% ee on **1b** [4]), two stereocomplementary enzymes are necessary to convert first intermediates **2a–b** to 2-oxo-acids **3a–b**. 2-Hydroxyisocaproate dehydrogenases from *Lactobacillus confusus* (L-Hic-DH) and *Lactobacillus casei* (D-Hic-DH) were selected and characterized in the oxidative reaction.[17] Both compounds (analytical standards of *rac*-**2a–b**) were successfully oxidized by the two enzymes using substrate concentrations of 1 and 10 mM (Fig. S2, ESI†).

Table 1 Characterization of $P450_{CLA}$ in fatty acid (**1a–b**) hydroxylation upon one-time addition of sub-stoichiometric quantities of H_2O_2 [a]

Entry	**1a–b** (10 mM)	Time (min)	[H_2O_2] (mM)	[**2a–b**] (mM)	Percentage yield[b] (%)	TON[c]	Specific activity[d] (U mg^{-1})
1	**1a**	5	0.8	0.69 ± 0.06	87	695	2.9
2	**1a**	15	0.8	0.71 ± 0.03	88	706	1.0
2	**1a**	30	1.6	0.88 ± 0.07	55	880	0.6
4	**1b**	5	0.8	0.49 ± 0.02	61	488	2.0
5	**1b**	15	0.8	0.51 ± 0.01	63	506	0.7
6	**1b**	30	1.6	0.94 ± 0.05	59	938	0.7

[a] Reactions performed in triplicate in Tris-HCl (pH 7.5, 100 mM) using 1 μM $P450_{CLA}$ and 5% (v/v) EtOH (co-solvent) at RT and 170 rpm shaking in closed glass vials. [b] $mol_{product}$ $mol_{peroxide}^{-1}$ (theoretical yield controlled by peroxide as limiting reagent). [c] Turnover number (at 0.8 and 1.6 mM H_2O_2 respectively, with 1 μM P450, TON_{max} = 800 and 1600, respectively). [d] MW_{P450} = 48 kDa, values only relevant at the initial rate.

Table 3 Characterization of L- and D-Hic-DHs in dehydrogenation of *rac*-2-hydroxyacids **2a–b** [a]

Entry	*rac*-**2a–b**	Concentration (mM)	Enzyme	Specific activity (U mg^{-1})
1	*rac*-**2a**	1	L-Hic-DH	1.5
2	*rac*-**2a**	10	L-Hic-DH	5.1
3	*rac*-**2b**	1	L-Hic-DH	1.3
4	*rac*-**2b**	10	L-Hic-DH	4.4
5	*rac*-**2a**	1	D-Hic-DH	2.7
6	*rac*-**2a**	10	D-Hic-DH	3.7
7	*rac*-**2b**	1	D-Hic-DH	3.5
8	*rac*-**2b**	10	D-Hic-DH	6.4

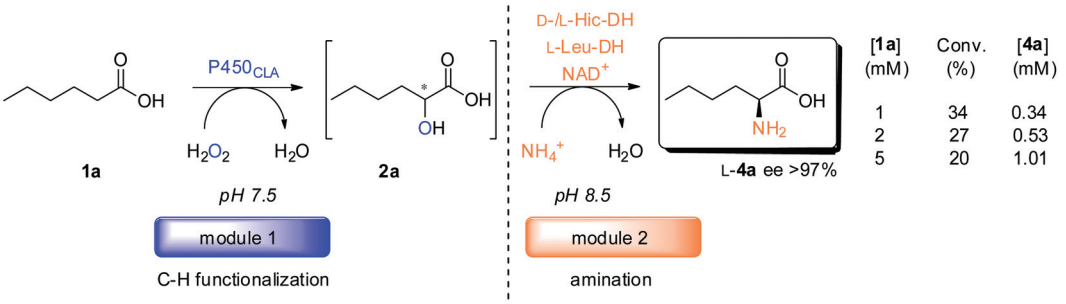
[a] Reactions performed in Tris-HCl (pH 8.5, 100 mM) using 0.01 mg mL^{-1} each of L- or D-Hic-DH (freeze-dried whole cells), 10 mM NAD$^+$, 5% (v/v) EtOH (co-solvent) and 1 or 10 mM **2a–b**; activity is determined by monitoring the increase in absorbance at 340 nm (EtOH is not oxidized by Hic-DHs).

Overall, both Hic-DHs[7] displayed good dehydrogenase activity at 10 mM (up to 5.1 U mg^{-1} on **2a** with L-Hic-DH and 6.4 U mg^{-1} on **2b** with D-Hic-DH, entries 2 and 8, respectively, Table 3), whereas lower substrate concentration decreased overall activity.

The basic pH required by Hic-DHs for oxidation of α-hydroxy acids necessitates a pH adjustment after completion of the hydroxylation step (**1** → **2**). This was performed by adding 5 M NaOH in 1 μL increments until the desired pH was reached. For the reductive amination step, L-Leu-DH was chosen, as it displays good activity on aliphatic substrates.[9] Activity of recombinant L-Leu-DH on L-norleucine (**4a**) was confirmed at pH 8.5 in the deamination direction (14.5 U mL^{-1} of activity determined for cell-free lysate, Fig. S3, ESI†). In addition, compatibility of the last two steps of the cascade (oxidation and amination) was tested (Fig. S4, ESI†), indicating that conversion of *rac*-**2a** to **4a** proceeded smoothly at 100 mM ammonium salt. Finally, a sequential one-pot protocol can be envisaged. The cascade to final amino acid products was designed in two modules occurring in the same vessel: after estimated completion of the hydroxylation step (module 1, Table 2) and addition of catalase (1200 U mL^{-1}), the pH was set to 8.5 (see above) and all components required for dehydrogenation (both Hic-DHs and NAD$^+$) and reductive amination (L-Leu-DH and ammonium salt) steps (module 2) were added at once. The reaction was investigated on **1a–b** and detection of amino acid products was performed by ninhydrin staining/thin layer chromatography. A positive response could be only observed in the reaction with **1a**, and formation of norleucine (**4a**) was confirmed with various control reactions and GC-MS analysis (Fig. S5, ESI†).

We finally focused on the conversion of hexanoic acid (**1a**) for quantification of final product **4a**, aiming at demonstrating the feasibility of a linear one-pot cascade toward L-norleucine (Scheme 2). Various substrate concentrations were tested (1–5 mM). Products were derivatized and analyzed by GC and chiral HPLC (Fig. S6–S8, ESI†). The amination reaction was confirmed to take place with high enantioselectivity, as formation of L-norleucine by L-Leu-DH in perfect enantiopurity (ee > 97%) could be confirmed (Fig. S8, ESI†). Up to 34% conversion to the amino acid product was reached at lower substrate concentration, and 1 mM **4a** could be formed starting from 5 mM **1a** (Scheme 2).

In the present design, despite theoretical hydride shuffling between **2a** and **4a** through NAD$^+$ (Scheme 1), the nicotinamide cofactor was added in stoichiometric amounts, as the reversibility of the reaction catalyzed by Hic-DHs otherwise favors formation of the hydroxy acid product.[18] Nevertheless, compared to other linear biocatalytic cascades to non-natural amino acids,[10c] the sequence hydroxylation-dehydrogenation-reductive amination allows access to enantiopure amino acids through a simplified reaction design, with initiation of the reaction by hydrogen peroxide, in the absence of a cofactor recycling system otherwise needed in other cascades, such as the transformation of substituted benzenes to tyrosine derivatives.[19] In comparison, oxy- and amino-functionalization of styrene derivatives in a modular cascade remains a complementary example of linear biocatalytic transformation, which elegantly relies on external glucose and ammonium salt for access to non-natural amino acids.[20] The recently reported combination of P450, ADH and amine dehydrogenase for amination of alkanes highlights the current interest in such biotransformations.[21]



Scheme 2 Conversion of **1a** to L-**4a** in a three-step one-pot cascade: the reaction proceeds in Tris-HCl buffer (pH 7.5, 100 mM) containing 3 μM P450$_{CLA}$, 5% (v/v) EtOH (co-solvent), and 0.8 mM H_2O_2 (from 320 mM stock) supplemented every 30 min (total 19.2 mM, module 1). After completion of the first step, catalase (1200 U mL^{-1}) is added and the pH is adjusted to 8.5, followed by addition of 0.07 U mL^{-1} Leu-DH (cell-free lysate), 0.01 mg mL^{-1} L- and D-Hic-DHs (freeze-dried whole cells), 50 mM $(NH_4)_2SO_4$ and 10 mM NAD$^+$ (module 2).

Conclusions

In this work, we could demonstrate as proof-of-principle the enantioselective biocatalytic formal α-amination of hexanoic acid to L-norleucine, which proceeds without intermediate purification in a linear one-pot three-step sequential cascade, with pH adjustment after the first step. The key step was the regioselective oxy-functionalization of non-activated C–H bonds by P450 monooxygenase, followed by dehydrogenation to the ketoacid and stereoselective reductive amination by amino acid dehydrogenase. Reductive amination of α-ketoacids to chiral amino acids by amino acid dehydrogenases is well-documented, with industrial examples showcasing the practical utility of the catalysts employed.[22] The combination with a P450 peroxygenase, reported here for the first time, appears promising for functionalization of bio-based chemicals to access relevant chiral synthons. Conversions to the final product are at present moderate; extension of this work to preparative-scale synthesis of non-natural amino acids with improved redox balance and final yield is currently under investigation.

Conflicts of interest

There are no conflicts to declare.

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Notes and references

‡In our previous work,[4] oxidation of 2-hydroxyacids was performed by two stereocomplementary (α-hydroxyacid oxidases, thereby releasing H_2O_2 required in the first step ('catalytic reagent' concept). This design was not selected for the present three-step cascade, as final NADH-dependent reductive amination by amino acid dehydrogenase would then require either stoichiometric amounts of reduced nicotinamide or sacrificial substrate for cofactor recycling, in turn increasing waste formation and affecting atom-economy.

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