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Preparative asymmetric synthesis of canonical and non-canonical α-amino acids through formal enantioselective biocatalytic amination of carboxylic acids

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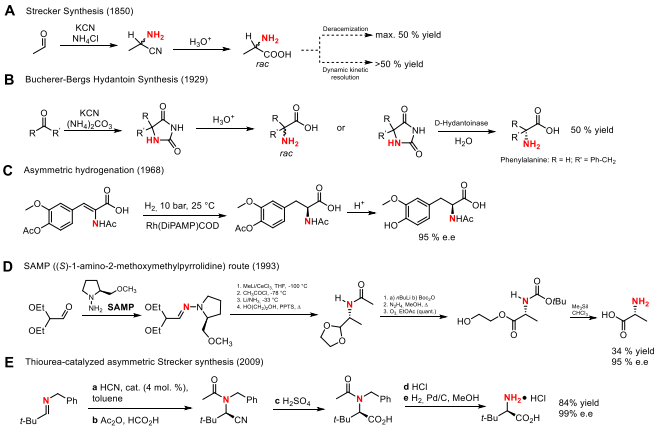
Abstract. Chemical and biocatalytic synthesis of non-canonical α-amino acids (ncAAs) from renewable feedstocks and using mild reaction conditions has not efficiently been solved. Here, we show the development of a three-step, scalable and modular one-pot biocascade for linear conversion of renewable fatty acids (FAs) into enantiopure L-α-amino acids. In module 1, selective α-hydroxylation of FAs is catalyzed by the P450 peroxygenase $P450_{CLA}$. By using an automated H_2O_2 supplementation system, efficient conversion (46 to >99%; TTN >3300) of a broad range of FAs (C6:0 to C16:0) into valuable α-hydroxy acids (α-HAs; >90 % α-selective) is shown on preparative scale (up to 2.3 g L^{-1} isolated product). In module 2, a redox-neutral hydrogen borrowing cascade (alcohol dehydrogenase/amino acid dehydrogenase) allowed further conversion of α-HAs into L-α-AAs (20 to 99%). Enantiopure L-α-AAs (e.e. >99%) including the pharma synthon L-homo-phenylalanine can be obtained at product titers of up to 2.5 g L^{-1}. Based on renewables and excellent atom economy, this biocascade is among the shortest and greenest synthetic routes to structurally diverse and industrially relevant ncAAs.

Keywords: catalysis; fatty acid; amino acid; P450; biocascade

Introduction

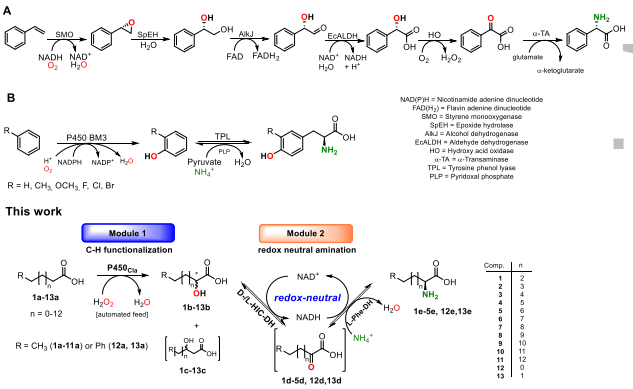
Refining and (steam) cracking of petroleum gas/naphtha provides a few intermediates (short-chain olefins and aromatics) that serve as current major feedstock for the chemical industry.[1] Despite the harsh production conditions (>500 °C; >10 bar; NiCr reactors)[1b, 1c] and unpredictable price fluctuations of fossil resources, alternative routes remain scarce.[1c, 1d] Environmental and economic concerns drive the exploration of alternative sources such as renewables or recycling of (organic) waste.[1d, 2] The substitution of fossil feedstocks can have a significant impact on achieving a safer and more sustainable production.[3] Saturated fatty acids (FAs) are promising feedstocks[4] since they are available in large quantities, from organic waste or fermentative processes and, in particular short chain FAs (C6:0-C8:0), do not compete with food. Oxy-functionalizations of FAs focused so far mainly on the ω-carbon to produce polyester synthons (fatty alcohols and diacids).[5] The

α-carbon has rarely been targeted for chemical/biological hydroxylation,[6] although selective α-functionalization affords versatile α-hydroxy acids (α-HAs; **1b-13b**; Scheme 1), e.g., for the production of biodegradable polyesters (PLA derivatives), pharmaceuticals, anti-microbial agents and natural products.[7] α-HAs can be further oxidized into α-keto-acids[8] to serve as precursors for α-amino acid (α-AAs).[9] Generally, α-AAs represent one of the five most important classes of natural compounds with myriad of applications.[9-10] In particular, non-canonical L-α-AAs (ncAAs) are of increasing interest and demand, driving innovations in the field of synthetic biology, pharma, bio-imaging, biosensors, medicinal applications or hybrid-/organo-catalysts design.[11] While fermentation processes for most proteinogenic L-α-AAs are well established,[9] ncAAs are often produced via chemical synthesis (Scheme 1).[10, 12] Despite the high efficiency and broad substrate scope, toxic cyanides (KCN), strong acids and petroleum-based aldehydes (derived from multi-step high energy processes involving rare-metals)[1b, 13] are typically required for chemical manufacturing (Scheme 1). The classical Strecker synthesis yields racemic products[9] and the high amount of generated waste drives innovation in this field.[14] Asymmetric chemical synthesis of α-AAs has been shown using pyrrolidine chiral auxiliaries, phosphine ligands and protection groups.[15] A chemo-enzymatic strategy combines efficiency of Bucherer-Bergs synthesis with enantioselective enzymatic kinetic resolution by hydantoinases yielding enantiopure α-AA (max. 50 % yield; Scheme 1 B).[10, 16]



Scheme 1. Selected chemical routes toward synthesis of α-amino acids. **A** = Strecker synthesis.[12a, 12b, 12d] **B** = Bucherer-Bergs synthesis (*hydantoin route*)[16a-c]. **C-E** = Asymmetric chemical synthesis using chiral auxiliaries/catalysts.[15a-c]

To overcome downsides in multi-step chemical synthesis of enantiopure α-AAs, biocatalytic cascades were developed that combine two or more enzymes for one-pot conversions in vivo or in vitro.[17] Still, many of these routes are based on petroleum-derived feedstocks such as benzenes, olefins, aldehydes or α-HAs (Scheme 2).[17c-e, 18] A direct route for α-amination of α-/ß-saturated organic/fatty acids has (so far) been restricted by efficient and selective oxy-functionalization of the poorly activated α-carbon, scalability of oxyfunctionalization reactions for preparative synthesis (in particular with O_2 as oxidant) as well as low conversion by the respective amination catalysts.[6, 8, 19] Herein, we show the development of a three-step linear biocascade for the atom-efficient formal asymmetric α-amination of saturated FAs into enantiopure L-α-AAs in one-pot, on preparative scale, using mild reaction conditions, renewable feedstocks, reagents and catalysts (Scheme 2).



Scheme 2. Selected biocatalytic cascades for the asymmetric synthesis of enantiopure α-AAs involving an initial oxy-functionalization step. **A** = Transformation of styrene and derivatives in six enzymatic steps into L-phenylglycines.[18b] **B** = Conversion of benzene and derivatives into L-tyrosines.[4b] **C** = One-pot cascade for regio- and enantioselective transformation of FAs and aromatic carboxylic acids into enantiopure L-α-AAs (this work). **Module 1:** $P450_{CLA}$ (P450 peroxygenase) catalyzed hydroxylation of **1a-13a** with H_2O_2 as oxidant yields the respective α-HAs **1b-13b**. **Module 2:** Redox-neutral hydrogen borrowing cascade (NAD^+ as electron shuttle) for oxidation a56nd reductive amination of α-HAs in one-pot. Potential side products and cascade intermediates (**1c-13c, 1d-5d, 12d, 13d**) are shown in square brackets.

Results and Discussion

The selective hydroxylation of **1a-11a** into α-HAs (**1b-11b**) is the key step for the envisioned cascade (Scheme 2; Module 1). Preparative scale synthesis has not been shown, as mostly µM substrate/product concentrations and small reaction scales (<5 mL) have been reported with P450s or fungal peroxygenases.[6, 19b-d, 20] Two regioselective α-hydroxylases ($CYP_{SPα}$ and $P450_{CLA}$)[19b, 19c], both from the cytochrome P450 family 152 (CYP152)[6], were selected and tested for hydroxylation of **1a-11a**. Noteworthy, both enzymes can utilize H_2O_2 as oxidant,[6, 19b, 19c, 20] which improves atom economy (no recycling of NAD(P)H or sacrificial co-substrates needed), simplicity as well as scalability of the reaction (no additional energy input required for gas to liquid transfer of O_2).[21] Further, H_2O_2 is a

Table 1. Selective hydroxylation of FAs (**1a-11a**) with $P450_{CLA}$ (0.06 mol%) and automated H_2O_2-feeding on 50 mL scale in module 1.

Entry	Substrate	Conv. [%]	α-HA [%]	ß-HA [%]	Others [%]	Isol. Yield [%]	Purity [% α-HA][a]	g L^{-1} [b]
1	**1a**	93	>99	0	0	68 (45 mg)	>95	0.9
2	**2a**	>99	91	0	9 (**1b**)	89 (65 mg)	>95	1.3
3	**3a**	>99	97.5	<0.5	2 (**2b**)	>95 (77 mg)	>95	1.5
4	**4a**	>99	81	0	19 (**3b**)	90 (78 mg)	>95	1.6
5	**5a**	>99	96	2	2 (**4b**)	>95 (92 mg)	>95	1.8
6	**6a**	>99	98	2	0	>95 (97 mg)	>95	1.9
7	**7a**	95	95	2	3 (**6a**)	>95 (106 mg)	>95	2.1
8	**8a**	>99	93	<1	7 (**7b**)	>95 (115 mg)	>95	2.3
9	**9a**	95	95	<1	2 (**8b**); 2 (**7b**)	85 (104 mg)	>95	2.1
10	**10a**	93	97	<1	<1 (**9b**); <1 (**8b**)	>95 (125 mg)	>95	2.5
11	**11a**	46	>99	0	0	n.a.	60[c]	---

All reactions (**1a-11a**; each 10 mM) were performed on 50 mL scale using automated H_2O_2 fed-batch supplementation (pulsing of 0.8 mM H_2O_2 every 30 min; totalling 2 mol equivalents). Conversion was determined by GC-MS (a sample was taken at 0 h and used as 100% reference) using 0.1 % (v/v) 1-octanol as internal standard. Selectivities are given in % GC-MS area. Isolated yields [in %] were calculated based on measured mass [mg] of isolated and dried products (α-HA, ß-OH and others) and the maximum theoretical yields [mg] obtained after full conversion of **1a-10a** into the respective α-HAs (**1b-10b**). [a] Purity of isolated materials was determined by ^{1}H-NMR. [b] Product titers were calculated based on isolated material. [c] Isolated material analyzed by ^{1}H-NMR contained ~40 % of **11a**. n.a. = not applicable.

renewable oxidant that can be produced efficiently from air/O_2 and renewable electron sources such as glucose or solar/wind energy.[22] Contrary, utilizing H_2O_2 in peroxygenase processes is a rather unsolved challenge as heme-thiolate containing proteins are rapidly inactivated by the oxidant even at low concentrations.[1a, 8, 20, 23] In this study, only $P450_{CLA}$ displayed desired properties for synthetic purpose: a) high/soluble expression in *E. coli*, b) simple purification, long term storage in liquid form and α-selective hydroxylation (>90 %; Table 1) of a broad range of FAs (**1a-11a**; see ESI).[8] Opposed to previous reports[19c, 20], $P450_{CLA}$ tolerates comparably high substrate (10 mM) and H_2O_2 concentrations (1.6 mM pulsing) at low catalyst loading (0.06 mol%). A specific activity of 5.5 U mg^{-1} (k_{cat} = 266 min^{-1}; 83% coupling [$mol_{product}$ mol_{H2O2}^{-1}]; Table S2) was determined for the conversion of **5a** with $P450_{CLA}$. A broad range of FAs with varying chain length (**1a-11a**) were hydroxylated efficiently and selectively (81 to 99% α-hydroxylation; Table 1). In contrast to other members of the CYP152 family such as $CYP_{SPα}$ and $CYP_{BSß}$ (both perfectly (*S*)-stereselective)[19a, 19b], $P450_{CLA}$ displayed low enantioselectivity in the conversion of **3a** (e.e. = 36% (*S*)-**3b**; Figure S2).[8] This might be related to its function in Nature e.g. for the neutralization of toxic FAs, similar to what is proposed for the bacterial fatty acid hydroxylase P450 BM3.[24] In order to show the practical applicability of $P450_{CLA}$, we developed and optimized a fully automated H_2O_2 fed-batch system (pulsing 0.8 mM every 30 min; Figure S1) for an initial scale up to 50 mL, to allow preparative synthesis and isolation of **1b-11b** (Scheme 1). Considering spontaneous disproportionation of H_2O_2[25] and a potential catalase activity of heme-iron enzymes,[26] we calculated a coupling efficiency of 50% (on 1 mL scale 2 mol equivalent H_2O_2 allowed full conversion of **1a**). To monitor product formation and efficiency in H_2O_2-utilization in detail, time studies were performed for the conversion of **1a**, **3a** and **5a** on 50 mL scale (see Figures 1, S13 and S42). All three substrates were hydroxylated efficiently showing linear time course for substrate depletion and product accumulation. Overall, the automated H_2O_2 feeding allowed excellent conversion of **1a-10a** (93 to 99%; Table 1) with intact selectivity for α-HA formation (81 to 99%; Table 1). Side products such as ß-HAs (**1c-11c**) or 2-keto acids (**1d-11d**) were formed in traces (<2%; Table 1). Only the conversion of **4a** yielded significant amount of side-product (19%) that was identified as **3b** (Table 1, entry 4; Figure S26). The formation of **3b** can be explained by oxidative FA carbon chain shortening (C-1) in excess of H_2O_2 (*vide infra*).[6] In case of **11a**, 54% of substrate was recovered after the reaction, indicating that a chain length of C16:0 is not suitable for efficient conversion by $P450_{CLA}$. Overall the automated H_2O_2-feeding allowed very efficient utilization of H_2O_2 resulting in total turnover numbers (TTNs) >1500 and excellent operational stability considering that most P450s are rapidly inactivated at much lower H_2O_2 concentrations.[1a, 27] The high and selective conversion by $P450_{CLA}$ enabled preparative product isolation in a single and simple solvent extraction step (no column chromatography required) yielding 45 to 125 mg of **1b-10b** (68 to >95% isolated yield, >95% purity) corresponding to product titers of 0.9 to 2.3 g L^{-1} (Table 1).

Optimization of module 1

To improve product titers, catalyst/substrate loading and oxidant concentration were further optimized (Table 2). All optimization steps were done on 50 mL scale with automated H_2O_2 feeding system and constant pH control (pH 7.0). When decreasing the catalyst loading to 0.01% the conversion of **7a** (10 mM) dropped to 6% and a TTN of 600 indicating inactivation of $P450_{CLA}$. Fast consumption of H_2O_2 at higher catalyst loading minimizes exposure of $P450_{CLA}$

Table 2. Optimization of module 1 applying varying reaction conditions.

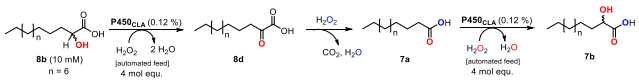
Entry	Substrate	Substrate [mM]	$P450_{CLA}$ [mol%]	H_2O_2 [mol. eq.]	Conv. [%]	α-HA [%]	ß-HA [%]	Side products [%]	TTN
1	**7a**	10	0.01	2	6	94	6	0	600
2	**7a**	10	0.03	2	47	98	2	0	1566
3	**7a**	10	0.03 (CFE)	2	<2	>99	0	0	33
4	**7a**	10	0.06	1.5	>99	97	<1	2 (**3b**)	1666
5	**4a**	20[a]	0.06	1.5	>99	95	1	4 (**3b**)	3330
6	**8a**	10	0.12	4	>99	67[b]	<1	19 (**7b**), <1 (**7c**), 12 (**6b**), <1 (**6c**)	n.d.
7	**8b**	10	0.06	1	17[d]	n.a.	<1	2 (**6b**), 13 (**7b**), <1 (**7c**)	n.d.
8	**7b**	10	0.06	1	10[d]	n.a.	<1	<1 (**6a**), 1 (**5b**), 1 (**7a**), 18 (**6b**), <1 (**6c**)	n.d.

All conversions were done on 50 mL scale (pH 7.0; 20 mM KPi) using automated H_2O_2 supplementation (0.8 mM H_2O_2 per 30 min) except for entry 5, which was done on 150 mL scale. Conversion was determined by GC-MS (a sample was taken at 0 h and used as 100% reference) using 0.1% (v/v) 1-octanol as internal standard. Selectivity was calculated as % GC-area. [a]Reaction on 150 mL scale yielded 0.49 g isolated product. [b]This value corresponds only to the amount of **8b** produced. [c]Substrates **7b**/**8b** were isolated from 50 mL conversion of the respective FAs **7a**/**8a** (Table 1). Detailed composition of the substrate is given in the supplementary information. [d] Conversion refers only to depletion of **7b**/**8b** in the reaction. TTN = total turnover number. CFE = cell free extracts. n.d. = not determined. n.a. = not applicable.

to the inactivating oxidant therefore increasing operational stability (Table 2, entries 1 and 2).

Denaturation and irreversible destruction of the heme-thiolate cluster[27] of $P450_{CLA}$ at increased H_2O_2 concentrations (>1.6 mM per pulse) was indicated by fast de-colorization of the reaction solution and accompanied by decreased conversion of FAs. Besides purified $P450_{CLA}$, we also tested cell-free extracts of the peroxygenase to avoid catalyst purification. Unfortunately, $P450_{CLA}$ reached only 2% conversion (Table 2, entry 3) even when supplementing more oxidant. One reason is that highly active *E. coli* catalases convert H_2O_2 rapidly into water and O_2 (strong foaming during H_2O_2 addition), therefore purification remains inevitable for peroxygenases. Finally, the amount H_2O_2 was reduced by 25% to 1.5 mol equivalents, which still allowed full conversion of **7a** (>99%; Table 2, entry 4). In addition, previously observed over-oxidation could be reduced by 4.8-fold for the conversion of **4a** (only 4% of **3b** formed; Table 2, entry 5).

To trace the origin of over-oxidation products the conversion of **8a** (10 mM) was performed with 4 mol equivalents of H_2O_2 (50x pulsing of 0.8 mM H_2O_2). A significant increase in formation of shorter chain side products was detected (see Table 1, entry 8 for comparison), including C-1/C-2 α-/ß-HAs, totaling ~33% of all reaction products (Table 2, entry 6). Heme-peroxygenase catalyzed formation of C-1 FAs proceeds via α-hydride abstraction from the respective α-HA intermediate[28] yielding an α-keto acid product, which can further undergo decarboxylation at elevated concentrations of H_2O_2 (Scheme 3).[8, 19d] Theoretical calculations suggested also a second route for peroxygenase-catalyzed alcohol oxidation via O-H cleavage.[6, 28] To prove the origin of C1-FAs/C1-α-HAs, prompted us to investigate conversion of isolated **7b**/**8b** (from module 1), which resulted in formation of 16 to 22% of over-oxidized/decarboxylated products (Table 2, entry 7 and 8). This can serve as a first experimental proof that α-HAs are intermediates for the proposed pathway for oxidative decarboxylation by CYP152 enzymes (Scheme 3). Overall the conversion of **8b** remained lower than for **8a**, even when adding equimolar amount of H_2O_2, indicating a complex dynamic/kinetic oxidation of FAs and α-HAs that needs to be carefully addressed in bioprocess development. As outlined by others[6, 19d], the synthesis of odd-chain FAs and derivatives might be synthetically attractive, however unwanted for module 2. By carefully optimizing module 1, reaction conditions leading to over-oxidation/decarboxylation[6, 19d] can be avoided (or enforced) while selectivity and productivity of $P450_{CLA}$ remains high for α-hydroxylation of FAs.



Scheme 3. Proposed biochemical route for over-oxidation of **8b** by $P450_{CLA}$ in excess of H_2O_2.

The significantly improved selectivity and robust conversion of FAs, in particular **4a**, prompted us to investigate this reaction in more detail. After full conversion of 10 mM **4a** (~1600 turnovers) with 0.06 mol% catalyst the reaction solution retained its typical red color originated from the prosthetic heme group. To our surprise 60% of the initial active P450 concentration (6 µM) was found in the reaction solution, indicating high operational stability of $P450_{CLA}$ under optimal H_2O_2 pulsing conditions (in total 15.2 mM). Subsequent supplementation of **4a** (10 mM, 237 mg, Table 2, entry 5) and pulsing of H_2O_2 for

additional 19 cycles (totaling 30.4 mM H_2O_2) to the same reaction lead again to full conversion of **4a**, which in the end corresponds to a TTN of ~3330. This is an outstanding value for a P450 peroxide-shunt catalyzed reaction and underlines the need for careful process design with these enzymes.[1a, 6, 8, 19c-e, 20] In total, 0.49 g of **4b** (3.2 g L^{-1}; 95%; only minor traces of **3b** found) could be isolated with the optimized reaction system, which corresponds to 93% isolated yield. The high/improved operational stability of $P450_{CLA}$ in excess of H_2O_2 can be explained by i) a controlled supplementation of the oxidant, ii) high catalyst activity and sufficient substrate loading and iii) minimized exposure times of the catalyst to inactivating concentrations of H_2O_2.[19c, 20] This system yields excellent product titers/concentrations for a peroxide-shunt catalyzed P450 reaction, comparable to values reported for fungal peroxygenases.[19d, 29] During all optimization steps, the high regioselectivity of $P450_{CLA}$ remained unaffected.

Optimization of module 2

With an optimized oxyfunctionalization system in hand, module 2 was examined to produce L-α-AAs directly from FAs in one-pot without intermediate purification steps. A redox-neutral "hydrogen borrowing" cascade was designed [18a, 18c, 18d] that would require only catalytic amount of NAD^+ as hydride shuttle for oxidation of **1b-11b** and NH_4^+ (amine donor) for the final reductive amination step (Scheme 2). To overcome the poor enantioselectivity of $P450_{CLA}$ in module 1,[8] alcohol dehydrogenases (ADHs) are required for oxidation of both enantiomers of **1b-11b** (Scheme 1, Module 2). Two stereo-complementary α-hydroxyisocaproate dehydrogenases (L- and D-HIC-DH)[18c, 18d, 30] could be identified that catalyze efficient oxidation of **1b-5b**. Longer chain α-HA (**6b-9b**) were also accepted by both ADHs, resulting in much slower oxidation rates or no detectable activity as in case of **10b**. As L- and D-HIC-DH can be produced easily in *E. coli* at high specific activity and without the need for purification (long-time storage as freeze-dried whole cell preparations), the requirement for two ADHs in module 2 can be regarded as minor drawback. For stereoselective reductive amination of α-keto acid intermediates, the L-phenylalanine dehydrogenase (L-Phe-DH) from *Rhodococcus sp.*[31] was selected due its broad substrate scope and high volumetric activity for reductive amination of **3d**. Here, initial conversions of **1a** (1 mM) with both modules in one pot mode (1 mL scale) yielded without further optimization 33% of **1e** while 77% of **1b** remained unconverted. To direct the equilibrium towards reductive amination, module 2 was optimized using *rac*-**1b** and *rac*-**3b** as model substrates. For this, concentrations of substrate, NH_4^+ and NAD^+ were varied as well as catalyst loading, pH and type of buffer (see Figures S69 to S80). The largest impact on the equilibrium between **1b**/**3b** and **1e**/**3e** was observed when modifying the pH and type of buffer. α-AA formation reached 79 to 89% after 24 h, when using either NH_4Cl or Na-carbonate buffered systems set to pH 8.0 (100 mM KPi buffer with pH of 8.5 to 10 was used for initial conversions). This can be explained by i) low solubility/precipitation of aliphatic α-AAs at neutral or slightly acidic/basic pH (isoelectric point of **1e**/**3e** is ~6), ii) better conditions for initial alcohol oxidation step by HIC-DHs[30] (note: reductive amination with amino acid dehydrogenases is often done at basic pH between 9 and 10) and iii) higher stability of NAD^+/NADH in absence of phosphate ions at slightly basic pH.[32] Other parameters did not significantly improve conversion or selectivity towards α-AA formation. Beneficially, the cascade intermediates **1d** and **3d** did not accumulate under optimized conditions indicating an excellent hydride shuttling via NAD^+/NADH ('closed loop').[18a] Unexpectedly, the conversion was found to proceed even in absence of commercial NAD^+ (traces of cofactor present in preparations of D-/L-HIC-DHs and L-Phe-DH) resulting in 27% conversion of *rac*-**1b**. For standardized reaction conditions, we decided to employ 6 mM NAD^+ leading to 95% conversion of *rac*-**1b** into **1e**. To improve economy of the reaction, up to 60-fold lower NAD^+ concentrations (0.1 mM or 0.5 mol%) can be employed to achieve 70% conversion of *rac*-**1b** into **1e** (Figure S77). This is 10-fold lower than comparable enzymatic hydrogen borrowing cascades reported recently for amine synthesis.[18a] To explore the substrate scope of the complete optimized cascade, 10 mM of **1a-11a** were converted by both modules initially on 1 mL scale (manual H_2O_2 feeding in module 1). Formation of the respective α-AAs was monitored by TLC and GC-FID/GC-MS analysis (control reactions did not contain $P450_{CLA}$). The whole cascade was most efficient for conversion of **1a-5a** yielding the respective α-AAs (minor traces detected for **6e-11e**; data not shown). Sterically more demanding substrates with increased hydrophobicity are not well accepted as substrate by the two ADHs.[30] Nonetheless, it appears that the L-Phe-DH likely becomes the limiting catalyst in the whole cascade.

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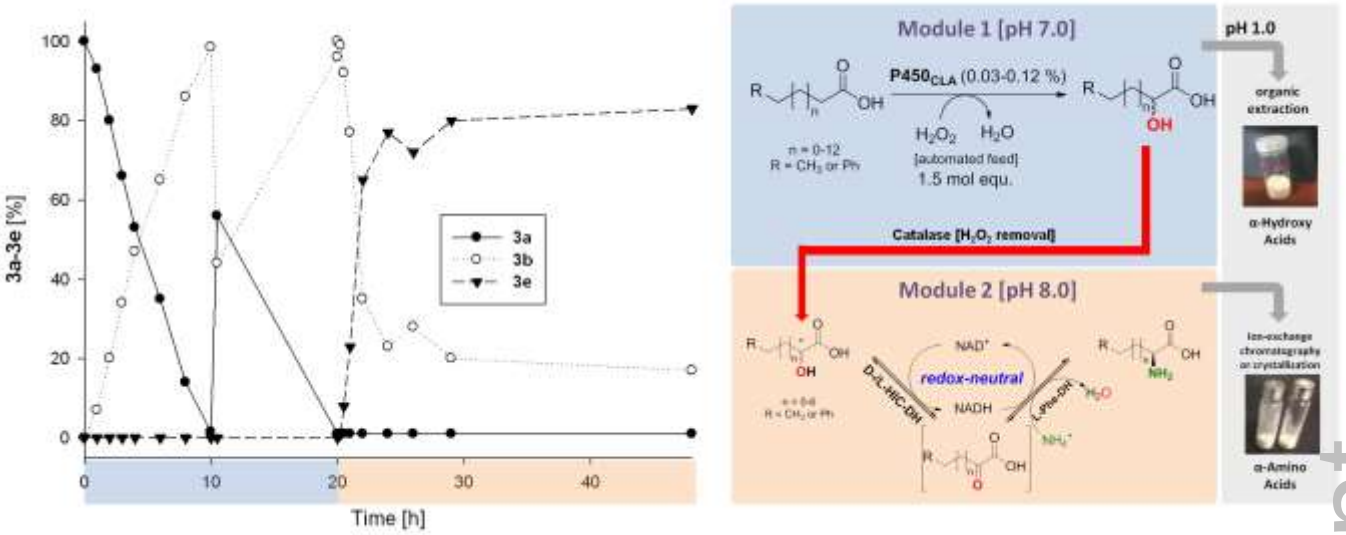

Figure 1. Time dependent conversion of 20 mM **3a** into **3e** on 150 mL scale. Initially, the reaction is started by adding 10 mM **3a**. After 10 h reaction time additional 10 mM of **3a** were added to the reaction (module 1). Module 2 was started after 20 h reaction time and pH adjustment (pH 8.0). Side products were not formed to a detectable amount (see supporting information). Isolation and purification yielded 257 mg (59% isolated yield) of L-α-**3e** in >95% purity (determined by ^{1}H-NMR) and e.e. >99% (see supporting information for analytical details). Both modules allow separate product down-streaming to access either α-HAs or α-AAs in high purity.

Practical use of the cascade on preparative scale

The practical use of the cascade was demonstrated on preparative scale (150 mL) with substrates **1a-5a** (20 mM, 348 to 516 mg starting material) under optimized conditions for both modules. Side product formation/over-oxidation(s) in module 1 (Scheme 2) was minimized by pulsing 1.5 molar equivalents of H_2O_2 for 19 cycles, which allowed under scale up conditions unchanged and excellent conversion (>99%) of 10 mM **1a-5a** with >98% selectivity towards **1b-5b**. To increase product titers further, additional 10 mM of **1a-5a** were supplemented after 10 h and the H_2O_2 feed was continued for 19 additional cycles (totaling 30.4 mM H_2O_2). After 24 h, conversion of **1a-5a** reached 73 to 99% (Table 3) and catalase (330 U mL^{-1}) was added to remove residual traces of H_2O_2 (30 min incubation). For stabilizing the pH at 8.0 (2nd pK_a of Pi = 7.2; 20 mM added in module 1), 20 mM of $(NH_4)_2CO_3$ and 200 mM NH_4Cl were added followed by the supplementation of remaining reagents. The pH was constantly controlled and stabilized by adding either base or acid. Conversion in module 2 was monitored using TLC and GC-MS. After a few minutes, formation of an insoluble white solid occurred (>C6 chain length) indicating rapid formation of poorly water soluble α-AAs (**2e-5e**). Other precipitation in the bulk phase (potentially related to protein denaturation) was not clearly visible. The precipitation of α-AAs provides a strong driving force in the reversible reaction, enforced by growing carbon chain length and hydrophobicity (Table 3). Overall, transformation of **1b-4b** into **1e-4e** proceeded with good to high conversion (77 to 96%) after three catalytic steps, while in case of **5b** selectivity for α-AA formation dropped to 20%, thereby marking the upper chain length for efficient amination (Scheme 2). Highest conversion was obtained for **4e** (>99%) that also displayed strongest visible precipitation during the amination step (**4a** or **4b** were recovered in minor traces; Figure S114 and S115). Operating module 2 with commercial *rac*-**1b**/**3b** yielded similar conversions (79 and 89%) indicating excellent functionality of module 1 and high compatibility with module 2. To monitor the reaction in more detail, a time course was recorded exemplarily for amination of **3a** (Figure 1). Hydroxylation of **3a** proceeds by efficient use of H_2O_2 (60 to 70% coupled to product) and the conversion was completed after 20 h (no substrate recovered; Figure 1) indicating high efficiency and facile scalability. The conversion of **3b** to **3e** (module 2) proceeded much faster reaching 65% conversion after only 2 h, whereas the equilibrium between **3c** and **3e** (Scheme 2) was reached after 24 h yielding 84% of the target α-AA. The undesired intermediate **3d** or other side products were not formed to a detectable amount indicating efficient electron shuttling with catalytic amount of NAD^+/NADH. Finally, **1e-5e** were isolated and purified by ion exchange chromatography or precipitation/crystallization followed by ^{1}H-/^{13}C-NMR analysis to confirm structures and determine the purity of products. Isolated yields ranged between 30 and 76% (156 to 372 mg) of highly pure material (>97% for **1e-5e**), which corresponds to product titers of 1 to 2.5 g L^{-1} (Table 3). For comparison, the asymmetric chemical route by Jacobsen et al. (Nature, 2009; Scheme 1, route E)[15b] yielded up to 2.1 g enantiopure *tert*-leucine. The optical purity of isolated α-AAs was checked by optical rotation measurements as well as chiral chromatography and confirmed that all α-AAs were obtained in perfect e.e. (>99% L-α-AA; *S*(+) configuration). Overall, the herein presented route can be considered as an excellent starting point for further

Table 3. Preparative-scale synthesis of L-α-AAs with combined module 1 and 2 in one-pot two-step mode.

General conditions			Module 1			Module 2					
Vol.[mL]	Sub.	Conc. [mM]	Conv. FA [%]	α-HA [%]	Side products	α-AA [%]	α-AA [*R/S*]	e.e. [%]	Iso. Yield [%]	Purity α-AA [%]	g L^{-1}
150	**1a**	20	86	>99	0	78	*S (+)*	>99	40 (156 mg)	>95	1.0
150	**2a**	20	>99	>99	0	77	*S (+)*	>99	76 (331 mg)	>95	2.2
150	**3a**	20	99	>99	0	84	*S (+)*	>99	59 (257 mg)	>95	1.7
150	**4a**	20	99	95	**3b** (4 %); **4c** (0.6 %)	>99[a]	*S (+)*	>99	72 (372 mg)	>95	2.5
150	**5a**	20	73	95	**4a** (3 %); **5c** (2%)	20	*S (+)*	>99	30[b] (171 mg)	>95	1.1
50	**12a**	10	56	>99	0	>99	n.a.	>99	n.a.[c] (14 mg)	n.a.	0.5[d]
50	**13a**	10	82	94	**13c** (6 %)	69	*S (+)*	>99	48 (44 mg)	>95	0.7

Conversion of **1a-5a** and selectivity for product formation was determined by GC-MS (values are given in % GC-area). Conversion of **12a** and **13a** was determined by HPLC and GC-MS. 1.5 mol equivalent of H_2O_2 were supplemented to the reactions via automated pulse feeding. All α-AAs were isolated, purified and structures/purity confirmed by ^{1}H- and ^{13}C-NMR. Chiral GC-FID and HPLC was used to determine enantiomeric excess (e.e.) of isolated products. Product titers (g L^{-1}) were calculated from isolated pure materials. [a] Minor traces of **3b** formed in module 1 were converted to **3e.** [b] High isolated yields can be explained by formation of insoluble/solid particles during formation of **5e**. Samples for conversion determinations were taken from liquid bulk. [c] Isolated yield cannot be stated due to impurities in the isolated material. [d] Determined by measuring concentration of **12e** (3.2 mM) in the liquid bulk. n. a. = not applicable.

process development in particular considering the simplicity in scaling module 1 that uses H_2O_2 as cheap oxidant.[33]

The substrate scope of the cascade was further extended to conversion of two aromatic acids **12a** and **13a**, which were converted on 50 mL scale (10 mM; 75 and 82 mg of **12a** and **13a**, respectively). The target products, L-phenylalanine (L-**12e**) and L-homo-phenylalanine (L-**13e**), represent key building blocks for fine chemicals and pharmaceuticals with substantial industrial significance.[34] Especially L-**13e** is widely used in the synthesis of ACE inhibitors/blood pressure regulators (e.g. Ramipril) for treatment of hypertension, cardiovascular diseases and congestive heart failure.[34] Initially, low conversion (<50%) was detected for **12a**/**13a** (10 mM; module 1), which could be improved by doubling the amount of peroxygenase (0.12% catalyst loading), reaching finally 56 and 80% conversion for **12a** and **13a**, respectively (Table 3). Both aromatic L-α-AAs were finally produced on 50 mL scale (combined module 1 and 2), followed by product isolation, yielding up to 44 mg (48% isolated yield; 0.3 g L^{-1}) of >95% pure L-**13e**.

In case of L-**12e,** a product concentration of 3.2 mM was measured in the bulk phase. Both aromatic L-α-AAs were obtained in perfect enantiopurity (e.e. >99%). Compared to other biocascades involving an oxy-functionalization step, e.g. for aromatic L-α-AAs synthesis, the present system can be operated already at fairly high substrate concentration with a minimal number of catalytic steps and reagents needed (Scheme 2). For example, Li et al. reported a whole cell-based cascade toward L-phenylglycines employing mainly petroleum-derived styrenes as starting materials (5 to 20 mM), reaching in six catalytic steps with two cofactors and glutamic acid as amine donor conversion of 29 to 91% (Scheme 2A).[18b] While fermentation processes for proteinogenic L-α-AAs such as L-**12e** can reach up to 52 g L^{-1}, these are usually limited to canonical derivatives.[9, 35] Compared to chemical catalysis or fermentative routes, the presented system is significantly simplified by less catalytic steps, the need of only two non-toxic reagents in excess (H_2O_2 and NH_4, no cyanide needed), advantageous internal cofactor recycling and liberating water as waste. For the current modular reaction system the calculated atom economy ranges between 88 to 94% (module 1) and 64 to 72% (module 1 and 2).[36] Moreover, intermediate purification is not required, which increases overall efficiency of the linear cascade and product yields.

Conclusion

The selective oxy-functionalization of non-activated C-H bonds (in particular for fatty acids/renewables) remains a synthetic challenge in chemistry. Here we show the development of an atom efficient, scalable linear biocascade for the formal enantioselective amination of FAs into L-α-AAs in one-pot via formation of valuable α-HA intermediates. Key-to-success was the efficient operation of the $P450_{CLA}$ peroxygenase with H_2O_2 as oxidant allowing α-selective hydroxylation (>95%), with high specific activity (5.5 U mg^{-1}), TTN of up to 3300 and product titers of 0.9 to 2.3 g L^{-1}. Controlled supplementation of H_2O_2 allowed the first preparative scale operation of a P450 at elevated concentration of the intrinsically harmful oxidant without significant and unwanted over-oxidation of the hydroxy acid products. Despite

those improvements, H_2O_2 remains a notoriously harmful and challenging reagent in enzyme catalysis. One way to solve this is to continue efforts in reaction/process engineering in addition to focused optimization/formulation of peroxygenases for specific process requirements. An optimized redox-neutral hydrogen borrowing cascade allowed one-pot transformation of α-HAs into enantiopure L-α-AAs (e.e. >99%), including industrially relevant L-homo-phenylalanine in preparative quantity (48 to 372 mg; 30 to 76% isolated yield) and excellent purity (>95%). The low to moderate solubility of α-AAs close to physiological pH and excess of NH_4^+ drives the reversible hydrogen borrowing cascade efficiently towards amine formation. Since most of the used reagents, catalysts and substrates are renewable, all reaction steps catalytic in aqueous medium, this concept represents a non-fermentative, sustainable synthesis of L-α-AAs under mild reaction conditions (RT, 1 atm) releasing H_2O as ideal waste product. The cascade can be run in one-pot in a sequential manner, with small pH adjustments between both modules. Obviously, the search for better compatible and more robust redox-partners in larger biocascades will be a future challenge to reach higher product titers for industrial production as shown e.g. for chemical synthesis combined with efficient deracemization techniques. A further point to address is the (re-)use of lower NAD^+ concentrations stabilization in order to make cascades economically more competitive. Nonetheless, this novel synthetic concept can be a true alternative to asymmetric chemical α-AA synthesis, with large potential as no fossil resources, toxic reagents (HCN), harsh reaction conditions, complex auxiliaries nor final deracemizations are required.

Experimental Section

Preparative conversion of **1a-13a** on 50 to 150 mL scale: Reactions were performed in a 250 or 500 mL round bottom flasks under continuous stirring (200 rpm, 1 atm, RT) and pH control using a pH stat and 0.5 M KOH or 10% HCl for automated titration. **Module 1:** The reaction mixture contained 6 µM purified $P450_{CLA}$ (0.03 to 0.06% catalyst), **1a-13a** substrate (10 or 20 mM), EtOH (5% v/v) in KPi buffer (pH 7.0, 20 mM). The pH was adjusted to 7.0 with 10 M KOH before starting H_2O_2 feeding. Conversion of 10 mM **1a-13a** was performed for 10 h, while pumping 19 times 125 µL of H_2O_2 (320 mM stock solution for 50 mL reaction volume; 960 mM stock solution for 150 mL) into the reaction vessel (totaling 15.2 mM H_2O_2). For higher product titers, additional 10 mM substrate (solubilized in 1.5 mL EtOH) were added to the reaction. The pH was re-adjusted to pH 7.0 and the H_2O_2 supplementation was re-started for additional 19 cycles (15.2 mM; totaling 30.4 mM). **Module 2:** Residual traces of H_2O_2 were removed by addition of 330 U mL^{-1} catalase (30 min incubation) before adding D- and L-HIC-DH (each 2 U mL^{-1}), L-Phe-DH (8 U mL^{-1}), 6 mM NAD^+ and 200 mM NH_4Cl. For better buffering capacity at pH 8.0, 20 mM of $(NH_4)_2CO_3$ were added followed by manual adjustment of the pH to 8.0 using 10 M KOH. Aromatic substrates **12a**/**13a** (each 10 mM) were converted under identical conditions (50 mL scale) using 12 µM of purified $P450_{CLA}$ in module 1. Detailed experimental information including catalyst preparation, reaction optimization and analytical data (traces for GC-MS, chiral GC-FID, HPLC, and ^{1}H-/^{13}C-NMR of isolated compounds) can be found in supplementary information.

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[36] Atom economy for route E in scheme B (g-scale synthesis of tert-leucine) is 52% and route A in scheme 2 (styrene conversion to phenylglycine) has an atom economy of 34%. Details on atom economy calculations can be found in Table S5 located in the supplementary information section.

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FULL PAPER

Preparative asymmetric synthesis of canonical and non-canonical α-amino acids through formal enantioselective biocatalytic amination of carboxylic acids

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Alexander Dennig*, Fabio Blaschke, Somayyeh Gandomkar, Erika Tassano, Bernd Nidetzky