Communication

Towards a scalable synthesis and process for EMA401. Part III: Using an engineered phenylalanine ammonia lyase enzyme to synthesize a non-natural phenylalanine derivative

Leo Albert Hardegger, Pascal Beney, Dominique Bixel, Christian Fleury, Feng Gao, Alexandre Grand-Guillaume Perrenoud, Xingxian Gu, Julien Haber, Tao Hong, Roger Humair, Andreas Kaegi, Michael Kibiger, Florian Kleinbeck, Van Tong Luu, Lukas Padeste, Florian A. Rampf, Thomas Ruch, Thierry Schlama, Eric Sidler, Anikó Udvarhelyi, Bernhard Wietfeld, and Yao Yang

Org. Process Res. Dev., **Just Accepted Manuscript** • DOI: 10.1021/acs.oprd.0c00217 • Publication Date (Web): 20 Aug 2020

Towards a scalable synthesis and process for EMA401. Part III: Using an engineered phenylalanine ammonia lyase enzyme to synthesize a non-natural phenylalanine derivative

Leo A. Hardegger[†], Pascal Beney[†], Dominique Bixel[†], Christian Fleury[†], Feng Gao[‡], Alexandre Grand-Guillaume Perrenoud[†], Xingxian Gu[‡], Julien Haber[†], Tao Hong[‡], Roger Humair[†], Andreas Kaegi[†], Michael Kibiger[†], Florian Kleinbeck[†], Van Tong Luu[†], Lukas Padeste[†], Florian A. Rampf[†], Thomas Ruch[†], Thierry Schlama[†], Eric Sidler[†¥], Anikó Udvarhelyi[¥], Bernhard Wietfeld[†], Yao Yang[‡]*

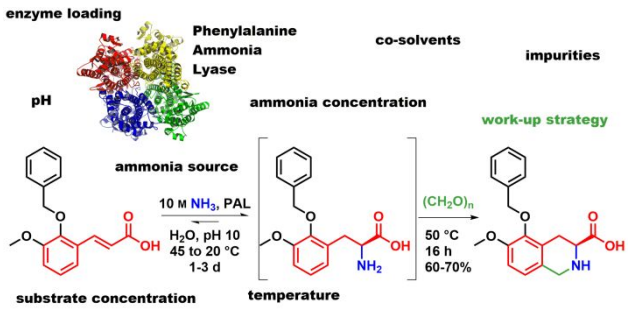
In memoriam Michael Kibiger

[†]Chemical and Analytical Development, Novartis Pharma AG, 4002 Basel, Switzerland.

[¥]Pharmaceutical and Analytical Development, Novartis Pharma AG, 4002 Basel, Switzerland.

‡Suzhou Novartis Technical Development Co., Ltd., Changshu City, 215537, China

For table of contents only



ABSTRACT A process using engineered phenylalanine ammonia lyase (PAL) enzymes was developed as part of an alternative route to a key intermediate of olodanrigan (EMA401). In the first part of the manuscript, the detailed results from a screening for the optimal reaction conditions are presented, followed by the discussion of several work-up strategies investigated. In the PAL catalyzed reaction, 70–80% conversion of a cinnamic acid derivative to the corresponding phenylalanine derivative could be achieved. The phenylalanine derivative was subsequently telescoped to a Pictet-Spengler reaction with formaldehyde and the corresponding tetrahydroisoquinoline derivative was isolated in 60–70% yield with >99.9:0.1 er. Based on our screenings, carbonate/carbamate buffered ammonia at 9–10 M NH_3 concentration and pH 9.5–10.5 were found as the optimal conditions. Enzyme loadings down to 2.5wt% (E:S 1:40 w/w) could be achieved and substrate concentrations between 3–9 v/w (1.17–0.39 M) were found to be compatible with the reaction conditions. A temperature gradient was applied in the final process: a pre-equilibrium was established at 45 °C, before making use of the temperature-dependence of the entropy term with subsequent cooling to 20 °C and achieving maximum conversion.

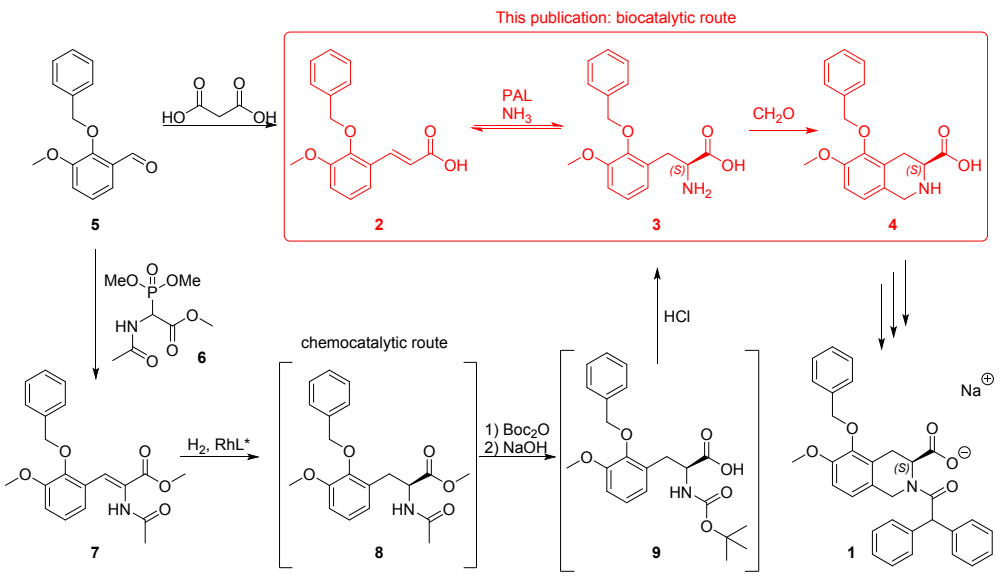
This temperature gradient also allowed balancing enzyme stability (low at 45 °C, high at 20 °C) with activity (high at 45 °C, low at 20 °C) in order to achieve optimal conversion (low at 45 °C, high at 20 °C). From the various work-up operations investigated, a sequence consisting of denaturation of the enzyme, followed by NH_3/CO_2 removal by distillation, acidification and telescoping to the subsequent Pictet-Spengler cyclization was our preferred approach. The process presented in this study is a more sustainable, shorter and more cost effective alternative to the previous process.

KEYWORDS. EMA401, phenylalanine ammonia lyase, scale-up, process development, biocatalysis, phenylalanine derivative

Introduction

In a route scouting exercise for the synthesis of EMA401 (**1**, olodanrigan), an angiotensin II type 2 antagonist used for the treatment of postherpetic neuralgia and neuropathic pain, the synthetic approach *via* the hydroamination of cinnamic acid **2** to amino acid **3** as the key step was identified as an attractive alternative to the existing chemocatalytic route (Scheme 1).[1] The chemocatalytic route relied on an asymmetric

hydrogenation step catalyzed by a chiral rhodium complex. The common late stage intermediate for both, the biocatalytic and chemocatalytic, routes is tetrahydroisoquinoline **4**, while the common starting material for both routes is benzylated ortho-vanillin **5**. For the preparation of amino acid **3** *via* the chemocatalytic route, aldehyde **5** was treated with Horner-Wadsworth-Emmons reagent **6** to obtain enamide **7**, which was subjected to asymmetric hydrogenation conditions to afford amide **8**, followed by functional group interconversion to carbamate **9** and finally deprotection to amino acid **3**. The synthetic approach using the biocatalytic route described in this publication follows a synthetic route requiring only two stages to the key intermediate phenylalanine **3** starting from benzylated ortho-vanillin **5**, which was reacted to cinnamic acid **2** and subsequently subjected to the PAL-catalyzed hydroamination to amino acid **3**.[2]



Scheme 1. Comparison of two synthetic routes to EMA401 (**1**) *via* the key intermediate amino acid **3** and tetrahydroisoquinoline **4**, either following the biocatalytic route (red) or the chemocatalytic route (black).

The key step of the biocatalytic route, the hydroamination to amino acid **3**, is catalyzed by a phenylalanine ammonia lyase (PAL, EC 4.3.1.24), an enzyme class which has attracted a lot of attention during the last decade in the academic and industrial community.[3,4] The advantages of the biocatalytic route are obvious: It is three steps shorter than the chemocatalytic route, uses readily available reagents, avoids the use of heavy metals, and is environmentally more sustainable.

PALs have been shown to catalyze the hydroamination reaction of various non-natural substrates,[5–14] and some examples for successfully scaled-up processes have been reported.[15,16] Two different mechanisms for the 4-methylideneimidazole-5-one (MIO)-dependent hydroamination with PALs have been proposed and appear to be operational depending on the electronic nature of the substrate.[6,8,14,17–19] Our own calculations of Gibbs free energies ΔG in the gas phase between cinnamic acid derivatives and amino acid derivatives indicated a strong correlation between the electronic nature of the aromatic ring and the equilibrium conversion, with smaller ΔG values predicted for electron-poor systems, which corresponds to an equilibrium position favoring product formation (Figure 1).[20–22] A similar trend was found when running the calculations simulating an aqueous environment (see Supporting Information (SI)). Our calculations indicated that both the enthalpy and entropy term were negative. Hence, the Gibbs free energy became negative at low temperatures only. In order to shift the equilibrium as much as possible on the amino acid product side, we aimed at applying a temperature gradient to lower temperatures towards the end of the reaction and applying Le Chatelier's principle by decreasing $\Delta\Delta S$ (*vide infra*).

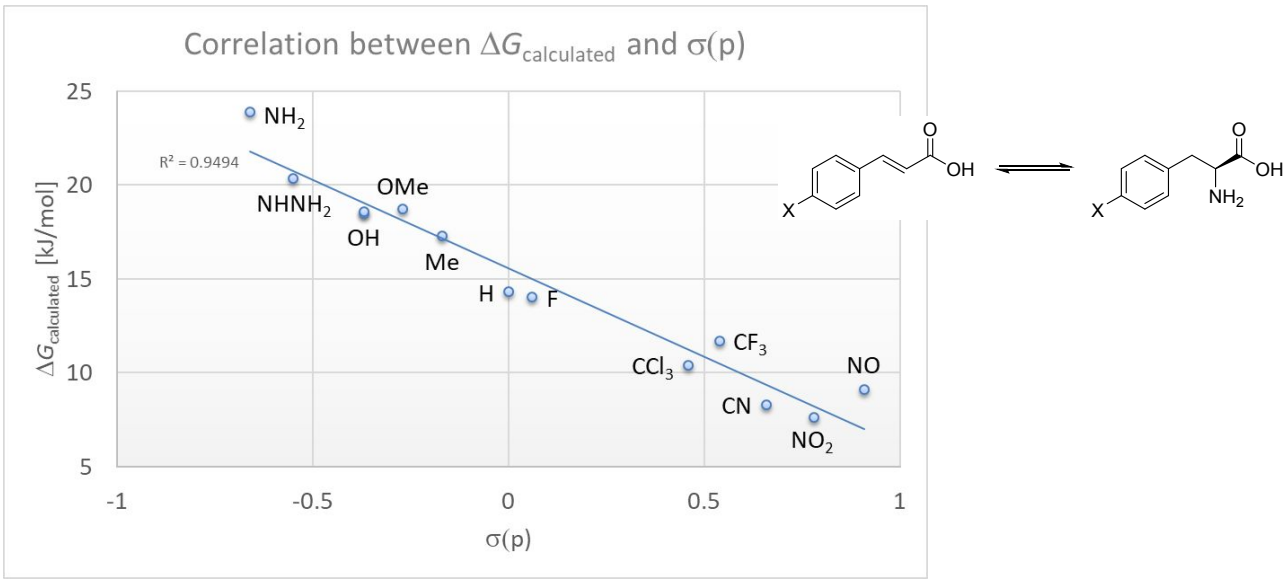


Figure 1. Correlation of calculated Gibbs free energy ΔG for the equilibrium between cinnamic acid derivatives and amino acid derivatives with the Hammett constant σ(p) in the gas phase. The ΔG values of electron-poor aromatic systems are smaller than those of electron-rich aromatic systems (see SI for more discussion on ΔG).

While whole cells were used in most of the previously published cases, we aimed at employing the isolated, lyophilized enzyme powders as the catalyst. Even though no conversion of cinnamic acid **2** could be detected in initial screening experiments with wild type PAL enzymes, presumably due to the bulky and electron-rich substituents on the substrate, we were convinced that the PAL-catalyzed route was the best option for

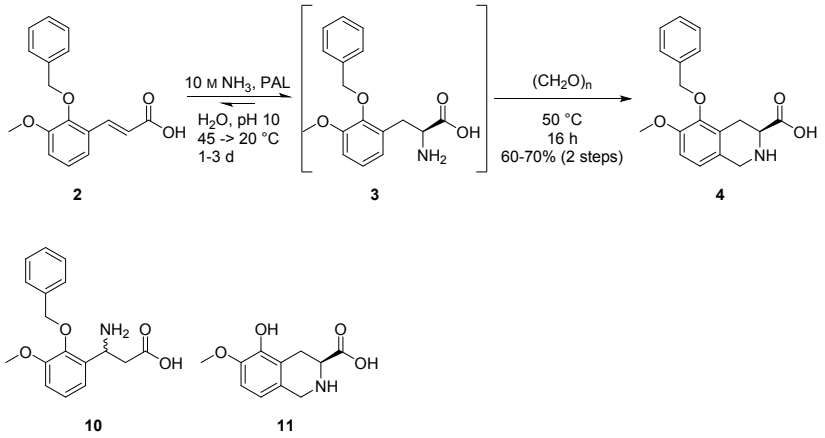
the synthesis of EMA401 (**1**) and initiated an extensive enzyme engineering program for the directed evolution of an enzyme starting from an engineered variant from *anabaena variabilis* specifically for the conversion of cinnamic acid **2** to amino acid **3**.[23] Enyzme engineering was performed at Codexis using the CodeEvolver® technology. After several rounds of enzyme evolution and more than 20 amino acid mutations from the starting point of evolution, engineered enzyme variants were obtained which could successfully be used in lab experiments and scale-up (*vide infra*, see Section 8 in the SI).

Results and discussion

Screening of reaction parameters

For the preparation of amino acid **3**, we initiated process development by screening the most important parameters (ammonia concentration and counter ion of ammonia buffer system, pH, substrate concentration, temperature profile of the reaction, enzyme loading and stability, and the use of co-solvents or additives), while enzyme evolution

was still ongoing in parallel. Most of the parameters screened were not strongly influenced by enzyme evolution, with the obvious exception of the enzyme loading, enzyme stability, and the use of co-solvents.



Scheme 2. Details of the synthetic route from cinnamic acid **2** to amino acid **3** and tetrahydroisoquinoline **4**. β-Amino acid **10** was observed as a by-product in the reaction to α-amino acid **3**. As a result of benzyl cleavage under the strongly acidic conditions of the Pictet-Spengler cyclization to tetrahydroisoquinoline **4**, phenol **11** was observed as the major impurity.

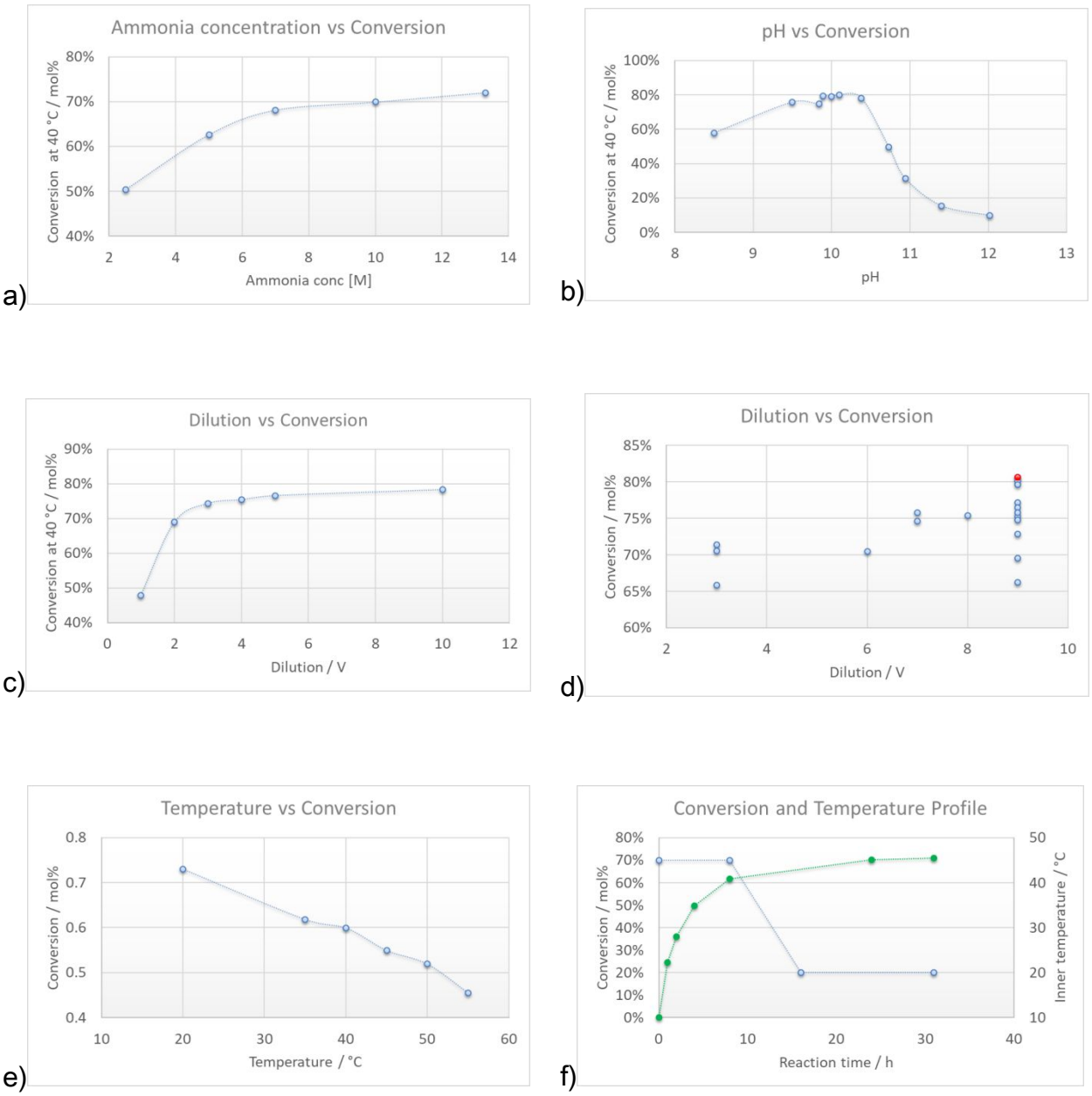


Figure 2. a) Dependence of conversion to amino acid **3** on the ammonia concentration; conditions: pH 9, 20 v/w, 40 °C, >40 h reaction time. Higher ammonia concentrations led to higher conversions; b) dependence of conversion on pH: the optimal pH range for

the conversion to amino acid **3** was found to be 9.0–10.0; conditions: 5wt% enzyme PAL-130, 40 °C, 10 v/w, >40 h reaction time; c) influence of the substrate concentration on the conversion to amino acid **3** obtained in screenings; conditions: 5wt% enzyme PAL-130, 40 °C, pH 10, 10.7 M NH_3, >40 h reaction time; d) conversion to amino acid **3** observed as a function of the substrate concentration in experiments on more than 10 g scale with at least 60 h total reaction time. The result from scale up on 2 kg of cinnamic acid **2** is highlighted in red; e) temperature-dependent conversion to amino acid **3**, a result of the temperature dependent entropy term; conditions: 5wt% enzyme PAL-130, 10 v/w, pH 9, 10 M NH_3, >40 h reaction time; f) temperature gradient employed during a standard PAL reaction (blue line), and respective conversion to amino acid **3** of a selected experiment with 2.5wt% PAL-131 enzyme (green line).

Ammonia concentration and pH: As expected for reactions driven against the thermodynamically favored direction, an excess of ammonia led to higher conversion of cinnamic acid **2** (Le Chatelier's principle). Our screenings confirmed that the higher the

ammonia concentration, the higher the conversion to amino acid **3** (Figure 2a).[22,24–28] The ammonia concentration was, however, limited by the solubility of the buffer system required due to the reduced stability of the enzyme above pH 10.5. At pH values below 8.5 (Figure 2b), little to no conversion was observed, presumably either due to the lack of free ammonia present in solution, which is required for the attack on the MIO group to initiate the catalytic cycle, or due to reduced solubility of the enzyme. As a result of our screening efforts, we selected a concentration of 10 M NH_3 at a pH = 10±0.5. The pH was found to be stable over the entire course of the reaction. Dosing of additional ammonia at different time points to replace the ammonia consumed by the formation of amino acid **3** did not have a significant influence on the equilibrium conversion (Figure SI3).

Ammonia source: In agreement with reports in the literature, carbonates and carbamates were found to be the optimal counter ions for the preparation of the buffer system used in the conversion of cinnamic acid **2** to amino acid **3** (Scheme 2).[22,28,29] The ammonia buffer with carbonates/carbamates could be prepared either by

dissolution of ammonium carbamate in water, by the addition of gaseous CO_2 to aqueous ammonia, or by the addition of aqueous ammonia to ammonium carbonate. While starting from ammonium carbamate was the simplest option in the lab for buffer preparation, its price and the low decomposition temperature of 35 °C did not make this the ideal reagent for handling, transport and scale-up. The addition of CO_2 to aqueous ammonia was a time-consuming process on scale, and preventing off-gassing during the addition of CO_2 to maintain full control over the composition of the buffered ammonia solution proved to be difficult. The preferred protocol consisted in the preparation of a suspension of ammonium carbonate in water and subsequent addition of concentrated aqueous ammonia to afford a solution at the desired concentration and pH. According to Raman studies found in the literature, an equilibrium of the carbonate/carbamate counter ion is established rapidly irrespective of the reagents used for its preparation.[30,31] Indeed, the results of the reaction were found to be independent of the method used for the preparation of the buffered ammonia solution. As a result of sublimation, ammonium salts were observed as a white precipitate at cold parts in the headspace of the reactors in lab reactions. Alternative counter ions tested

delivered lower conversion as compared to the carbonate/carbamate system. We reasoned that a weak counter ion was required to maintain enzyme stability. The use of unbuffered, diluted ammonia was tested with the penultimate PAL enzyme generation PAL-130 at a loading of 50wt% enzyme, providing a satisfactory conversion of 68% at 25 °C, pH 12.6 and 6.3 M ammonia concentration (Table SI3). The conversion dropped to 19% when the enzyme loading was reduced to 5wt% with respect to the cinnamic acid substrate **2** in unbuffered conditions. We hypothesized that the enzyme stability was reduced at higher pH as shown in Figure 2b for buffered systems.

Substrate concentration: Screenings proved that satisfactory conversion as well as acceptable stirring properties could be achieved when reactions were concentrated to 3 v/w. It is important to note that cinnamic acid **2** in our case did not dissolve in the ammonia buffer, but formed an emulsion upon addition of the buffered ammonia solution. This behavior was observed at all dilutions of interest for commercial production and did not appear to have a detrimental influence on the outcome of the reaction. The conversion was found to be proportional to the dilution of the substrate in

the range of 3–9 v/w, corresponding to 1.17–0.39 M, as evidenced in screenings (Figure 2c) and reactions with more than 10 g substrate (Figure 2d).[24,27–29]

Temperature profile: The desired reaction was thermodynamically not favored (*vide supra*). We used the temperature dependence of the entropy term ΔS, as confirmed in screenings at different temperatures (Figure 2e), following investigations of the reaction kinetics and applying a temperature gradient as shown in Figure 2f.[32] The mixture was stirred at 45 °C for 8 h, to establish a pre-equilibrium (criterion: less than 2mol% change in conversion per hour). Subsequently the mixture was cooled to 20 °C over 8 h. Kinetic modelling indicated that the nature of the cooling ramp does not have a major influence on the equilibrium established at 20 °C after 24 h reaction time, with a conversion of 65–70% achieved in 9 v/w dilution. When applying this gradient, higher enzyme activity and lower enzyme stability at 45 °C was balanced with lower enzyme activity and higher enzyme stability at 20 °C.

Enzyme loading: The enzyme loading could be significantly optimized throughout the enzyme evolution. For the latest enzyme generation PAL-131, 2.5wt% with respect to

the substrate cinnamic acid **2** was used in standard experiments to achieve more than 60% conversion after 8 h at 45 °C. Similar results were obtained for PAL-130, the enzyme generation mainly used for development, at 5wt% loading. Screening experiments showed that conversion deteriorated at loadings of less than 3wt% for PAL-130 (Table SI4). When increasing the enzyme loading to 25wt%, a conversion of up to 68% amino acid **3** could be achieved within 2 h at 40 °C (Table SI4). For simplified enzyme handling and charging in the scale-up, the lyophilized enzyme powders were charged as a colloidal solution in a mixture of the buffered ammonia solution and water. The engineered PAL enzymes proved to be stable for several hours when stored in solution at room temperature. Expectedly, the reduction of the enzyme loading with every new enzyme generation led to faster clear filtrations for the enzyme removal. Clear filtrations could also be expedited with some of the co-solvents tested, for example DMSO. First attempts of immobilization of the PAL enzymes were in our case only successful with epoxy-substituted beads, which led to a significant reduction of the enzyme activity and processing issues with the epoxy beads due to long wetting times.

Co-solvents: An extensive screening of co-solvents, surfactants and additives did not indicate any hit with a significant influence on the equilibrium conversion to amino acid **3**. Some of the solvents tested, however, were found to increase the initial reaction rate. While for early generations of the engineered PAL enzymes, DMSO or glycols were required to achieve good conversion,[25–27] co-solvents were no longer necessary for the latest generations, PAL-130 and PAL-131.

Impurities: A streamlined manufacturing process for the manufacture afforded the substrate cinnamic acid **2** with around 4–5mol% morpholine content.[2] Use-tests and screenings showed that morpholine in cinnamic acid **2** was not critical, and up to 10mol% morpholine did not have an impact on the conversion (Table SI5). The only by-product observed by HPLC in the PAL-catalyzed conversion of cinnamic acid **2** to α-amino acid **3** was the corresponding β-amino acid **10** in amounts below 0.5a% (Scheme 2). As the formation of the β-amino acid **10** by a background 1,4-addition was not observed when the substrate was subjected to the standard reaction conditions in the

absence of an enzyme, the impurity is potentially generated *via* a phenylalanine amino mutase (PAM) pathway catalyzed by the PAL enzyme.[18,21]

In summary, the screening results of the key parameters for the PAL-catalyzed hydroamination of cinnamic acid **2** to α-amino acid **3** resulted in the following conditions for the standard PAL-catalyzed reaction: The reaction was run in 9–10 M buffered ammonia solution at pH 10±0.5, at a dilution of 9 v/w and with 5wt% (PAL-130) or 2.5wt% (PAL-131) enzyme loading for 24–96 h, applying the temperature gradient in Figure 2f. Under these conditions, an equilibrium conversion of 70–80% was achieved on lab scale.[33] The conditions were then successfully scaled up to a batch size of 2 kg of cinnamic acid **2**, resulting in 81% conversion to amino acid **3** after 71 h with 100 g of enzyme PAL-130.

Work-up of the PAL-catalyzed reaction

With the reaction conditions established, we focused our attention on different work-up strategies. Applying the above conditions, approximately 1–1.5 kg of ammonia had to be used for each kg of substrate **2**; and 20–30% of the substrate **2** could not be converted to the desired product. For a multi ton product such as EMA401 (**1**), this would have resulted in significant amounts of ammonia and cinnamic acid **2** waste streams. During design of the work-up of the PAL reaction, we therefore evaluated the generated waste streams and thoroughly assessed the potential for recycling of ammonia, cinnamic acid **2** and enzyme. Some of the work-up strategies are summarized in Figure 3 and described in more detail below.

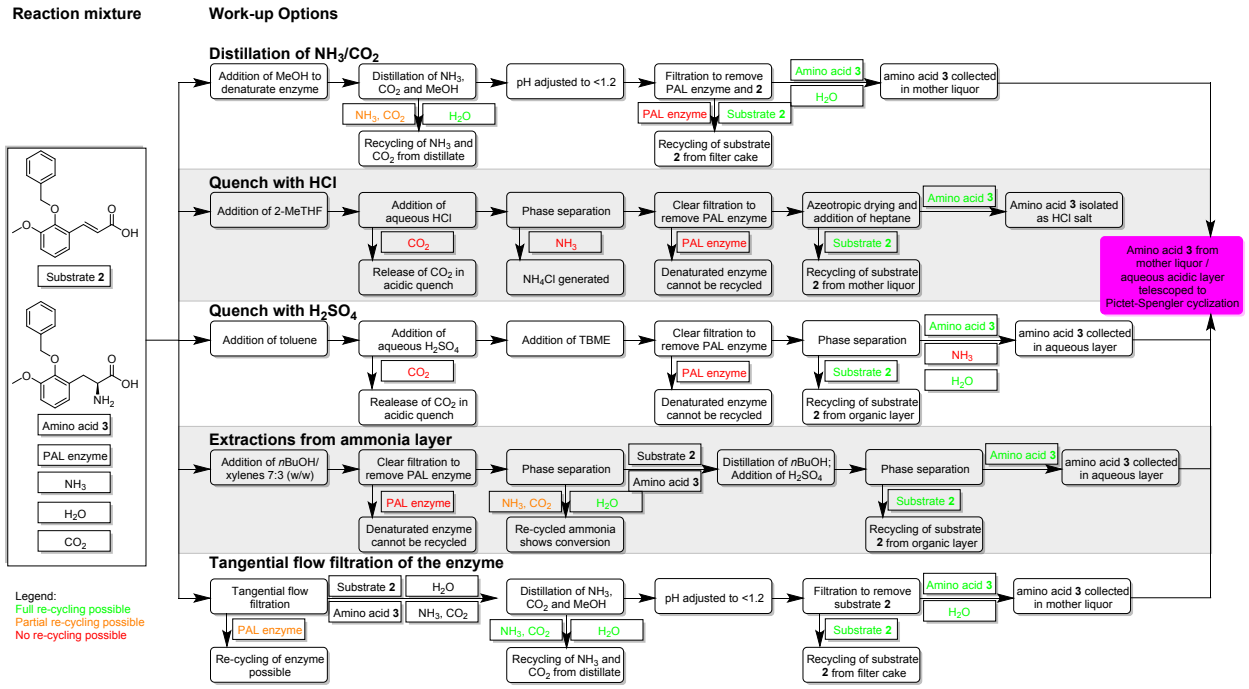


Figure 3. Work-up strategies investigated for the PAL-catalyzed reaction. For details, see the respective sections of the manuscript.

Distillation of NH_3/CO_2: Distillation allowed for the recycling of ammonia and CO_2 by condensation into a second reactor for the next batch. As the hydroamination reaction is reversible, it was of obvious importance to remove or denaturate the enzyme before distillation to avoid the back reaction. As mentioned above, the use of immobilized enzymes as a straightforward way to remove the enzyme from the reaction mixture prior to distillation was not yet practical in our case. We thus evaluated enzyme denaturation

by heat, taking into consideration that the chiral stability of amino acid **3** had previously been found to deteriorate at temperatures above 70 °C under the reaction conditions. As an additional challenge, the stability of the enzyme had been concomitantly improved over the course of the extensive protein engineering, and we found that full denaturation could not be ensured at temperatures below 70 °C, posing the risk of a back reaction upon distillation of ammonia. We therefore investigated the suitability of denaturating *via* chaotropic reagents. For a first scale-up, we selected MeOH to denaturate the PAL enzymes, as MeOH could be removed in the subsequent distillations. Experiments had shown that the PAL-130 enzyme retained activity after addition of 20v% of MeOH, and 50v% MeOH was required to ensure full denaturation of the enzyme (Figure SI4). Even though this approach did not allow for the recycling of ammonia due to contamination with MeOH, denaturation with MeOH was found to be acceptable as a temporary solution, in line with the phase-dependent development targets. Under the applied reaction conditions, the reaction mixture was stirred for 1 h after addition of MeOH to ensure close to full denaturation of the enzyme, followed by subsequent removal of ammonia, CO_2 and MeOH by distillation. The resulting

suspension was then acidified with aqueous H_2SO_4 to pH below 1.2 at 40 °C. To ensure stirrability of the resulting suspension thus obtained, an anchor stirrer was required. The suspension was particularly thick around pH 5.9, corresponding to the iso-electric point of amino acid **3**, and pH 4.2, corresponding to the pK_a of cinnamic acid **2** (Figure 4). At lower pH values, amino acid **3** started to dissolve again, while the enzyme and substrate **2** were suspended and removed *via* clear filtration. The use of filter aids led to significant losses of amino acid **3** in the clear filtration. Collection of the substrate in the filter cake gave the potential for recycling with a simple process. Residual amino acid **3** in the filter cake was extracted by re-slurrying with 1 M aqueous H_2SO_4. The mother and wash liquors were combined and subsequently telescoped to the Pictet-Spengler cyclization to tetrahydroisoquinoline **4** (*vide infra*). This work-up procedure was the preferred option for the scale-up and could be implemented successfully at a batch size of 2 kg of cinnamic acid **2**. Distillations ran smoothly without observation of major sublimation of ammonium salts. An Apovac pump was used in the distillations to trap ammonia. An agitated filter drier with a diameter of 20 cm was used to re-slurry the filter

cake, leading to filtration times of 2 h for the clear filtration and 1 h each for the filtrations after re-slurry.

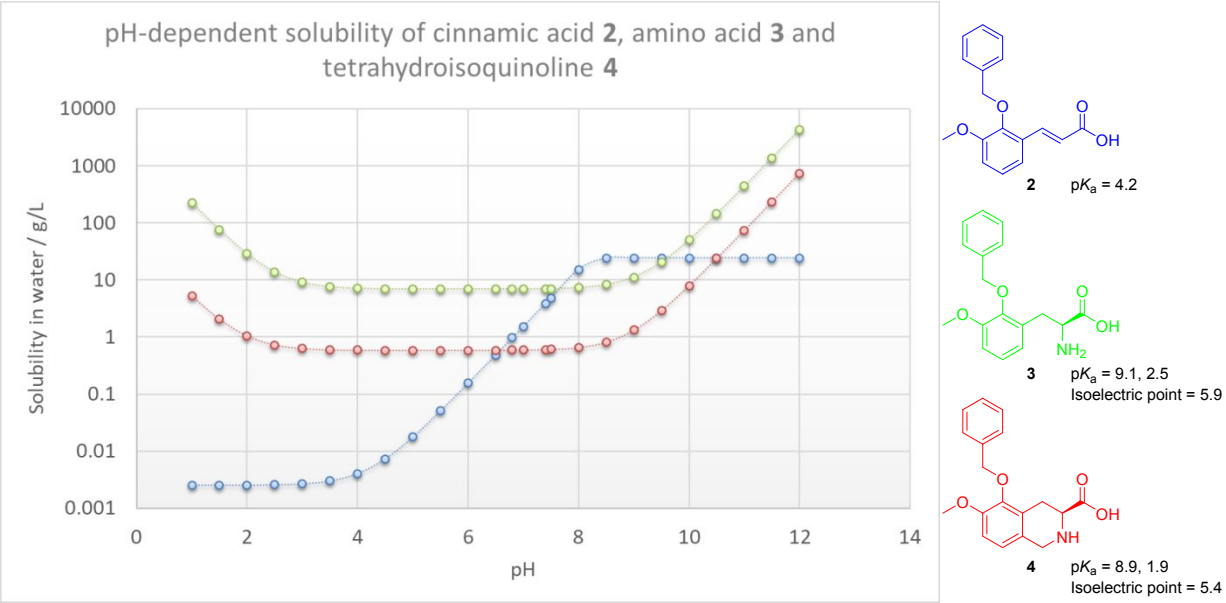


Figure 4. pH-dependent solubility of cinnamic acid **2**, amino acid **3** and tetrahydroisoquinoline **4** in water determined with a Sirius T3 instrument.

Quench with acids: Very high or very low pH is known to denaturate many enzymes. In the PAL-catalyzed hydroamination reaction, the addition of NaOH led to the formation of by-products and was thus abandoned. When acids were added, full denaturation of the PAL enzyme could be ensured, however at the cost of a strong exotherm, the violent

release of large amounts of CO_2 and hence concomitant foaming. In addition, recycling of the thus formed ammonium salts was not as straightforward as compared to the distillation of the ammonium carbonate/carbamate system.

When the reaction mixture was quenched with concentrated aqueous HCl, 2-MeTHF was added before the quench to suppress foaming during the addition of HCl, and to extract the resulting hydrochloride salt of amino acid **3** along with cinnamic acid **2** into the organic layer. Though low, the solubility of the hydrochloride salt of amino acid **3** exceeded the solubility of the zwitterion, and large amounts of 2-MeTHF were required for the extraction. A clear filtration to remove the enzyme buffer layer prior to phase separation was not implemented due to major losses of amino acid **3** in the filter cake, which could only be recovered with larger amounts of 2-MeTHF. The enzyme buffer layer made the phase separations tedious, leading to the presence of residual enzyme in the organic layer and the need for clear filtration of the organic layer. The hydrochloride salt of amino acid **3** was isolated from the organic layer after azeotropic

distillation and addition of heptane. Cinnamic acid **2** was collected in the mother liquor and could be isolated by concentration.

In an alternative process, the buffered ammonia solution obtained at the end of the reaction was quenched with aqueous H_2SO_4. In contrast to the quench with aqueous HCl, we aimed at retaining amino acid **3** in the aqueous acidic layer. This approach allowed to extract cinnamic acid **2** in the organic layer and perform a clear filtration before the phase separation. Though the quench was again strongly exothermic, heat generation and CO_2 release occurred dose-controlled. An organic solvent had to be added to suppress foaming. The solvent of choice was toluene, as other solvents (for example TBME) were either not compatible with the process conditions, or led to racemization in the subsequent step to tetrahydroisoquinoline **4** (for example THF and 2-MeTHF).[34] After the acidic quench, TBME was added to the toluene layer to solubilize cinnamic acid **2** in the organic layer. A clear filtration allowing the removal of the enzyme was implemented, followed by a phase separation . This approach allowed extraction of amino acid **3** into the aqueous acidic layer, while cinnamic acid **2** could be

recycled from the organic layer. In addition, the ammonium sulfate generated during the quench ensured complete denaturation of the enzyme. The aqueous layer was subsequently treated with *para*-formaldehyde, and amino acid **3** was telescoped to the subsequent transformation to tetrahydroisoquinoline **4** (*vide infra*).

Extractions from ammonia buffer: As the quench with acids did not allow for the simple recycling of ammonia, we also investigated an approach relying on extraction of the basic ammonia buffer, potentially even providing a handle to further drive the equilibrium between cinnamic acid **2** and amino acid **3**. A first round of solvent screening indicated that alcoholic solvents were capable of extracting amino acid **3** from the reaction mixture. In a subsequent screening of more than 20 alcohols or solvent mixtures with alcohols, a mixture of *n*BuOH/xylenes 7:3 (w/w) was chosen to extract amino acid **3** from the ammonia buffer, along with cinnamic acid **2**. To our dismay, none of the solvents or solvent mixtures tested favored the extraction of amino acid **3** over the extraction of cinnamic acid **2** from the buffered ammonia layer. Addition of *n*BuOH/xylenes 7:3 (w/w) to the reaction mixture resulted in a biphasic mixture with an

enzyme buffer layer, which was removed by clear filtration. The organic layer containing the cinnamic acid **2** and amino acid **3** was then separated. Attempts to run a continuous extraction with a perforator were stopped as precipitation at the inlet of the organic solvent into the aqueous layer led to clogging. Using *n*BuOH/xylenes led to the co-extraction of significant amounts of the aqueous ammonia buffer into the organic layer, and titrations indicated that the concentration of the aqueous ammonia buffer dropped to only 3.4 M NH_3 when starting from 10 M NH_3. Hence, recycling of ammonia proved difficult with this work-up strategy. The ammonia buffer thus recovered was nevertheless treated with substrate **2** and enzyme PAL-130, leading to 28% conversion in 27 h (Figure SI5). The organic *n*BuOH/xylenes phase was concentrated by distillation to remove residual water, ammonia and *n*BuOH, resulting in a suspension of amino acid **3** and cinnamic acid **2** in xylenes. Aqueous H_2SO_4 was added to dissolve amino acid **3** while cinnamic acid **2** remained dissolved in xylenes at higher temperature. This allowed re-cycling of cinnamic acid **2** from xylenes after phase separation. The aqueous acidic layer containing amino acid **3** was telescoped to the subsequent Pictet-Spengler cyclization to tetrahydroisoquinoline derivative **4** (*vide infra*).

Tangential flow filtration of the enzyme: In parallel to the traditional work-up strategies presented in this study, implementation of an ultrafiltration was investigated to separate the enzyme from the small molecules cinnamic acid **2** and amino acid **3**, taking advantage of the significant size difference between the enzyme (61.5 kDa/subunit of the tetramer), product and residual substrate. The main drivers for the investigations were a potential cost reduction, achieved by recycling of the enzyme, process intensification as a result of increased enzyme loading (*vide supra*) and simplified downstream work-up due to the absence of the enzyme (see discussion of the NH_3/CO_2 distillation). In trial reactions, the reaction was run with PAL-130 for 8 h at 45 °C, followed by cooling to 25 °C. Subsequently, the ultrafiltration with a cellulose membrane (Ultracel from Millipore 10 kDa, 45 cm^2) was investigated and filtration experiments were run for 7–8 h. As a proof of concept, three subsequent batches were successfully run on 50 mL scale, affording 52% yield and complete collection of cinnamic acid **2** and amino acid **3**. In between the individual batches, half of the initial amount of enzyme had to be replaced due to its relatively short half-life of 10 h at 45 °C (three days at 25 °C). While the process was technically robust, the main challenge was the short half-life of the

enzyme. The positive economic impact of the enzyme recycling strategy would substantially benefit from an enzyme evolution directed towards enhanced stability.[35]

Telescope to tetrahydroisoquinoline 4: The preferred work-up options for the PAL step resulted in an aqueous acidic solution of amino acid **3**, which was telescoped into the next step. The aqueous acidic solution was treated with *para*-formaldehyde and warmed to 50 °C until full conversion was achieved. Tetrahydroisoquinoline **4** usually precipitated at 50 °C. The pH was then adjusted to 5.4, corresponding to the isoelectric point of tetrahydroisoquinoline **4** (Figure 4). In order to maintain a well stirrable suspension, the pH adjustment was carried out at 40 °C, and tetrahydroisoquinoline **4** was collected by filtration after cooling to 20 °C. The filter cake was washed with water and acetone affording tetrahydroisoquinoline **4** in greater than 60% isolated yield and an er greater than 99.9:0.1. Cinnamic acid **2** was the major impurity in tetrahydroisoquinoline **4** at levels around 0.5 a% and was found to be purged in the subsequent synthetic step to EMA401 (**1**). In addition, the strongly acidic conditions (pH

0.5–1.0) of the Pictet-Spengler cyclization caused the cleavage of the benzyl group, leading to formation of phenol **11** at levels below 0.2 a% (Scheme 2). The telescope to the Pictet-Spengler cyclization was performed successfully on kilogram scale and provided a total of 1.3 kg (59%) of tetrahydroisoquinoline **4** in high purity (99.5 a%) and excellent optical purity (99.9:0.1 er). Unlike in the Pictet-Spengler process in an aqueous buffer system used for the preparation of tetrahydroisoquinoline **4** from the isolated phenylalanine derivative **3**, no floating of tetrahydrisoquinoline **4** on the aqueous buffer layer was observed in the scale-up.[36]

Experimental procedure for the PAL-catalyzed synthesis of (3*S*)-5-(benzyloxy)-6-methoxy-1,2,3,4-tetrahydroisoquinoline-3-carboxylic acid (**4**)

To an emulsion of cinnamic acid **2** (20 g, 1.0 eq.) in ammonia buffer (170 mL, 9.3 M, prepared by suspending ammonium carbonate (70 g) in H_2O (120 mL) and adjustment of the pH to 10 with 25wt% aqueous NH_3 (40 g)) was added a colloidal solution of enzyme PAL-130 (1 g) in a 1:1 mixture of the aqueous ammonium carbonate solution (5

mL) and H_2O (5 mL). The resulting turbid emulsion was warmed to 45 °C and stirred for 8 h at this temperature, then cooled to 20 °C in 8 h and stirred for another 80 h (76% conversion by HPLC, UV factor corrected). MeOH (60 mL) was added, and the reaction mixture was stirred for 1 h. H_2O (60 mL) was added, then 60 mL of solvent were distilled out of the reactor (70 °C, 300–600 mbar). This procedure was repeated two more times. The resulting suspension was cooled to 40 °C followed by addition of 2.5 M aq. H_2SO_4 (37.7 g) until pH <1.2 (1.08 measured). The resulting mixture was cooled to 25 °C and stirred for 16 h, then filtered over a K-900 filter plate (Seitz). The filter cake was re-slurried with 1 M H_2SO_4 (50 g), followed by filtration. Re-slurrying and filtration were repeated once more. The combined mother and wash liquors were treated with *para*-formaldehyde (3.2 g), warmed to 50 °C and stirred for 16 h. After cooling to 40 °C, the pH was adjusted to 5.5 with 50wt% aq. NaOH solution (26.4 g). The suspension was cooled to 20 °C, filtered, and the filter cake was washed with H_2O (2 × 60 g) and acetone (2 × 60 g). The wet product was dried at 50 °C under vacuum to afford tetrahydroisoquinoline **4** (15 g, 68%) as a white solid.

HPLC purity: 99.5a%: chiral purity: 99.9:0.1 er; ^{1}H NMR (400 MHz, D_3CCO_2D): δ = 3.02 (dd, J = 17.6, 11.0 Hz, 1 H), 3.48 (dd, J = 17.6, 5.4 Hz, 1 H), 3.89 (s, 3 H), 4.16 (dd, J = 11.0, 5.3 Hz, 1 H), 4.33–4.51 (m, 2 H), 5.05 (d, J = 1.7 Hz, 2 H), 6.90–7.02 (m, 2 H), 7.26–7.40 (m, 3 H), 7.42–7.51 ppm (m, 2 H); ^{13}C NMR (101 MHz, D_3CCO_2D): δ = 25.05, 45.21, 56.40, 57.16, 75.26, 112.89, 121.68, 123.13, 127.27, 129.04, 129.34 (2 C), 129.40 (2 C), 138.62, 146.19, 153.39, 174.36 ppm; ESI-MS: *m/z* = 314.2 ($[M + H]^+$, calcd for $C_{18}H_{20}NO_4^+$: 314.1).

Conclusion

In summary, we have presented the development of conditions for the use of (engineered) phenylalanine ammonia lyase enzymes, which were successfully employed for the hydroamination reaction of cinnamic acid derivative **2** to amino acid **3**. The telescoped process from cinnamic acid **2** *via* amino acid **3** to tetrahydroisoquinoline **4** was successfully scaled up to a batch size of 2 kg of substrate **2**, resulting in 81% conversion to amino acid **3** and an isolated yield of 59% for tetrahydroisoquinoline **4**,

with a chiral purity of 99.9:0.1 er. The biocatalytic transformation is part of an optimized synthetic route of EMA401 and reduces the length of the synthesis by three steps compared to the previous chemocatalytic route. In addition, the process uses readily available reagents, is environmentally more sustainable than the chemocatalytic route and economically attractive. We hope that our study will contribute to future applications of this enzyme class in chemical development.

ASSOCIATED CONTENT

Supporting Information. The following files are available free of charge.

Computational details, additional screening results, experimental part and ^{1}H and ^{13}C NMR spectra of tetrahydroisoquinoline **4**. (PDF)

AUTHOR INFORMATION

Corresponding Author

*Dr Leo A. Hardegger, Chemical & Analytical Development, WSJ-360.10.02, Novartis Campus, 4002 Switzerland; leo.hardegger@novartis.com

Present Addresses

≠Eric Sidler, Department of Chemistry, University of Basel, St. Johanns-Ring 19, 4056 Basel, Switzerland.

Author Contributions

The manuscript was written by L.A.H through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENT

Foremost, we are very grateful to Dr Scott Novick, Dr David Entwistle, Dr Mandy Vink and Dr Oscar Alvizio (all Codexis) for the excellent collaboration, fruitful discussions, and for providing us with many different PAL variants. We thank Dr Stéphane Rodde

(NIBR) for determining the pH-dependent solubility values. We would like to thank Michael Marti and Vladimir Milovanovic for analytical support, Dr Pascale Hoehn and Marian Lanz for running the safety measurements, and the group of Prof. Liese at TUHH and Dr Radka Snajdrova, Dr Elina Siirola and Fabian Eggimann (all Novartis) for fruitful discussions. We want to thank Nikola Kovacic, Luca Hermann and Manfred Schadock for performing the scale-up. We thank Dr Greg Mann for helpful comments on the manuscript.

ABBREVIATIONS

Er: enantiomeric ratio; 2-MeTHF 2-methyltetrahydrofuran; MIO, 4-methylideneimidazole-5-one; PAL, phenylalanine ammonia lyase; THF, tetrahydrofuran.

REFERENCES

(1) Gao, F.; Hardegger, L. A.; Humair, R.; Kleinbeck-Riniker, F. K.; Rampf, F. A.; Ruch, T.; Schlama, T.; Wietfeld, B. Biocatalytic Synthesis of Olodanrigan (EMA401) from 3-(2-(Benzyloxy)-3-methoxyphenyl)propenoic Acid with

Phenylalanine Ammonia Lyase. WO2020/012266, 16 Jan 2020.

(2) Sidler, E.; Humair, R.; Hardegger, L. A. Towards a Scalable Synthesis and Process for EMA401. Part II: Development and Scale-up of a Pyridine- and Piperidine-free Knoevenagel-Doebner Condensation *Org. Process Res. Dev.* **2020**, *ZZ*, ZZ-ZZ.

(3) Ahmed, S. T.; Weise, N. J.; Parmeggiani, F.; Turner, N. J. Applications of Phenylalanine Ammonia Lyases in Synthesis Access to Enantiopure Arylalanines from Laboratory Scale to Industrial Production. *Chim. Oggi/Chemistry Today* **2017**, *35*, 30–33.

(4) Parmeggiani, F.; Weise, N. J.; Ahmed, S. T.; Turner, N. J. Synthetic and Therapeutic Applications of Ammonia-lyases and Aminomutases. *Chem. Rev.* **2018**, *118*, 73–118.

(5) Renard, G.; Guilleux, J. C.; Bore, C.; Malta-Valette, V.; Lerner, D. A. Synthesis of L-Phenylalanine Analogs by *Rhodotorula glutinis*. Bioconversion of Cinnamic Acid

Derivatives. *Biotechnol. Lett.* **1992**, *14*, 673–678.

(6) Gloge, A.; Zoń, J.; Kövári, Á.; Poppe, L.; Rétey, J. Phenylalanine Ammonia-Lyase: The Use of Its Broad Substrate Specificity for Mechanistic Investigations and Biocatalysis–Synthesis of L-Arylalanines. *Chem. - A Eur. J.* **2000**, *6*, 3386–3390.

(7) Bartsch, S.; Bornscheuer, U. T. Mutational Analysis of Phenylalanine Ammonia Lyase to Improve Reactions Rates for Various Substrates. *Protein Eng. Des. Sel.* **2010**, *23*, 929–933.

(8) Lovelock, S. L.; Lloyd, R. C.; Turner, N. J. Phenylalanine Ammonia Lyase Catalyzed Synthesis of Amino Acids by an MIO-Cofactor Independent Pathway *Angew. Chem. - Int. Ed.* **2014**, *53*, 4652–4656.

(9) Parmeggiani, F.; Lovelock, S. L.; Weise, N. J.; Ahmed, S. T.; Turner, N. J. Synthesis of D-and L-Phenylalanine Derivatives by Phenylalanine Ammonia Lyases: A Multienzymatic Cascade Process. *Angew. Chem. - Int. Ed.* **2015**, *54*,

4608–4611.

(10) Ahmed, S. T.; Parmeggiani, F.; Weise, N. J.; Flitsch, S. L.; Turner, N. J. Chemoenzymatic Synthesis of Optically Pure L- and D-Biarylalanines through Biocatalytic Asymmetric Amination and Palladium-Catalyzed Arylation. *ACS Catal.* **2015**, *5*, 5410–5413.

(11) Ahmed, S. T.; Parmeggiani, F.; Weise, N. J.; Flitsch, S. L.; Turner, N. J. Synthesis of Enantiomerically Pure Ring-Substituted L-Pyridylalanines by Biocatalytic Hydroamination. *Org. Lett.* **2016**, *18*, 5468–5471.

(12) Rowles, I.; Groenendaal, B.; Binay, B.; Malone, K. J.; Willies, S. C.; Turner, N. J. Engineering of Phenylalanine Ammonia Lyase from *Rhodotorula graminis* for the Enhanced Synthesis of Unnatural L-Amino Acids. *Tetrahedron* **2016**, *72*, 7343–7347.

(13) Ahmed, S. T.; Parmeggiani, F.; Weise, N. J.; Flitsch, S. L.; Turner, N. J. Engineered Ammonia Lyases for the Production of Challenging Electron-Rich

L-Phenylalanines. *ACS Catal.* **2018**, *8*, 8–11.

(14) Paizs, C.; Katona, A.; Rétey, J. The Interaction of Heteroaryl-Acrylates and Alanines with Phenylalanine Ammonia-Lyase from Parsley. *Chem. - Eur. J.* **2006**, *12*, 2739–2744.

(15) De Lange, B.; Hyett, D. J.; Maas, P. J. D.; Mink, D.; Van Assema, F. B. J.; Sereinig, N.; De Vries, A. H. M.; De Vries, J. G. Asymmetric Synthesis of (*S*)-2-Indolinecarboxylic Acid by Combining Biocatalysis and Homogeneous Catalysis. *ChemCatChem* **2011**, 289–292.

(16) Dreßen, A.; Hilberath, T.; Mackfeld, U.; Rudat, J.; Pohl, M. Phenylalanine Ammonia Lyase from *Arabidopsis thaliana* (At PAL2): A potent MIO-enzyme for the Synthesis of Non-canonical Aromatic alpha-Amino Acids. *J. Biotechnol.* **2017**, *258*, 158–166.

(17) Gloge, A; Langer, B.; Poppe, L.; Rétey, J. The Behavior of Substrate Analogues and Secondary Deuterium Isotope Effects in the Phenylalanine Ammonia-Lyase

Reaction. *Arch. Biochem. Biophys.* **1998**, *359*, 1–7.

(18) Weise, N. J.; Parmeggiani, F.; Ahmed, S. T.; Turner, N. J. The Bacterial Ammonia Lyase EncP: A Tunable Biocatalyst for the Synthesis of Unnatural Amino Acids. *J. Am. Chem. Soc.* **2015**, *137*, 12977–12983.

(19) Viergutz, S.; Poppe, L.; Tomin, A.; Rétey, J. Mechanistic Investigation of Phenylalanine Ammonia Lyase by Using N-Methylated Phenylalanines. *Helv. Chim. Acta* **2003**, *86*, 3601–3612.

(20) Hansch, C.; Leo, A; Taft, R. W. A. A survey of Hammett Substituent Constants and Resonance and Field Parameters. *Chem. Rev.* **1991**, *91*, 165–195.

(21) Szymanski, W.; Wu, B.; Weiner, B.; De Wildeman, S.; Feringa, B. L.; Janssen, D. B. Phenylalanine Aminomutase-Catalyzed Addition of Ammonia to Substituted Cinnamic Acids: a Route to Enantiopure α- and β-Amino Acids. *J. Org. Chem.* **2009**, *74*, 9152–9157.

(22) Weise, N. J.; Ahmed, S. T.; Parmeggiani, F.; Siirola, E.; Pushpanath, A.; Schell,

U.; Turner, N. J. Intensified Biocatalytic Production of Enantiomerically Pure Halophenylalanines from Acrylic Acids using Ammonium Carbamate as the Ammonia Source. *Catal. Sci. Technol.* **2016**, *6*, 4086–4089.

(23) Novick, S. J; García R. D.; Ching, C.; Dellas, N.; Entwistle, D.; Mitchell, V. Engineered Phenylalanine Ammonia Lyase Polypeptides. WO2020/013951, 16 Jan 2020.

(24) Yamada, S.; Nabe, K.; Izuo, N. Production of L-Phenylalanine from trans-Cinnamic Acid with *Rhodotorula glutinis* Containing L-Phenylalanine Ammonia-lyase Activity. *Appl. Environ. Microbiol.* **1981**, *42*, 773–778.

(25) De Vries, A. H. M.; De Vries, J. G.; Van Assema, F. B. J.; De Lange, B.; Daniel, M.; Hyett, D. J.; Maas, P. J. D. Process for the Preparation of Enantiomerically Enriched Indoline-2-carboxylic Acid. WO2006/069799, 06 Jul 2006.

(26) Van Assema, F. B. J.; Sereinig, N. Methods for Producing Optically Active Phenylalanine Compounds from Cinnamic Acid Derivatives Employing a

Phenylalanine Ammonia Lyase Derived from *Idiomarina loihiensis*. WO2008/031578, 20 Mar 2008.

(27) Hauer, B.; Schneider, N.; Drew, D.; Ditrich, K.; Turner, N. J.; Nestl, B. M. Biocatalyst for Catalytic Hydroamination. WO2011/012632. 03 Feb 2011.

(28) Parmeggiani, F.; Ahmed, S. T.; Weise, N. J.; Turner, N. J. Telescopic One-pot Condensation-hydroamination Strategy for the Synthesis of Optically Pure L-Phenylalanines from Benzaldehydes. *Tetrahedron* **2016**, *72*, 7256–7262.

(29) Zhao, J.-S.; Yang, S.-K. Red Yeast Catalyzed Amination of Olefin Bonds and Synthesis of Optically Pure S-Amino Acids. *Chinese J. Chem.* **1995**, *13*, 241–245.

(30) Halstensen, M.; Jilvero, H.; Jinadasa, W. N.; Jens, K. J. Equilibrium Measurements of the NH_3-CO_2-H_2O System: Speciation Based on Raman Spectroscopy and Multivariate Modeling. *J. Chem.* **2017**, *2017*.

(31) Wen, N.; Brooker, M. H. Ammonium Carbonate, Ammonium Bicarbonate, and Ammonium Carbamate Equilibria: A Raman Study. *J. Phys. Chem.* **1995**, *99*,

359–368.

(32) Swann, W. E. Production of L-Phenylalanine. EP0165757, 27 Dec 1985.

(33) Conversion was monitored by HPLC. As the UV factors vary significantly between substrate and product, the relative mol% is given based on the UV factor at a given wavelength.

(34) Up to 6wt% of THF or MeTHF were telescoped with the aqueous acidic layer into the subsequent Pictet-Spengler cyclization. Whenever THF or 2-MeTHF were used in the extraction, an erosion of the enantiomeric purity of isolated tetrahydroisoquinoline **4** was observed.

(35) Stimple, S. D.; Smith, M. D.; Tessier, P. M. Directed Evolution Methods for Overcoming Trade-offs Between Protein Activity and Stability. *AIChE J.* **2020**, *6*.

(36) Hardegger, L. A.; Mallet, F.; Bianchi, B.; Cai, C.; Grand-Guillaume-Perrenoud, A.; Humair, R.; Kaehny, R.; Lanz, S.; Li, C.; Li, J.; Rampf, F. A.; Shi, L.; Spoendlin, C.; Teng, S.; Stäuble, J.; Tian, X.; Wietfeld, B.; Yang, Y.; Yu, B.; Zepperitz, C.;

Zhang, X.; Zhang, Y. Towards a Scalable Synthesis and Process for EMA401. Part I: Late Stage Process Development, Route Scouting and ICH M7 Assessment *Org. Process Res. Dev.* **2020**, *YY* (YY), YY.