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Biocatalysis Very Important Paper

Engineered Phenylalanine Ammonia-Lyases for the Enantioselective Synthesis of Aspartic Acid Derivatives

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Abstract: Biocatalytic hydroamination of alkenes is an efficient and selective method to synthesize natural and unnatural amino acids. Phenylalanine ammonia-lyases (PALs) have been previously engineered to access a range of substituted phenylalanines and heteroarylalanines, but their substrate scope remains limited, typically including only arylacrylic acids. Moreover, the enantioselectivity in the hydroamination of electron-deficient substrates is often poor. Here, we report the structure-based engineering of PAL from *Planctomyces brasiliensis* (*Pb*PAL), enabling preparative-scale enantioselective hydroaminations of previously inaccessible yet synthetically useful substrates, such as amide- and ester-containing fumaric acid derivatives. Through the elucidation of cryo-electron microscopy (cryo-EM) *Pb*PAL structure and screening of the structure-based mutagenesis library, we identified the key active site residue L205 as pivotal for dramatically enhancing the enantioselectivity of hydroamination reactions involving electron-deficient substrates. Our engineered PALs demonstrated exclusive α-regioselectivity, high enantioselectivity, and broad substrate scope. The potential utility of the developed biocatalysts was further demonstrated by a preparative-scale hydroamination yielding *tert*-butyl protected L-aspartic acid, widely used as intermediate in peptide solid-phase synthesis.

Biocatalysis is an increasingly useful approach for the synthesis of natural and non-canonical amino acids (ncAAs), capitalizing on mild reaction conditions in aqueous buffers and tunable biocatalysts allowing effective control of regio- and enantioselectivities. Particularly, both native and engineered members of carbon-nitrogen (C–N) lyases family (EC 4.3.X.X) have emerged as powerful biocatalysts for the production of enantiopure ncAAs via asymmetric hydroaminations of α,β-unsaturated mono- or di-carboxylic acids at elevated ammonia concentrations.[1,2] Despite offering distinct advantages, only a small number of C–N lyases, namely aspartate ammonia-lyases (DALs, EC 4.3.1.1) and phenylalanine ammonia-lyases (PALs, EC 4.3.1.24) (Figure 1A), have found applications in biotechnological and industrial contexts. DALs, naturally catalyzing the reversible deamination of L-aspartic acid, are used industrially for producing L-aspartic acid from fumaric acid at high concentration of ammonium salts.[3,4] Recently, to broaden its synthetic versatility, the catalytic pocket of DAL was computationally redesigned to accommodate substituted acrylates (Figure 1B).[5,6] PALs are employed in industrial hydroamination processes such as the multi-ton scale production of 2-chloro-L-phenylalanine by DSM[7] and the synthesis of a crucial intermediate for EMA401 by Novartis.[8] Most significantly, in contrast to the β-regioselectivity observed with DALs, PALs catalyze the addition of ammonia to the α-position of the acrylic acid with anti-Michael regioselectivity. This reaction, challenging to achieve with current synthetic or enzymatic methods, has a high synthetic value.

Enzyme engineering efforts to enhance the practical applications of PALs focused on increasing substrate versatility for synthesizing various non-natural ortho-, meta-, and para-substituted L-phenylalanines, utilizing PALs from various sources, including *Petroselinum crispum* (*Pc*PAL)[9–15], *Anabaena variabilis* (*Av*PAL),[8,16–18] *Arabidopsis thaliana* (*At*PAL),[19,20] and *Planctomyces brasiliensis* (*Pb*PAL)[21] (Figure 1B). While engineered PALs efficiently catalyze hydroamination reactions on various substituted aromatic arylacrylic acids, they still clearly exhibit a narrow specificity for this substrate class. Phenylalanine analogues, such as L-(2,5-dihydrophenyl)alanine[22] and β-cyclooctatetraenylalanine,[23] were studied in ammonia elimination reactions, demonstrating the acceptance of non-aromatic substrates by wild-type PALs. However, efforts to utilize PALs in the synthesis of non-aromatic α-amino acids have been met only with limited success. Notably, engineered *Pc*PAL catalyzed the hydroamination of (2*E*,4*E*)-5-phenylpenta-2,4-dienoic acid to L-styrylalanine with a 19% isolated yield,[9] and immobilized *Pc*PAL enabled the addition of ammonia to (*E*)-pent-2-ene-4-ynoate, producing L-propargylglycine in a modest

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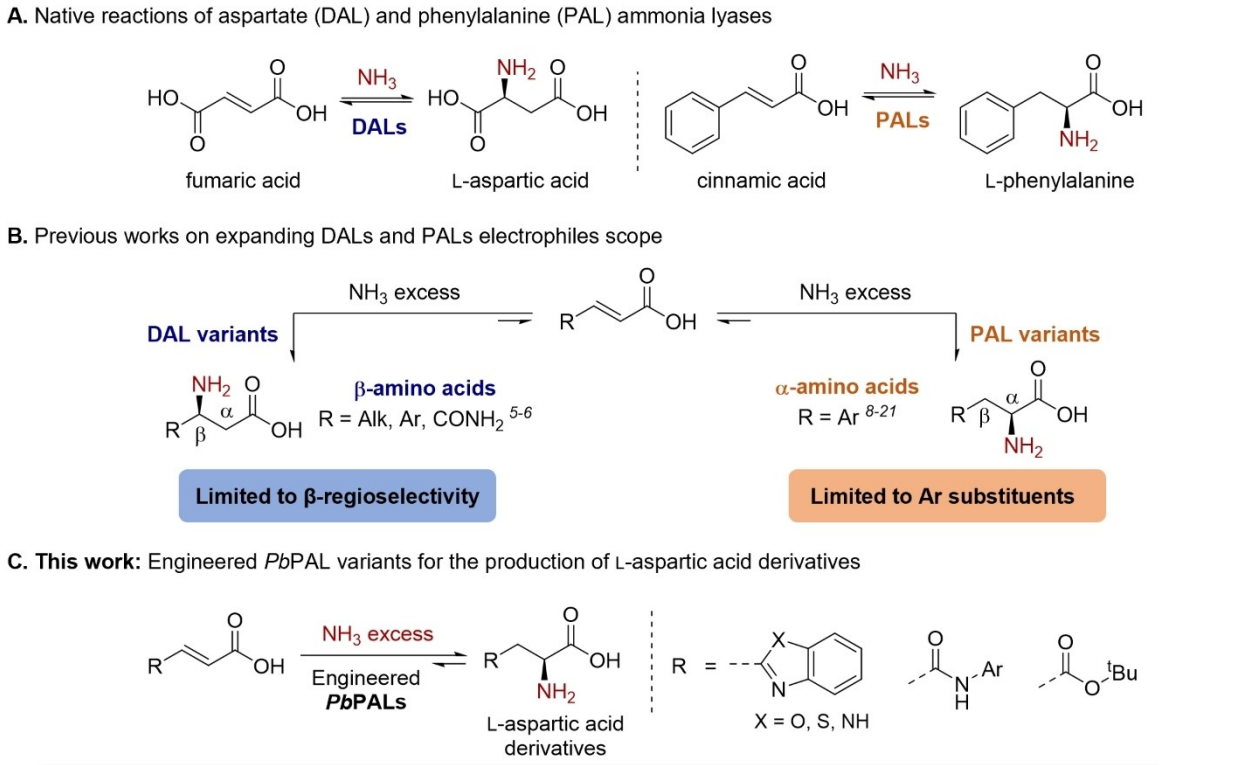

Figure 1. Synthesis of α- and β-amino acids via hydroamination catalyzed by C–N lyases. A. Native reactions of aspartase (DALs) and phenylalanine ammonia-lyases (PALs). **B**. Previous works on expansion of DALs and PALs electrophile scope. **C**. This work: engineered *Pb*PALs with improved stereoselectivity for the hydroamination of fumaric acid derivatives.

isolated yield.[24] Consequently, broadening electrophiles substrate scope of PALs represents a significant challenge.

We envisioned that expanding PAL substrate scope to include fumaric acid derivatives would represent a highly advantageous development, enabling the synthesis of valuable aspartic acid derivatives with α-selectivity, which would complement the existing β-selective DALs (Figure 1C).

We hypothesized that fumaric acid derivatives featuring electron-withdrawing groups attached to the β-carbon would favor α-regioselective ammonia addition due to the directing effect of these groups, similar to the preferred α-regioselectivity observed previously in bacterial PAL[25] and phenylalanine aminomutase-mediated hydroaminations of electron-poor aryl acrylates.[26–28] Moreover, we anticipated that fumaric acid derivatives, being more activated substrates compared to cinnamic acid, would display variations in hydroamination enantioselectivity, as observed with 4-nitro-cinnamic[16] acid and 2- and 4-pyridinylacrylic acids.[29] To address this challenge, we selected highly stable, promiscuous *Pb*PAL and devised a structure-guided engineering strategy. This approach involved the identification of key active side residues L90, F93 and L205 responsible for the stereoselectivity of hydroamination, followed by the creation of a targeted library of variants. Although the position corresponding to L205 in *Pb*PAL (L256 in *Pc*PAL, L257 in *At*PAL2, L266 in PAL from *Rhodosporidium toruloides* (*Rt*PAL), respectively) was previously subjected to mutagenesis in eukaryotic PALs to improve the yields of hydroamination,[11,20] it has not been used to improve the enantioselectivity of these reactions. Likewise, in a study uncovering *Av*PAL variants with altered enantioselectivity for 4-nitrocinnamic acid hydroamination conducted by Turner and co-workers, although 48 residues near the substrate binding site were subjected to NNK site-saturation mutagenesis, the corresponding L219 residue (equivalent to L205 in *Pb*PAL) was omitted.[30] Thus, our strategy based on utilizing only the hydrophobic pocket residues to modulate enantioselectivity stands as a groundbreaking method for enhancing the stereoselective properties of PALs.

We postulated that by screening wild-type PALs from various biological sources using heterocyclic substrate[31] (*E*)-3-(benzo[d]oxazol-2-yl)acrylic acid (**1a**), we could identify potential candidates for engineering PALs with the ability to perform efficient hydroamination on fumaric

acid derivatives. Substrate **1a** features a constrained amide conjugated to the phenyl ring. Beyond its potential as a promising starting point for investigating wild-type PALs promiscuity, the corresponding hydroamination product **2a** represents a valuable pharmacophore. Indeed, the benzoxazole heterocycle is a common structural motif in ligands designed for a wide spectrum of receptors,[32,33] including benzoxazole-alanine ones.[34–36]

We first selected two PALs from eukaryotic sources, namely, *Petroselinum crispum* (*Pc*PAL) and *Arabidopsis thaliana* (*At*PAL2) PALs and two PALs from bacterial sources, namely, *Anabaena variabilis* (*Av*PAL) and *Planctomyces brasiliensis* (*Pb*PAL) PALs as candidates since they previously demonstrated broad substrate scope range and high potential in evolvability. After expression and purification as recombinant proteins in *Escherichia coli*, all wild-type PALs demonstrated catalytic efficiencies in the hydroamination of the natural substrate, cinnamic acid, into L-phenylalanine (Table S5). The thermal stabilities of the purified wild-type PALs were subsequently evaluated, unveiling that PALs sourced from bacterial origins exhibited superior stability compared to their eukaryotic counterparts, with *Av*PAL displaying a T_m of 70.0 ± 0.3 °C and the remarkably stable *Pb*PAL showing a T_m of 81.5 ± 0.6 °C (Table S4).

The reaction was first conducted with 2.5 mM of substrate **1a** (Figure 2) and 0.1 mol % of wild-type PALs (in all cases mol % refers to the monomeric form of PALs). Both *Av*PAL and *Pb*PAL produced the desired amination product **2a**, with a high conversion (> 95 %). The wild-type PALs exhibited elevated K_m and k_{cat} values during the ammonia addition to **1a** compared to cinnamic acid (Table S6). Upon increasing **1a** concentration to 50 mM only *Pb*PAL achieved a 96 % conversion rate while *Av*PAL afforded a moderate conversion of 54 %. All of the screened wild-type PALs exhibited low enantioselectivity, with enantiomeric excess (*ee*) of (*S*)-benzoxazole alanine ranging from 10 to 38 %. Given its high thermal stability and high conversion rate of **1a**, we decided to use *Pb*PAL as a promising scaffold for further engineering.

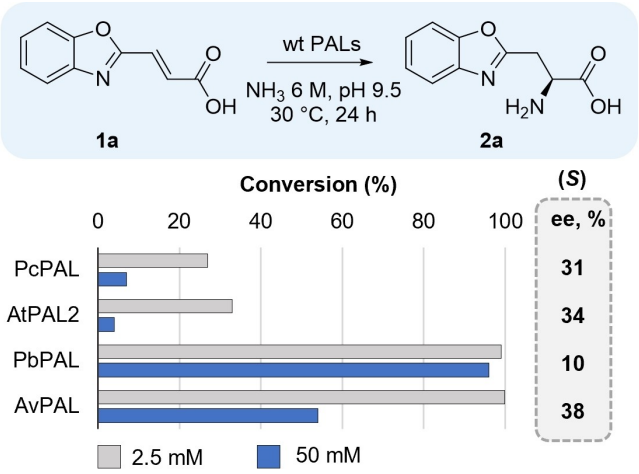

Figure 2. Selected wild-type PALs for the hydroamination of (*E*)-3-(benzo[d]oxazol-2-yl)acrylic acid (1a). Experimental conditions: **1a** 2.5 mM, 0.1 mol % wild-type PALs, ammonium carbamate 6 M, pH 9.5, 30 °C, 24 h (grey bars); Experimental conditions: **1a** 50 mM, 5 wt % wild-type PALs, ammonium carbamate 6 M, pH 9.5, 30 °C, 24 h (blue bars). Conversion and enantiomeric excess were determined by HPLC.

In the absence of an elucidated X-ray structure of the *Pb*PAL enzyme, we turned our attention towards cryo-electron microscopy (cryo-EM). Cryo-EM has become highly potent and is increasingly utilized in enzyme engineering owing to progress in both hardware and software utilized for data analysis.[37] Notably, the approach offered advantages by circumventing the tedious process of crystallization, and allowed freely selecting the buffer for measurement. To gain insights into the catalytic pocket, we formed a complex of *Pb*PAL in 50 mM Tris buffer, 300 mM NaCl at pH 8.8 with the previously characterized strong inhibitor (*R*)-(1-amino-2-phenylethyl)phosphonic acid ((*R*)-APEP), which serves as phosphonic acid analog of the natural substrate L-phenylalanine.[38] The corresponding cryo-EM structure was successfully obtained (PDB ID: 9EQ5) as shown in Figure 3A. Our structural analysis revealed a tetrameric assembly in the overall structure. Superposition of the Cα coordinates of all residues displayed an overall root mean square deviation (rmsd) of 0.664 Å when compared to a previously reported X-ray structure of *Av*PAL (PDB: 2NYN).[39] Notably, the catalytic pockets were fully occupied by (*R*)-APEP, and we could observe the flexible loop stabilized in the catalytically relevant "loop-in" conformation. The structural alignment with previously reported complex of eukaryotic *Pc*PAL with (*R*)-APEP[38] reveals remarkable congruence, despite a sequence similarity of only 33 % (Figure S13). The significant difference in the hydroamination of **1a** between *Pc*PAL and *Pb*PAL underscores the complexity of enzyme function and emphasizes the potential impact of factors beyond mere structural features, such as dynamic alterations or allosteric modulation.

To facilitate the elucidation of key factors influencing hydroamination enantioselectivity in *Pb*PAL, we conducted flexible docking of **1a** into the enzyme's active site using AutoDock Vina.[40] Interestingly, both pro-*R* and pro-*S* **1a** orientations were among poses with highest affinities (Figure 3B) (see the Supporting Information Section 7 for details.). The 4.8 Å proximity of L205 to the β-carbon of **1a** suggested its potential to influence ligand orientation, thereby possibly impacting the enantioselectivity of the hydroamination reaction. Following our docking study, the hydrophobic amino acid residues L90, F93 situated within < 4.0 Å to the phenyl ring of **1a**, and L205 were chosen for site-directed mutagenesis. The mutagenesis of these residues involved replacing L205 with smaller residues, either alanine or valine (L205 A and L205 V), L90 with alanine (L90 A), and F93 with valine (F93 V), resulting in the generation of 4 mono-, 4 double-, and 2 triple-mutants of *Pb*PAL, designated as **P1** to **P10** (Figure 3C). Hydroamination of **1a** with **P1**-**P10** variants unveiled that the triple mutants **P9** (L90 A/F93 V/L205 V) and **P10** (L90 A/F93 V/L205 A) generated

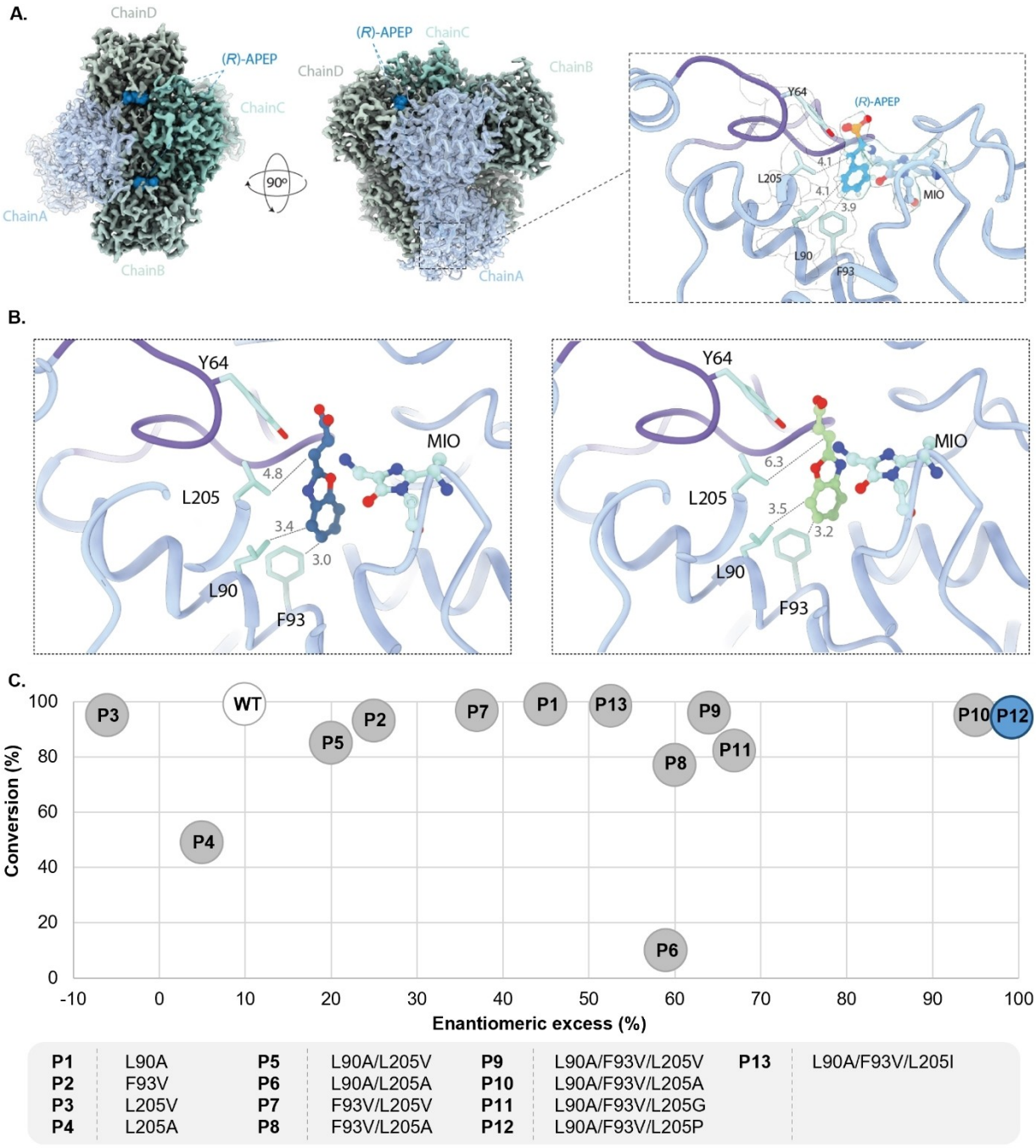

Figure 3. Structure-guided engineering of *Pb*PAL. **A**. Cryo-EM structure of *Pb*PAL (blue gradiant) in complex with (*R*)-APEP (deep sky blue, shown as spheres). **B**. *Pb*PAL with pro-*S* (left) and pro-*R* (right) **1a** modeled into the active site showing distances in Å from L205 to the β-carbon, and from L90 and F93 to the aromatic carbons. **C**. Structure-guided library of *Pb*PAL variants and **1a** hydroamination screening results. Experimental conditions: 2.5 mM **1a**, 0.1 mol% *Pb*PAL variants, NH_3 6 M, pH 9.5, 30 °C, 24 h. Conversion and enantiomeric excess were determined by HPLC. MIO = 3,5-dihydro-5-methylidene-4H-imidazole-4-one.

L-**2a** with enhanced enantiomeric excesses of 64 % and 93 %, respectively (Table S12). Given the enhancement in enantioselectivity achieved with both triple mutants, we decided to introduce three additional small hydrophobic amino acid residues (G, P, I) at position L205 resulting in variants **P11**-**P13**. A further increase in enantioselectivity was achieved with **P12** (L90 A/F93 V/L205P) with an enantiomeric excess of 96 %.

We then explored the preparative-scale hydroamination of **1a** and of both related aromatic thioamide (**1b**) and imidoamide (**1c**) fumaric acid derivatives (Figure 4). Using 5.0 wt % of **P12**, **2a**, and **2b**[41] were prepared in 83 % and 85 % yields, and 96 % and 98 % *ee*, respectively.

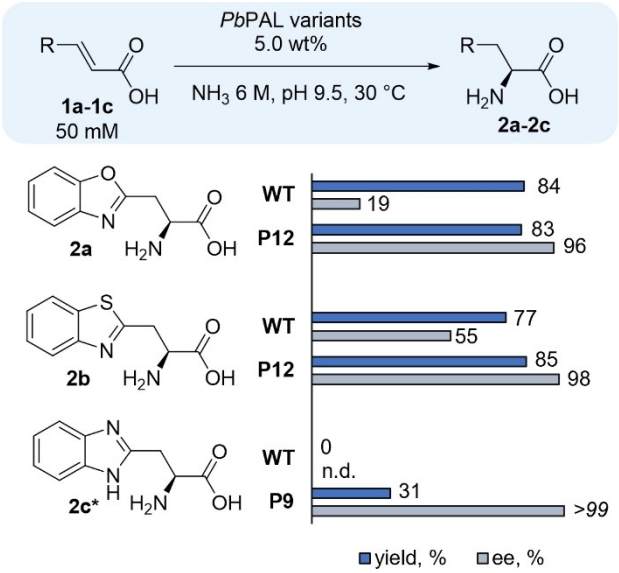

Figure 4. Preparative-scale hydroaminations by engineered *Pb*PAL variants. Experimental conditions: **1a–1b** 50 mM, 5.0 wt% PAL, NH_3 6 M, pH 9.5, 30 °C, 12 h. Isolated yields, 0.5 mmol scale. *__1c__ 50 mM, 10.0 wt% PAL, NH_3 6 M, pH 9.5, 30 °C, 48 h. Isolated yield, 0.5 mmol scale.

In contrast, while their syntheses with wild-type *Pb*PAL, afforded comparable yields, the enantioselectivity of the reaction decreased significantly with *ee* of 19 % and 55 % for **2a** and **2b**, respectively. Such drastic difference in enantioselectivity demonstrates the efficiency of our engineering strategy. Benzimidazolylalanine[42] **2c** was obtained with >99 % *ee* and with moderate yield using 10 wt % of **P9**. Notably, wild-type *Pb*PAL did not afford the desired product **2c** (Figure 4). With these encouraging results in hand, we aimed to employ the developed *Pb*PAL variants for the hydroamination of the fumaric acid monoamides (**1d–1m**) into the corresponding amination products, namely, N^4-aryl-L-asparagines (**2d–2m**) (Figure 5). N^4-aryl-L-asparagines, have several applications including their use as therapeutic agents[43,44] and fluorogenic probes[45] for enzymatic assays and organocatalysts.[46]

Conventional synthetic methodologies for their syntheses rely on amide coupling reactions and require the use of toxic dimethylformamide (DMF) as a solvent, along with the employment of protective groups.[44,46] Our objective was to improve atom efficiency by utilizing biocatalytic hydroamination and readily available reagents. For hydroamination of these substrates, we screened the five triple mutants **P9-P13** in hydroamination of fumaric acid monoanilide **1d** at 2.5 mM and 0.1 mol % of enzyme loading (Table S15). We were pleased to discover that two variants, **P9** and **P13**, exhibited efficacy in the hydroamination of **1d**, with 76 % and 92 % conversions, respectively. We thus decided to employ **P13** to evaluate the generality and limitations of hydroamination of substituted fumaric acid monoamides (Figure 5).

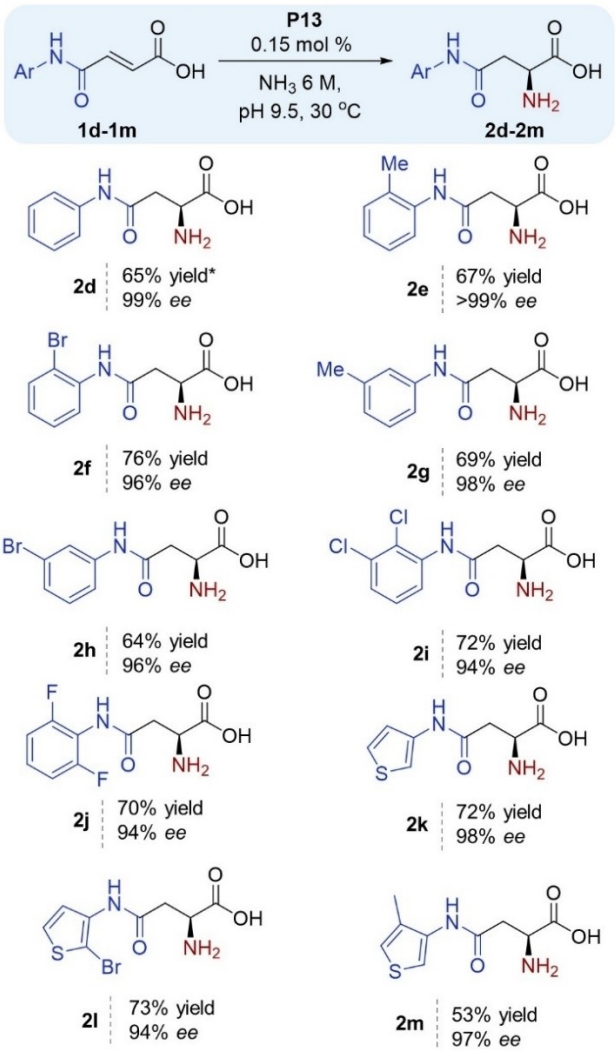

Figure 5. Substrate scope of N^4-arylasparagines. Experimental conditions: **1d–1m** 10 mM, 6 M NH_3, pH 9.5, 30 °C, 24 h. *0.1 mmol scale, isolated yields.

To achieve satisfactory yields of *N*-arylasparagines, using 0.15 mol % of **P13** was required. Employing the optimized hydroamination protocol resulted in the production of ortho-substituted N^4-(*o*-tolyl)-asparagine and N^4-(2-bromophenyl) asparagines (**2e–f**), meta-substituted N^4-(*m*-tolyl)- and N^4-(3-bromophenyl)asparagines (**2g–h**), as well as the 2,3- and 2,6-disubstituted *N*-phenylasparagines (**2i** and **2j**). Five-membered ring heterocyclic N^4-(thiophen-3-yl)asparagine (**2k**) and 2- and 4-substituted N^4-(thiophen-3-yl)asparagines (**2l–2m**) were also produced with good yields and 98 %, 94 % and 97 % *ee*, respectively. It is worth noting that amides derived from para-substituted anilines, 2-naphthylamine and alkylamines revealed to be poor substrates for **P13** (Figure S14).

Finally, we aimed to employ our engineered *Pb*PAL variants for the hydroamination of fumaric acid esters (Figure 6). We identified mono-*tert*-butyl fumarate **1n** as a particularly valuable substrate, upon hydroamination

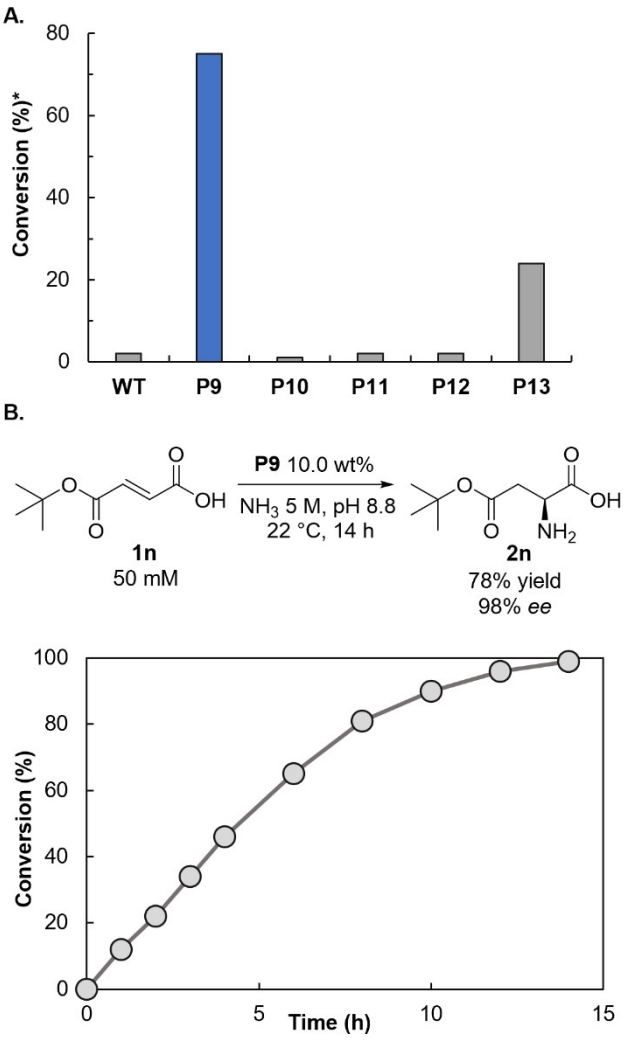

Figure 6. Engineered *Pb*PAL variants for L-aspartic acid 4-*tert*-butyl ester (2n) synthesis. A. Hydroamination of **1n** using **P9-P13**. Experimental conditions: **1n** 50 mM, 5.0 wt% PAL, 5 M NH_3, pH 8.8, 22 °C, 24 h. *Conversions were determined by ^{1}H NMR. **B**. Preparative scale synthesis of L-aspartic acid 4-*tert*-butyl ester **2n** and kinetics of **1n** 50 mM hydroamination using 10.0 wt% of **P9**.

yielding L-aspartic acid 4-*tert*-butyl ester **2n**—a precursor for Fmoc-L-Asp(^{t}*Bu*)–OH utilized in solid-phase peptide synthesis. Conventionally, **2n** can be prepared by a 3-step protocol from aspartic acid via alkylation of oxazolidinone or boroxazolidinone derivatives,[47,48] or through anhydride method.[49] To improve overall atom-efficiency and reduce the number of steps in the preparation of **2n**, we started with the synthesis of **1n** from maleic anhydride and potassium *tert*-butoxide. In the hydroamination step, to avoid undesired background aza-Michael addition of ammonia to **1n**, we adjusted the pH to 8.8 by using ammonium carbonate-bicarbonate buffer and conducted the reaction at 22 °C.

Measuring the production of **2n** from **1n** revealed that two variants, **P9** and **P13**, showed activity, with **P9** being the most active design (Figure 6A). Using 10 wt % loading of **P9**, preparative-scale hydroamination of mono-*tert*-butyl fumarate (50 mM) afforded L-aspartic acid 4-*tert*-butyl ester in 78 % yield and 98 % *ee* (Figure 6B). The exhibited absolute α-regioselectivity, high enantioselectivity, and promising catalyst productivity in the described hydroamination route indicate its potential for industrial applicability. This was underscored by the particularly atom-efficient synthesis of **2n** in just two steps without the need for protective groups, showcasing distinct advantages of our approach over conventional synthesis.

In summary, our study highlights the significant potential of engineered phenylalanine ammonia-lyases (PALs) for expanding their substrate scope to fumaric acid derivatives, offering a versatile platform for the synthesis of non-canonical amino acids.

We employed a structure-guided engineering strategy that identified key residues in the catalytic pocket of *Pb*PAL responsible for enhanced enantioselectivity in hydroamination reactions. Notably, the cryo-EM structure of *Pb*PAL provided crucial insights into the catalytic pocket, guiding our targeted mutagenesis efforts. The resulting triple mutants exhibited remarkable improvements in enantiomeric excess, paving the way for preparative-scale hydroamination of various fumaric acid derivatives with excellent α-regio- and high L-enantioselectivity. This work represents a significant advancement in the utilization of PALs and sets the stage for their broader integration into biocatalytic processes for amino acid synthesis.

Supporting Information

The authors have cited additional references within the Supporting Information.[50–55] The data supporting this work are accessible in Zenodo, doi: 10.5281/zenodo.11127746.

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Conflict of Interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: ammonia-lyases · biocatalysis · enantioselectivity · enzyme engineering · non-canonical amino acids

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Communications

Biocatalysis

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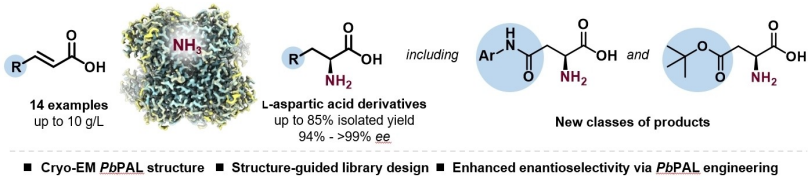
Engineered Phenylalanine Ammonia-Lyases for the Enantioselective Synthesis of Aspartic Acid Derivatives



A structure-based engineering of phenylalanine ammonia-lyase from *Planctomyces brasiliensis* (*Pb*PAL) is conducted for preparative-scale enantioselective hydroaminations of previously inaccessible yet synthetically useful substrates, such as amide- and ester-containing fumaric acid derivatives. Our engineered *Pb*PALs exhibit exclusive α-regioselectivity, high enantioselectivity, and broad substrate scope. The potential utility of the developed biocatalysts is demonstrated by a preparative-scale hydroamination yielding *tert*-butyl protected l-aspartic acid, widely used as intermediate in peptide solid-phase synthesis.