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Tailored Mutants of Phenylalanine Ammonia-Lyase from *Petroselinum crispum* for the Synthesis of Bulky L- and D-Arylalanines

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Tailored mutants of phenylalanine ammonia-lyase from *Petroselinum crispum* (*Pc*PAL) were created and tested in ammonia elimination from various sterically demanding, non-natural analogues of phenylalanine and in ammonia addition reactions into the corresponding (*E*)-arylacrylates. The wild-type *Pc*PAL was inert or exhibited quite poor conversions in both reactions with all members of the substrate panel. Appropriate single mutations of residue F137 and the highly conserved residue I460 resulted in *Pc*PAL variants that were active in ammonia elimination but still had a poor activity in ammonia addition onto bulky substrates. However, combined mutations that involve I460 besides the well-studied F137 led to mutants that exhibited activity in ammonia addition as well. The synergistic multiple mutations resulted in substantial substrate scope extension of *Pc*PAL and opened up new biocatalytic routes for the synthesis of both enantiomers of valuable phenylalanine analogues, such as (4-methoxyphenyl)-, (napthalen-2-yl)-, ([1,1′-biphenyl]-4-yl)-, (4′-fluoro-[1,1′-biphenyl]-4-yl)-, and (5-phenylthiophene-2-yl)alanines.

Introduction

The synthesis of natural and unnatural aromatic amino acids in the homochiral form is an important challenge in preparative chemistry, highlighted by the significant interest towards these building blocks in the development of therapeutic peptides and proteins.[1–3] An attractive enzymatic route to enantiomerically pure α- or β-aromatic amino acids involves the use of aromatic ammonia-lyases (ALs) and 2,3-aminomutases (AMs),[4] which act by the aid of a 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO) electrophilic prosthetic group formed autocatalytically. Among the so-called MIO enzymes, phenylalanine ammonia-lyases (EC 4.3.1.24/25) from *Petroselinum crispum* (*Pc*PAL),[5–7] *Rhodotorula glutinis* or *graminis* (*Rg*PAL or *Rgr*PAL),[8–10] and *Anabaena variabilis* (*Av*PAL)[11,12] have been most used as biocatalysts in whole cells or as purified enzymes for ammonia elimination or for the reverse ammonia addition to yield a wide range of L- and D-arylalanines.

Although wild-type PALs exhibited a broad substrate tolerance towards various cinnamic acid derivatives in the ammonia addition, synthetically valuable 4-substituted cinnamic acids with electron-withdrawing but bulky (4-NO_2, 4-Ph)[13,14] or electron-donating (4-CH_3, 4-OCH_3, 4-NH_2)[9,15,16] substituents proved to be poor substrates. Protein engineering strategies have focused mainly on residue F137 in the hydrophobic binding pocket of *Pc*PAL[13,17] or on the sterically analogous F107 and H143 of *Av*PAL[14] and *Rgr*PAL,[10] respectively. These mutations enhanced the catalytic properties of PALs in ammonia addition onto 4-NO_2, 4-Br, and 4-F-substituted cinnamic acids[13,14] and in the ammonia elimination of styrylalanines,[17] however, none of the PAL mutants showed activity in ammonia addition onto (4-phenyl)cinnamic acid[14] or styrylacrylic acids.[17] The screening of new PALs from various organisms revealed variants that favored the acceptance of substituted cinnamic acids with electron-donating substituents, but the conversion of valuable 4-methoxyphenylacrylate still remains a challenge.[16]

Herein we report a thorough mutational analysis of the hydrophobic binding pocket of PcPAL with the aim to develop PAL biocatalysts for the production of bulky and valuable L- and D-arylalanines. These compounds offer new possibilities to extend the chemical space available to biomedicine (peptides, proteins, peptidomimetics) and chiral small-molecule drugs. The envisaged ([1,1′-biphenyl]-4-yl)alanines are constituents of various bioactive peptides,[18] such as peptidase, protease,[14]

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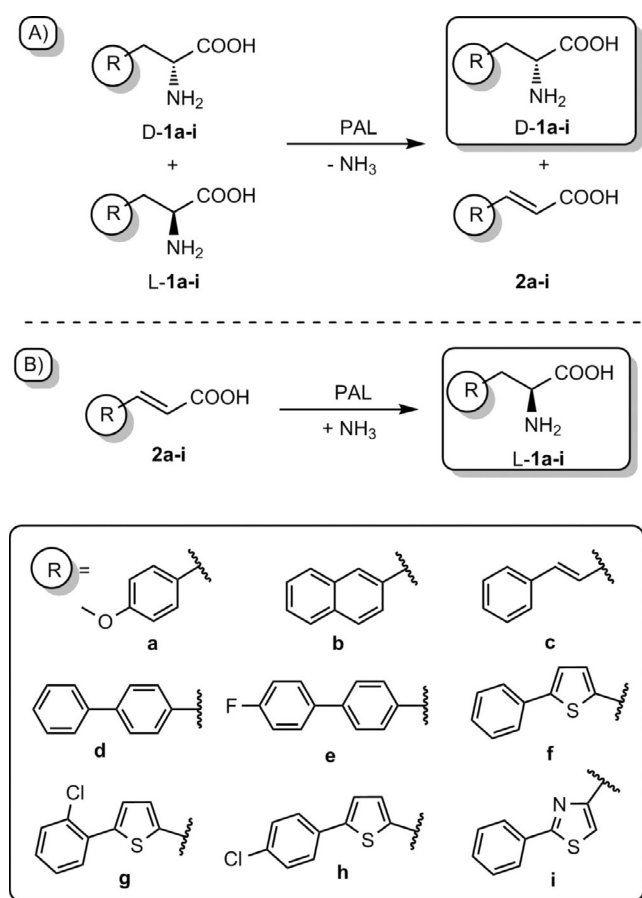
and cancer-related histone lysine demethylase KDM4A[19] inhibitors. (Naphthalen-2-yl)alanine is used frequently as a phenylalanine analogue in the development of peptides,[20,21] whereas (4-methoxyphenyl)alanine is a chiral intermediate in the synthesis of the antihypertensive drug tamsulosin.[22] (5-Phenylthiophene-2-yl)alanine and its new derivatives represent attractive phenylalanine analogue targets because of the heteroaryl motif that feature in endothelin convertase[23] and Factor IX/XI inhibitors.[24] Amide derivatives of styrylalanine were identified as potent peptidyl-prolyl isomerase (PPIase) inhibitors at Pfizer.[25] The origin of the potency was attributed to the (*E*)-ethene-1,2-diyl linker that increases the distance between the aromatic moiety and the chiral alanine moiety.

Notably, some of the target compounds (Scheme 1) have been tested before and exhibited little or no conversions or even inhibitory activity with wild-type PALs.[9,14,15,17] Moreover, no mutant PAL variants were known to possess activity in the corresponding ammonia elimination and ammonia addition reactions, except for the deamination of styrylalanines.[17] The initial tests of this study also confirmed the insufficient catalytic activity of wt-*Pc*PAL on the substrate panel.



Scheme 1. A) Ammonia elimination and B) ammonia addition reactions tested by the *Pc*PAL variants.

Results and Discussion

CASTing[26] or directed evolution methods, that require high-throughput enzyme assays (HTS) of large mutant libraries, to generate mutants of *Pc*PAL that accept bulky target compounds as substrates were avoided because of cell membrane penetration issues of the large hydrophobic substrates with whole-cell PAL biocatalysts. Instead of such HTS-based methods, the structure-driven approach was selected and based on steric clash reduction concepts[14,17] residues L134, F137, L138, L206, L256, and I460 from the hydrophobic binding site of *Pc*PAL (Figure 1) were exchanged to smaller amino acids (i.e., valine (V) or alanine (A)) to provide a limited number of single or multiple residue mutants of *Pc*PAL (Table S1).

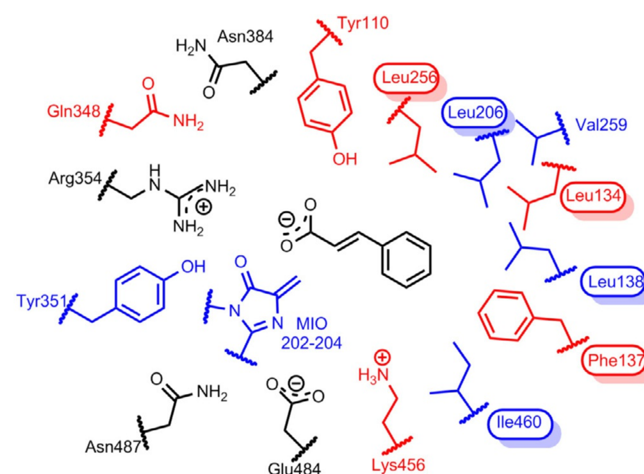


Figure 1. Active site model of *Pc*PAL with (*E*)-cinnamic acid as a model ligand and the surrounding residues within less than 5 Å distance. The colors of the amino acid side chains refer to their position with respect to the plane of the substrate: black—within, red—above, blue—below the plane. Hydrophobic binding pocket residues in boxes were exchanged individually or in combination with smaller hydrophobic amino acids V or A.

To exclude the influence of mutation-induced improper folding on enzyme activity, the oligomerization state and thermal unfolding of the isolated and purified mutants were compared with those of the wt-*Pc*PAL. Retention volumes obtained by using size-exclusion chromatography (Figure S2) revealed that all *Pc*PAL variants were folded properly and existed in the native, tetrameric form, similar to the wild-type enzyme. The slight variations in the thermal unfolding temperatures (T_m), determined by using differential scanning fluorimetry, indicated that the mutations did not affect protein folding (Table S3). The only exception was the I460A mutation that decreased the T_m value significantly (from 75 ± 1.5 to $51\pm1.2\,^{\circ}C$; Table S4 and Figure S5) without affecting the tetrameric fold detected by using size-exclusion chromatography. As later tests showed that I460A-*Pc*PAL was catalytically active (Tables S2–S19), we presumed that the mutation corrupted only the thermal stability without disrupting the main folding patterns related to enzyme activity.

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Next, the generated single-mutant *Pc*PAL library (Table S1) was tested with the targeted substrate panel in ammonia elimination from arylalanines *rac*-**1 a**–**i** (Scheme 1 A) and in ammonia addition onto arylacrylates **2 a**–**i** (Scheme 1 B). Results from the ammonia eliminations revealed that besides the known F137 to V or A mutations,[13,17] the mutation of another highly conserved residue I460 (Figure S1) to V or A increased the activity significantly towards almost all substrates compared to the wild-type enzyme (Table 1 and Tables S5–13).

Although wt-*Pc*PAL could convert the members of the tested substrate panel quite poorly ($c_{rac\text{-}1a}$ = 3%; $c_{rac\text{-}1b}$ = 6%; $c_{rac\text{-}1c\text{-}i}$ < 1%, even after long reaction times up to 48 h), mutants I460V and F137V/A provided medium to high conversions from *rac*-**1 a**–**i** (Table 1). The lowest enhancement was achieved from *rac*-**1 i**, a structural analogue of the known wt-*Pc*PAL inhibitors (benzo[*b*]furan-3-yl)- and (benzo[*b*]thiophene-3-yl)alanines,[6] for which only mutant F137A provided the arylacrylate **2 i**, but regrettably at a low conversion ($c_{rac\text{-}1i}$ = 6%).

Despite the flourishing extension of the substrate scope of wt-*Pc*PAL in ammonia elimination, in the reverse ammonia addition reaction the single mutants of *Pc*PAL showed improved activity only with 4-methoxyphenyl- (**2 a**) and napthalen-2-ylacrylic acid (**2 b**), whereas for more bulky substrates **2 d**–**h** and styrylacrylate (**2 c**) no conversion was detected (Tables S14–S22).

Table 1. Activity of wt-*Pc*PAL compared to the best *Pc*PAL single mutants in the ammonia elimination reaction of *rac*-**1 a**–**i**.

Substrate	R group	*Pc*PAL variant[a]	*c*[b]
rac-**1 a**	4-methoxy	wt	3
rac-**1 a**	4-methoxy	F137	37
rac-**1 a**	4-methoxy	**I460V**	**39**
rac-**1 b**	napthalen-2-yl	wt	6
rac-**1 b**	napthalen-2-yl	I460V	37
rac-**1 b**	napthalen-2-yl	**F137V**	**39**
rac-**1 c**	styryl	wt	< 1
rac-**1 c**	styryl	**I460V**	**≈50**
rac-**1 c**	styryl	**F137V**	**≈50**
rac-**1 d**	biphenyl-4-yl	wt	< 1
rac-**1 d**	biphenyl-4-yl	I460V	8
rac-**1 d**	biphenyl-4-yl	**F137A**	**35**
rac-**1 e**	4′-fluorobiphenyl-4-yl	wt	< 1
rac-**1 e**	4′-fluorobiphenyl-4-yl	F137V	37
rac-**1 e**	4′-fluorobiphenyl-4-yl	**F137A**	**39**
rac-**1 f**	5-phenylthiophen-2-yl	wt	< 1
rac-**1 f**	5-phenylthiophen-2-yl	F137V	35
rac-**1 f**	5-phenylthiophen-2-yl	**F137A**	**44**
rac-**1 g**	2′-chloro-5-phenylthiophen-2-yl	wt	< 1
rac-**1 g**	2′-chloro-5-phenylthiophen-2-yl	F137V	19
rac-**1 g**	2′-chloro-5-phenylthiophen-2-yl	**F137A**	**≈50**
rac-**1 h**	4′-chloro-5-phenylthiophen-2-yl	wt	< 1
rac-**1 h**	4′-chloro-5-phenylthiophen-2-yl	I460V	10
rac-**1 h**	4′-chloro-5-phenylthiophen-2-yl	**F137A**	**≈50**
rac-**1 i**	2-phenylthiazol-4-yl	wt	< 1
rac-**1 i**	2-phenylthiazol-4-yl	**F137A**	**6**

[a] *Pc*PAL variant: 50 μg, reaction volume: 500 μL, medium: Tris buffer (100 mM Tris·HCl, pH 8.8, 20 mM β-cyclodextrin), substrate concentration: 1 mM; assays were performed in 1.5 mL glass vials sealed with a PTFE septum at 30 °C, 200 rpm for 16 h; [b] conversion values [%].

To explore the advantageous synthetic potential of ammonia addition onto arylacrylates (100% theoretical yield; the use of synthetically accessible, achiral starting materials), further mutations of neighboring residues were introduced into the single mutants with the best activities. In this way, a focused library of double and triple mutants that involve I460, F137, and L138 (Table S1) was obtained and tested in both kinds of PAL-mediated reactions (Tables S5–S22). Analogously to the single-mutant *Pc*PALs, the I460A mutation altered the thermal unfolding profile of double and triple mutants as well (Figure S5), but the detected catalytic activities and the native tetrameric fold indicated that the overall folding was not altered seriously even in case of double and triple mutants.

Multiple mutations of *Pc*PAL could result in a moderate enhancement of the conversion in ammonia elimination from (4′-fluoro-[1,1′-biphenyl]-4-yl)alanine (*rac*-**1 e**: 39% with F137A/I460V-*Pc*PAL, 18% with F137A/L138V-*Pc*PAL vs. 15% with F137A-*Pc*PAL; Table S9) and for (5-phenylthiophen-2-yl)alanine (*rac*-**1 f**: 48% with F137A/L138V-*Pc*PAL vs. 44% with F137A-*Pc*PAL; Table S10). Similarly, in the case of ammonia addition onto **2 a** and **b** no significant increase in conversion was provided by multiple mutations (Tables S14 and S15).

However, *Pc*PALs that have simultaneous mutations of F137 and I460 gave promising results in ammonia additions onto **2 c** and **d**. In these cases, no product was detected with wt-*Pc*PAL or either single mutants of *Pc*PAL, but 22 and 27%, respectively, conversion could be achieved with the F137(V,A)/I460V double mutants (Table 2; Tables S16 and S17). Arylacrylates **2 e**–**h** were moderate substrates even for the F137A/I460V double mutant (conversions of 3–8% after 20 h; Tables S18–S21), although no conversion of (2-phenylthiazol-4-yl)acrylic acid (**2 i**) could be achieved with the investigated multiple mutant *Pc*PALs (Table S22).

Table 2. Activity of wt-*Pc*PAL compared to the best *Pc*PAL mutants in the ammonia addition reaction of **2 a**–**h**.

Substrate	R group	*Pc*PAL variant[a]	*c*[b]
2 a	4-methoxy	wt	< 1
2 a	4-methoxy	**F137V/I460V**	**32**
2 b	napthalen-2-yl	wt	< 1
2 b	napthalen-2-yl	**F137V**	**55**
2 c	styryl	wt	< 1
2 c	styryl	**F137V/I460V**	**22**
2 d	biphenyl-4-yl	wt	< 1
2 d	biphenyl-4-yl	**F137A/I460V**	**27**
2 e	4′-fluorobiphenyl-4-yl	wt	< 1
2 e	4′-fluorobiphenyl-4-yl	**F137A/I460V**	**8**
2 f	5-phenylthiophen-2-yl	wt	< 1
2 f	5-phenylthiophen-2-yl	**F137A/I460V**	**6**
2 g	2′-chloro-5-phenylthiophen-2-yl	wt	< 1
2 g	2′-chloro-5-phenylthiophen-2-yl	**F137A/I460V**	**3**
2 h	4′-chloro-5-phenylthiophen-2-yl	wt	< 1
2 h	4′-chloro-5-phenylthiophen-2-yl	**F137A/I460V**	**2**

[a] *Pc*PAL variant: 50 μg, reaction volume: 500 μL, medium: 6 M NH_3 buffer (pH 10, adjusted with CO_2), substrate concentration: 1 mM; assays were performed in 1.5 mL glass vials sealed with PTFE septum at 30 °C, 200 rpm for 20 h; [b] conversion values [%].

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Our mutational analysis revealed that in most cases single mutations of F137 and I460 in *Pc*PAL were sufficient to perform ammonia elimination from bulky amino acids decently and additional mutations did not improve the conversions significantly. However, double mutants of *Pc*PAL that involve F137 and I460 were required to achieve adequate ammonia addition activity with bulky arylacrylates **2c**–**h**. In the case of less bulky substrates **2a** and **b**, active single mutants of *Pc*PAL could be identified as well. These data demonstrated clearly that multiple mutations exhibited a strong, nonadditive cooperative effect[27] on *Pc*PAL activity in the ammonia addition reaction.

The fact that individual mutations of L134, L206, L256, and L138 as well as the double and triple mutants of L138 with the neighbor, activity-modulator-residues F137 and I460 did not provide any increase in the conversion of the tested substrate panel in either reaction direction highlighted the importance of residue I460, besides the well-studied residue F137, and their combined mutations for the substrate specificity modulation of *Pc*PAL, especially in the synthetically valuable ammonia addition reaction.

With the most active mutants in hand (Tables 1 and 2), the reaction conditions in terms of activity and selectivity were optimized using ([1,1′-biphenyl]-4-yl)alanine (*rac*-**1d**) and (napthalen-2-yl)acrylic acid **2b** as models for ammonia elimination and addition, respectively.

Additionally, reactions of *rac*-**1d** and **2b** were investigated using whole cells of *E. coli* to express the corresponding *Pc*PAL mutants to take advantage of the possible lower production costs and increased stability, characteristic for whole-cell PAL biocatalysts compared to purified enzymes. In spite of our all efforts (the use of living or lyophilized whole cells, various biocatalyst/substrate ratios, and different temperatures), the results of whole-cell biotransformations with these bulky and hydrophobic substrates were irreproducible even within the same batch of cells, which suggests cell internalization difficulties with the bulky hydrophobic substrates. The reproducibility of experiments with cell lysates supported this hypothesis but provided poor-quality analytical data (with the appearance of additional signals in HPLC chromatograms). As different batches of purified enzymes exhibited negligible biocatalytic variability and clean analytical data, all further experiments were performed with isolated *Pc*PALs.

The low solubility (<1 mM) of substrates **1b** and **d**–**i** in the reaction buffer of ammonia elimination was addressed during the initial screening tests. Although the solubility of *rac*-**1d** could be increased to 2–3 mM by using DMSO or MeOH as cosolvents (5, 10, 20 vol%), conversions decreased from 37% after 16 h to 16% at 10% cosolvent level and down to zero at 20% cosolvent level. Finally, the solubilization of *rac*-**1d** up to 2.5 mM concentration was achieved by forming an inclusion complex with 5–20 mM β-cyclodextrin[28] without altering the enzyme activity. Thus, activity screening in ammonia elimination with *rac*-**1a**–**i** was performed in the presence of 20 mM β-cyclodextrin (Table 1).

Unfortunately, low substrate solubility prevented the determination of Michaelis–Menten curves approaching substrate saturation. Despite the apparent solubility increase by β-cyclodextrin, the unknown actual concentrations of uncomplexed substrate and product hindered the determination of kinetic constants.

The solubility of acrylic derivatives **2a**–**i** in the high-concentration ammonia buffer was higher without any additive (2.5 mM; Table 2), but still not enough to obtain full Michaelis–Menten curves.

Next, the influence of various ammonium sources was tested (2, 4, 6 M ammonia or ammonium carbamate) on the *Pc*PAL-catalyzed ammonia addition onto **2b**. The best results in terms of conversion and enantiotopic selectivity were achieved using 4 M ammonium carbamate (Table S23), in accordance with the optimal conditions reported for the PAL-catalyzed ammonia addition onto 3-fluorocinnamic acid.[12]

Finally, the *Pc*PAL-catalyzed reactions of the entire substrate panel (*rac*-**1a**–**i**, **2a**–**i**) were performed under the optimal reaction conditions to monitor the conversions and enantiomeric excess values of D- and L-**1a**–**i**.

The maximal conversions in the kinetic resolutions (KRs) as well as the maximal *ee* of the unreacted D enantiomer (D-**1a**–**h**) were reached in all but one case in relatively short reaction times (14–40 h; Table 3). In ammonia elimination from (2-phenylthiazol-4-yl)alanine (*rac*-**1i**) the reaction stopped at a low conversion (10%), which suggests product inhibition. This hypothesis was confirmed by the inhibitory effect of **2i** upon the wt-*Pc*PAL-catalyzed ammonia elimination from L-Phe (Supporting Information). Furthermore, ammonia addition onto **2i** with wt- or mutant *Pc*PALs did not succeed, which suggests that structural analogues of (benzo[*b*]furan-3-yl)- and (benzo[*b*]thiophene-3-yl)acrylates, known wt-PAL-inhibitors[6] still behave as competitive inhibitors also for mutant *Pc*PALs.

The ammonia addition reaction onto **2a**–**h** proceeded somewhat slower (Table 4), and after longer incubation times (>20–24 h), the deactivation of *Pc*PALs was observed because of the harsh reaction conditions. This issue was solved by supplementing the reactions with fresh batches of enzyme after each 24 h of incubation. In almost all cases 80–85% of the final conversions could be reached within the first 28–36 h, followed by a slow increase of the conversion until 70 h reaction time (Fig-

Table 3. Yield and enantiomeric excess of D-**1a**–**h** after the *Pc*PAL-catalyzed ammonia eliminations from *rac*-**1a**–**h** at approximately 50% conversion.

cc	*Pc*PAL variant	$t_{reaction}$ [h]	$Y^{[a]}$ [%]	$ee_{D\text{-}1a\text{–}h}$ [%]
rac-**1a**	I460V	16	45	>99
rac-**1b**	F137V	17	46	>99
rac-**1c**	F137V	14	43	>99
rac-**1d**	F137A	40	47	>99
rac-**1e**	F137A/I460V	40	45	>99
rac-**1f**	F137A/L138V	16	46	>99
rac-**1g**	F137A	16	42	>99
rac-**1h**	F137A	16	39	>99

[a] The reaction yields were determined from the preparative-scale ammonia eliminations (for reaction conditions see Supporting Information, Section 6.6.)

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Substrate	*Pc*PAL variant	*c* [%]	$Y^{[a]}$ [%]	$ee_{L\text{-}1a\text{–}h}$ [%]
2a	F137V/I460V	74	65	>99
2b	F137V	73	61	>99
2c	F137V/I460V	23	19	>99
2d	F137A/I460V	68	59	82
2e	F137A/I460V	50	43	95
2f	F137A	6	nd	nd
2g	F137A/I460V	9	nd	nd
2h	F137A/I460V	3	nd	nd

Table 4. Conversion of *Pc*PAL-catalyzed ammonia additions onto **2a–h** and yield and enantiomeric excess of the products L-**1a–h** after 70 h reaction time.

[a] The reaction yields were determined from the preparative-scale ammonia additions (for reaction conditions see Supporting Information, Section 6.7)

ures S7–S12, progression curves of ammonia addition reaction). The fact that after an additional 48 h no further reaction progress could be detected indicated that an equilibrium state was reached.

Accordingly, compounds **2a** and **2b** were transformed with a good conversion (74 and 73%) and excellent enantiomeric excess ($ee_{L\text{-}1a}$ and $ee_{L\text{-}1b} > 99\%$), whereas the conversion of **2c** by F137V/I460V-*Pc*PAL remained low (23%) but selective ($ee_{L\text{-}1c} > 99\%$). (1,1′-Biphenyl)acrylates **2d** and **e** were transformed with good to moderate conversions (68 and 50%) but with incomplete enantiotopic selectivity ($ee_{L\text{-}1d} = 82\%$, $ee_{L\text{-}1e} = 95\%$; Table 4). The fact that $NaBH_4$-reduced *Pc*PALs or the MIO-less S203A/F137A/I460V-*Pc*PAL variant proved to be inactive in these reactions ruled out the occurrence of the competing, D-selective MIO-less reaction route.[29] The (5-phenylthiophen-2-yl)acrylates **2f–h** were transformed much more slowly to reach conversions of only 3–9% within 70 h.

The enhanced conversions of L-**1a–h** with the appropriate mutants can be rationalized by the better substrate affinities and also by the higher turnover numbers because of the better stabilization of the reaction transition states, in accordance with our previous computer-aided results.[17] The modeling of the *N*-MIO covalent enzyme–substrate complexes, obtained with induced-fit covalent docking, confirmed that substrate affinities increased dramatically with the most active mutants over wt-*Pc*PAL in almost all cases (Table 5).

The reason for the affinity increase of L-**1e** in which F137A/I460V-*Pc*PAL provides a larger active site volume and much lower area of close contact of the *N*-MIO covalent enzyme–substrate complex with sites A137 and V460 is shown in Figure 2C and a significant shift of the ligand position is shown in Figure 2B as compared to wt-*Pc*PAL shown in Figure 2A. Com-

Substrate	Most active *Pc*PAL mutant	${}_b^R\Delta E_{WT}$ [a] [kcal mol^{-1}]	${}_b^R\Delta E_{MA}$ [a] [kcal mol^{-1}]	${}_b^R\Delta E_{MA} - {}_b^R\Delta E_{WT}$ [kcal mol^{-1}][a]
L-**1a**	I460V	2.9	3.7	0.8
L-**1b**	F137V	4.9	−11.2	−16.1
L-**1c**	F137V	13.7	−4.0	−17.7
L-**1d**	F137A	29.8	−3.5	−33.3
L-**1e**	F137A/I460V	35.5	12.6	−22.9
L-**1f**	F137A/L138V	19.7	−5.3	−25.0
L-**1g**	F137A	32.1	0.3	−31.8
L-**1h**	F137A	40.6	7.5	−33.1

Table 5. Calculated relative binding energies (${}_b^R\Delta E$) of L-**1a–h** in wt-*Pc*PAL and in the most active *Pc*PAL variants. The subscripts WT and MA correspond to the wild-type and most active mutant, respectively. Notably, these quantities are not meant to determine actual binding energies computationally but to approximate them only.

[a] Binding energies are related to the same property of L-Phe with wt-*Pc*PAL in the form of ${}_b^R\Delta E = {}_b\Delta E - {}_b\Delta E_{L\text{-Phe;wt-}PcPAL}$. For a detailed description of the calculation method and reasoning of the necessary relativization, see Supporting Information.

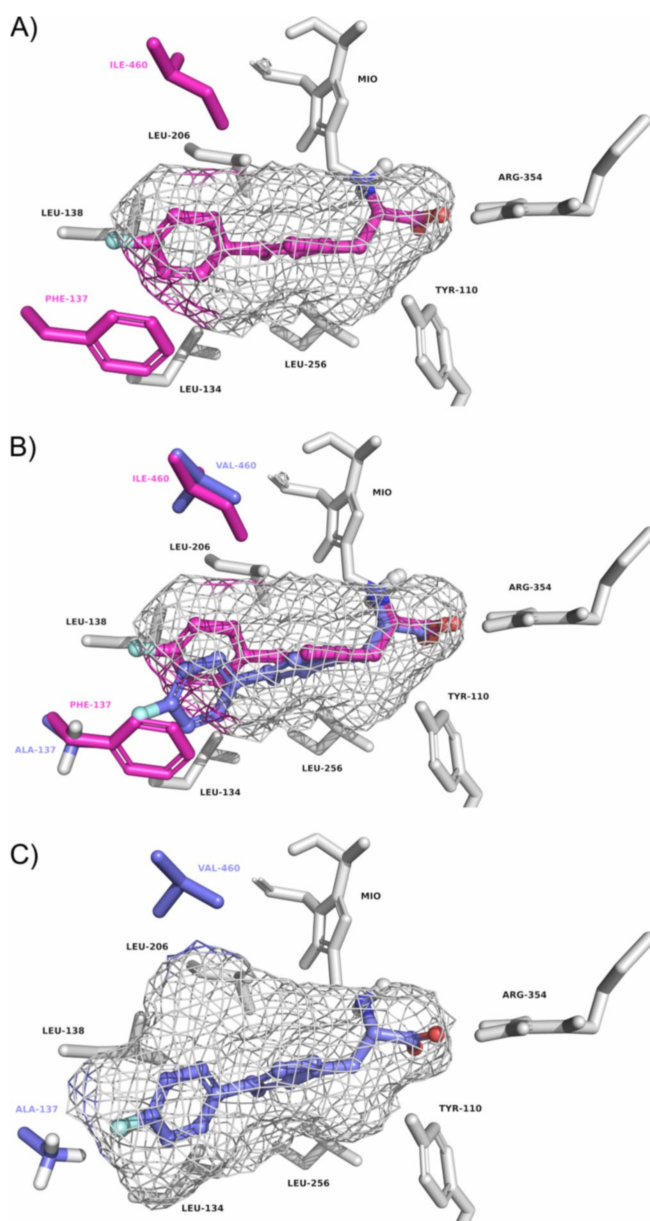

Figure 2. Catalytically active *N*-MIO intermediate models of L-**1e** within A) wt-*Pc*PAL and C) F137A/I460V-*Pc*PAL. Volumes inside the mesh represent the cavity provided by the active site of the corresponding enzyme. B) The combination of A and C depicted with the mesh for wt-*Pc*PAL only. Mutational sites and ligand pose are colored magenta in wt-*Pc*PAL and blue in F137A/I460V-*Pc*PAL. The coloring of the mesh represents close contact with the corresponding residues of the mutational site.

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parisons of the *N*-MIO intermediates from the other L-arylalanines L-**1b**–**d** and **f**–**h** indicated similar situations. The only exception was L-**1a**, with a nearly unaffected affinity, which in turn suggested that the catalytic enhancement stemmed solely from the other presumed factor, a higher turnover number.

Computational results, however, proved to be inconsistent with the experimental results of ammonia additions. Apart from potential parameterization problems of atomic interactions in our model, two reasons can rationalize this observation. One reason is the Hammond's postulate that states that in an exothermic reaction the high-energy intermediate and thus the transition state (TS) resembles the substrate state better, whereas the TS of an endothermic reaction resembles the product state better. In our case, the reverse ammonia addition reaction is endothermic, therefore, the *N*-MIO intermediate structure is not appropriate to draw conclusions on the affinity situations for the arylacrylates. Moreover, the enzyme most probably adopts a different conformation under high ammonia concentrations, which invalidates our computational results for ammonia addition. This was supported by the analysis of the thermal unfolding profiles of wt-*Pc*PAL (Figure S6) and F137A/I460V-*Pc*PAL (Figure 3) at different ammonia concentrations, which indicated shifts of the melting temperature (T_m) by 10–12 °C at the highest ammonia concentration as compared to that determined in Tris buffer.

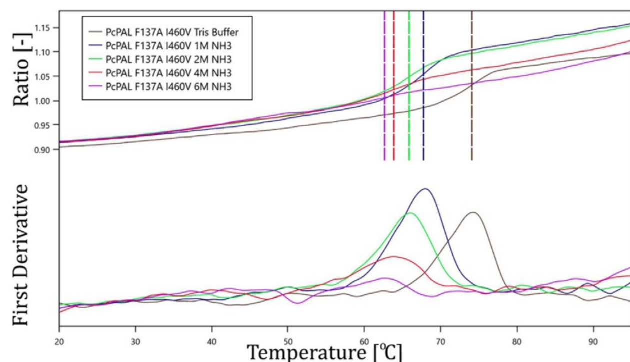

Figure 3. Thermal unfolding temperature (T_m) of F137A/I460V-*Pc*PAL in media with different ammonia contents (20 mM Tris·HCl, pH 9; and 1, 2, 4, and 6 M NH_3-buffer, with pH 9.5 adjusted by CO_2) determined by nanoDSF (Prometheus NT.48). Fluorescence intensity ratios F_{350}/F_{330} and their first derivatives are presented as a function of the applied linear thermal ramp.

Finally, the synthetic applicability of the tailored *Pc*PAL mutants was demonstrated by performing KRs from racemic arylalanines *rac*-**1a**–**h** by ammonia elimination to digest the L enantiomers (Scheme 1A) and by the enantiotopic selective ammonia addition reactions onto **2a**–**e** (Scheme 1B) at a larger scale (0.25 mmol substrate; for details, see Supporting Information). Preparative-scale reactions of **2f**–**h** were omitted because of the quite low equilibrium conversions. During the preparative-scale reactions, no significant alterations of conversions, reaction times, and enantiomeric excess values were observed as compared to the analytical-scale bioconversions. The corresponding unreacted D enantiomers, D-arylalanines D-**1a**–**h** (Table 3), and the produced L-arylalanines L-**1a**–**e** (Table 4) were isolated conveniently by Dowex cation-exchange chromatography in good to moderate yields.

Notably, before this work, the stereoconstructive ammonia addition onto the bulky arylacrylates **2a**–**h** was unprecedented, and no data were reported on PAL-mediated routes to amino acids L-**1a**–**e**. Similarly, no reports existed on successful PAL-mediated ammonia elimination reactions from ([1,1′-biphenyl]-4-yl)-, (napthalen-2-yl)-, (4-methoxyphenyl)-, and (5-phenylthiophen-2-yl)alanines (**1a**,**b**,**d**–**h**) to yield the corresponding amino acids D-**1a**,**b**,**d**–**h**.

The fact that PALs are known to present difficulties in the transformation of substrates with electron-donating ring substituents and the recent efforts that focus on the discovery of new PALs with such activity[16] highlights the excellent results obtained for the synthesis of both L- and D-4-methoxyphenylalanine (L- and D-**1a**).

Pharmaceutically important ([1,1′-biphenyl]-4-yl)alanines were the subject of recent *Av*PAL-mediated biotransformations in which the studied *Av*PAL variants showed no activity in ammonia addition onto 4-phenylcinnamic acid, thus a chemoenzymatic procedure was required that involves the *Av*PAL-mediated synthesis of L-(4-bromophenyl)alanine followed by Pd-catalyzed Suzuki-coupling.[14] In this frame, the tailored multiple mutant *Pc*PAL-based processes reported here represent the first direct enzymatic route towards both enantiomers of ([1,1′-biphenyl]-4-yl)alanines L- and D-**1d** and **e**.

As future perspectives, the combination of tailored *Pc*PAL mutants with the immobilization techniques reported recently can lead to their use in continuous-flow reactors,[30–32] which provides accessibility for the industrial synthesis of sterically demanding non-natural arylalanines.

Conclusions

The substrate scope of phenylalanine ammonia-lyase from *Petroselinum crispum* (*Pc*PAL) has been expanded towards a series of sterically demanding phenylalanine analogues by tailored mutations of the hydrophobic substrate binding pocket based on a simple concept of steric clash reduction. Although single mutations of residues F137 and I460 were sufficient to enhance the phenylalanine ammonia-lyase (PAL) activity in the ammonia elimination reactions, combined mutations of F137 and I460, which result in a cooperative, nonadditive effect, was required to create PAL biocatalysts that are active in the reverse ammonia addition reactions of bulky substrates. This work highlights the importance of residue I460, besides the F137 residue explored already, in the modulation of the substrate specificity of *Pc*PAL and demonstrates the importance of the non-additive effects of combined mutations on PAL activity.

The new *Pc*PAL mutants enabled unprecedented PAL-mediated biocatalytic routes to the D and L enantiomers of (napthalen-2-yl)alanine **1a** and 4-methoxyphenylalanine **1b**. With the aid of tailored multiple mutations of *Pc*PAL, direct biocatalytic routes could be developed towards D- and L-([1,1′-biphenyl]-4-

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yl)alanines, D- and L-**1 d**,**e**, valuable chiral intermediates for several drugs under development, as well as towards new non-natural amino acids D-(5-phenylthiophen-2-yl)alanines D-**1 f**–**h**.

Experimental Section

For all experimental details see the Supporting Information.

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Conflict of interest

The authors declare no conflict of interest.

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