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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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Abstract: 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 onto the corresponding (*E*)-arylacrylates. The wild-type *Pc*PAL was inert or exhibited quite poor conversion in both reactions with all members of the substrate panel. Proper single mutations of residue F137 and the highly conserved residue I460 resulted in *Pc*PAL variants being active in ammonia elimination but having still poor activity in ammonia additions onto bulky substrates. However, combined mutations involving I460 besides the well-studied F137 led to mutants exhibiting activity in ammonia addition as well. The synergistic multiple mutations resulted in substantial substrate scope extension of *Pc*PAL and opened up novel 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 homochiral form is an important challenge of 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] acting by the aid of an autocatalytically-formed 3,5-dihydro-5-methylidene-4*H*-imidazol-4-one (MIO) electrophilic prosthetic group. 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] were used most of the time as biocatalysts in whole cells or as purified enzymes for ammonia elimination or for the reverse ammonia addition yielding a wide range of L- and D-arylalanines.

Although wild-type PALs exhibited 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 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[[14]3,[13]4] and in the ammonia elimination of styrylalanines[[17]7], however, none of the PAL mutants showed activity in the ammonia addition onto (4-phenyl)cinnamic acid[14] or styrylacrylic acids[[17]7]. Screening novel PALs from various organisms revealed variants which favored the acceptance of substituted cinnamic acids with electron-donating substituents, but conversion of valuable 4-methoxyphenylacrylate still remained 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] or cancer-related histone lysine demethylase KDM4A[19] inhibitors. (Naphthalen-2-yl)alanine is frequently used as a phenylalanine analogue in the development of peptides,[20,21] while (4-methoxyphenyl)alanine is a chiral intermediate in the synthesis of the antihypertensive drug tamsulosin.[22] (5-Phenylthiophene-2-yl)alanine and its novel derivatives represent also attractive phenylalanine analogue targets due to the heteroaryl motif featured 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 potency was attributed to the (*E*)-ethene-1,2-diyl linker increasing the distance between the aromatic moiety and the chiral alanine moiety.

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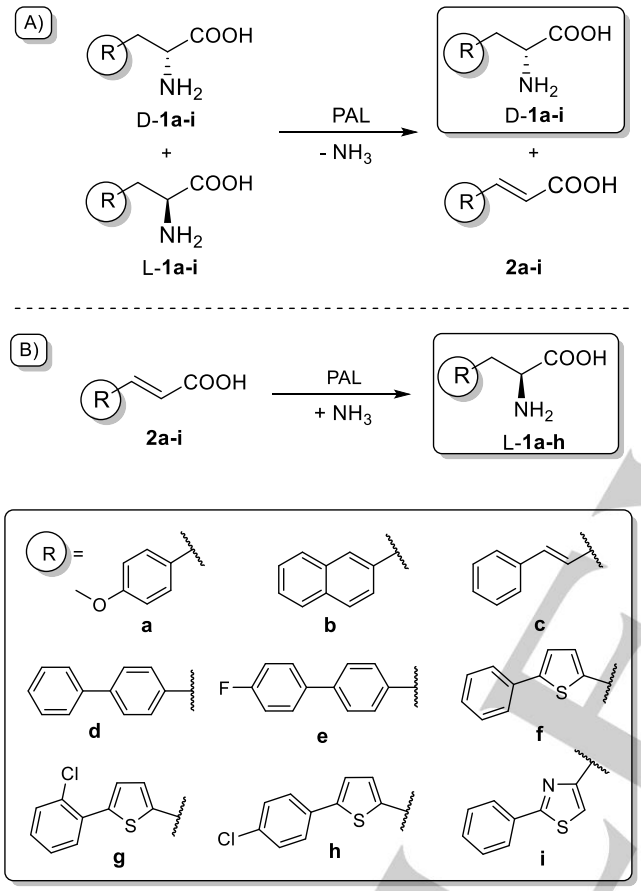
It is worth noting that some of the target compounds (Scheme 1) had already been tested before and exhibited little or no conversions or even inhibitory activity with wild-type PALs.[9,14,15,17] Moreover, no mutant PAL variants were known to possess activity in the corresponding ammonia elimination and 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 upon the substrate panel.



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

Results and Discussion

CASTing[26] or directed evolution methods requiring high-throughput enzyme assays (HTS) of large mutant libraries to generate mutants of *Pc*PAL accepting the bulky target compounds as substrates were avoided, due to 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]4,[17]7], 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. V, A) providing a limited number of single or multiple residue mutants of *Pc*PAL (Table S1).

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 from size-exclusion chromatography (ESI, figure S2) revealed that all *Pc*PAL variants were properly folded and existed in the native, tetrameric form, similarly to the wild-type enzyme. The slight variations in thermal unfolding temperatures (T_m), determined by differential scanning fluorimetry measurements, indicated that the mutations did not affect protein folding (see Table S3 in ESI). The only exception was the I460A mutation which decreased the T_m value significantly (from 75±1.5 °C to 51±1.2 °C, see Table S4, Figure S5 in ESI) without affecting the tetrameric fold detected by size exclusion chromatography. Since later tests showed that I460A-*Pc*PAL was catalytically active (Tables S2-S19 in ESI), we presumed that the mutation corrupted only the thermal stability without disrupting the main folding patterns related to enzyme activity.

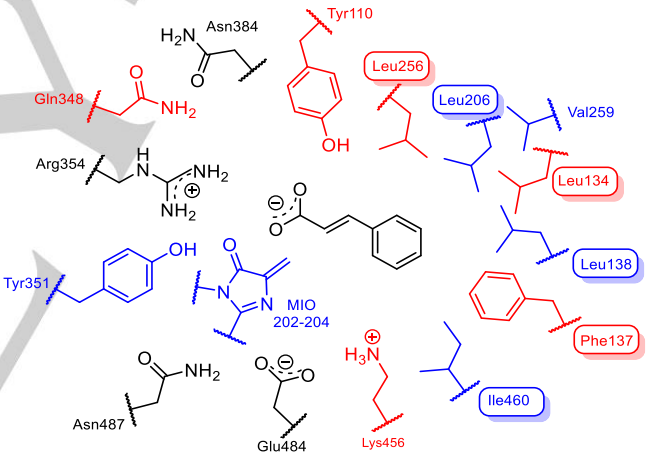


Figure 1. Active site model of *Pc*PAL with (*E*)-cinnamic acid as a modeled ligand and the surrounding residues within less than 5 Å distance. Colors of amino acid side chains refer to their position compared to the plane of substrate: black – within, red – above, blue – below the plane. Hydrophobic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

Next, the generated single mutant *Pc*PAL library (Table S1) was tested with the targeted substrate panel in ammonia elimination from arylalanines *rac*-**1a-i** (Scheme 1A) and in ammonia addition onto arylacrylates **2a-i** (Scheme 1B). Results from the ammonia eliminations revealed that besides the known F137 to V or A mutations,[13,[17]7] mutation of another highly conserved residue I460 (see Figure S1 in ESI) to valine or alanine increased the activity significantly towards almost all substrates compared to the wild-type enzyme (Table 1 and Tables S5-13 in ESI).

Whilst wt-*Pc*PAL could convert the members of the tested substrate panel quite poorly ($c_{rac\text{-}1a}$= 3%; $c_{rac\text{-}1b}$= 6%; $c_{rac\text{-}1c\text{-}i}$ <

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

Substrate	R group	*Pc*PAL variant[a]	*c*[b]
rac-**1a**	4-methoxy	wt	3
rac-**1a**	4-methoxy	F137	37
rac-**1a**	4-methoxy	**I460V**	**39**
rac-**1b**	napthalen-2-yl	wt	6
rac-**1b**	napthalen-2-yl	I460V	37
rac-**1b**	napthalen-2-yl	**F137V**	**39**
rac-**1c**	styryl	wt	<1
rac-**1c**	styryl	**I460V**	**~50**
rac-**1c**	styryl	**F137V**	**~50**
rac-**1d**	biphenyl-4-yl	wt	<1
rac-**1d**	biphenyl-4-yl	I460V	8
rac-**1d**	biphenyl-4-yl	**F137A**	**35**
rac-**1e**	4'-fluorobiphenyl-4-yl	wt	<1
rac-**1e**	4'-fluorobiphenyl-4-yl	F137V	37
rac-**1e**	4'-fluorobiphenyl-4-yl	**F137A**	**39**
rac-**1f**	5-phenylthiophen-2-yl	wt	<1
rac-**1f**	5-phenylthiophen-2-yl	F137V	35
rac-**1f**	5-phenylthiophen-2-yl	**F137A**	**44**
rac-**1g**	2'-chloro-5-phenylthiophen-2-yl	wt	<1
rac-**1g**	2'-chloro-5-phenylthiophen-2-yl	F137V	19
rac-**1g**	2'-chloro-5-phenylthiophen-2-yl	**F137A**	**~50**
rac-**1h**	4'-chloro-5-phenylthiophen-2-yl	wt	<1
rac-**1h**	4'-chloro-5-phenylthiophen-2-yl	I460V	10
rac-**1h**	4'-chloro-5-phenylthiophen-2-yl	**F137A**	**~50**
rac-**1i**	2-phenylthiazol-4-yl	wt	<1
rac-**1i**	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 PTFE septum at 30 °C, 200 rpm for 16 h;

[b] conversion values (%)

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

Despite the flourishing extension of 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 (**2a**)-and napthalen-2-ylacrylic acid (**2b**), while for more bulky substrates **2d**-**h** and styrylacrylate (**2c**) no conversions could be detected (Tables S14-22 in ESI).

To explore the advantageous synthetic potential of ammonia addition onto arylacrylates (100% theoretical yield; use of synthetically accessible, achiral starting materials), further mutations of neighboring residues were introduced to the single mutants with the best activities. In this way, a focused library of double and triple mutants involving I460, F137 and L138 (Table S1 in ESI) was obtained and tested in both kinds of PAL mediated reactions (Tables S5-22 in ESI). 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 overall folding was not seriously altered even in case of double and triple mutants.

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

However, *Pc*PALs having simultaneous mutations of F137 and I460 gave promising results in ammonia additions onto **2c**,**d**. In these cases no product could be detected with wt-*Pc*PAL or either single mutants of *Pc*PAL, but 22%, respectively 27% conversion could be achieved with the F137(V,A)/I460V double mutants (Table 2; Tables S16,S17 in ESI). Arylacrylates **2e**-**h** proved to be moderate substrates even for the F137A/I460V double mutant (conversions between 3-8% after 20 h, Tables S18-21 in ESI), while no conversion of (2-phenylthiazol-4-yl)acrylic acid (**2i**) could be achieved with the investigated multiple mutant *Pc*PALs (Table S22 in ESI).

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 significantly improve conversions. However, double mutants of *Pc*PAL involving F137, I460 were required to achieve adequate ammonia addition activity with bulky arylacrylates **2c-h**. In case of less bulky substrates **2a,b** active single mutants of *Pc*PAL could be identified as well. These data clearly demonstrated that multiple mutations exhibited a strong, non-additive, cooperative effect[27] on *Pc*PAL activity in the ammonia addition reaction.

The fact that individual mutations of L134, L206, L256, and L138 L138 as well as the double and triple mutants of L138 with the neighbor, activity modulator residues F137 and I460 didn't provide any increase in conversion of the tested substrate panel in neither 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.

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

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

[a] *Pc*PAL variant: 50 µg, reaction volume: 500 µL, medium: 6M 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 (%)

With the most active mutants in hands (Tables 1,2), 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 the ammonia elimination and addition, respectively.

Reactions of *rac*-**1d** and **2b** were investigated also using whole cells of *E. coli* expressing 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 (using living or lyophilized whole cells, various biocatalyst-substrate ratio or temperature), results of whole cell biotransformations with these bulky and hydrophobic substrates were irreproducible even within the same batch of cells suggesting cell internalization difficulties of the bulky hydrophobic substrates. The reproducibility of experiments with cell lysates supported this hypothesis, but provided poor quality analytical data (appearance of additional signals in HPLC chromatograms). Since 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**,**d**-**i**, in the reaction buffer of ammonia eliminations was addressed already during the initial screening tests. Although by using DMSO or MeOH as cosolvents (5,10,20 v/v%) the solubility of *rac*-**1d** could be increased to 2-3 mM, conversions dropped from 37% after 16 h to 16% at 10% cosolvent level and down to zero at 20% cosolvent level. Finally, solubilization of *rac*-**1d** up to 2.5 mM concentration was achieved by forming inclusion complex with 5-20 mM β-cyclodextrin[28] without altering the enzyme activity. Thus, activity screens in ammonia elimination with *rac*-**1a-i** were performed in the presence of 20 mM β-cyclodextrin (Table 1).

Unfortunately, the low substrate solubility prevented us from the determination of Michaelis-Menten curves approaching substrate saturation. Despite the apparent solubility increase by β-cyclodextrin, the unknown actual concentrations of non-complexed 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 of (napthalen-2-yl)acrylic acid (**2b**). The best results in terms of conversion values and enantiotopic selectivities were achieved using 4M ammonium carbamate (Table S23 in ESI), 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, monitoring 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**) have been 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 low conversion (10%) suggesting product inhibition. This hypothesis was confirmed by the inhibitory effect of **2i** upon the wt-*Pc*PAL catalyzed ammonia elimination from L-Phe (see ESI). Furthermore, ammonia additions onto **2i** with wt- or mutant *Pc*PALs did not succeed suggesting 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 reactions onto **2a-h** proceeded somewhat slower (Table 4) and, after longer incubation times (>20-24 h), deactivation of *Pc*PALs was observed due to 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 slow increase of conversions until 70 h reaction time (see Figure S7-S12, progression curves of ammonia addition reaction) .The fact that after additional 48 h no further reaction progress could be detected indicated that an equilibrium state was reached.

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

cc	*Pc*PAL variant	t_{react} (h)	Y [a] (%)	$ee_{D\text{-}\mathbf{1a\text{-}h}}$ (%)
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 ESI, chapter 6.6.)

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.

Substrate	*Pc*PAL variant	c (%)	Y[a] (%)	$ee_{L\text{-}\mathbf{1a\text{-}h}}$ (%)
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	n.d	n.d
2g	F137A/I460V	9	n.d	n.d
2h	F137A/I460V	3	n.d	n.d

[a] the reaction yields were determined from the preparative scale ammonia additions (for reaction conditions see ESI, chapter 6.7)

Accordingly, 4-methoxycinnamic acid (**2a**) and (napthalen-2-yl)acrylic acid (**2b**) were transformed with good conversion (74% and 73%) and excellent enantiomeric excess ($ee_{L\text{-}\mathbf{1a}}$ and $ee_{L\text{-}\mathbf{1b}}$ >99%), while conversion of styrylacrylate (**2c**) by F137V/I460V-*Pc*PAL remained low (23%) but selective ($ee_{L\text{-}\mathbf{1c}}$ >99%). (1,1'-Biphenyl)acrylates **2d,e** were transformed with good to moderate conversions (68% and 50%) but with non-complete enantiotopic selectivity ($ee_{L\text{-}\mathbf{1d}}$= 82%, $ee_{L\text{-}\mathbf{1e}}$= 95%; see 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 slower, reaching conversions of only 3-9% within 70 h.

The enhanced conversions of L-**1a-h** with the proper mutants can be rationalized by the better substrate affinities and also by the higher turnover numbers due to better stabilization of the reaction transition states, in accordance with our previous computer-aided results.[17] 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 the cases (Table 5).

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. Subscripts WT and MA correspond to *wild-type* and *most active mutant,* respectively. Worth to note that these quantities are not meant to computationally determine actual binding energies but to approximate them only.

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

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

Figure 2 illustrates the reason for affinity increase of L-**1e** where F137A/I460V-*Pc*PAL provides a larger active site volume and much less area of close contact of the *N*-MIO covalent enzyme-substrate complex with sites A137 and I460 (Figure 2C) and a significant shift of the ligand position (Figure 2B) as compared to wt-*Pc*PAL (Figure 2A). Comparisons of the *N*-MIO intermediates from the other L-arylalanines L-**1b-d,f-h**, indicated similar

situations (see ESI). 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.

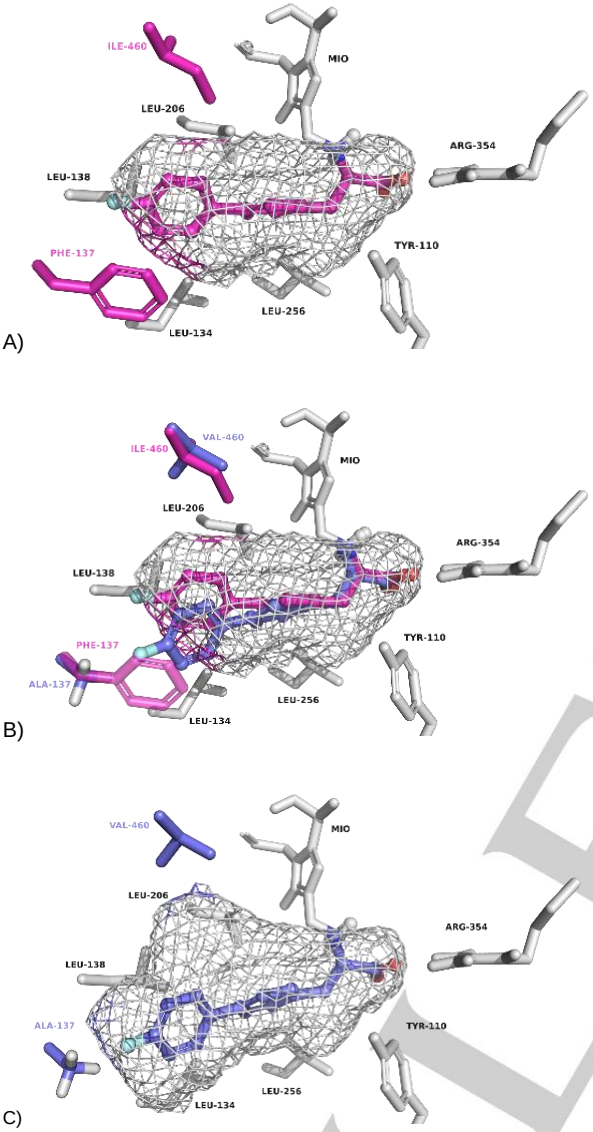


Figure 2. Catalytically active *N*-MIO intermediate models of L-**1e** within wt-*Pc*PAL (panel **A**) and F137A/I460V-*Pc*PAL (panel **C**). Volumes inside the mesh represent the cavity provided by the active site of the corresponding enzyme. Panel **B** represents the combination of panel **A** and **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. Coloring of the mesh represents close contacts with the corresponding residues of the mutational site.

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 which states that in an exothermic reaction the high energy intermediate and thus the transition state (TS) resembles better to the substrate state, while the TS of an endothermic reaction resembles better to the products state. In our case, the reverse ammonia addition reaction is known to be 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 invalidating our computational results for ammonia addition. This was supported by analysis of the thermal unfolding profiles of wt-*Pc*PAL (Figure S6) and F137A/I460V-*Pc*PAL (Figure 3) at different ammonia concentrations indicating shifts of melting temperature (T_m) by 10-12 °C at the highest ammonia concentration as compared to that determined in Tris buffer.

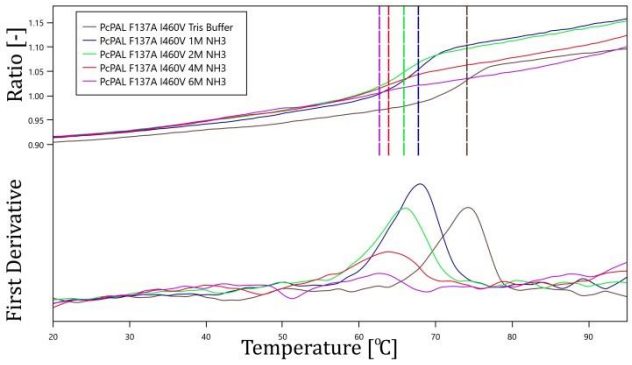


Figure 3. The thermal unfolding temperature (T_m) of F137A/I460V-*Pc*PAL in media with different ammonia content (20 mM Tris.HCl, pH 9; and 1 M, 2 M, 4 M and 6 M NH_3-buffer, with pH 9.5 adjusted by CO_2) determined by nanoDSF (Prometheus NT.48). Fluorescence intensity ratios F350/F330 and their first derivative are represented as a function of the applied linear thermal ramp.

Finally, synthetic applicability of the tailored *Pc*PAL mutants was demonstrated by performing KRs from racemic arylalanines *rac*-**1a**-**h** by ammonia elimination digesting the L-enantiomers (Scheme 1A) and by the enantiotopic selective ammonia addition reactions onto **2a**-**e** (Scheme 2B) at larger scale (0.25 mmol substrate, for details, see ESI). The preparative scale reactions of **2f**-**h** were omitted due to the quite low equilibrium conversions. During the preparative scale reactions no significant alterations of conversions, reaction times, and enantiomeric excess values have been 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.

Worth to note that prior to this work, the stereoconstructive ammonia addition onto the bulky arylacrylates **2a-h** were 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**) yielding the corresponding amino acids D-**1a,b,d-h**.

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

Pharmaceutically important ([1,1'-biphenyl]-4-yl)alanines were subjects of recent *Av*PAL mediated biotransformations where the studied *Av*PAL variants showed no activity in ammonia addition onto 4-phenylcinnamic acid, thus a chemoenzymatic procedure was required involving 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,e**.

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

Conclusions

The substrate scope of *Pc*PAL has been expanded towards a series of sterically demanding phenylalanine analogues by tailored mutations of the hydrophobic substrate binding pocket based on simple, steric clash reduction concept. While single mutations of residues F137 and I460 proved to be sufficient to enhance the PAL activity in the ammonia elimination reactions, combined mutations of F137 and I460 – resulting in a cooperative, non-additive effect – was required to create PAL biocatalysts being active in the reverse ammonia addition reactions of bulky substrates. This work highlights the importance of residue I460 – besides the already explored residue F137 – in the modulation of the substrate specificity of *Pc*PAL and demonstrates the importance of non-additive effects of combined mutations on PAL activity.

The novel *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**. By the aid of tailored multiple mutations of *Pc*PAL, direct biocatalytic routes could be developed to D- and L- ([1,1'-biphenyl]-4-yl)alanines, D- and L-**1d,e**, valuable chiral intermediates for several drugs under development as well as to novel non-natural amino acids D-(5-phenylthiophen-2-yl)alanines D-**1f-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.

Keywords: biocatalysis • phenylalanine ammonia-lyase • non-natural amino acids • protein engineering • substrate scope extension

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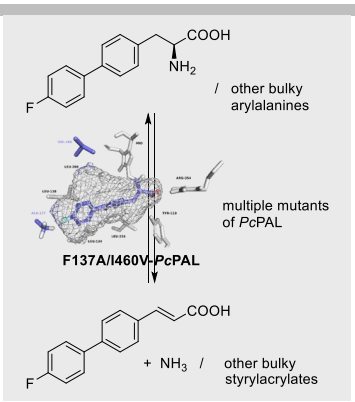
Entry for the Table of Contents

FULL PAPER

Alone is not enough. Tailored mutants of phenylalanine ammonia-lyase from *Petroselinum crispum* (*Pc*PAL) were created and tested in ammonia elimination reactions of sterically demanding, non-natural analogues of phenylalanine and in ammonia addition reactions onto the corresponding (*E*)-arylacrylates. The synergistic multiple mutations resulted in substantial substrate scope extension of *Pc*PAL.



Alina Filip, Emma Z. A. Nagy, Souad D. Tork, Gergely Bánóczi, Monica I. Toșa, Florin D. Irimie, László Poppe, Csaba Paizs,* and László C. Bencze**

1 – 8

Tailored mutants of phenylalanine ammonia-lyase from *Petroselinum crispum* for the synthesis of bulky L- and D-arylalanines