Babeş-Bolyai University
Faculty of Chemistry and Chemical Engineering
Doctoral School of Chemistry

Wild-type and tailored phenylalanine ammonia-lyases for the synthesis of unnatural L- and D-arylalanines

PhD Thesis Abstract

PhD candidate: Alina FILIP
Scientific advisor: Prof. Habil. Dr. Csaba PAIZS

Cluj-Napoca
2019

„ BABEŞ-BOLYAI“ UNIVERSITY

CLUJ-NAPOCA

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Public defense: 21st June 2019, Cluj-Napoca

Thesis Table of Contents

Scientific activity

I. Articles on which the thesis is based on.

1. **Filip A.**,‡ Nagy E.Z.A.,‡ Tork S.D., Bánóczi G., Toşa M.I., Irimie F.D., Poppe L., Csaba P., Bencze L.C., Tailored mutants of phenylalanine ammonia-lyase from *Petroselinum crispum* for the synthesis of bulky L- and D-arylalanines, *ChemCatChem*, **2018**, *10*, 2627-2633.

IF 4.674
ISSN 1867-3880
Relative score of influence = 1.899

2. Csuka P., Juhász V., Kohári Sz., **Filip A.**, Varga A., Sátorhelyi P., Bencze P., Barton H.A., Paizs C., Poppe L., *Pseudomonas fluorescens* strain R124 encodes three different MIO-enzymes, *ChemBioChem*, **2018**, *19*, 411-418.

IF 2.774
ISSN 1439-4227
Relative score of influence = 1.840

3. Bencze L.C.,‡ **Filip A.**,‡ Bánóczi G., Toşa M.I., Irimie F.D., Gellért Á., Poppe L., Paizs C., Expanding the substrate scope of phenylalanine ammonia-lyase from: *Petroselinum crispum* towards styrylalanines, *Organic and Biomolecular Chemistry*, **2017**, *15*, 3717-3727.

IF 3.423
ISSN 1477-0520
Relative score of influence = 1.798

4. Dima N.A., **Filip A.**, Bencze L.C., Oláh M., Sátorhelyi P., Vértessy B.G., Poppe L., Paizs C., Expression and purification of recombinant Phenylalanine ammonia-lyase from *Petroselinum crispum*, *Studia UBB Chemia*, **2016**, *LXI*, 2, 21-34.

IF 0.244
ISSN 1224-7154
Relative score of influence = 0.026

5. **Filip A.**, Bencze L.C., Paizs C., Poppe L., Irimie F.D., MIO-enzyme toolbox: cloning, expression and purification of recombinant *Rt*PAL, *Stud. Univ. Babeş-Bol.* Sp. Iss. **2015,** 39-43.

BDI
Short communication

‡ These authors contributed equally to this work.

Key words: site directed mutagenesis, expression and purification, phenylalanine ammonia-lyase, *wild-type*, tailored mutants, MIO-enzymes, styrylalanines, arylalanines, biocatalysis, enzymatic kinetic resolution.

Introduction

Amino acids are the building blocks of proteins, a class of biomacromolecules with a wide range of functions. Furthermore, they are involved in different metabolic pathways of fundamental processes of living organisms such as, growth, development, reproduction, immune protection, cellular and organism homeostasis.[1,2] Accordingly, the term *"bricks of life"* associated with amino acids is well justified.

The practical use of natural and unnatural amino acids in the production of biologically valuable products or intermediates of fine organic synthesis useful for various industries provides a continuous interest towards their production.

Natural amino acids are accesible through their isolation from biological sources or by chemical/biochemical synthesis. Thus, the alternative for their production is mostly determined by economical and sustainability factors. Unnatural amino acids can be obtained only by synthesis. Their chiral nature and structural complexity, but also their similarity with natural amino acids, guide their synthesis towards more active, selective, environmently friendly, thus more efficient biocatalytic routes.

The use of enzymatic preparations, with different degree of purification level, is a well-established practice both in academic research and in industrial manufacturing. Their capacity to assist reactions catalytically, with high activity and selectivity, provides a solid base for clean synthetic procedures. Therefore, the use of biocatalysts is based on the dichotomic compromise between selectivity and promiscuity. The proper function of an enzyme has been optimized over millions of years/generations by repetitive mutation/selection cycles. Such enzymes function perfectly for their natural substrates. Obviously, their performances towards an unnatural substrate analogues, will be inferior. Therefore, in case if an alternative, enzymatic synthetic route is required, the biocatalyst must be adapted to the unnatural substrate analogue. This adaption process can be accomplished either through rational means, employed within this thesis, or directed evolution methods. Since, each one of them present advantages/disadvantages results in the complexity of independent variables, making the choice specific for the studied case.

The research described within this thesis aimed to adapt, through rational means, the functionality of phenylalanine ammonia-lyase originary from *Petroselinum crispum* (*Pc*PAL) towards bulky substrates. The range of substrates accepted by *Pc*PAL is limited, bulky biaryl, heteroaryl substrate analogues are poorly or not transformed by the enzyme. Modifications focusing on the hydrophobic region of the catalytic site of *Pc*PAL increased its tolerance towards bulky L- and D-arylalanines.[3, 4]

Tailored mutants of *Pc*PAL such as variant F137V/A, I460V/A, with single mutations[5] or with multiple mutations such as F137V/L138V, F137V/I460V, F137A/I460V or F137V/L138V/I460V have shown considerable enhancement of their catalytic properties compared to the *wild-type* enzyme, in reactions with bulky substrates, such as styrylalanines, (4-methoxyphenyl)-, (4'-fluoro-[1,1'-biphenyl]-4-yl)-, ([1,1'-biphenyl]-4-yl)-, (napthalen-2-yl)- and (5-pheniltiophen-2-yl) alanines and their corresponding aryl-acrylic derivatives yielding a wide range of L- and D-arylalanines.[6]

Besides the specific procedures of organic synthesis used for subtrates preparation, the genetic engineering and microbiology techniques, providing the recombinant PAL variants, the biotransformations, yielding the L- and D-arylalanines, other instruments such as, computational studies and X-ray crystallography were also used to provide molecular level insights of the PAL-catalyzed processes. The obtained data open new perspectives on the rational design of the hydrophobic binding pocket of *Pc*PAL.

Through the presented reuslts, the thesis contributes to a better understanding of phenylalanine ammonia-lyase (PAL) originary from *Petroselinum crispum*, by extending its range of utility and providing particular methodological contributions to adaption process of the enzyme for non-natural substrates of interest.

Aims of the thesis

This thesis is structured in two main chapters: Systems of expression and purification (I) and Chemical synthesis of styrylalanines and biotransformations mediated by *wild-type* and *Pc*PAL mutant variants (II). Each chapter is divided in three subchapters. Each subchapter is divided in: literature survey, materials and methods, results and discussion and conclusions.

1. Modification of the catalytic site of phenylalanine ammonia-lyase from *Petroselinum crispum* through genetic engineering in order to provide novel mutant variants with superior biocatalytic activity (Subchapter I A).

2. His-tag removal of several *Pc*PAL mutant enzymes (I460V, F137A, F137A/I460V and Y110F/F137/I460V) using a TEV endopeptidase (Tobacco Etch Virus) in order to provide highly purified protein samples for crystallization studies (Subchapter I B).

3. Expression, isolation and purification of three nevel MIO enzymes, phenylalanine 2,3-aminomutase (*Pf*PAM), phenylalanine/histidine ammonia-lyase/tyrosine (*Pf*XAL) and histidine ammonia-lyase (*Pf*HAL) identified in the genome of the bacterium *Pseudomonas fluorescens* R124 (Subchapter I C).

4. Chemical synthesis of styrylalanines as novel substrates for phenylalanine ammonia-lyase (Subchapter II A).

5. The rational development, using site-directed mutagenesis, of useful *Pc*PAL mutants F137/G/A/V-*Pc*PAL for the stereoselective synthesis of L-styryl-alanines (Subchapter II B).

6. Synthesis of synthetically valuable, bulky L- and D-arylalanines through rationally designed PcPAL mutants, bearing single or multiple mutations of the hydrophobic substrate binding region (Subchapter IIC).

CHAPTER I. Systems of expression and purification

Subchapter A. Expression and purification of recombinant, *wild-type* and mutant forms of phenylalanine ammonia-lyase from *Petroselinum crispum*

I. A.2. Results and discussion

I. A.2.1. Site-directed mutagenesis

Site-directed mutagenesis is a fast and reliable method used to alterate in a desired way a gene at the level of the desired codon. Replacement of an amino acid with a desired one is accomplished by changing a nucleotide (or two/three nucleotides) of the original codon with the nuclotide/nucleotides corresponding to the new codon encoding the desired amino acid.

The sequence alteration is performed through PCR reaction (Polymerase Chain Reaction), using a pair of specifically designed mutagenic primers, which contain the desired nucleotide modification.[6]

Figure 1 shows the agarose 1% gel in which the spots corresponding to some polymerization products can be observed (as described) at about 8.000 base pairs.

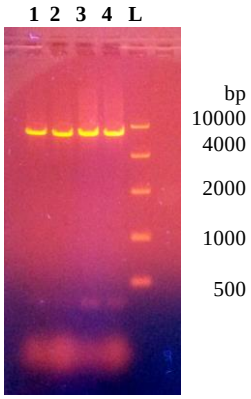


Figure 1. Agarose gel electrophoresis of the PCR products products from the site-directed mutagenesis experiments: **1** – **pET-19b** vector harbouring the gene of *wt-Pc*PAL (7817 kpb), **2** – PCR reactions performed for mutation for **F137V**, **3** – product of PCR reaction performed for mutation **F137A**, **4** – PCR reactions for mutation **F137G**, **L** – DNA Ladder**.**

The polymerization products have been successfully transformed into *E.coli* XL1Blue competent cells for plasmid storage, followed by extraction of the plasmid.

External sequencing services were used to confirm the presence of the desired mutations. Once the mutations were confirmed, the corresponding plasmids were transformed succesfully into expression host *E.coli* Rossetta (DE3) pLysS cells.

I. A.2.2. Expression and purification of proteins

For the expression of *wild-type* and mutant phenylalanine ammonia-lyases, *Escherichia coli* Rosetta (DE3) pLyS host cells harbouring the pET-19b_*Pc*PAL **(Figure 2a** and **2b)** expression vector were used.[7]

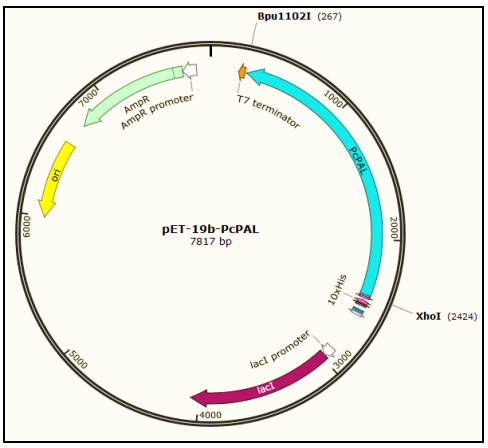


Figure 2a. Plasmid map of pET-19b-*Pc*PAL

MGHHHHHHSGHIDDDDKHMLEMENGNGATTNGHVNGNGMDFCMKTEDPLY
WGIAAEAMTGSHLDEVKKMVAEYRKPVVKLGGETLTISQVAAISARDGSG
VTVELSEAARAGVKASSDWVMDSMNKGTDSYGVTTGFGATSHRRTKQGGA
LQKELIRFLNAGIFGNGSDNTLPHSATRAAMLVRINTLLQGYSGIRFEIL
EAITKFLNQNITPCLPLRGTITASGDLVPLSYIAGLLTGRPNSKAVGPTG
VILSPEEAFKLAGVEGGFFELQPKEGLALVNGTAVGSGMASMVLFEANIL
AVLAEVMSAIFAEVMQGKPEFTDHLTHKLKHHPGQIEAAAIMEHILDGSA
YVKAAQKLHEMDPLQKPKQDRYALRTSPQWLGPQIEVIRSSTKMIEREIN
SVNDNPLIDVSRNKAIHGGNFQGTPIGVSMDNTRLAIAAIGKLMFAQFSE
LVNDFYNNGLPSNLSGGRNPSLDYGFKGAEIAMASYCSELQFLANPVTNH
VQSAEQHNQDVNSLGLISSRKTSEAVEILKLMSTTFLVGLCQAIDLRHLE
ENLKSTVKNTVSSVAKRVLTMGVNGELHPSRFCEKDLLRVVDREYIFAYI
DDPCSATYPLMQKLRQTLVEHALKNGDNERNLSTSIFQKIATFEDELKAL
LPKEVESARAALESGNPAIPNRIEECRSYPLYKFVRKELGTEYLTGEKVT
SPGEEFEKVFIAMSKGEIIDPLLESLESWNGAPLPIS-AEQ-LA-
PLGASKR

Figure 2b. The translation sequence of *wt-Pc*PAL with the *N*-terminal 6xHis-tag (green) and the enterokinase cleavage site (cyan).

The protein expression was performed at 37 °C by inducing the culture medium with 0.5 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) at OD_{600} of 0.7-0.8, then followed by maintaining the cell mass at 25 °C overnight.

Purification was performed on a Ni-NTA affinity chromatography column as described in the manufacturer's protocol. The *Pc*PAL protein with the *N*-terminal His-tag was eluted with 250 mM imidazole.

I. A.2.2.1. SDS-PAGE analysis

The steps of enzyme expression and purification were monitored by SDS-PAGE with Coomassie Blue R 250 staining.

In lane 13 of the 10% polyacrylamide gel **(Figure 3)** a pronounced band around 80 kDa corresponding to the *Pc*PAL monomer, with a high purity can be observed.

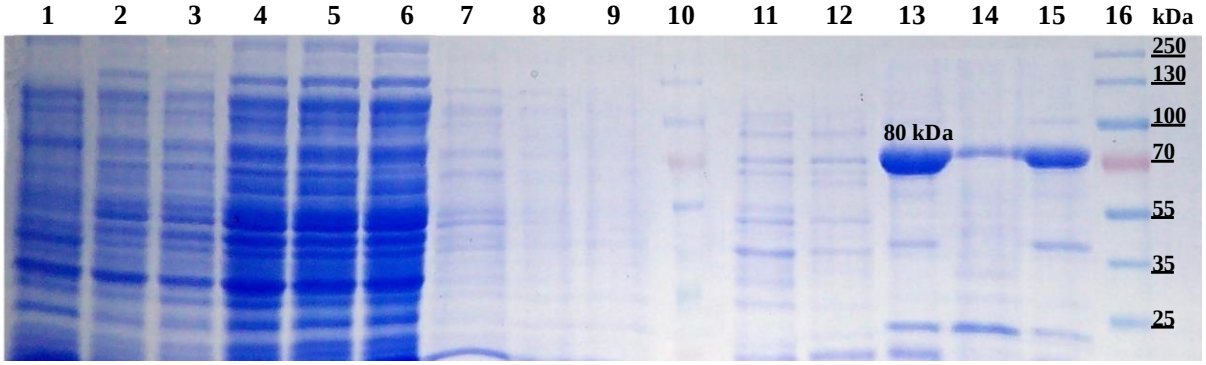


Figure 3. SDS-PAGE 10% gel containing samples from the purification steps of *Pc*PAL F137V. **1** – cells before induction, **2** – cells after induction, **3** – cell lysate, **4** – pellet of the cells lysate, **5** – supernatant after centrifugation of the lysate, ***6** – flow-through fraction from the Ni-NTA column, ***7** – fraction eluted with a weak saline solution (30 mM KCl and 50 mM HEPES), ***8** – fraction eluted with a strong saline solution (300 mM KCl and 50 mM HEPES), ***9** – fraction eluted with a weak saline solution (30 mM KCl and 50 mM HEPES), ***10** – molecular weight marker, ***11** – fraction eluted with 25 mM fraction of imidazole, ***12** – fraction eluted with 50 mM imidazole, ***13** – fraction eluted with 250 mM imidazole (pure protein fraction), ***14** – fraction eluted with 1 M imidazole, **15** – the fraction with the pure protein after dialysis and **16** – molecular weight marker.

*samples from the Ni-NTA affinity purification step

I. A.2.2.2. Size exclusion chromatography

The purity of each enzyme obtained, was analyzed by size exclusion chromatography (analytical column Superdex 200 5/150 GL). Preparative scale purification of the enzymes was performed (on a preparative column Superdex 200 10/300 GL) in order to perform kinetic studies, which require the use of biocatalysts with a high degree of purity.

For most of the *Pc*PAL mutants obtained, the degree of purity was high. In **Figure 4** below, the chromatogram profile for the mutants *Pc*PAL I460V- and *Pc*PAL F137A/L138A/I460A can be observed.

According to the equation of the calibration curve determined in advance, the signal between 1.3–1.6 mL with a maximum intensity at 1.48 mL, corresponds to the tetrameric *Pc*PAL enzyme with a molecular weight of approximately 320 kDa.

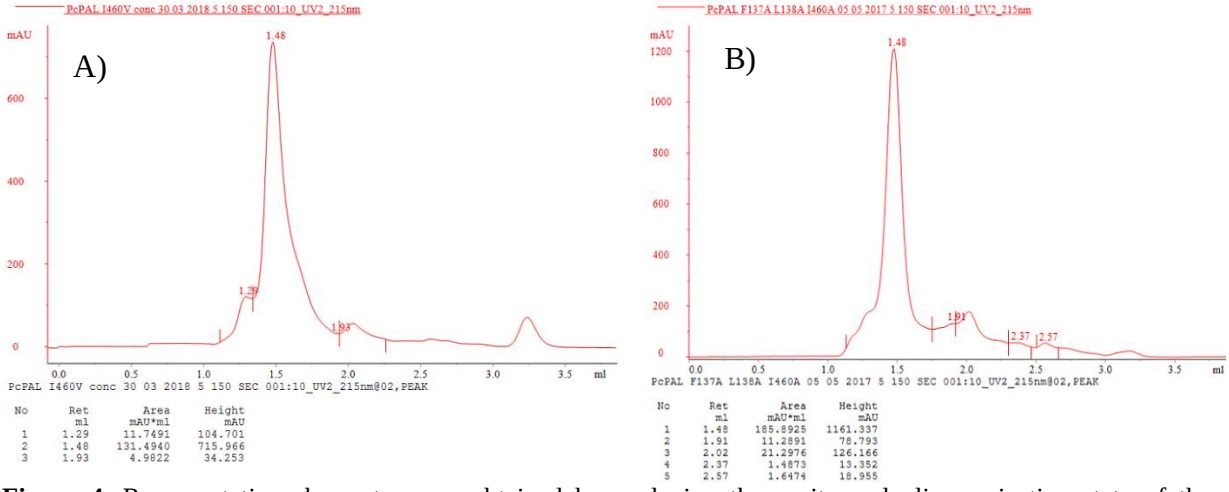


Figure 4. Representative chromatograms obtained by analyzing the purity and oligomerization state of the enzymes using the steric exclusion column Superdex 200 5/150 GL, **A.** *Pc*PAL I460V, **B.** *Pc*PAL F137A/L138A/I460A after the dialysis and concentration steps (3 mg/mL).

I. A.2.3. Analyzing thermal unfolding of *Pc*PAL mutants using the Prometheus NT.48

Nano differential scanning fluorimetry (NanoDSF)[8] was used to determine the protein stability of *wt-Pc*PAL and its mutants by measuring the melting temperature (T_m). As a result of the mutation, enzymes can suffer significant changes, which can be observed by comparing the T_m value to that of the *wild-type* enzyme.

Table 1. Melting temperature (T_m) of *Pc*PAL variants.

Entry	***Pc*PAL**	T_m (ºC)
1	*wild-type*	75.1±0.2
2	L134A	70.2±0.5
3	L134V	72.6±0.2
4	F137G	74.2±0.3
5	F137A	76.2±0.2
6	F137V	73.5±0.4
7	L138A	72.3±0.2
8	L138V	68.4±0.3
9	L206A	72.6±0.8
10	L206V	74.4±0.5
11	L256A	72.9±0.7
12	L256V	73.1±0.5
13	I460A	51.3±0.8
14	I460V	74.2±0.2
15	F137A/I460V	74.4±0.4
16	F137A/ I460A	51.8±0.9
17	F137V/I460A	51.8±0.8
18	F137V/I460V	71.7±0.3
19	F137A/L138V	70.9±0.4
20	F137A/L138A	75.3±0.3
21	F137V/L138A	73.0±0.3
22	F137V/L138V	71.1±0.5
23	L134V/F137A	73.8±0.2
24	F137V/L138V/I460V	70.7±0.6
25	F137A/L138V/I460V	72.3±0.2
26	F137A/L138A/I460V	69.8±0.5
27	F137A/L138A/I460A	62.1±0.7
28	F137A/L138V/I460A	52.2±0.9
29	F137A/S203A/I460V	72.2±0.2

As it can be seen in **Table 1**, the mutants showed different degrees of thermal stability. The melting temperature of the native enzyme was recorded at 75 °C. *Pc*PAL F137A showed an unfolding at a slightly higher temperature compared to the *wild-type* enzyme (76 °C vs. 75 °C), indicating an improved thermostability, although not statistically significant.

T_m values of mutants F137V-, F137A-, I460V- and F137A/I460V- *Pc*PAL were lower only by 2-3 ºC than the *wt-Pc*PAL control, these being the most active enzymes in transforming the tested substrates.

Instead, mutants I460A-, F137A/I460A-, F137V/I460A- and F137A/L138V/I460A *Pc*PAL showed a drastic decrease in thermal stability with a melting temperature by 23-24 ºC lower than the control and turned out to be less useful in the biotransformation of unnatural phenylalanine analogs **(Figure 5)**.

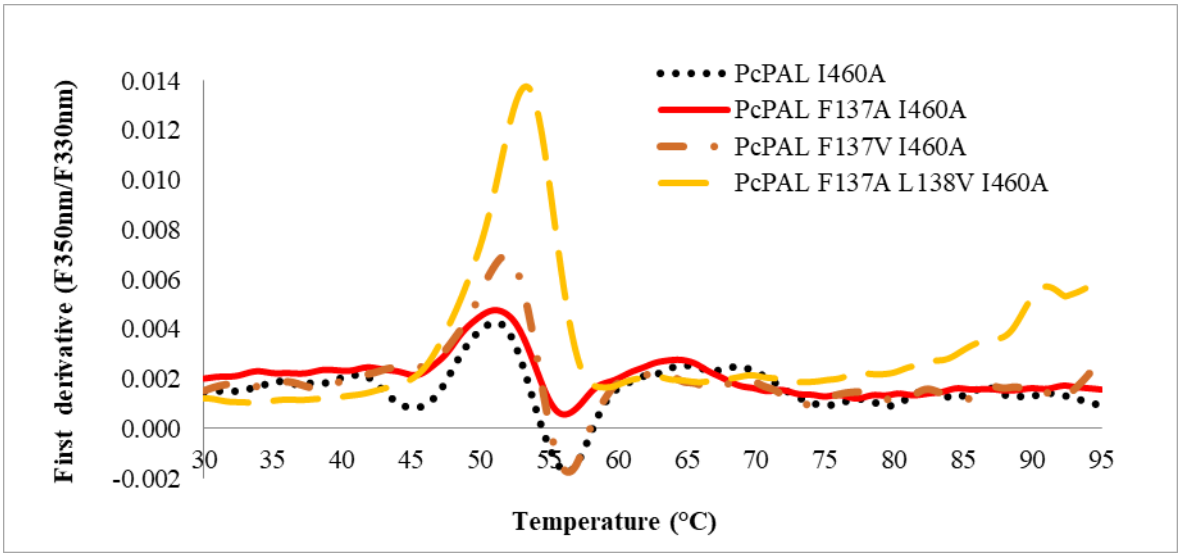


Figure 5. Thermal unfolding of *Pc*PAL variants involving mutation I460A. Melting temperatures (T_m) range from 51 to 52 ºC.

Other singular mutations, L134, L138, L206, L256, didn't cause probably a global conformational change of the protein, since T_m values between 69-74 ºC are only slightly lower than that of the native enzyme.

I. A.3. Conclusions

A library of 28 mutants of phenylalanine ammonia-lyase from *Petroselinum crispum* have been successfully obtained through site-directed mutagenesis. All the mutants have been expressed and purified to high purity and homogenity, as demonstrated by SDS-PAGE and size exclusion chromatography.

Differential fluorimetric measurements of single and multiple mutations containing the I460A residue, revealed a significant decrease in T_m (51 °C) compared to *wild-type* *Pc*PAL (75.1 °C ± 0.2). The most active *Pc*PAL mutant variants have a thermal stability in a high range of 72-76 °C.

Subchapter B. Obtaining mutant *Pc*PAL variants without hexa histidine-tag in order to crystallize the protein in its most natural form

I. B.2. Results and discussion

I. B.2.1. Site-directed mutagenesis, plasmid isolation and the required transformations

Based on the pET15b_*Pc*PAL template, with a cleavage site for the endopeptidase TEV, we tried to obtain four different types of mutants. The mutagenic primers used were the same as in case of pET19b_*Pc*PAL template.

The main purpose of this section is to obtain mutant *Pc*PAL enzymes with removable 6xHis-tag.

The recombinant *Pc*PAL encoded in plasmid construct pET19b_*Pc*PAL, contains an additional *N*-terminal 21 amino acids long sequence, including the 6xHis-tag, which might affect the protein folding. Therefore, for crystallization studies, we considered the development of recombinant *Pc*PALs with removable *N*-terminal His-tag, more resembling the original protein sequence.

After the expression, purifaction and the TEV mediated removal of 6xHis-tag, the *wt*- and mutant *Pc*PALs will be used for the crystallization of phenylalanine ammonia-lyases in the presence of several synthetic phenylalanine analogs or the corresponding cinanamates (for example, styryl-, 4-methoxyphenyl-, 4-nitrophenyl-, naphthalen-2-yl- or biphenyl-4-yl-), which were accepted as substrates by *Pc*PAL mutants, but not by *wt-Pc*PAL. These results will provide information about the enzyme-substrate interactions.

I. B.2.3. Expression and purification of TEV S219V enzyme

The steps of enzyme expression and purification were monitored by SDS-PAGE method.

The TEV enzyme having a molecular weight of approximately 29 kDa was eluted with 300 mM-1M imidazole. As it can be observed on column 9 of gel 2, the enzymatic solution after dialysis has a high purity (~ 90% on SDS-PAGE) - **Figure 6**.

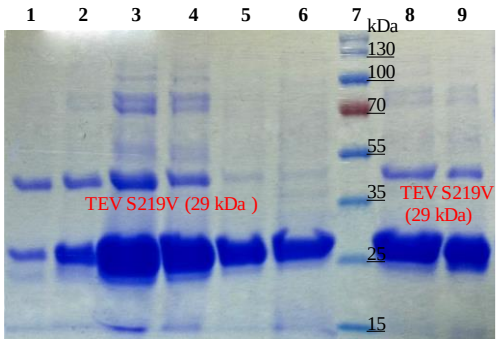


Figure 6. SDS-PAGE 10%, gel containing samples from the optimization of the purification steps of **TEV S219V**: **1 –** fraction eluted with 100 mM imidazole, **2 –** fraction eluted with 200 mM imidazole, **3 –** fraction eluted with 300 mM imidazole, **4 –** fraction eluted with 400 mM imidazole, **5 –** fraction eluted with 500 mM imidazole, **6 –** fraction eluted with 1M imidazole, **7 –** marker, **8** – the mixture of fractions **2**, **3**, **4** and **5** before dialysis, **9** – the point **8** after dialysis.

Therefore, the enzyme was expressed in a large amount (2 mg/mL) and used immediately to remove histidine labels of *wt-Pc*PAL and *Pc*PAL mutants or was stored at -80 °C in 20% glycerol until further use.

I. B.2.4. The removal of 6xHis-tag through digestion with TEV S219V

The removal of the affinity tag was carried out at 4 °C, using 2 mg of *Pc*PAL enzyme and 0.2 mg of enzyme TEV were added in a final volume of 2 mL of Tris buffer (50 mM Tris and 300 mM NaCl, 20% glycerol, pH 8.0) without stirring. The *Pc*PAL without His-tag was purified by Ni-affinity column, with a yield of 50-55%, followed by dialysis in 20 mM Tris and 100 mM NaCl, pH 8.0.

I. B.2.5. Analysis of the protein oligomerization by steric exclusion chromatography

Using a Superdex 200 5/150 GL column the oligomerization state[9] of the enzymes was verified. The elution profile for mutant proteins can be seen in the chromatograms below **(Figure 7)**. According to the equation in the calibration curve the signal between 1.4-1.6 mL, where the maximum intensity is at 1.49 mL, corresponds to the tetrameric *Pc*PAL enzyme with a molecular weight of 320 kDa.

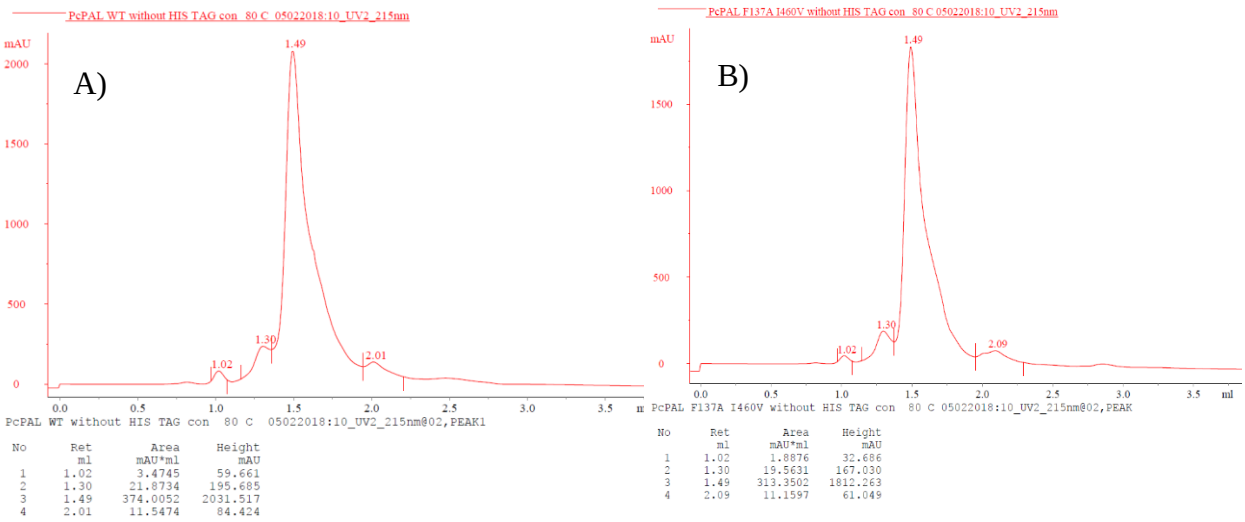


Figure 7. Representative chromatogram of the enzyme after the dialysis and concentration step using Superdex 200 5/150 GL size-exclusion column: **A)** *wt-Pc*PAL without His-tag (10 mg/mL) and **B)** *Pc*PAL F137A/I460V without His-tag (8 mg/mL).

I. B.2.6. Analyzing the thermal unfolding of *Pc*PAL mutants without the 6×His label.

The unfolding *of Pc*PAL *wild-type* and *Pc*PAL mutant (I460V, F137A, F137A/I460V and Y110F/F137/I460V) proteins devoided of their **6×His-tag** was performed in the range of 73-74.6 °C, where *Pc*PAL *wt* and *Pc*PAL F137A had a T_m value lower by 2 °C than the protein counterparts with hexa histidine tag.

Table 2. Melting temperatures (T_m) of *wt*- and I460V-, F137A-, F137A/I460V- and Y110F/F137/I460V-*Pc*PAL.

Nr.	***Pc*PAL**	**T_m (°C)**
1	native *(wt)*	73.0±0.2
2	I460V	74.2±0.5
3	F137A	74.6±0.5
4	F137A/I460V	73.7±0.2
5	Y110F/F137A/I460V	73.5±0.2

I. B.3. Conclusions

The expression, purification and the removal of *N*-terminal 6xHis-tag has been successfully achieved in case of *wt*- and several mutant *Pc*PALs, obtaining protein fractions with high degree of purity and homogeneity. No significant changes in the thermal stability of these enzymes after removing the affinity tag.

The purified, recombinant *Pc*PALs without the label 6×His label allowed the initiation of proteins crystallization experiments with different ligands.

Subchapter C. Expression and purification of novel MIO enzymes from *Pseudomonas fluorescens* R124

I. C.2. Results and discussion

I. C.2.1. Expression and purification of aromatic phenylalanine 2,3-aminomutase (PAM), phenylalanine/histidine or tyrosine ammonia-lyase (XAL) and histidine ammonia-lyase (HAL) all isolated from *Pseudomonas fluorescens* R124

I. C.2.1.1. Expression and purification of PAM, XAL and HAL

Using the general method for fermentation, isolation and purification of MIO enzymes the *Pf*HAL, *Pf*XAL and *Pf*PAM proteins with the *N*-terminal His-tag were obtained and stored in aqueous solution with 15% glycerol at - 20 °C without losing their activity.

The purity of all enzymes was verified using a 12% polyacrylamide gel **(Figure 8)**.

For each enzyme the fractions eluted from Ni-NTA, corresponding to the pronounced bands around 60 kDa, were analyzed also by size exclusion chromatography, in order to check the protein homogeneity **(Figura 9)**. All the expressed and isolated enzymes showed a high degree of purity and homogenity ~ >85%.[11]

Molecular weight of MIO enzymes was determined based on the calibration curve determined in advance **(Table 3).**

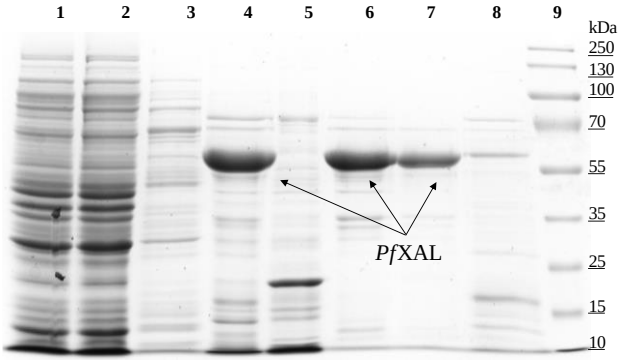


Figure 8. SDS-PAGE gel of samples from the purification steps of *Pf*XAL. **1** – supernatant after centrifugation of the lysate, 2 – flow-through after application of supernatant on the Ni-NTA column, **3** – fraction eluted with a weak saline solution (30 mM KCl and 50 mM HEPES), **4** – fraction eluted with 300 mM imidazole, **5** – fraction eluted with 50 mM imidazole, **6** – dialyzed, **7** – dialyzed, **8** – fraction eluted with 1 M imidazole, **9** – marker.

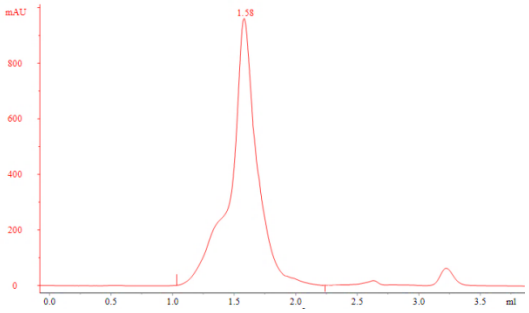


Figure 9. Representative chromatogram of *Pf*HAL, purification step using Superdex 200 5/150 GL size-exclusion column. The fraction with a retention volume of 1.2-1.45 mL represents the aggregated forms of the isolated enzyme, while the fraction with 1.5-1.8 mL represents the tetrameric form.

Table 3. MIO enzyme parameters obtained from the calibration curve and steric exclusion chromatography analysis using the Superdex 200 150/5 GL column.

Code	Experimentally determined molecular weight (Daltons)	Calculated molecular weight of tetramer (Daltons)	Calculated molecular weight of monomer (Daltons)
***Pf*HAL**	204046	222000	55500
***Pf*XAL**	211948	228000	57000
***Pf*PAM**	228681	233600	58400

I. C.2.2. Melting temperatures (T_m) of *Pf*HAL, *Pf*XAL and *Pf*PAM enzymes

Table 4. Melting temperatures (T_m) of *Pf*HAL, *Pf*XAL and *Pf*PAM

Enzyme	T_m (°C)
***Pf*PAM**	81.8 ±0.3
***Pf*XAL**	87.0±0.4
***Pf*HAL**	not detectable (>90)

I. C.3. Conclusions

The MIO enzymes, phenylalanine 2,3-aminomutase (PAM), phenylalanine/histidine or tyrosine ammonia-lyase (XAL) and histidine ammonia-lyase (HAL) identified in the genome of the bacterium *Pseudomonas fluorescens* R124 were successfully expressed and isolated using the *Escherichia coli* Rosetta strain cells (DE3) pLyS.

CHAPTER II. Chemical synthesis and biotransformations mediated by *wt*- and *Pc*PAL mutant variants

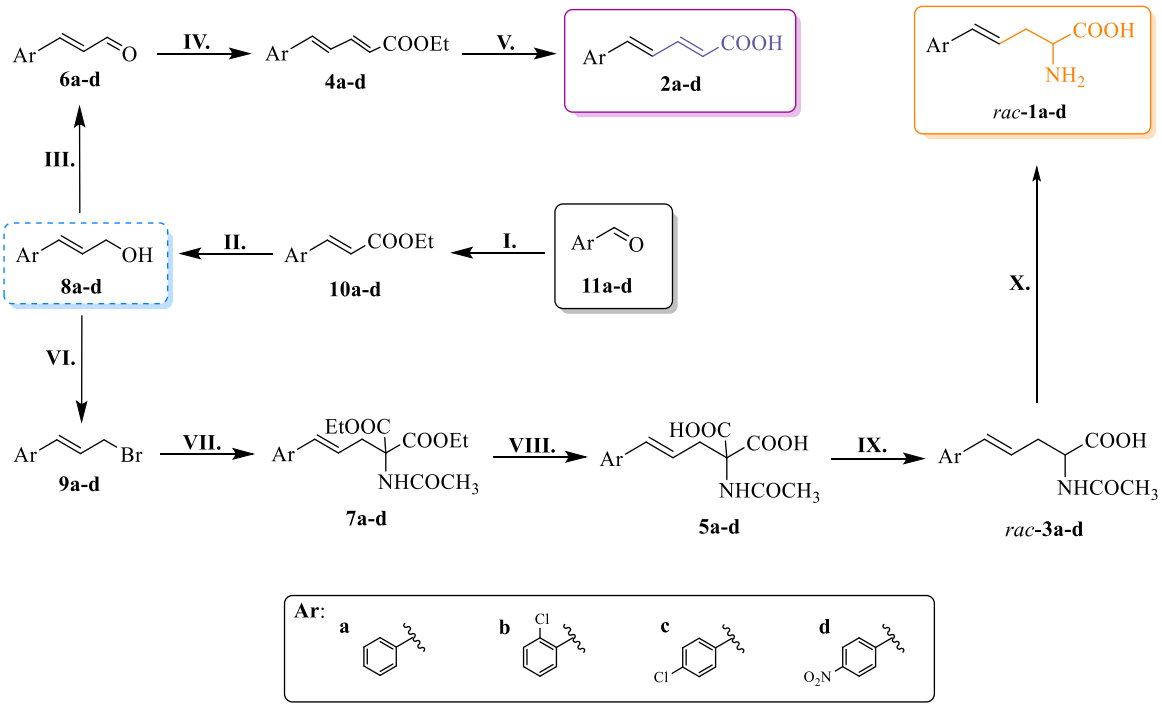
Subchapter A. Chemical synthesis of styrylalanines as novel substrates for phenylalanine ammonia-lyase

II. A.2. Results and discussion

II. A.2.1. Chemical synthesis of styrylalanine substrates *rac*-1a-d and 2a-d

Starting from the commercially available aldehydes **11a-d** acrylic esters **10a-d** were obtained through Wittig reaction, which by reduction with DIBAL-H afforded the derivatives **8a-d**. Further oxidation with manganese-dioxide, followed by another Wittig reaction with triphenyl-phosphoylide afforded **4a-d**, which finally through mild alkaline hydrolysis yielded the styrylic acrylate **2a-d.**

The styrylic alcohols **8a-d** were converted into the diethyl-acetamido malonate derivatives **7a-d** *via* malonic acid coupling of brominated compounds **9a-d**. Then, through a mild alkaline hydrolysis of **7a-d** followed by the subsequent decarboxylation of **5a-d**, the *N*-acylated amino acids *rac*-**3a-d** were obtained. Finally, deprotection of *rac*-**3a-d** afforded the racemic amino acids *rac*-**1a-d**. (**Scheme 1)**.



Scheme 1. Synthesis of (2*E*,4*E*) styrylacrylates **2a-d**. Reagents and conditions: **I.** Ph_3P=CH-CO_2Et, toluene, reflux, 24 h; **II.** DIBAL-H/CH_2Cl_2 at - 60 °C, 1 h; **III.** MnO_2/CH_2Cl_2, RT, 48 h; **IV.** Ph_3P=CH-CO_2Et, toluene, reflux, 24 h; **V.** 10% KOH, reflux, 20 h. Synthesis of racemic styrylalanines *rac*-**1a-d**. Reagents and conditions: **VI.** Ph_3P=CH-CO_2Et, $C_4H_4BrNO_2$/CH_2Cl_2, 25 °C, 2 h; **VII.** NaH, $CH_3CONHCH(COOEt)_2$/DMF, 60 °C, 3 h; **VIII.** 10% NaOH in water/MeOH, 60 °C, 5 h; **IX.** toluene, reflux, 20 h; **X.** dioxane/18% HCl, reflux, 4 h.

II. A.3. Conclusions

The chemical synthesis of styrylic acrylates **2a-d** and of the corresponding racemic amino acids *rac*-**1a-d** were performed successfully. Infurther experiments they are used in the ammonia elimination and ammonia addition reactions mediated by *Pc*PAL and its F137X mutant variants.

Subchapter B. Inclusion of styryl-alanines in the substrate domain of *Pc*PAL through rational design driven protein engineering.

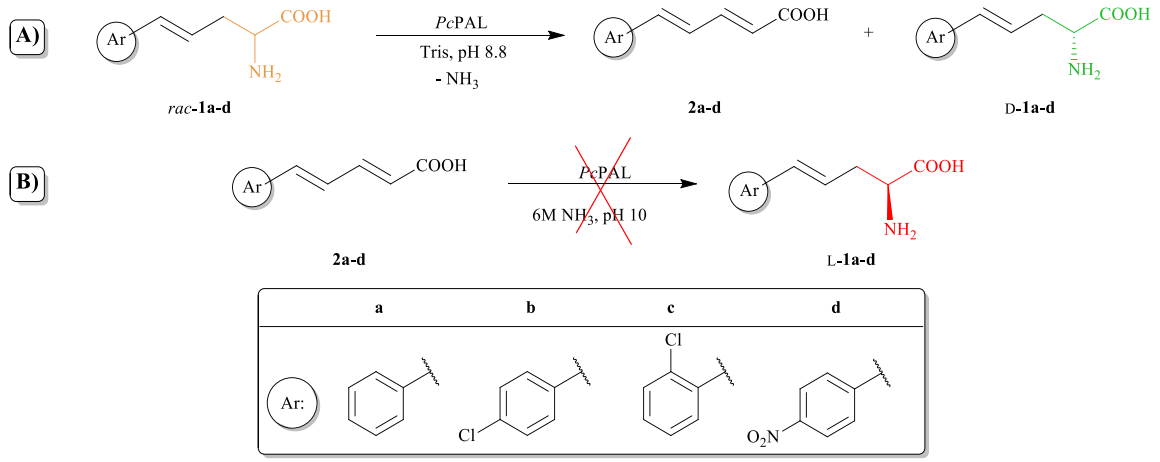
II. B.2. Results and discussion

II. B.2.1. Initial studies on the activity of *Pc*PAL and its F137X mutant variants towards styrylalanines

Knowing the ability of *Pc*PAL enzymes to accept a wide range of (hetero)arylalanines, we first focused on testing the *wt*-*Pc*PAL on styrylalanines (*rac*-**1a-d**), in order to expand the substrate scope of this enzyme towards compounds with side chains of extended length.

In the fist step, a Michaelis-Menten kinetics was measured for the deamination reaction of styrylalanines L-**1a-d** catalyzed by *wt*-PAL. The reaction rate was determined by monitoring the product formation (2*E*, 4*E*)-5-phenylpenta-2,4-dienoic acid (**2a**), using both UV spectroscopy and HPLC chromatography. Moreover, the presence of the reaction product **2a** in the reaction medium was confirmed also by ^{1}H-NRM spectroscopy.

The k_{cat} value for the ammonia elimination reaction from L-**1a** was **14** times smaller than the k_{cat} measured for the natural substrate (L-Phe), while the k_{cat}/K_M value for the deamination reaction of L-**1a** was 777 times smaller than the calculated value for the deamination of L-Phe **(Table 5)**.



Scheme 2. (**A**) Synthesis of enantiomerically pure styrylalanines D-**1a-d** by deamination reaction of *rac*-**1a-d** and **(B)** L-**1a-d** by ammonia addition on styrylacrylates **2a-d**, with *wt*- and mutant *Pc*PALs as biocatalysts.

Molecular data provided an explanation for the low enzymatic activity towards the new substrate: the detrimental interaction between the aromatic ring of the ***N*-MIO-L-1a**

reaction intermediate and the phenyl group of the amino acid F137, phenylalanine, which is part of the aromatic binding pocket of the *wild-type* enzyme.

Panels A and **B** from **Figure 10** illustrate three possible arrangements of the reaction intermediate ***N*-MIO-L-1a**, two of which are apparently active and resemble the binding of the natural substrate, phenylalanine, to the active site of *Pc*PAL.[5]

Thus, the (*2E,4E*)-diene product can be obtained from the two *N*-MIO intermediate conformers, namely from the *s-cis* [referred to as *pro-s-cis (psc)*] – abbreviated L-**1a**$_{psc}$ – and *s-trans* [referred to as *pro-s-trans* (*pst*)] – abbreviated L-**1a***pst*, respectively. From the third conformer, (L-**1a**$_{u}$) illustrated in **panel B**, which adopts a more relaxed arrangement, that experiences less strain from the F137 side chain, the (2*Z*, 4*E*)-5-phenylpenta-2,4-dienoic acid can be obtained. Unfortunately, this species could not be detected in the reaction mixture. Thus, L-**1a**$_{u}$ may be considered an unproductive state.

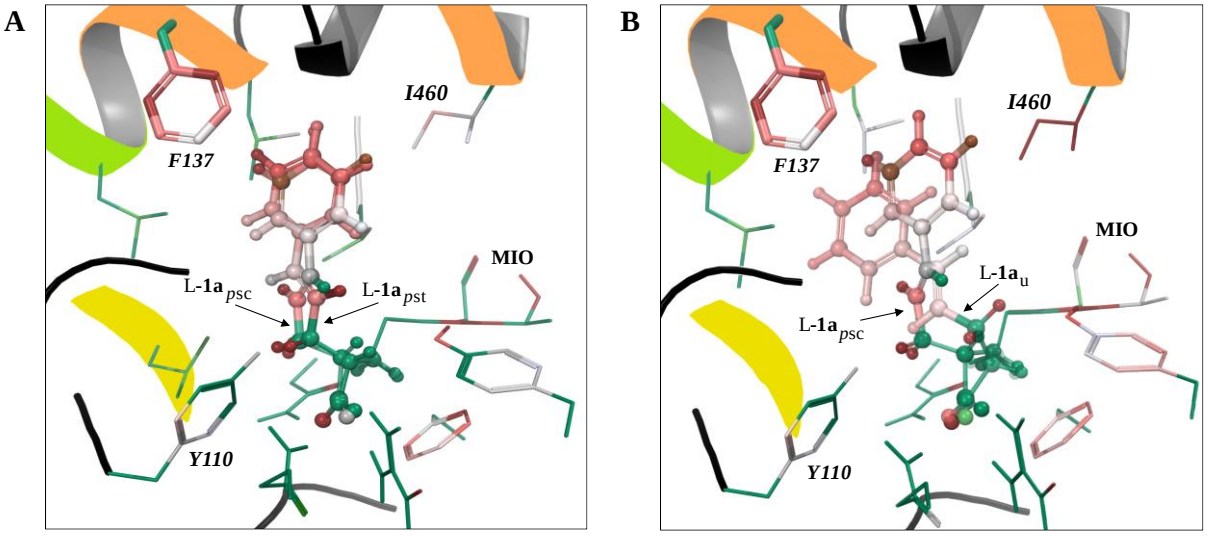


Figure 10. Models of the three major confromers of the L-**1a** – *N*-MIO reaction intermediate within the active center of *wt-Pc*PAL. Colors illustrate the contributions of various atoms to the total energy of the system with green indicating a beneficial contribution (≤-5 kcal mol^{-1}) and red denoting a detrimental contribution (≥ 2 kcal mol^{-1}). **(A)** The two conformations that presumably exhibit catalytic activity: L-**1a**$_{psc}$, enabling the formation of the product **2a** in the ***s-cis*** (synperiplanar) conformation and L-**1a**$_{pst}$, enabling the synthesis of the product **2a** in the ***s-trans*** (antiperiplanar) arrangement. **(B)** The L-**1a**$_{u}$ conformation appears to exhibit no catalytic activity as compared to the L-**1a**$_{psc}$ conformation.

Consequently, the above-mentioned results indicate that the clashes manifested between the aromatic ring of the *N*-MIO–L-**1a** intermediate and the F137 residue are responsible for the decrease in the reaction rate.

In order to expand the hydrophobic binding pocket of the *wild-type* enzyme and therefore, loosen up the interaction between the ***N*-MIO-L-1a** reaction intermediate and F137, this amino acid was replaced, in turn, by smaller hydrophobic amino acid residues, namely valine, alanine and glycine. The design of *Pc*PAL mutants F137V, F137A and F137G, respectively, was justified also by the fact that F137X-*Pc*PAL mutants have proven to be highly efficient biocatalysts in the amination reactions of several *para*-substituted cinnamates.[4]

II. B.2.2. Enzyme kinetics

The catalytic efficiency or the specificity constant of an enzyme is given by the k_{cat}/K_M ratio indicating the efficiency of the biocatalyst towards natural/non-natural substrates or competing substrates.[12]

The catalytic efficiency of the *wild-type* enzyme and its mutant forms towards L-Phe and L-**1a** substrates is presented in **Table 5**.

In the deamination reaction of L-**1a** substrate, F137V-*Pc*PAL had a remarcable catalytic efficiency, with the k_{cat}/K_M ratio being 240 times larger than the one calculated in case of *wt*-*Pc*PAL.

Table 5. Kinetic parameters for ammonia elimination reactions with *wt*-*Pc*PAL and F137X-*Pc*PAL mutants for L-phenylalanine and for L-styrylalanine (L-**1a**).

***Pc*PAL**	**L-phenylalanine**			**L-styrylalanine**		
	K_M (μM)	$k_{cat} \times 10^{-3}$ (s^{-1})	$k_{cat}/K_M \times 10^{-3}$ ($nM^{-1}\ s^{-1}$)	K_M (μM)	$k_{cat} \times 10^{-3}$ (s^{-1})	$k_{cat}/K_M \times 10^{-3}$ ($nM^{-1}\ s^{-1}$)
wt	83 ± 5	694 ± 20	8361 ± 291	4384 ± 158	47.1 ± 0.3	10.7 ± 0.4
F137V	86 ± 10	173 ± 1	2011 ± 131	186 ±6	422 ± 28	2269± 168
F137A	1732 ± 15	283 ± 1	163 ± 2	1173 ± 70	132 ± 2.6	112.5 ± 7.2
F137G	4969 ± 153	52 ± 3	10.4 ± 0.9	4120 ± 270	34.5 ± 3.8	8.3 ± 0.2

Table 6. Kinetic parameters for the ammonia elimination reactions with *wt*- and F137V-*Pc*PALs for racemic styrylalanines *rac*-**1a-d.**

Substrate	***wt*-*Pc*PAL**			**F137V-*Pc*PAL**		
	K_M (μM)	$k_{cat} \times 10^{-3}$ (s^{-1})	$k_{cat}/K_M \times 10^{-3}$ ($nM^{-1}\ s^{-1}$)	K_M (μM)	$k_{cat} \times 10^{-3}$ (s^{-1})	$k_{cat}/K_M \times 10^{-3}$ ($nM^{-1}\ s^{-1}$)
rac-**1a**	395 ± 6	6.2 ± 0.7	15.6 ± 0.9	201 ± 12	276 ± 13	1373 ± 97
rac-**1b**	154 ± 7	0.24 ± 0.01	1.5 ± 0.03	78.3 ± 2	78.6 ± 4.4	1004 ± 86
rac-**1c**	28 ± 1	0.34 ± 0.02	12.1 ± 0.6	94.7 ± 2	156.7 ± 0.02	1667 ± 10
rac-**1d**	287 ± 3	3.2 ± 0.03	11.1 ± 0.5	326 ± 10	9.9 ± 0.01	30.2 ± 1.9

When using L-**1a** as substrate, the mutant F137V-*Pc*PAL showed a 9-fold increase in k_{cat}, while the F137A-*Pc*PAL mutant showed a 2,8-fold increase in k_{cat} when compared to *wt*-PAL. The F137G mutation proved to be inefficient from an enzymatic catalytic capacity point of view.

As it can be seen in **panel A, Figure 10**, in the active site of the native enzyme the aromatic moiety of the *N*-MIO – L-**1a**$_{psc}$ intermediate is forced towards the I460 residue, causing its conformational change. In turn, this local conformational change can reshape the whole active site, which could lead to the decrease of the energy level of *N*-MIO-L-**1a**$_{psc}$ reaction intermediate. Due to F137V mutation, the styryl residue is more relaxed and thus, the energy level of the reaction intermediate is lower. The F137V mutation does not influence the spacial display of the alanine residue of L-**1a** within the catalytic site of F137V-*Pc*PAL enzyme as against to *wt*-PAL.

II. B.2.3. Kinetic resolution and synthetic applications

The promising enzyme kinetics measurements of L-**1a** deamination, but also the molecular data obtained this far regarding the energy level of *N*-MIO-L-**1a** reaction intermediate, have qualified F137V-*Pc*PAL mutant as an efficient biocatalyst for preparative scale production of enantiopure styrylalanines. **(Panel A, Figure 10)**.

The styrylalanines *rac*-**1a-d** were all accepted as substrates in the ammonia elimination reactions in the presence of *wt*-*Pc*PAL or F137V-*Pc*PAL. Unfortunately, these

biocatalysts were inactive in the reverse ammonia addition reactions of styrylacrylates **2a-d.** Consequently, emphasis is placed on the ammonia elimination reaction from the racemic styrylalanines.

The kinetic parameters for the ammonia elimination reaction of *rac*-**1a-d** with *wt*- and F137V-*Pc*PAL were determined by UV-based enzymatic assays of styryl acrylates products accumulation in time **(Table 7).**

In all cases, the F137V-*Pc*PAL mutant proved to be a superior alternative to the *wild-type* enzyme, the *turnover number* (k_{cat}) and specificity constant (k_{cat}/K_M) values being significantly enhanced for the mutant-mediated reactions in comparison with the *wild-type* enzyme **(Table 7)**.

In order to confirm the synthetic usefulness of F137V-*Pc*PAL mutant for the kinetic resolutions (KRs) of racemic styrylalanines *rac*-**1a-d**, the deamination reactions were tested on a semi-preparative scale starting from a 0.1 mmol to a 5 mM substrate concentration with 0.5-1 mg purified *wt*- and F137V-*Pc*PAL enzyme **(Table 7)**.

Table 7. Conversion of styrylalanines *rac*-**1a-d** and enantiomeric excess of the products D-**1a-d** in the ammonia elimination reactions catalyzed by *Pc*PAL variants.

Entry	*Pc*PAL	Substrate	Time (h)	*c* (%)	ee_{theor} (%)[a]	ee_{obs} (%)
1	F137V	*rac*-**1a**	24	50	100	>99
2		*rac*-**1b**	274	50	100	>99
3		*rac*-**1c**	134	50	100	>99
4		*rac*-**1d**	300	50	100	>99
5	*wild-type*	*rac*-**1a**	274	50	100	>98
6		*rac*-**1b**	504	29	41	41
7		*rac*-**1c**	600	36	56	55
8		*rac*-**1d**	600	31	45	41

[a] $ee_{theor} = c/(1-c)$ for a fully selective kinetic resolution

In **Figure 11**, the time conversions of the racemic styrylalanines *rac*-**1a-d** are presented, while the enantiomeric excess (ee) values for the D-**1a-d** compounds, corresponding to the ammonia elimination reactions catalyzed by the two *Pc*PAL (mutant and native) are listed in **Table 7**. After each 48 hours, a new batch of enzyme was added to the reaction mixture, compensating the enzyme inactivation in the applied reaction conditions. In all cases, the reaction progress was monitored by HPLC.

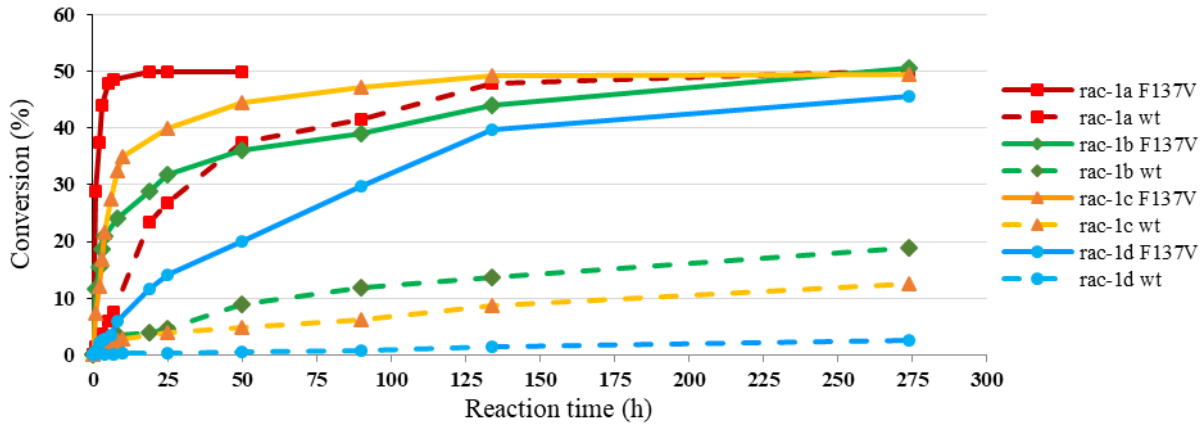


Figure 11. Comparative time profiles of conversions of *rac*-**1a-d** in the ammonia elimination reactions catalyzed by *wt-Pc*PAL (dashed lines) and F137V-*Pc*PAL (continuous lines).

The obtained time course profile of the conversion of *rac*-**1a-d (Figure 11)** also supported the catalytic superiority of the F137V-*Pc*PAL mutant compared to the *wild-type* enzyme, a characteristic that had also been confirmed by Michaelis-Menten kinetic measurements.

In the presence of F137V-*Pc*PAL enzyme, an exclusively enantioselective kinetic resolution with a conversion of ~ 50% was achieved after 24 hours in case of *rac*-**1a**, after 134 hours for *rac*-**1c** or after a longer period of time for *rac*-**1b,d** substrates (274 hours and 300 hours, respectively).

Wt-*Pc*PAL proved to be efficient only for the kinetic resolution of *rac*-**1a (Table 7, Entry 5)**. As for substituted styrylalanines *rac*-**1b-d**, the activity of the same enzyme was too low, and moderate conversion values were registred even after longer reaction times **(Figure 11, Table 7, Entries 6-8)**.

It is important to note the fact that the stereoselective aspect of L-**1a-c** biotransformations was preserved, and the values of the enantiomeric excess for D-**1a-c** at a given conversion were in accordance with the theoretical values corresponding to the measured conversion, as regard to the initial *rac*-**1a-d** concentrations.

In case of D-**1d** fraction obtained using the *wt*-PAL, the enantiomeric excess was lower than its theoretical value, thus in accordance with the results that demonstrate the low stereoselectivity of various phenylalanine ammonia-lyases in the synthesis of both nitrophenylalanine enantiomers.

It should be emphasized that, in the case of *rac*-**1a-d** substrates catalyzed by F137V-*Pc*PAL, the compounds D-**1a-d** were obtained with enantiomeric excesses higher than 99%. This result is supported by the obtained time course profiles of conversions, which asymptotically reach the value of 50%.

Therefore, F137V-*Pc*PAL proved to be an active and stereoselective biocatalyst for the synthesis of D enantiomers of styrylalanines.

Comparing the kinetic data obtained in the *wt*-*Pc*PAL and F137V-*Pc*PAL catalyzed reactions using as substrates L-**1a (Table 5)** and *rac*-**1a (Table 6)** respectively, as substrates, it can be concluded that ammonia elimination from L-**1a** styrylalanine is inhibited by the presence of the D-amino acid in both catalysed reactions.

If D-**1a** would not be a competitive inhibitor for the two enzymes, then the apparent K_M of the racemate *rac*-**1a** would be comparable to the K_M of the L-**1a** enantiomer, while the calculated k_{cat} value would be similar in both cases.

However, the registred kinetic data in the presence of *wt*-*Pc*PAL show a decrease by an order of magnitude of the K_M value and a 7.6-fold decrease of v_{max} and implicitly k_{cat}, when the enzyme is incubated with *rac*-**1a** instead of L-**1a**.

These differences become less significant when F137V-*Pc*PAL is used, with k_{cat} showing only a 1.5-fold decrease when the enzyme is incubated with *rac*-**1a** instead of L-**1a**. Experimental data obtained for the semipreparative scale synthesis of D-**1a-c** mediated by F137V-*Pc*PAL, with an ee >99% at conversions of 50% with regard to the starting materials *rac*-**1a-c (Table 7)**, certifies the fact that D-**1a-c** were not processed by the enzyme. This fact suggest that D-**1a**, and also probably D-**1b-d**, act as reversible inhitors, forming an unproductive D-**1a-d**–*N*-MIO-F137V-*Pc*PAL reaction intermediate.

Molecular modelling data confirm the possible formation of D-**1a**–*N*-MIO intermediate. Overlaid structures of L-**1a**$_{pst}$ - *N*-MIO and unproductive covalent intermediate D-**1a** - *N*-MIO within the active site of *wt-Pc*PAL are presented in **Panel B, Figure 12.**

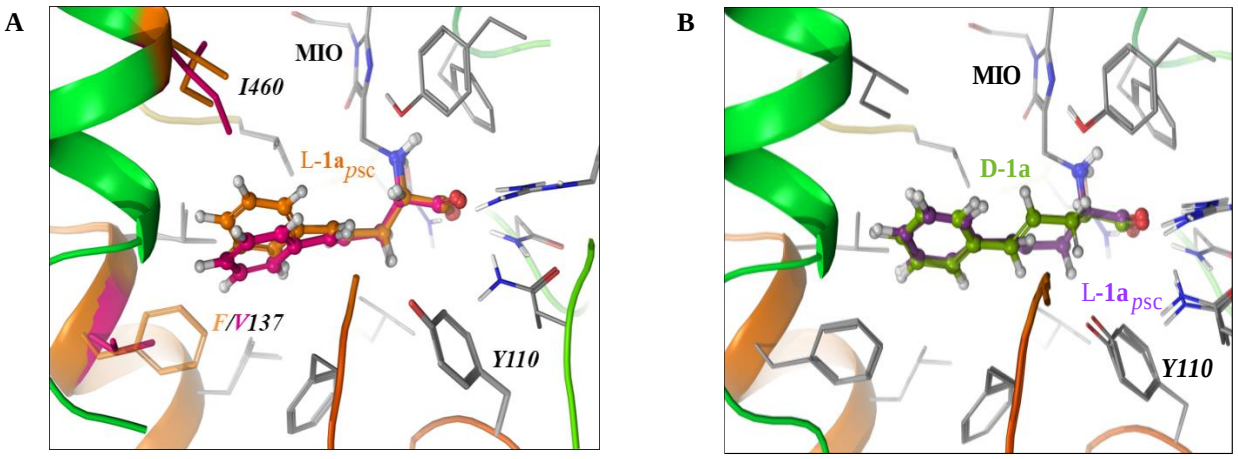


Figure 12. Arrangements of **1a**-*N*-MIO covalent reaction intermediates in the active site of *Pc*PAL variants. **(A)** The L-**1a**$_{psc}$-N-MIO conformer shown in the active site of al *wt-Pc*PAL și F137V-*Pc*PAL. The differences in the arrangement of the I460 residue in both enzymes can be observed. **(B)** The contrast between the arrangement of the covalent unproductive *wt-Pc*PAL-D-**1a** complex (shown in green) and that of the covalent productive *wt-Pc*PAL – L-**1a**$_{psc}$ complex (shown in purple).

II. B.2.5. The ammonia addition of styrylacrylates 2a-d mediated by *Pc*PAL for the synthsis of L-arylalanines

Styrylacrylates **2a-d** were not accepted as substrates in enzymatic ammonia addition reaction neither by *wild-type* or mutant enzyme variants. The products **1a-d** could not be detected by HPLC even after 20 days reaction time.

Molecular modelling studies could not offer an undeniable explanation of the experimental results. Plausible causes could be: poor ligand affinities of **2a-d** to the enzyme or their binding to the catalytic site in unproductive states.

The poor binding of styrylic acids to the catalytic site of *wt-Pc*PAL, but much stronger in case of F137V-*Pc*PAL, was confirmed by calculated relative binding energies.

Molecular modelling has also revealed that, regardless of the enzyme used, unproductive energetically favorable conformers have greater binding affinity in comparison with the supposed reactive, but energetically unfavorable, conformers of **2a-d**.

II. B.3. Conclusions

Michaelis-Menten kinetics showed that the value of k_{cat} for the ammonia elimination reaction of L-styryl-alanines L-**1a** using the *wt-Pc*PAL enzyme was 14 times lower than the k_{cat} obtained for same reaction but in presence of the natural substrate (L-Phe), while the k_{cat}/K_M value for the deamination reaction of L-**1a** was 777 times smaller than the corresponding value for the deamination of L-Phe.

Molecular data have illustrated three possible arrangements (L-**1a**$_{psc}$, L-**1a**$_{pst}$ - active state and L-**1a**$_{u}$ - unproductive state) of the ***N*-MIO - L-1a** reaction intermediate in the active site of *wild-type* enzyme, and highlighted the repulsion between the phenyl ring of the ***N***-

MIO - L-1a intermediate and the phenyl group of the amino acid phenylalanine F137 from the hydrophobic pocket of the catalytic site of *wild-type* enzyme.

It has been concluded that this interaction is responsible for the decrease of the reaction rate displayed by *wt-Pc*PAL to L-**1a**.

Replacement of the residue F137 with smaller hydrophobic amino acids (valine, alanine and glycine), led to an enlargement of the hydrophobic binding pocket of the native enzyme and consequently, to the increase of the affinity of the mutant enzyme towards L-**1a.** The mutant F137V-*Pc*PAL proved to be the most efficient biocatalyst for the removal of ammonia from styrylalanine L-**1a,** but also from *rac*-**1a-d.**

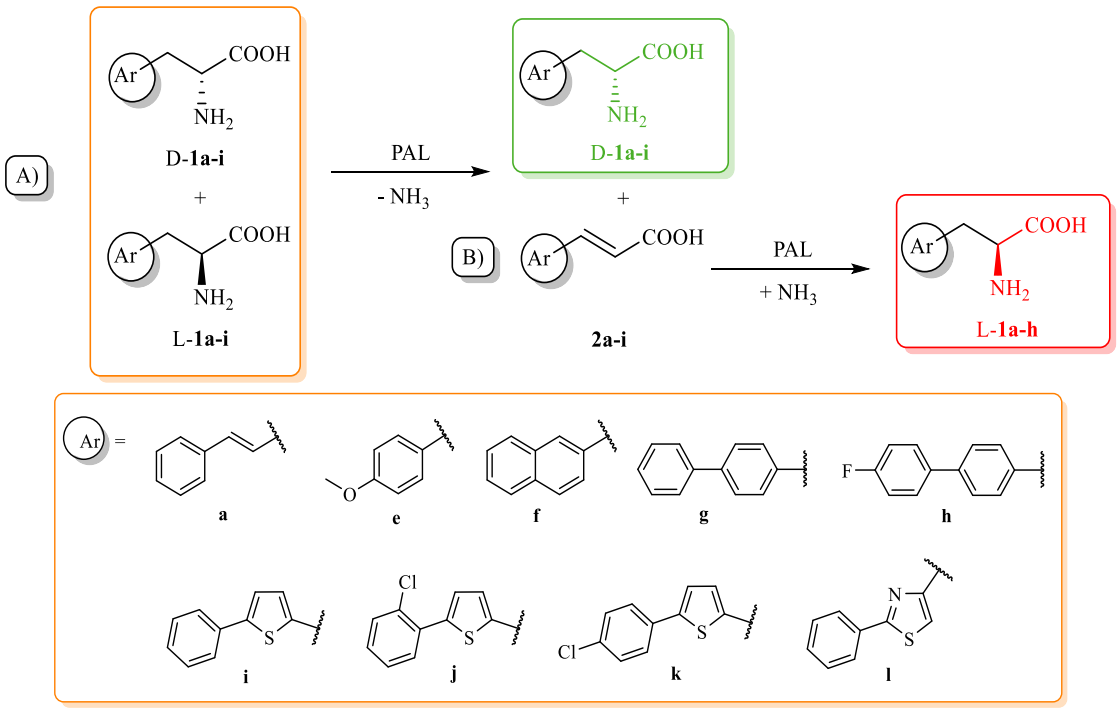
Nevertheless, neither *wild-type* enzyme or its mutant variants were active in the ammonia addition reactions to (*E*)-styrylacrylates **2a-d**. It is presumed that the main reason for this drawback lies in the formation of unproductive binding states of compounds **2a-d** in the enzymatic catalytic site.

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

II. C.2. Results and Discussion

II. C.2.1. Rational design, stability and activity of mutant *Pc*PALs

Some substrates, namely styryl **(2a)**, 4-methoxyphenyl (**2e)**, napthalen-2-yl- **(2f)** and biphenyl-4-yl **(2g)** acrylic acid presented in **Scheme 3**, were previously tested with *wild-type* *Pc*PAL, but the conversions achieved were either very low or nonexistent or the target compounds proved to be enzyme inhibitors. [4, 5, 14, 15]



Scheme 3. The ammonia elimination (A) and ammonia addition (B) reactions tested using *Pc*PAL variants.

In the second subchapter we witnessed that in the ammonia addition reaction no *Pc*PAL mutant variants are known to exhibit such activity, except in case of the styrylalanines deamination where mutant F137V showed activity.[5]

In order to obtain PAL biocatalysts capable of transforming a series of bulky and sterically demanding valuable L- and D-arylalanines, we focused on the mutational analysis and rational design of the hydrophobic binding pocket to *Pc*PAL.

According to earlier reported substrate domain expansion of PAL enzymes based on steric clash reduction concepts,[4,5] we selected several amino acids residues from the hydrophobic binding site of *Petroselinum crispum* PAL, namely L134, F137, L138, L206, L256 and I460 residues (highly conserved residue) and exchanged them to smaller amino acids such as valine or alanine, in order to obtain both single and double/triple mutants of *Pc*PAL (**Figure 13**).

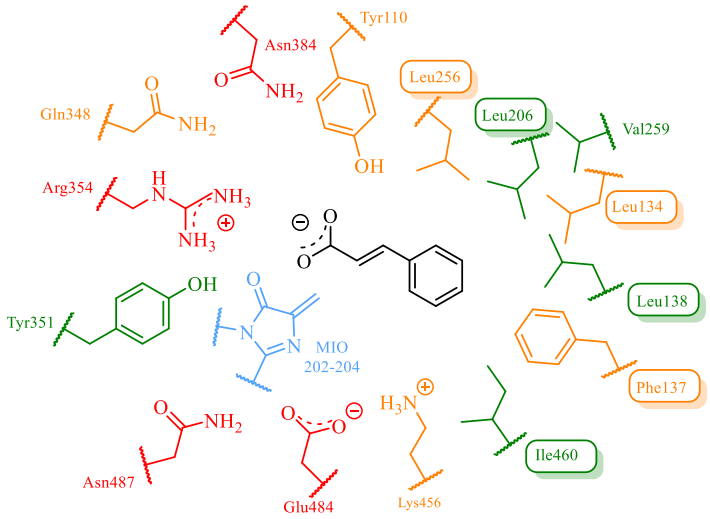


Figure 13. Model of the active center of *Pc*PAL with (*E*)-cinnamic acid as a modeled substrate. The distance between the neighboring amino acid side chains and the substrate is less than 5 Å. The positions of these residues relative to the plane of the ligand are differentiated by the use of various colors: **black** and red – within, green– below and orange – above the plane. MIO grup is above the plane. The hydrophobic amino acids depicted in boxes were replaced either independently or in combination with smaller hydrophobic amino acids, such as valine or alanine.

II. C.2.2. Testing of *Pc*PAL single mutant variants in the elimination reaction of arylalanines *rac*-1a,e-l

The activity of the generated *Pc*PAL single mutant variants was tested in both ammonia elimination and addition reactions, using arylalanines *rac*-**1a,e-l (Scheme 3A)** and arylacrylates **2a,e-l (Scheme 3B)** respectively, as substrates. Besides the well-known beneficial effect of the frequently mutated F137 to Ala and Val[3, 5] in the deamination reactions of *rac*-**1a,e-l**, the importance in changing the highly conserved isoleucine within the hydrophobic pocket (position 460 in *Pc*PAL) with less bulkier apolar amino acids V and A was revealed in this thesis.

Thus, I460V/A mutant provided significantly increased catalytic activity towards almost all tested substrates compared to *wild-type Pc*PAL enzyme **(Table 8)**. Moreover, both F137V/A and I460V mutants provided good (c=35% in case of *rac*-**1g**) to excellent (c=50% in case of *rac*-**1a,j,k)** conversions after 16 h. Unfortunately, there was one substrate, namely *rac*-**1l**, whose structure resembles the benzo[b]furan-3-yl- and benzo[b]thiophene-3-yl alanines,[16] the *wt*-PAL-inhibitors, that still behaved as a competitive inhibitor for both *wild-type* and mutant variants.

Surprisingly, F137A-*Pc*PAL turned out to be active in the ammonia elimination reaction of *rac*-**1l**, but regrettably with very low conversion (c=6%) even after a longer reaction time.

Table 8. The ammonia elimination reactions of *rac*-**1a,e-l** – conversions of the best *Pc*PAL mutants compared to the *wild-type* (*wt*) *Pc*PAL.

Substrate	Ar. group	*Pc*PAL variant[a]	*c* (%)	Substrate	Ar. group	*Pc*PAL variant[a]	*c* (%)
rac-**1a**	styryl	*wt*	<1	*rac*-**1i**	5-phenylthiophen-2-yl	*wt*	<1
rac-**1a**	styryl	**I460V**	**~50**	*rac*-**1i**	5-phenylthiophen-2-yl	F137V	35
rac-**1a**	styryl	**F137V**	**~50**	*rac*-**1i**	5-phenylthiophen-2-yl	**F137A**	**44**
rac-**1e**	4-methoxy	*wt*	3	*rac*-**1j**	2'-chloro-5-phenylthiophen-2-yl	*wt*	<1
rac-**1e**	4-methoxy	F137V	37	*rac*-**1j**	2'-chloro-5-phenylthiophen-2-yl	F137V	19
rac-**1e**	4-methoxy	**I460V**	**39**	*rac*-**1j**	2'-chloro-5-phenylthiophen-2-yl	**F137A**	**~50**
rac-**1f**	napthalen-2-yl	*wt*	6	*rac*-**1k**	4'-chloro-5-phenylthiophen-2-yl	*wt*	<1
rac-**1f**	napthalen-2-yl	I460V	37	*rac*-**1k**	4'-chloro-5-phenylthiophen-2-yl	I460V	10
rac-**1f**	napthalen-2-yl	**F137V**	**39**	*rac*-**1k**	4'-chloro-5-phenylthiophen-2-yl	**F137A**	**~50**
rac-**1g**	biphenyl-4-yl	*wt*	<1	*rac*-**1l**	2-phenylthiazol-4-yl	*wt*	<1
rac-**1g**	biphenyl-4-yl	I460V	8	*rac*-**1l**	2-phenylthiazol-4-yl	**F137A**	**6**
rac-**1g**	biphenyl-4-yl	**F137A**	**15**				
rac-**1h**	4'-fluorobiphenyl-4-yl	*wt*	<1	[a] *Pc*PAL variant: 50 µg; reaction volume: 500 µL; reaction medium: Tris-buffer (100 mM Tris.HCl, pH 8.8, 20 mM *β*-cyclodextrin), substrate concentration: 1 mM; reaction time: 16 h			
rac-**1h**	4'-fluorobiphenyl-4-yl	F137V	37				
rac-**1h**	4'-fluorobiphenyl-4-yl	**F137A**	**39**				

II. C.2.3. Testing of *Pc*PAL single mutant variants in the ammonia addition reactions onto 2a,e-l cinnamic acids

In case of the ammonia addition reactions onto **2a,e-k** cinnamic acid analogues, the single *Pc*PAL mutants were, in general, not very active biocatalysts. Still, there were two exceptions, namely the amination of 4-methoxyphenyl- **2e** catalyzed by I460V-*Pc*PAL (c=29%) and napthalen-2-yl-acrylic acid **2f** catalyzed by F137V-*Pc*PAL (c=55%) **(Tabel 9)**.

It is notable that, individual mutations of residues L138, L134, L206, L256 to valine or alanine did not improve the catalytic performance of the *wild-type* enzyme in neither of the investigated cases **(Table 9)**.

Table 9. Conversion values of the ammonia addition reaction onto **2a,e-l** substrates after 20 h.

Nr.	***Pc*PAL**	**Conversions (%)**								
		2a	**2e**	**2f**	**2g**	**2h**	**2i**	**2j**	**2k**	**2l**
1	*wild-type*	<1	<1	<1	<1	<1	<1	<1	<1	<1
5	F137A	<1	<1	<1	<1	<1	4	<1	<1	<1
6	F137V	<1	26	55	<1	<1	2	<1	<1	<1
14	I460V	<1	29	50	<1	<1	<1	<1	<1	<1
15	F137A/I460V	4	4	9	27	8	6	3	2	<1
16	F137A/ I460A	<1	<1	<1	8	<1	<1	<1	<1	<1
17	F137V/I460A	<1	<1	<1	<1	<1	<1	<1	<1	<1
18	F137V/I460V	22	32	39	9	<1	3	<1	<1	<1
19	F137A/L138V	<1	<1	<1	<1	<1	2	<1	<1	<1
24	F137V/L138V/I460V	14	27	21	4	<1	<1	<1	<1	<1
25	F137A/L138V/I460V	<1	3	<1	8	<1	3	<1	<1	<1

[a] Single mutants L134A, L134V, F137G, L138A, L138V, L206A, L206V, L256A, L256V, I460A, double F137A/L138A, F137V/L138A, F137V/L138V, L134V/F137A and triple F137A/L138A/I460V, F137A/L138A/I460A, F137A/L138V/I460A proved to be totally inefficient in transforming the substrates **2a,e-l**.

II. C.2.4. Testing of *Pc*PAL double and triple mutant variants in the elimination (*rac*-1a,e-l) and addition reactions (2a, e-l)

In the ammonia elimination reactions of *rac*-**1a,e-l**, the double and triple mutants of *Pc*PAL managed to improve the conversion values only in case of two substrates: 4'-fluorobiphenyl-4-ylalanine, *rac*-**1h** (from 15% conversion with F137A-*Pc*PAL **(Table 8)** to 39% with F137A/I460V mutant), and 5-phenylthiophen-2-yl-alanine *rac*-**1i** (from 44% conversion with F137A-*Pc*PAL **(Table 8)** to 48% conversion with F137A/L138V mutant).

In case of ammonia addition reactions onto **2a,e-l** acrylates, double mutants of F137V/A showed increased activity in case of **2a** and **2g (Table 10)** to whom single mutants proved to be inactive **(Table 9)**. This result can be explained by the strong non-additive, cooperative effect[5] of point mutations of the neighboring residues F137 and I460.

Table 10. Activity of the *wild-type* (*wt*) *Pc*PAL compared to the best *Pc*PAL mutants in the ammonia addition reaction of **2a,e-l**.

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

[a] *Pc*PAL variant: 50 µg; reaction volume: 500 µL; reaction 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.

Unfortunately, F137A/I460V showed low activity in the presence of **2h-k** (c=3-8% after 20 h) **(Table 9)** were observed using F137A/I460V enzyme as biocatalyst. Moreover, using *Pc*PAL enzymes with multiple mutations resulted in no significant increase in conversion when compared to the *Pc*PAL single mutants in case of **2e** and **2f** arylacrylates **(Table 9)**, while **2l** (2-phenylthiazol-4-yl) acrylic acid could not be transformed by neither of these enzymes **(Table 9)**.

Combining L138V/A mutation with F137V/A and/or I460 A/V in order to afford double and triple mutants, was unsuccessful in increasing the catalytic activity in either elimination or addition reactions. These results point out the importance of I460 residue in obtaining a *Pc*PAL mutant with increased activity and which combined with the mutation of F137 amino acid, may generate valuable biocatalysts in ammonia addition reactions for the production of important L-arylalanines in the synthesis of biologically active compounds.

Once the best mutants were obtained (high conversion values and enantiomeric excess), we further focused our attention on the optimization of the reaction conditions, using as model substrates biphenyl-4-yl-alanine *rac*-**1g** in ammonia elimination reaction and napthalen-2-yl-acrylic acid **2f** in ammonia addition reaction.

II. C.2.7. Computational results and the thermal unfolding profile of *wild-type Pc*PAL and F137A/I460V-*Pc*PAL in the presence of high concentrations of ammonia

According to our previously computational analysis, the improved results in conversions and ee in case of L-**1a,e-k** substrates are possible due to the enhanced affinity of the corresponding *Pc*PAL mutant variants for these compounds, but also due to a better reaction transition states stabilization, which can be seen in higher turnover numbers.[5] This increase in substrate affinity was confirmed/analysed by molecular modelling of the *N*-MIO enzyme-substrate covalent reaction intermediate.

Figure 14 illustrates three situations for the increased affinity for L-**1h** of F137A/I460V-*Pc*PAL in comparison to *wt*-*Pc*PAL. The binding of L-**1h** substrate to the catalytic site of the native enzyme **(Figure 14A)** and to F137A/I460V-*Pc*PAL **(Figure 14C)** are presented. The double mutation led to an increase of the hydrophobic binding pocket of the catalytic site and therefore, the *N*-MIO intermediate is more sterically relaxed.

Figure 14B illustrates an overlapping of the two above described situations, where a visible significant displacement of the biarylic residue in the expanded hydrophobic pocket of F137A/I460V-*Pc*PAL as against to its position, is sterically hindered in the active site of *wt*-*Pc*PAL.

Similar situations have also been reported for L-arylalanines L-**1f-g,i-k**, with the exception of L-**1e**, whose affinity to the enzyme was less affected by the mutations.

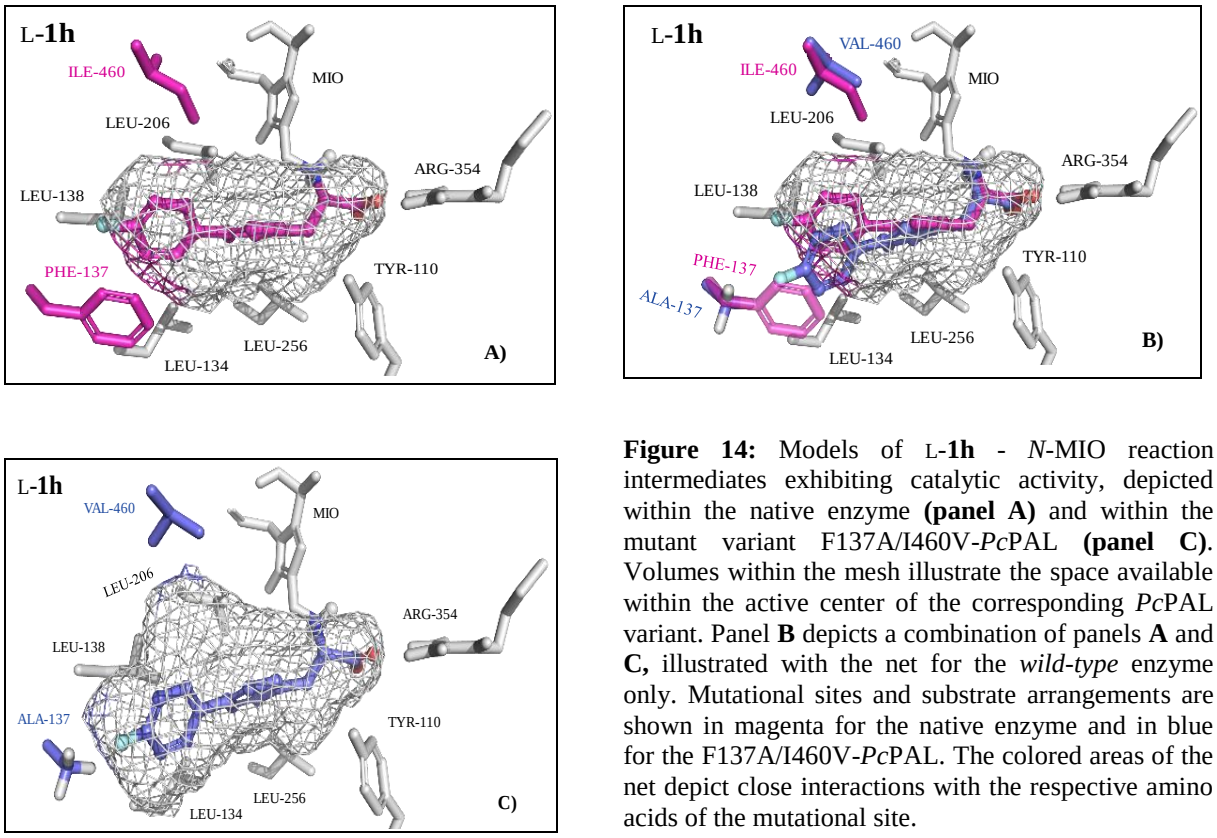


Figure 14: Models of L-**1h** - *N*-MIO reaction intermediates exhibiting catalytic activity, depicted within the native enzyme **(panel A)** and within the mutant variant F137A/I460V-*Pc*PAL **(panel C)**. Volumes within the mesh illustrate the space available within the active center of the corresponding *Pc*PAL variant. Panel **B** depicts a combination of panels **A** and **C,** illustrated with the net for the *wild-type* enzyme only. Mutational sites and substrate arrangements are shown in magenta for the native enzyme and in blue for the F137A/I460V-*Pc*PAL. The colored areas of the net depict close interactions with the respective amino acids of the mutational site.

Analyzing the thermal unfolding profile of *wild-type Pc*PAL and F137A/I460V-*Pc*PAL, a decrease in ther thermostability can be observed in the presence of high ammonia concentrations **(Figure 15 and 16)**. A decrease of the melting temperature T_m with up to 20 °C was observed in case of the native enzyme and 13 °C in case of the double mutant, when compared to temperatures of 74-75 °C measured for the same enzymes in 0.1 M Tris buffer **(Table 11)**.

Enzymes most likely adopt another conformation, a reason why the obtained computational results cannot be correlated with protein thermostability experiments.

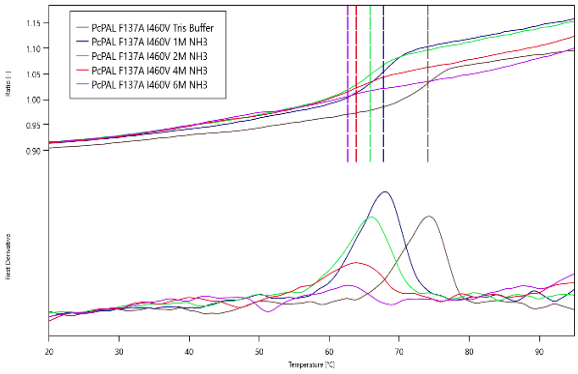


Figure 15. Thermal unfolding of F137A/I460V-*Pc*PAL in buffers with different ammonia content.

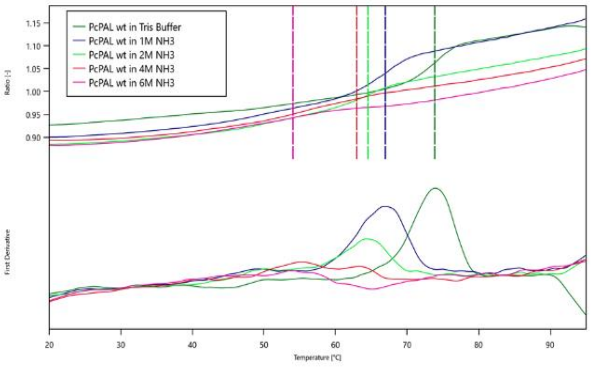


Figure 16. Thermal unfolding of *wt*-*Pc*PAL in buffers with different ammonia content.

Table 11. Melting temperatures (T_m) of *wt*- and F137A/I460V-*Pc*PAL variants in buffers with different ammonia concentrations.

Buffer	**T_m (ºC) *wt*-*Pc*PAL**	**T_m (ºC) F137A/I460V-*Pc*PAL**
0.1 M Tris	75.1±0.2	74.4±0.4
1M NH_3	68.9±0.2	67.0±0.2
2M NH_3	64.7±0.5	65.1±0.2
4M NH_3	55.1±0.5	63.4±0.3
6M NH_3	54.4±0.9	61.9±0.7

II. C.3. Conclusions

The exchange of only one of the F137 and I460 residues to a smaller hydrophobic amino acid residue provided enhanced catalytic activity of *Pc*PAL in the deamination reaction.

Combined mutations of F137 and I460 residues with valine and alanine, with a non-additive favorable effect, allowed the creation of efficient PAL-biocatalysts also for the stereoselective ammonia addition reactions of bulky cinnamic acid analogues.

While the importance of the F137 residue and its modified variants regarding the enzyme's substrate tolerance has been known, the beneficial effect on catalytic activity of mutations of residue I460, and its cooperative, non-additive effect with mutations of F137, is firstly reported.

The developed novel mutants, F137V/A and I460V/A, act as remarkable biocatalysts, facilitating the synthesis of D- and L- enantiomers of bulkier, synthetically valuable phenylalanine analogues, such as (naphtalen-2-yl) alanine **1f** and 4-methoxy-phenylalanine **1e**.

General Conclusions

1. Site-directed mutagenesis was employed in order to successfully obtain a considerable number of *Pc*PAL mutant variants. Thermal stability measurements concluded that the T_m was influenced only by I460A mutation (T_m = 74 $\rightarrow$51 °C), but this did not affect the yield of the catalyzed reaction.

2. *Wt*- and mutant variants (F137A, I460V, F137A/I460V and Y110F/F137A/I460V) of recombinant *Pc*PALs without an *N*-terminal His-tag have been obtained through TEV-protease mediated digestions. The high purity and homogenous recombinant protein samples, resembling the original *Pc*PAL sequence, have been used in crystallization studies.

3. The MIO-enzyme collection was expanded with novel recombinant phenylalanine 2,3-aminomutase (PAM), 2,3-aminomutases XAL (phenylalanine/histidine/tyrosine ammonia-lyase) and histidine ammonia-lyase (HAL) originary from *Pseudomonas fluorescens* R124.

4. The chemical synthesis of novel substrates for *Pc*PAL, such as the styrylacrylates **2a-d** and their corresponding racemic styrylalanines, *rac*-**1a-d**, was achieved.

5. The molecular modeling data has established the repulsive interactions between the F137 residue of *Pc*PAL and the aromatic ring of **L**-styrylalanine L-**1a** in the case of *N*-MIO-L-1a intermediate formation. A library of F137X-*Pc*PAL mutants was created (with X representing valine, alanine or glycine) from which F137V-*Pc*PAL mutant turned out to be the most efficient in both the deamination of L-**1a** and of the styrylalanine racemic mixtures *rac*-**1a-d.** In contrast to the native enzyme, F137V-*Pc*PAL mutant proved to be a useful biocatalyst for preparative scale synthesis of D-styryl-alanines with high enantiomeric purity.

6. Point mutations were employed in order to obtain a more relaxed enzyme-substrate complex also in the case of bulky analogues of phenylalanine. It was demonstrated that the replacement of F137 and I460 residues with smaller hydrophobic amino acids facilitated the removal of ammonia from bulky arylalanines in a stereoselective manner.

7. For the succesfull stereoselective ammonia addition on the bulky cinnamic acid analogues, combined mutations of F137 and I460 residues was necessary. Thus, active and stereoselective biocatalysts were developed for the synthesis of D- and L-([1, 1'–biphenyl]-4-yl) and D- 5-phenylthiophen-2-yl) alanines, key intermediates in the synthesis of compounds with pharmaceutical activity.

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