Babeş-Bolyai University
Faculty of Chemistry and Chemical Engineering
Doctoral School of Chemistry
Enzymology and Applied Biocatalysis Research Center

DOCTORAL THESIS

ENZYME ENGINEERING AND IMMOBILIZATION STRATEGIES FOR THE EFFICIENT BIOCATALYTIC SYNTHESIS OF D-PHENYLALANINES

PhD candidate: **Krisztina (Boros) Kelemen**
Scientific supervisor: **Prof. Habil Dr. Monica-Ioana Toșa**

Cluj-Napoca
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Facultatea de Chimie și Inginerie Chimică
Școala Doctorală de Chimie
Centrul de Cercetare Enzimologie și Biocataliză Aplicată

TEZĂ DE DOCTORAT

STRATEGII DE INGINERIE PROTEICĂ ȘI DE IMOBILIZARE PENTRU SINTEZA BIOCATALITICĂ EFICIENTĂ A D-FENILALANINELOR

Candidat: **Krisztina (Boros) Kelemen**
Coordonator științific: **Prof. Habil Dr. Monica-Ioana Toșa**

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CHAPTER 1

Among the biological small molecules, aromatic D-amino acids have attracted increasing attention as chiral building blocks for various pharmaceuticals and fine chemicals.[1] D-Phenylalanine (D-Phe) has been explored for its therapeutic potential in the treatment of pain and Parkinson's disease;[2] its derivatives have been employed in the treatment of type 2 diabetes,[3] they are precursors for antibiotics, spermicides[3,4], Pin1 inhibitors[5], cyclic plasmin inhibitors[6], anti-obesity therapeutics[7], SET domain-containing lysine methyltransferase 7 (SETD7) inhibitors[8], and botulinum neurotoxin[9], kinesin-14 motor protein KIFC1[10], and amyloid-β (Aβ) peptide aggregation inhibitors[11] (**Figure 1**).

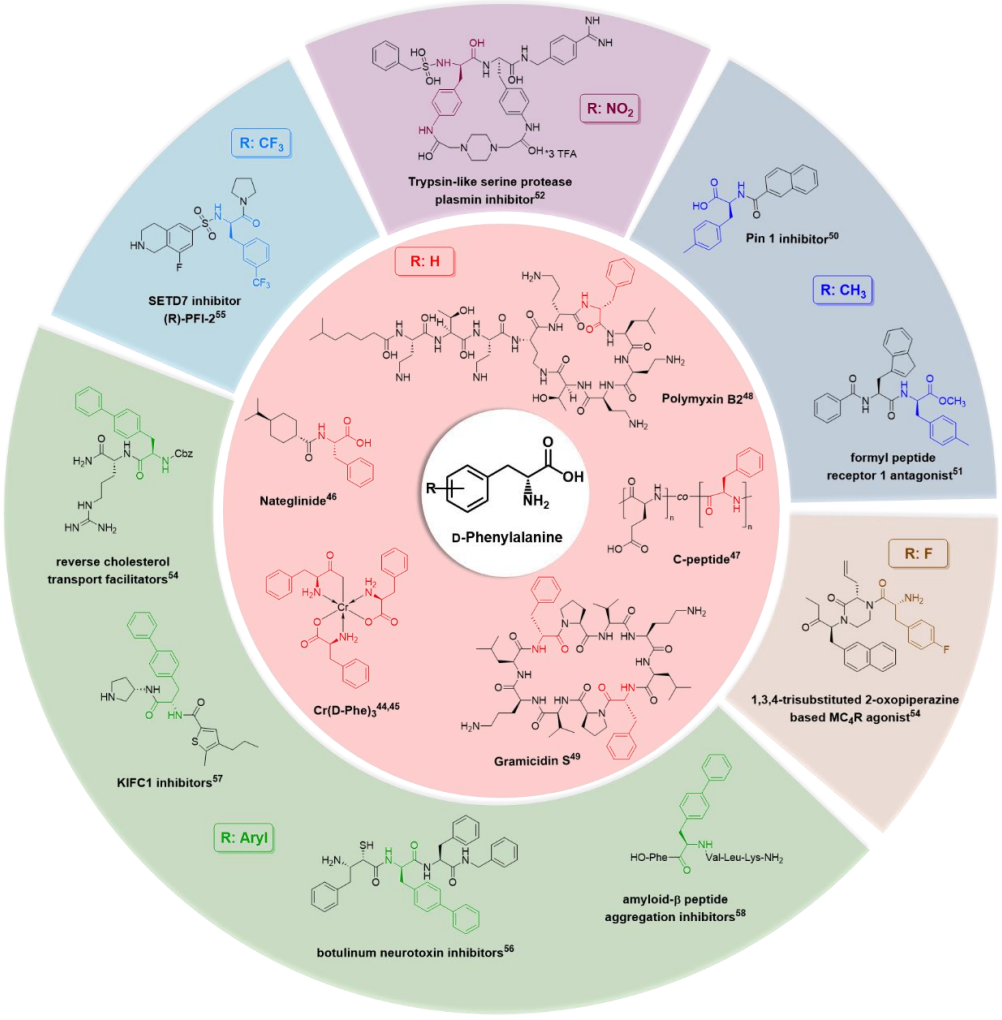

Figure 1. D-Phenylalanine and its derivatives as active pharmaceutical ingredients.

CHAPTER 2

The thesis provides a comprehensive analysis and comparison of the biosynthetic procedures and (chemo)enzymatic cascades dealing with the asymmetric synthesis of D-phenylalanines, with special focus on the protein engineering efforts resulting efficient biocatalysts. Biocatalyzed procedures for the production of D-AAs include the conversion of

D-hydantoin through the coupled action of D-hydantoinase and D-carbamoylase[12,13], the hydrolysis of *N*-acyl-D-amino acids mediated by *N*-acyl-D-amino acid amidohydrolases[12], the kinetic resolution of racemic mixtures using L-amino acid oxidases (or deaminases), and the asymmetric procedures like the reductive amination of α-keto acids by D-amino acid dehydrogenases or amino acid aminotransferases[1], and the ammonia addition to cinnamic acids catalyzed by phenylalanine ammonia-lyases[14,15] (**Figure 2**). In addition, chemoenzymatic and multi-enzyme cascade systems have emerged as important strategies for the enantiopure synthesis of several high-value molecules like D-AAs.[1]

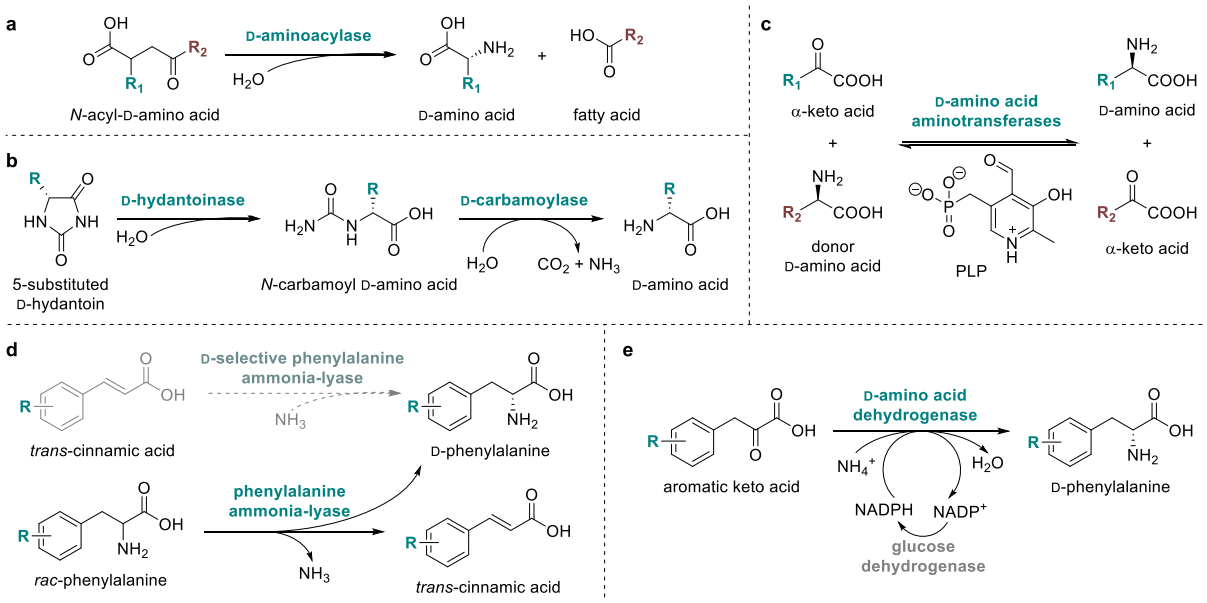

Figure 2. Biocatalyzed procedures for the production of D-amino acids: **a.** hydrolysis of *N*-acyl-D-amino acids catalyzed by D-aminoacylases, **b.** transformation of the racemic 5-substituted hydantoins through the coupled activity of D-hydantoinase and *N*-carbamoyl-D-amino acid amidohydrolase (D-carbamoylase), **c.** amino group transfer catalyzed by the PLP-dependent D-amino acid aminotransferases, **d.** hydroamination of *trans*-cinnamic acid or stereoselective deamination of *rac*-phenylalanine catalyzed by phenylalanine ammonia-lyases, and **e.** asymmetric reductive amination of aromatic keto acids catalyzed by D-amino acid dehydrogenases.

In our conclusion, the procedures mediated by D-amino acid dehydrogenases (DAADHs) or phenylalanine ammonia-lyases (PALs) appear to be the most promising in terms of industrial applicability. Consequently, in the following chapters the focus was oriented towards further optimization of these processes.

CHAPTER 3

In the case of PALs, a highly attractive strategy is the reversal of the enzyme's stereoselectivity in the synthetically important hydroamination reaction,[1] which enables the conversion of cinnamic acid analogues into D-phenylalanines. For the stereoselectivity reversal, a rational design approach was targeted within the thesis. The thorough analysis of the existing structural data on aromatic ammonia-lyases suggested the absence of catalytically competent *Pc*PAL structures under hydroamination reaction conditions, complexed with a *trans*-cinnamic acid analogue. Having at our disposal *Pc*PAL and *At*PAL mutants known to exhibit reduced enantiomeric excess in the transformation of trans-cinnamic acid analogues

bearing strong electron-withdrawing substituents, we attempted to crystallize the single mutant variants F137V and I460V of *Pc*PAL and F136V and I461V of *At*PAL. During our experiments, the engineered *Pc*PAL I460V variant co-crystallized with D-*m*-CF_3-phenylalanine yielded a ligand-bound structure with an unreported catalytic site orientation of the corresponding cinnamic acid analogue (**Figure 3**), providing valuable insights for further rational design studies.

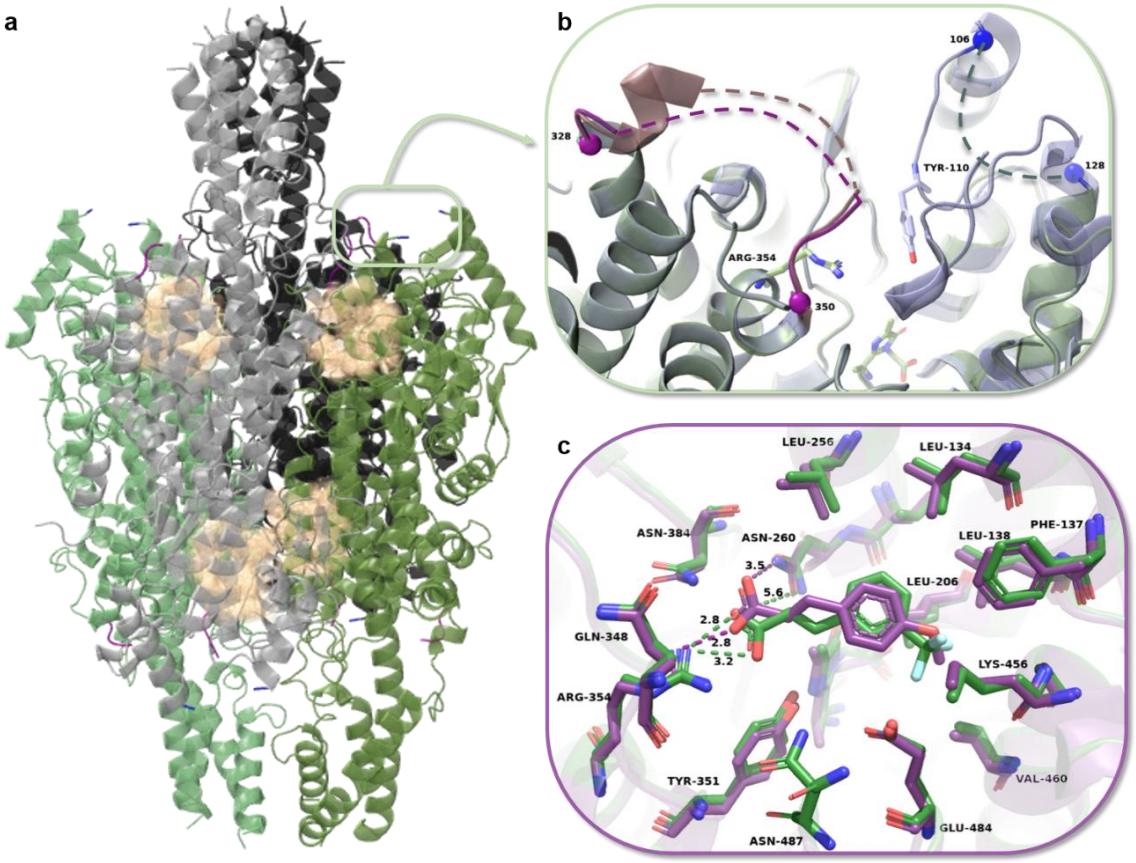

Figure 3. Novel *Pc*PAL structure with *m*-CF_3-cinnamic acid bound in the catalytic site: **a)** overall homotetrameric fold, with the four subunits colored in light/dark green and grey/black, and the four catalytic sites in each chain highlighted in sand; **b)** overlay of the novel structure with 6HQF[16] structure (light blue), highlighting the presence or absence (dashed lines) of the Tyr-loop (grey for the novel structure, light blue for 6HQF) and Arg-associated loop (deep purple for the novel structure and light pink for 6HQF); **c)** overlay of the catalytic site of the novel structure with previously solved *Pc*PAL structures co-crystallized with *p*-methoxycinnamic acid (PDB ID: 6RGS[17]; dark purple).

However, the crystal structure lacked structural information on several functionally significant loops; therefore, we focused on obtaining a complete PAL structure in complex with a cinnamic acid analogue. Screening cinnamic acid analogues as potential PAL inhibitors, we identified *N*-phenylglycine (NPG) as potent *Pc*PAL inhibitor. Using NPG as a ligand in co-crystallization experiments provided a complete crystal structure of *Pc*PAL, with both catalytically essential loops, Tyr110-loop and Arg354-associated loop, fully resolved in their closed, catalytically active conformations (**Figure 4**). These novel structures serve as a solid starting point for the rational design of D-selective PALs.

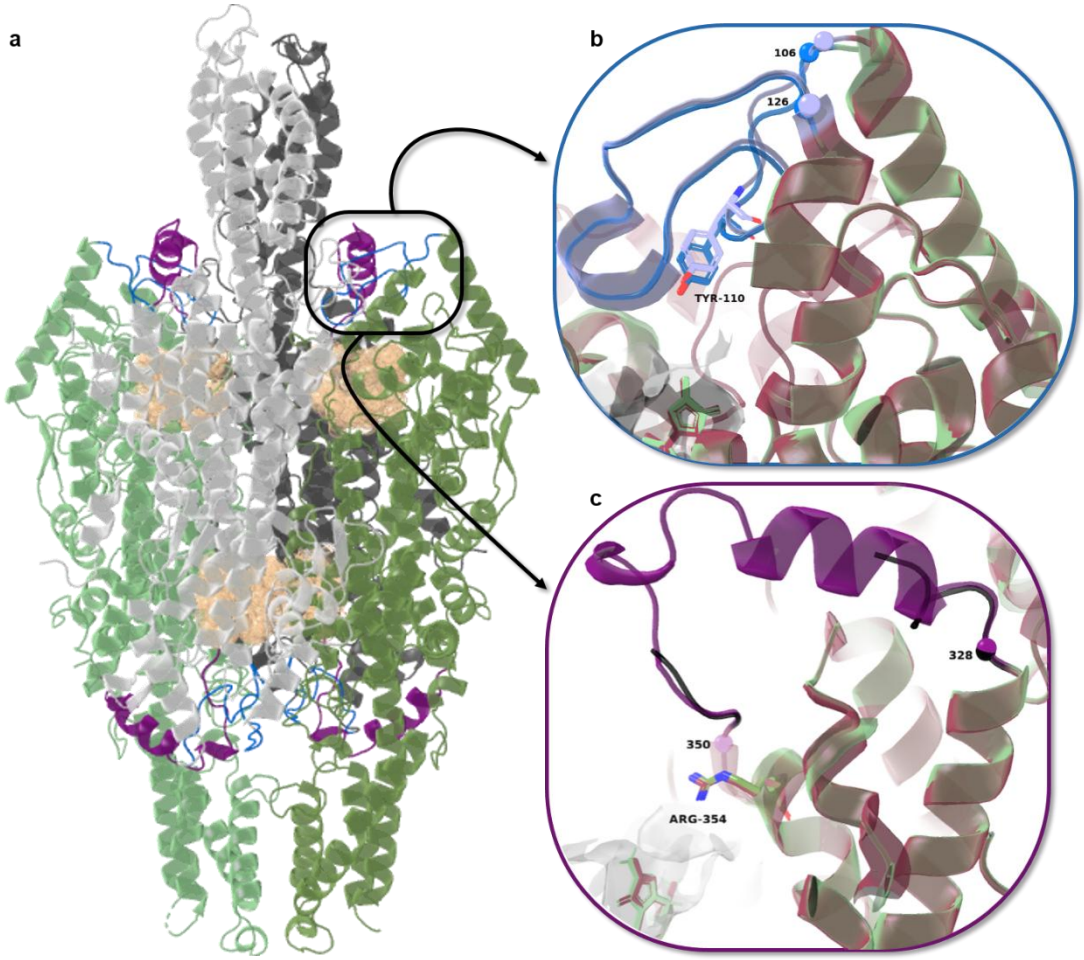

Figure 4. Novel *Pc*PAL structure with both catalytically essential loops in the closed, catalytically competent conformation. **a)** Overall fold of *Pc*PAL in its homotetrameric form, with the four subunits colored in light/dark green and grey/black, and the four catalytic sites in each chain are highlighted in sand. Overlay of the novel structure with the (S)-(1-amino-2phenylallyl)phosphonic acid-bound 6F6T[16] structure (red), highlighting the differences in the **b)** Tyr-loop and positioning of the Tyr110 residue (blue for the novel structure and light blue for 6F6T); and differences in case of the **c)** Arg-associated loop (deep purple for the novel structure and black for 6F6T).

CHAPTER 4

Since the enantioselectivity reversal of PALs is a challenging task and exceeded the timeframe of the thesis, we also focused on optimizing the second most promising biocatalytic route for the synthesis of D-Phes, the D-amino acid dehydrogenase-catalyzed reductive amination of keto acids. While the engineering of DAADHs has been well established, their immobilization – crucial for industrial applicability – has been less explored. Therefore, within this thesis, several commercially and in-house available supports and procedures were tested for the immobilization of the engineered DAADH from *Ureiabacillus thermosphaericus* (*Ut*DAADH) (**Figure 5**). Based on the conversions in the reductive amination of phenylpyruvate, as well as recyclability, batch-to-batch reproducibility, and immobilization costs, DAADH covalently attached to Purolite® ECR8415F resin or co-immobilized with GDH on polyethylenimine-coated agarose were selected for further optimizations.

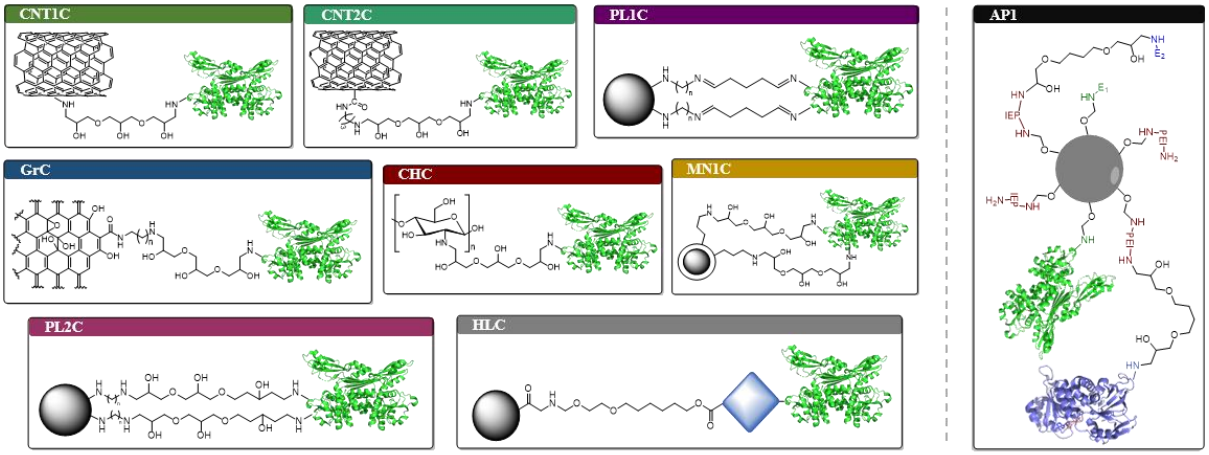

Figure 5. DAADH covalently immobilized on amino-functionalized single-walled carbon nanotubes (SWCNTs) (CNT1C), on carboxy-functionalized SWCNTs (CNT2C), on reduced graphene oxide (GrC), on magnetic nanoparticles (MN1C), on chitosan (CHIC), on Purolite® ECR8415F resin *via* glutaraldehyde linker (PL1C), on Purolite® ECR8415F resin *via* glycerol diglycidyl ether linker (PL2C), through the fusion HaloTag® (blue rhombus) (HLC), and co-immobilized with GDH on polyethyleneimine-coated agarose beads (AP1). The monomeric structures of *Ut*DAADH (PDB ID: 5GZ6) and *Bs*GDH, (PDB ID: 2CDA) have been represented in green and blue, respectively.

The more readily prepared immobilization procedure, DAADH adsorbed onto the Purolite® resin, also proved applicable. Although this preparation exhibited a significant drop of 58.8% of its specific activity over 10 reaction cycles, it still provided conversions comparable to those of the covalently immobilized variant (**Figure 6**). As the third effective preparation, the DAADH–GDH co-immobilized system showed no loss of activity over 10 reaction cycles. In this case, the additional co-immobilization of the $NADP^+$ cofactor provided self-sufficient biocatalysts, but only for three cycles, after which the conversions dropped by ~60%, due to cofactor leakage.

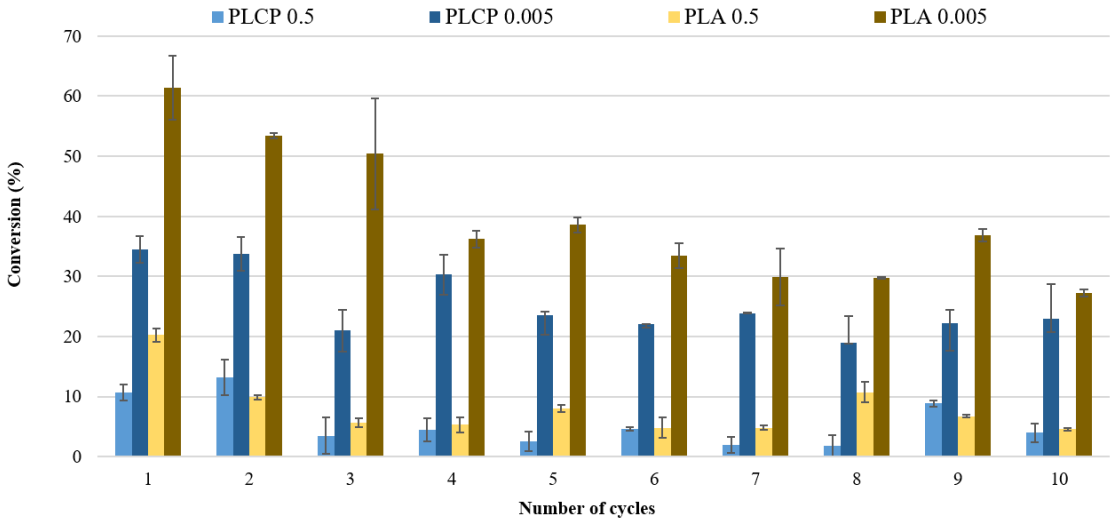

Figure 6. Conversion values obtained after 4 h reaction time using *Ut*DAADH immobilized either by covalent binding (PLCP) or by adsorption (PLA), at both the highest and the optimal enzyme-to-support *ratios* (the latter determined for the PLCP preparation).

The synthetic utility of the immobilized DAADH was demonstrated by the 100 mg-scale reductive amination of phenylpyruvate, obtaining D-phenylalanine with 86% isolation yield.

The most robust immobilization strategies rely on the covalent attachment of the enzyme to solid supports; however, non-specific multipoint attachments can rigidify the inherently flexible structure of biomolecules, often leading to a significant loss of activity compared to the free enzyme.[18–20] Thus, in a parallel study, we investigated the development of site-specifically immobilized biocatalysts. Having in hand a robust, covalently immobilized PAL biocatalyst, we tested and compared its site-specifically immobilized variant, employing the maleimide/thiol coupling through solvent-exposed cysteine residues of the enzyme. The immobilization method allowed modulation of enzymatic activity by appropriate selection of the binding site, thereby controlling the orientation of the enzyme attached to the support. The resulting robust biocatalysts were evaluated in the synthetically challenging ammonia addition reaction catalyzed by PALs. The optimization of immobilization and reaction conditions was carried out, while the best performing biocatalyst, $SWCNT_{NH2}$-SS-*Pc*PAL exhibited high catalytic efficiency, providing excellent conversion (~90% in 10 hours) of cinnamic acid to L-phenylalanine and maintained the conversion values over >7 reaction cycles. Furthermore, site-specifically immobilized mutant *Pc*PAL variants were successfully employed in the synthesis of several L-phenylalanine analogues of high synthetic value (**Figure 7**).

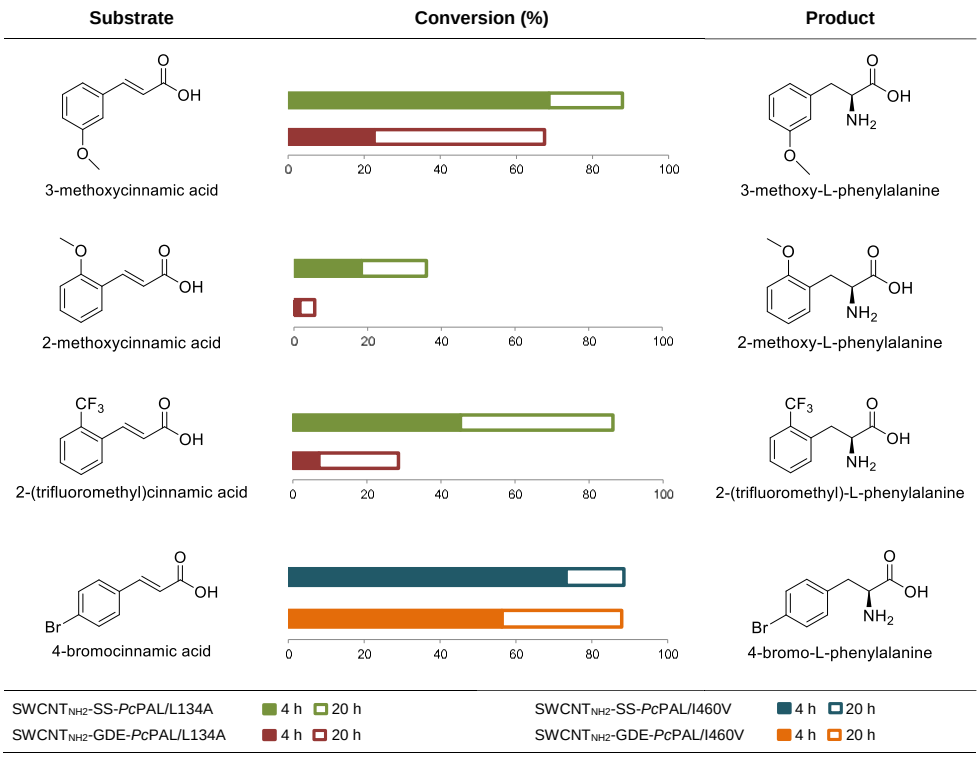

Figure 7. Conversion values for the ammonia addition reactions of cinnamic acid derivatives catalyzed by site-specifically and non-specifically immobilized engineered *Pc*PAL variants L134A and I460V, after 4 hours (filled bars) and after 20 hours reaction time (empty bars).

With the optimal procedure for the covalent immobilization of DAADH and the optimal conditions for the site-specific immobilization in hand, we implemented the site-specific immobilization of DAADHs. The individual substitution of surface-exposed serines to

cysteines at positions 2, 58, 92, 185, 192 and 317 of *Ut*DAADH enabled their site-specific immobilization onto the maleimide-functionalized Purolite® ECR8415F methacrylic support. The highest specific activities were achieved through immobilization *via* Cys2 and Cys192, showing 2.1- and 2.2-fold increase, respectively, when compared with the covalently, but non-specifically immobilized *Ut*DAADH. The recyclability of the immobilized preparations was evaluated over 10 reductive amination cycles. Based on the retained conversions and specific activities, *Ut*DAADH immobilized *via* S2C emerged as the best-performing biocatalyst, maintaining 65-70% of the initial conversion value and 50% of the initial enzyme activity after the 10th cycle. After determining the optimal enzyme-to-substrate *ratio*, the immobilized S2C *Ut*DAADH was tested in three consecutive 200 mg-scale reactions, providing enantiopure D-phenylalanine with complete conversion and excellent >88% isolation yields (**Figure 8**).

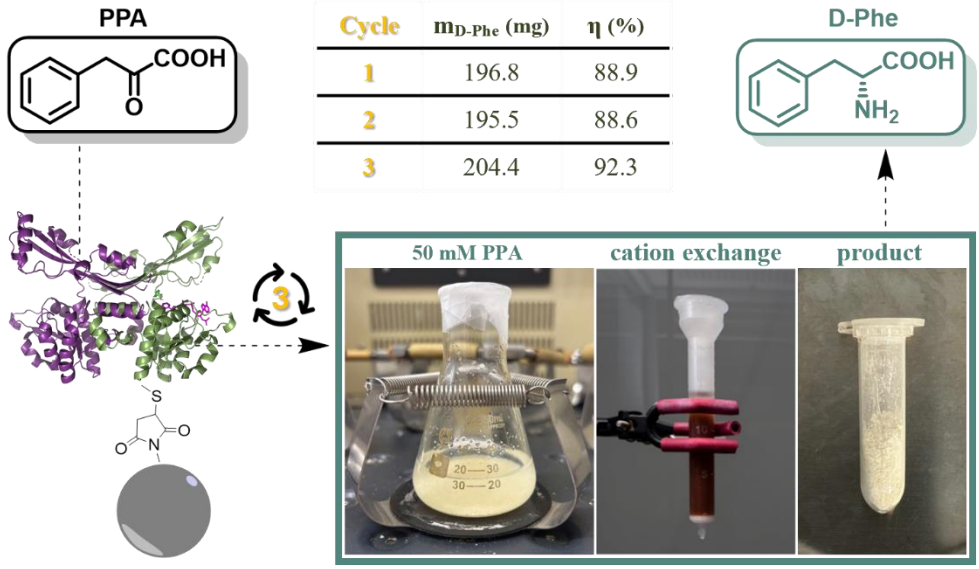

Figure 8. Reaction setup for the three consecutive preparative-scale reductive amination cycles using phenylpyruvate (PPA) as substrate, catalyzed by the site-specifically immobilized *Ut*DAADH S2C variant. The resulting D-phenylalanine was isolated *via* AmberChrom™ 50WX2 cation exchange resin, affording the enantiopure amino acid in high yields (η).

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