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Mapping the Hydrophobic Substrate Binding Site of Phenylalanine Ammonia-Lyase from *Petroselinum crispum*

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Abstract

Modification of the hydrophobic binding pocket of phenylalanine ammonia-lyase from *Petroselinum crispum* (*PcPAL*) enables increased activity and selectivity towards phenylalanines and cinnamic acids mono-substituted with both electron donating ($-\text{CH}_3$, $-\text{OCH}_3$) and electron withdrawing ($-\text{CF}_3$, $-\text{Br}$) groups at all positions (*o*-, *m*-, *p*-) of their aromatic ring. The results reveal specific residues involved in accommodating substituents at *o*-, *m*-, *p*-positions of the substrate's phenyl ring. The predicted interactions were validated by crystallographic analysis of the binding mode of *para*-methoxy cinnamic acid complexed at the active site of *PcPAL*. The biocatalytic utility of the tailored *PcPAL* mutants was demonstrated by the efficient preparative scale synthesis of (*S*)-*m*-bromo-phenylalanine (ee: > 99%, yield: 60%) and (*R*)-*p*-methyl-

phenylalanine (ee: 97%, yield: 49%), using the corresponding ammonia addition and ammonia elimination reactions catalyzed by the L134A and I460V *PcPAL* variants, respectively. Overall, the results reveal the potential for structure based protein engineering of PALs to provide enzymes with enhanced catalytic properties and which are specifically tailored for differently substituted phenylalanine analogues of high synthetic value.

Keywords: biocatalysis; protein engineering; phenylalanine ammonia-lyases; active site modification; D- and L-phenylalanine analogues

Introduction

Unnatural amino acids are valuable building blocks for the pharmaceutical and agrochemical industries. They are also increasingly being incorporated into proteins and peptides^{1,2}. Phenylalanine analogues have been successfully incorporated into

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4 peptidomimetics aimed at addressing the increasing antimicrobial resistance^{3–5}, as well
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7 those with antifungal properties⁶ and oxytocin analogues⁷.
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10 Phenylalanine ammonia-lyases (PALs, EC 4.3.1.24/25) have considerable potential
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14 as biocatalysts for the production of optically pure D- and L-phenylalanine derivatives,⁸
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17 as demonstrated by the implementation of PAL-based industrial processes such as the
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21 multi-ton scale production of (*S*)-2,3-dihydro-1*H*-indole-2-carboxylic acid by DSM
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24 (Netherlands).⁹
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28 Phenylalanine ammonia-lyase from *Petroselinum crispum* (*Pc*PAL) is amongst the
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31 most studied PALs and has been used in both ammonia addition and elimination
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34 reactions (**Figure 1a**), providing a wide range of L- and D-phenylalanine analogues.^{10–14}
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38 The substrate scope of *Pc*PAL has been significantly extended to bulky substituents
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41 located, especially at the *para*-position of the substrate's aromatic ring, by individual and
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44 combined substitutions of the active site residues I460 and F137.¹⁵ Previous
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47 substitutions of sequence-analogous residues for I460 and F137 have been shown to
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50 change the substrate selectivities of phenylalanine and tyrosine ammonia-lyases
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53 (PAL/TAL), providing variants with increased PAL-activity.^{16,17}
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4 Inspired by these results, we directed interest towards exploring possible spatial
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7 orientations of differently positioned, *ortho*- (*o*-), *meta*- (*m*-), *para*- (*p*-) substituents of
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10 the substrate, towards neighboring active site residues (**Figure 1b**). We envisaged that
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13 identification and modification of *Pc*PAL residues responsible for the active
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16 accommodation of the substrate's ring-substituents, would enable tailoring of the *Pc*PAL
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19 towards non-natural substrates of high synthetic value that are poorly transformed by
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24 *wild-type* PALs.
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28 Several studies on engineered PALs have recently been reported, including ones
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31 focusing on tailoring towards accommodating the *p*-substituted and/or bulky
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34 substrates^{15,18,19} or substrates with electron donating groups²⁰ and extended aliphatic
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37 chain²¹. However, to our knowledge no systematic studies have been carried out on
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41 correlations between the position (i.e. *o*-, *m*-, *p*-) of the substrate aryl ring substituent
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45 and specific active site residues involved in the aryl ring accommodation.
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is also shown (black). Residues placed in boxes were selected for alanine and valine scanning with the aim of accommodating of ring substituents. Side chains in dashed boxes are positioned below the plane of the substrate's aromatic ring, while those with normal boxes are positioned above this plane.

Results and Discussion

Mutagenesis of the hydrophobic binding pocket

Prior to this work no structure of *PcPAL* with a substrate/product complexed had been reported, moreover, the only eukaryotic PAL structure with a bound product (PAL from *Rhodospiridium toruloides* – PDB ID: 1T6J), manifests 'relatively weak' and 'incomplete' electron density for the *trans*-cinnamic acid ligand²⁴. In order to inform on the active site orientations of substituents attached to the aromatic ring of the substrate we employed a systematic mutational analysis of the hydrophobic substrate binding pocket of our *PcPAL* model.^{15,21,22}

First, a set of *PcPAL* single mutants was prepared, by the individual replacement of all the residues of the hydrophobic binding pocket of the active site (Figure 1b, Table S1),

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4 to less sterically demanding alanine (A) and/or valine (V) residues, aiming to increase
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7 the volume available for substrates with ring substituents. All these substitutions have
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10 been found to be non-essential for PAL-activity¹⁵, and - despite decreasing the original
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13 PAL-activity in the natural reaction (ammonia elimination from L-Phe, **Table S2**) –
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16 substantially increased the activities for different bulky, unnatural substrates.^{15,21}
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21 Modifications of active site residues from the polar active site region were omitted, since
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24 R354 (fixing the carboxylate of substrate), E484 (and presumably also their neighboring,
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27 ‘counter-ion’ residues Q348, K456) are involved in a H-bonding network that is essential
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31 for activity^{18,25}. Y110 and Y351 were also shown to be essential within the MIO-group
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34 assisted reaction mechanism^{26,27}. Besides the substitutions of the selected active site
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38 residues, the C704S and C716S substitutions of surface cysteines were also present
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42 within all tested PAL variants²⁸, with the aim of decreasing aggregation tendency during
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45 isolation and crystallization of recombinant PALs^{28,29}.
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48 *Substrate library*

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52 To explore the correlation between the position of the aromatic substituent and of the
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55 catalytically beneficial mutation of the hydrophobic substrate binding pocket (**Figure 1b**),
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we searched for substrates substituted at different positions of the aromatic ring, that are challenging to be transformed by the *wt*-PALs. Substrates with electron donating substituents are poorly converted, if at all, by *wt*-PALs, while *p*-substituted substrates with electron withdrawing and bulky substrates (*p*-Br-, *p*-NO₂-Phe) present steric clashes with catalytic residues of *wt*-PALs^{15,18,19}. Accordingly, for testing the PAL-activity of the mutant library, cinnamic acid and phenylalanine analogues, mono-substituted at all positions (*o*-, *m*-, *p*-) of the aromatic ring with either electron donating (-CH₃, -OCH₃) or electron withdrawing (-CF₃, -Br) substituents, were investigated as substrates (Figure 2).

Figure 2. Substrates with monosubstituted phenyl moieties bearing electron donating or electron withdrawing groups in all positions of the aromatic ring for probing the *PcPAL* mutant library.

PcPAL mediated biotransformations

Initially, the set of *PcPAL* single mutants was screened for ammonia addition and ammonia elimination reactions of substrates **1a-l** and *rac*-**2a-l** (see **Tables S3-S26** for results of all *PcPAL* mutants tested). The *PcPAL* single mutants which showed the highest conversions were identified and used in further selectivity screens. In the subsequent tests, besides the conversions of substrates, the enantiomeric excesses (ee_p) of products were also monitored (**Tables 1-3**).

Overall, despite the substantial differences between the reaction medium of the ammonia addition (4-6 M NH_3 , pH 10, buffered with CO_2) and ammonia elimination (Tris.HCl, pH 8.8) reactions, similar mutations provided enhanced catalytic properties in both types of reaction (**Tables 1-3**). This observation suggests that the hydrophobic pocket of the active site adopts a similar fold relative to the substrate in both reaction

types. Thus, it is reasonable to propose that crystal structures obtained in a low ammonia environment can be used for the rational design of PALs with increased efficiency in the synthetically more important ammonia addition reaction. The electronic effects of different *o*- and *m*-substituents provided no significant influence on enzyme activity (**Table 1** and **2**). In case of *p*-substitutions, in accordance with recent reports using PAL from *Planctomyces brasiliensis*²⁰, substrates with electron donating groups (**1c,f** and *rac*-**2c,f**) clearly underperformed in the *Pd*PAL-catalyzed reactions, when compared to substrates with electron withdrawing groups (**1i,l** and *rac*-**2i,l**) (**Table 3**).

More specifically, in the case of *o*-substituted substrate analogues *wt-Pd*PAL was an efficient catalyst, suggesting that accommodation of, at least, moderately sized (Me-, CF₃- and Br-) *o*-substituents in the hydrophobic binding site is not sterically hindered in *wt-Pd*PAL (**Table 1**). However, mutation of L256 in *Pd*PAL to smaller side-chain amino acids resulted in higher conversions of *o*-substituted substrates in both reaction types, suggesting its interference with the *o*-substituents on binding of the substrate to the active site. One exception is the case of *o*-methoxy substituted substrates **1d** and *rac*-**2d**, where in comparison to the *wt-Pd*PAL only the L134A and L134V variants provided

significantly increased conversions, supporting a spatial orientation of the *o*-methoxy methyl group towards residue 134.

Table 1. Conversion and enantiomeric excess values for the ammonia addition and elimination reactions catalyzed by *wild-type* and the three best performing mutant *PcPALs* in case of substrates with an *o*-substituted phenyl ring.

Ammonia addition*						Ammonia elimination*					
Substrate / EWG ^a						Substrate / EWG ^a					
1j / <i>o</i> -Br			1g / <i>o</i> -CF ₃			<i>rac</i> - 2j / <i>o</i> -Br			<i>rac</i> - 2g / <i>o</i> -CF ₃		
<i>PcPAL</i>	c%	ee _(S) -2	<i>PcPAL</i>	c%	ee _(S) -2	<i>PcPAL</i>	c%	ee _(R) -2	<i>PcPAL</i>	c%	ee _(R) -2
L256V	91.5	>99	L134A	78.4	>99	L256V	49.1	97.2	L256V	50.8	>99
L256A	89.8	>99	L256V	77.6	>99	L256A	44.8	84.8	L206V	51.8	>99
<i>wt</i>	89.0	>99	<i>wt</i>	76.2	>99	I460V	44.7	79.3	L256A	50.0	>99
F137V	85.9	>99	L206V	76.1	>99	<i>wt</i>	42.0	72.4	<i>wt</i>	49.0	94.0
Substrate / EDG ^b						Substrate / EDG ^b					
1a / <i>o</i> -CH ₃			1d / <i>o</i> -OCH ₃			<i>rac</i> - 2a / <i>o</i> -CH ₃			<i>rac</i> - 2d / <i>o</i> -OCH ₃		
<i>PcPAL</i>	c%	ee _(S) -2	<i>PcPAL</i>	c%	ee _(S) -2	<i>PcPAL</i>	c%	ee _(R) -2	<i>PcPAL</i>	c%	ee _(R) -2
L256V	62.1	>99	L134A	47.9	>99	L256V	54.9	>99	L134A	29.3	41.1
<i>wt</i>	54.3	>99	L134V	18.5	>99	<i>wt</i>	52.8	>99	I460V	1.3	1.3
L134A	52.4	>99	L206V	5.7	>99	I460V	50	>99	<i>wt</i>	1.0	1.1
I460V	47.4	>99	<i>wt</i>	<1	-	I460A	38.6	62.8	L256A	<1	-

* Reactions were performed under the conditions of biotransformation screenings (see Experimental Section)

^a – Electron withdrawing group; ^b – Electron donating group

The proposed orientation of the *o*-OCH₃ substituent towards L134 is further supported by the low conversions of *m*-substituted substrates when using the *wild-type* *PcPAL* (**Table 2**), with the exception of moderate conversions of *m*-bromo-substituted substrates **1k** and *rac*-**2k**. Indeed, as for *o*-OCH₃-substituted substrates **1d** and *rac*-**2d**, the L134A variant provided significantly improved conversions compared to *wt-PcPAL* for all the *m*-substituted substrates (**Table 2**).

Unexpectedly, *PcPAL* I460V - reported recently as improved mutant variant for *p*-substituted substrate analogues¹⁵ - also enhanced the catalytic properties of the enzyme to a similar degree and even surpassing L134A, in terms of activity and/or selectivity in the case of **1k**, **1h** and *rac*-**2h**, bearing electron withdrawing groups (-Br, -CF₃) as substituents. These results support our model (**Figure 1b**), in which substitution of either residues L134 or I460 could provide more space for *m*-substituents; moreover, the presence of the polar residue K456 in proximity of I460, might contribute to the observed higher transformation rates of substrates bearing polar *m*-substituents, when using mutant I460V.

Table 2. Conversion and enantiomeric excess values for the ammonia addition and elimination reactions catalyzed by *wild-type* and the three best performing mutant *PcPALs* in case of substrates with a *m*-substituted phenyl ring.

Ammonia addition*						Ammonia elimination*					
Substrate / EWG ^a						Substrate / EWG ^a					
1k / <i>m</i> -Br			1h / <i>m</i> -CF ₃			<i>rac</i> -2k / <i>m</i> -Br			<i>rac</i> -2h / <i>m</i> -CF ₃		
<i>PcPAL</i>	c%	ee _(S) -2	<i>PcPAL</i>	c%	ee _(S) -2	<i>PcPAL</i>	c%	ee _(R) -2	<i>PcPAL</i>	c%	ee _(R) -2
L134A	79.6	99	I460V	36.9	>99	L134A	48.9	94.1	I460V	31.4	49.5
I460V	68	>99	L134A	17.5	>99	I460V	42.4	77.8	L134A	26.2	38.5
I460A	29.6	>99	L138V	11.1	>99	<i>wt</i>	31.4	47.0	<i>wt</i>	9.1	9.9
<i>wt</i>	28.9	>99	<i>wt</i>	<1	-	F137V	24.5	33.0	F137V	6.9	7.5

Substrate / EDG ^b						Substrate / EDG ^b					
1b / <i>m</i> -CH ₃			1e / <i>m</i> -OCH ₃			<i>rac</i> -2b / <i>m</i> -CH ₃			<i>rac</i> -2e / <i>m</i> -OCH ₃		
<i>PcPAL</i>	c%	ee _(S) -2	<i>PcPAL</i>	c%	ee _(S) -2	<i>PcPAL</i>	c%	ee _(R) -2	<i>PcPAL</i>	c%	ee _(R) -2
L134A	22.7	>99	L134A	73.0	>99	L134A	23.8	31.2	L134A	51.2	>99
I460V	12.7	>99	F137V	24.5	>99	I460V	14.6	20.4	L134V	29.5	40.3
L134V	9.8	>99	<i>wt</i>	16.9	>99	L134V	9.1	10.2	<i>wt</i>	16.1	20.2
<i>wt</i>	4.9	>99	I460V	4.2	>99	<i>wt</i>	7.1	7.7	I460V	7.9	8.7

* Reactions were performed under the conditions of biotransformation screenings (see Experimental Section)

^a – Electron withdrawing group; ^b – Electron donating group

The low or moderate conversions obtained with *wt-PcPAL* in the case of *p*-substituted substrates (Table 3) is consistent with a steric clash between *p*-substituent and active site residues, as described in previous reports^{15,18}. Two mutants, namely

F137V and I460V, proved to be superior in terms of conversions (**Table 3**). With the substrates *p*-substituted with EWG (e.g. for **1l** and **1i**), higher conversions could be achieved with the F137V variant than with the I460V mutant; however *Pc*PAL F137V provided incomplete enantiotopic selectivity, while enantiopure products (*ee*_p >99%) resulted when using *Pc*PAL I460V

Table 3. Conversion and enantiomeric excess values for the ammonia addition and elimination reactions catalyzed by *wild-type* and the three best performing mutant *Pc*PALs in case of substrates with a *p*-substituted phenyl ring.

Ammonia addition*						Ammonia elimination*					
Substrate / EWG ^a						Substrate / EWG ^a					
1l / <i>p</i> -Br			1i / <i>p</i> -CF ₃			<i>rac</i> - 2l / <i>p</i> -Br			<i>rac</i> - 2i / <i>p</i> -CF ₃		
<i>Pc</i> PAL	c%	<i>ee</i> _{(S)-2}	<i>Pc</i> PAL	c%	<i>ee</i> _{(S)-2}	<i>Pc</i> PAL	c%	<i>ee</i> _{(R)-2}	<i>Pc</i> PAL	c%	<i>ee</i> _{(R)-2}
F137V	61.2	97	F137V	73.9	91.0	I460V	49.8	>99	F137V	48.8	81.5
I460V	51.1	>99	I460V	20.4	>99	F137V	45.0	95.9	I460V	36	51.7
L138V	13.2	>99	F137A	16.6	>99	wt	34.2	54.3	F137A	30.1	42.2
wt	7.6	>99	wt	5.7	>99	L206V	7.9	8.6	wt	5.9	6.3
Substrate / EDG ^b						Substrate / EDG ^b					
1c / <i>p</i> -CH ₃			1f / <i>p</i> -OCH ₃			<i>rac</i> - 2c / <i>p</i> -CH ₃			<i>rac</i> - 2f / <i>p</i> -OCH ₃		
<i>Pc</i> PAL	c%	<i>ee</i> _{(S)-2}	<i>Pc</i> PAL	c%	<i>ee</i> _{(S)-2}	<i>Pc</i> PAL	c%	<i>ee</i> _{(R)-2}	<i>Pc</i> PAL	c%	<i>ee</i> _{(R)-2}
F137V	16.6	>99	I460V	11.8	>99	I460V	22.1	25.8	F137V	21	26.1
I460V	16.2	>99	F137V	5.4	>99	F137V	10.7	23.8	I460V	16.8	19.1
L134V	3.7	>99	L138A	2.9	>99	wt	3.0	3.1	I460A	12.0	15.1

<i>wt</i>	6.0	>99	<i>wt</i>	<1	-	I460A	1.9	1.9	<i>wt</i>	<1	-
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* Reactions were performed under the conditions of biotransformation screenings (see Experimental Section)
^a – Electron withdrawing group; ^b – Electron donating group

Kinetic analyses

Kinetic parameters (K_M , k_{cat} , k_{cat}/K_M) determined for the best performing and *wild-type* *PcPAL* variants in the ammonia elimination reactions of representative *o*-, *m*-, *p*-CF₃-phenylalanines **rac-2g,h,i**, revealed valuable insights regarding the effect of mutations upon the catalytic efficiency of *PcPAL* in the reactions of ring-substituted substrates (**Table 4**).

In conformity with the results of the whole cell biotransformations, the catalytic activities reflected by k_{cat} values for the best performing mutants incubated with **rac-2g,h,i** were significantly higher than those found for *wt-PcPAL*. These data associated with increased K_M values in case of *o*-, *m*-CF₃-phenylalanines **rac-2g,h**, revealing the simultaneous increased activity and lowered affinity of the *PcPAL* mutants towards these substrates. Thus, in accordance with other similar studies²¹ a more relaxed arrangement of the substrate within the catalytic site for the tested mutant variants can be stated. As exception, I460V *PcPAL* displays significantly increased activity and affinity towards *m*-CF₃-Phe, which is consistent with the used model and, supports the proposed interaction of the electronwithdrawing substituents with residue K456, which is likely proximal to I460 (**Figure 1b**).

In case of *p*-CF₃-phenylalanine **rac-2i**, compared to *wt-PcPAL*, both mutant variants F137V and I460V displayed increased affinity and catalytic activity, resulting up to 27 fold improved catalytical efficiency (k_{cat}/K_M) compared with *wt-PcPAL* (**Table 4**). For the ammonia elimination from *o*-CF₃-Phe (**rac-2g**), despite the high conversion values obtained with the L206V and *wt-PcPAL* (52% and 49%, respectively, comparable with those for L256V (50%))

(Table 1)), mutant L256V presented a significantly higher k_{cat} (3.6 fold compared to *wt*- or L206V), albeit the k_{cat}/K_M values are in similar range for all three enzymes (61.7 for *wt*-, 64.8 for L206V and 54.2 for L256) (Table 4). These observations suggests that during long-term biotransformations besides intrinsic enzyme activity, other factors such as enzyme stability, solubility and product inhibition influence the conversion values.

Tabel 4. Kinetic parameters for the ammonia elimination reaction of *rac*-2g,h,i.

Compound <i>PcPAL</i>	<i>rac</i> -2g / <i>o</i> -CF ₃			<i>rac</i> -2h / <i>m</i> -CF ₃			<i>rac</i> -2i / <i>p</i> -CF ₃		
	<i>wt</i> -	L206V	L256V	<i>wt</i> -	I460V	L134V	<i>wt</i> -	F137V	I460V
K_M (μ M)	523	743	2733	533	163	912	2490	151	901
k_{cat} (s ⁻¹)	0.042	0.041	0.148	0.057	0.200	0.203	0.25	0.42	0.55
k_{cat}/K_M (s ⁻¹ × M ⁻¹)	61.7	64.8	54.2	103.2	1227.0	222.8	100.4	2781.4	611.1

Crystallization studies

To investigate whether our strategy had correctly identified the substrate substituent orientation relative to the hydrophobic residues in the active site of *PcPAL* active site, we pursued crystallographic analyses. These resulted in a crystal structure (resolution of 2.4 Å) of the I460V mutant liganded with *p*-methoxycinnamic acid 1f at the active site (PDB ID: 6RGS). As reported for *wt-PcPAL*²³, *PcPAL* I460V crystallizes at neutral pH in the space group *C*222₁ with two chains (A, B) in the asymmetric unit, with symmetry related molecules making up a tetramer, as observed in solution²³.

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4 The crystallographic tetramer contains four active sites and shows high similarity to
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7 the reported structure of *wt-PcPAL* with an overall RMSD of 0.271. Several loops could
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10 not be modeled, with two of the loops (residues 105-127 and 331-346) being close to
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13 the active site (**Figure S34**). These loops are proposed to be highly mobile and residues
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17 105-127 have been proposed by Ritter *et al.* to be involved in catalysis²³. In our
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20 structure we do not see evidence for the proposed²³ induced fit of these loops upon
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23 substrate binding, though this may occur in solution.
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28 Electron density was observed in one of the active sites corresponding to the presence of
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30 substrate **1f**. Analysis of the binding mode of *p*-methoxycinnamic acid **1f** in *PcPAL* I460V
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33 shows the *para*-position of the aromatic ring in close proximity of the substituted residue 460.
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35 Overlay with the reported structure of the *wt-PcPAL*²³ reveals a potential steric clash with the
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37 I460 side chain (**Figures 3** and **S35**). It should, however be noted that significantly weaker
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39 electron density is observed in our structure for the methoxy group, compared to the rest of the
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41 molecule (**Figure S37**), likely indicating a degree of disorder for this substituent (resulting in an
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43 increase of B-factor by about 10% compared to the rest of the molecule). Nonetheless, in accord
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45 with our model (**Figure 1b**) and the biotransformation results, the structure suggests orientation
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47 of the methyl group of methoxy towards V460. The positions of the other hydrophobic active site
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49 residues (F137, L138, L134, L206 and L256) are very similar to those within our model (**Figure**
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52 **1b**). An exception is K456, which is flanked between residues V460 and F137 (**Figure 3**),
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exposing the hydrophobic part of its side chain towards the *p*- and *m*-positions of the substrate. The missing electron density for the NH₂- group of K456 hinders the validation of the polar interactions with the *m*-CF₃-substituent, as proposed based on the biotransformation and enzyme kinetic data.

Figure 3: View of *p*-methoxy-cinnamic acid **1f** at the active site of *PdPAL* I460V (PDB ID: 6RGS). Side chains of active site residues (white) involved in substrate binding, including those in the hydrophobic pocket are shown. The side chain of I460 as observed in the active site of *wt-PdPAL* (PDB ID: 1W27) is overlaid in magenta. The

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4 I460V substitution prevents the indicated steric clash of this side chain with the *p*-
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7 substituent of the substrate. See **Figure S36** for full overlay of the two active sites.
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11 Prior to our work no PAL structure with a ring substituted substrate-like ligand/product
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14 complexed in the active site has been reported. The structure of *Rhodospiridium*
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17 *toruloides* PAL (*RtPAL*) with *trans*-cinnamic acid bound to the active site (PDB ID:
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19 1T6J), represented the only previously reported structure of fully catalytically viable PAL
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22 complexed with a substrate/product, albeit with the reported electron density
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25 corresponding to the ligand being non-optimal²⁴. Although Weise *et al.*³⁰ have reported
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28 a structure of the *Anabaena variabilis* (*AvPAL*) bound to cinnamate (PDB ID: 5LTM),
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31 this represents a structure for a catalytically inactive Y78F-C503S-C565S mutant of a
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34 prokaryotic PAL. Overlay of the active sites of this structure and the structure of the
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37 I460V *PcPAL*-ligand complex reported here shows, although there is otherwise good
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40 agreement of the most active site residues, some differences in the binding-mode of the
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43 substrate carboxylate moiety, as well as in the orientation of R354 which is involved in
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46 the interaction with the substrate carboxylate, are manifest (**Figure 4a** and **Figure S38**).
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Comparison of our structure with that of cinnamate complexed with the structurally related phenylalanine aminomutase from *Taxus canadensis* (*TcPAM*) (PDB ID: 3NZ4³¹) presents good agreement. However, there are shown minor differences in the substrate interactions. Interestingly, the *p*-methoxycinnamic acid **1f** ligand as observed in our structure would sterically clash with Y80 of *TcPAM*, the equivalent of Y110 in *PcPAL*, which has been proposed to be involved in catalysis and which was not observed in our structure (Figure 4b and Figure S38).

Figure 4. Overlay of active sites of I460V *PcPAL* – *p*-methoxy cinnamic acid **1f** (cyan) complex and reported structures: **a)** overlay with structure of Y78F/C503S/C565S *AvPAL* with “cinnamate”, apparently refined into hydro-cinnamic acid (magenta) (PDB ID: 5LTM)³⁰ **b)**

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3 overlay with the active site of TcPAM in complex with cinnamic acid (yellow) (PDB ID:
4 3NZ4)³¹, amino acids 76-96 are located in the active site and indicated as cartoon.
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10 Although the present work is focused on biocatalytic application of PAL, the substrate
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Although the present work is focused on biocatalytic application of PAL, the substrate
complexed protein structure is of interest from a mechanistic perspective. Two general
mechanisms for PAL have been proposed^{25,26}: one involving reaction of a substrate
amino group/ammonia with MIO and one involving reversible Friedel-Crafts type
reaction at the *ortho*-position of the substrate aryl-ring. Whilst the solution situation is
almost certainly more complex than manifested in static crystal structures, our structure
is more consistent with the former mechanism. The distance from the MIO methylene
carbon to the C-2 carbon of *p*-methoxycinnamic acid is 3.8 Å, (while the closest *ortho*-
position of *p*-methoxycinnamic acid is located at a distance of 4.5 Å), suggesting the
viability of the reaction of the substrate amino group with MIO.

Synthetic applications

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To test the synthetic applicability of the PALs with improved catalytic properties,
preparative scale biotransformations were attempted for production of phenylalanine
derivatives of high synthetic interest. (*S*)-*m*-Bromo-phenylalanine L-2k, through the

amber suppression approach, has been used to expand the chemical diversity of bioactive peptides, such as lasso peptide microcin J25 (MccJ25)³² or Cmpd-15³³, a negative allosteric modulator for the β_2 -adrenergic receptor, while (*R*)-*p*-methyl-phenylalanine **D-2c** has been incorporated in anti-inflammatory formyl peptide receptor 1 antagonists³⁴, as well as Pin1 inhibitors³⁵ (**Figure S39**). To determine the optimal reaction times for the preparative scale reactions, we monitored the conversions and the enantiomeric excess of the product in the ammonia addition reactions of *m*-bromocinnamic acid **1k** (**Figure 5a**) and of the ammonia elimination from racemic *p*-methyl-phenylalanine *rac*-**2c** (**Figure 5b**). The reaction progress reached stationary phase at conversion values of ~80% in case of ammonia addition onto **1k** after 8 hours reaction, while the ammonia elimination from *rac*-**2h** reached the optimal 50% conversion of the kinetic resolution process after 50 hours reaction time. Both reactions were highly selective, the enantiomeric excess values of products approximated the theoretical maximum of a fully selective process (**Figure 5a,b**). The preparative scale reactions were performed on a 500 mg scale, allowing the isolation of phenylalanine analogues

(*S*)-**2k** and (*R*)-**2c** in high yields (49% and 60%, respectively) and optical purity ($ee_{(S)-2k}$: >99% and $ee_{(R)-2c}$: 97%) (Figure S39).

Figure 5. Conversion and enantiomeric excess progression curves for **a)** the ammonia addition onto *m*-Br-cinnamic acid **1k** using L134A *PcPAL* and for **b)** the ammonia elimination from *rac*-*p*-methyl-phenylalanine *rac*-**2c** using I460V *PcPAL*.

Experimental Section

Site-directed mutagenesis

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4 Mutant PAL genes were obtained through site-directed mutagenesis using *wt-pcpal*
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7 with pET-19b vector as template. The protocol as well as the list of primers used for
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10 mutagenesis are provided in the Supporting Information.

11 12 13 14 *Protein production and purification*

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17 The purification of the recombinant *wild-type* PcPAL (containing surface mutations
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20 C704S/C716S, shown to decrease the aggregation tendency of PALs²⁹), and active site
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24 mutant variants with the additional C704S/C716S substitution were performed using the
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28 protocol described in our previous work²⁸. The protein purity and homogeneity were
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31 determined using SDS-PAGE electrophoresis and size-exclusion chromatography.

32 33 34 35 *Whole cell-assay plate preparation*

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38 The overnight preculture was prepared in a 2.2-ml 96-deep-well plate containing 600
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41 μ L/well of LB (Lysogenic Broth) medium supplemented with carbenicillin (50 μ g/mL) and
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45 chloramphenicol (30 μ g/mL) and inoculated with glycerol stocks of *E. coli* Rosetta (DE3)
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49 pLysS cells harbouring the pET19b vector carrying the *wt* or mutant *pal* gene, followed
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52 by overnight incubation at 37 °C and shaking at 200 rpm.
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4 The obtained preculture was further used to inoculate the assay plate with 1.2 mL/well
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7 of LB medium grown at 37 °C, 200 rpm until OD₆₀₀ reached 0.6-0.8, at which point
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10 protein production was induced via the addition of 0.1 mM IPTG (final concentration),
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13 and the cell growth was maintained at 25 °C for another 16 h. Cell densities of OD₆₀₀
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16 were measured for each mutant variant and wild-type *PcPAL*, and duplicate wells were
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19 prepared containing cells of OD₆₀₀ of ~ 1. The cells were harvested by centrifugation at
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22 4000 rpm (2270 × g) and 4 °C for 20 min and washed with PBS buffer (20 mM
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25 phosphate, 150 mM NaCl, pH 8.0) (4000 rpm, 2270 × g, 4 °C, 20 min) and stored at -
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28 20 °C until further use.
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35 *Biotransformation screening using 96-well assay plates for ammonia elimination*
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38 *reactions*
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42 Initial screenings in case of the ammonia elimination reactions were measured in 96-
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45 well UV-Microplates (Corning Inc.), using Tecan Infinite Spark 10M microplate reader.
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49 Stock solutions of 5 mM substrate solutions of *rac-2a-I* were prepared in Tris-buffer (100
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52 mM Tris-HCl, 120 mM NaCl, pH 8.8) while using isolated *wild-type* and mutants *PcPAL*
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56 enzyme solutions. Reactions were done in triplicate at 30 °C with the ratio of substrate
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concentration: biocatalysts of 1:0.005 (1 mM substrate and 5 μ g enzyme), monitoring the production of specific cinnamic acid analogues through absorbance at 290 nm (Tables S15-S26).

The first five *Pc*PAL variants with the highest relative activity were selected for whole-cell biotransformations, monitoring the conversions after 16 h. In cases where the conversion-based ranking of *Pc*PAL variants significantly differed from their relative activity-based order, the whole-cell biotransformations with all *Pc*PAL variants were performed (Table S15-S26). Based on the obtained conversion values, the biotransformations of *wt-Pc*PAL and the three best performing mutant variants were further selected and the enantiomeric excess of D-**2a-I** products was also determined, resulting in data presented within Tables 1-3.

The whole cell biotransformations were performed in 96-well deep-well plates of 2.2 mL, containing the whole cell *Pc*PAL-biocatalysts, prepared as described above. The bacterial pellet was resuspended to an OD₆₀₀ of $\sim$ 2, in 200 μ L of Tris-buffer (100 mM Tris-HCl, 120 mM NaCl, pH 8.8) solutions containing amino acids *rac*-**2a-I** in 2 mM concentration. The plates were incubated at 30 $^{\circ}$ C, 250 rpm, samples were taken at

different reaction times, were quenched by adding an equal volume of MeOH, vortexed and centrifuged (13400 rpm, 12000 × g, 10 min). The supernatant was filtered through a 0.22 µm nylon membrane filter and analyzed by HPLC.

Biotransformation screening using 96-well assay plates for ammonia addition reactions

The screens for ammonia addition reactions were performed in duplicate using 96-well deep-well plates of 2.2 mL, containing the whole cell *Pc*PAL-biocatalysts. The bacterial pellet was resuspended to an OD₆₀₀ of ~ 2, in 200 µL of 6 M NH₄OH buffer (pH 10, adjusted with CO₂) solutions containing cinnamic acids **1a-l** in 2 mM concentration. The plates were incubated at 30 °C, 250 rpm. Conversions were monitored using reversed phase high performance liquid chromatography (HPLC). Samples taken at different reaction times, were quenched by adding an equal volume of MeOH, vortexed and centrifuged (13400 rpm, 12000 × g, 10 min). The supernatant was filtered through a 0.22 µm nylon membrane filter and analyzed by HPLC (Tables S3-S14).

Kinetics measurements

Enzyme assays were carried out in triplicate at 30 °C in 96-well Corning UV-microplates using 100 mM Tris.HCl, 120 mM NaCl (pH 8.8) as buffer, at constant

concentration of the specific *PcPAL* (0.3125 μ M), varying the substrate concentrations from 0.025 and 8 mM, until substrate saturation occurred. The kinetic measurements were based on UV-spectroscopy by monitoring the production of the acrylic derivatives **2a-d** for 10 min, at wavelengths where the corresponding amino acids *rac-1a-d* showed no absorption (Table S29). Kinetic constants (K_M , v_{max}) were obtained from the Michaelis-Menten curves by non-linear fitting (see ESI, Figures S25-S33).

Ammonia addition reaction at preparative scale

In a 100 mL flask, *E. coli* Rosetta (DE3) pLysS cells harbouring the pET19b vector carrying the *pcpal* L134A mutant gene were resuspended in 6 M NH_4OH -solution (pH 9.8 adjusted with CO_2) to give a final OD_{600} of ~ 10 . To the cell suspension was added *m*-bromocinnamic acid **1k** (0.5 g, 2.21 mmol) at a final concentration of 10 mM and the reaction was incubated at 200 rpm, 30 $^\circ\text{C}$ for 48 h, with monitoring the conversion values by reversed phase HPLC. When stationary conversions were reached the reaction mixture was acidified to pH 1.5 by dropwise addition of aqueous H_2SO_4 (50% w/v). The formed precipitate was removed by centrifugation at 10000 rpm ($10900 \times g$), 4 $^\circ\text{C}$ for 20 min.; the non-reacted cinnamic acid **1k** was removed by extraction with ethyl

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3 acetate (3 x 10 mL). The amino acid found within the aqueous phase was purified by ion
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7 exchange chromatography using Dowex® 50WX2 resin and 2M NH₄OH solution for
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10 elution. The fraction containing the final product was evaporated in a centrifugal
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13 evaporator affording the pure L-amino acid L-**2k** (0.4 g, 1.64 mmol and 74.5% isolation
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17 yield, [α]_D: -3.9 (25°C, 10 mg/mL, in MeOH).
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20 *Ammonia elimination reaction at preparative scale*

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22 In a 100 mL flask, *E. coli* Rosetta (DE3) pLysS cells harbouring the pET19b vector carrying
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24 the the *pcpa* l460V mutant gene were resuspended in 0.1 M NH₄OH (pH 10 adjusted with
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26 CO₂) to give a final OD₆₀₀ of ~ 10. To the cell suspension was added the racemic *p*-methyl-
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29 phenylalanine *rac*-**2c** (0.5 g, 2.79 mmol) in a final concentration of 10 mM and the reactions
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32 were incubated at 200 rpm, 30 °C for 48 hours, monitoring the conversion values by reversed
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35 phase HPLC. When stationary conversions were reached the reaction mixtures were purified
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38 using the same method described above in the preparative scale ammonia addition section,
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41 yielding the pure D-amino acid D-**2c** (0.24 g, 1.34 mmol and 49% isolation yield, [α]_D: +7.8
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46 (25°C, 10 mg/mL, in MeOH)– in accordance with data from literature³⁶).
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49 *HPLC methods for the determination of conversion and enantiomeric excess values*

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51 High performance liquid chromatography (HPLC) analyses were conducted with an Agilent
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54 (Santa Clara, CA, USA) 1200, 1260 and 1100 systems.
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For the determination of conversion values samples from biotransformations were injected onto a Gemini NX-C18 column (150 × 4.5 mm; 5 μm), and eluted with a flow rate of 1.0 mL min⁻¹, at 25 °C using different gradients of the mobile phase: A: NH₄OH buffer (0.1 M, pH 9.0) / B: MeOH (see detailed description in ESI).

Enantiomeric excess values were determined using a chiral HPLC separation of the *rac*-**2a-l**, developed using Crownpak CR-I (+) chiral column (150 × 3 mm; 5 μm) and HClO₄ (pH=1.5): ACN as mobile phase, at different flow rate (see detailed description in ESI). The absolute configuration of the eluted enantiomers was assessed from their elution order from the chiral CROWNPAK CR-I(+)column, according to the manufacturer's instructions, which was also confirmed by the obtained retention times for commercial D- and L-Phe standards ($R_{t,D-Phe} = 3.05$ min, $R_{t,L-Phe} = 5.03$ min).

Crystallisation experiments

A solution of recombinant *PcPAL* I460V/C704S/C716S (10 mg/mL) purified according to a previously reported procedure²⁸, in (20 mM Tris, 100 mM NaCl, pH 8.0) was saturated with substrate **1f** and left to incubate at 4 °C for 1 h prior to crystallisation. Crystallization was performed at 4 °C using the sitting-drop vapor diffusion method. Initial crystallisation conditions were identified using a Peglon broadscreen (Hampton Research) set up in low reservoir Intelli-Plate 93-3 (Art Robbins Instruments) using a Rigaku Phoenix RE Drop setter instrument (Art Robbins Instrument) mixing 100 nL of

protein solution with 100 nL of precipitant. Small crystals were obtained after 2 weeks in condition C6 (0.2 M ammonium acetate, 20 % PEG 3350). Larger crystals were obtained by using larger drops (2 μ L protein and 2 μ L precipitant) in combination with micro-seeding. A data-set was collected using beamline I03 at the Diamond Light Source. The structure was solved in the space group C2221 via molecular replacement using Phaser with the reported structure of *Pd*PAL (PDB ID: 1W27²³) as a search model. Refinement was carried out using Phenix and iterative fitting using Coot software packages^{37,38}. The tetramer, obtained using the symmetry related molecules, contains four active sites which each involve residues of three different enzyme molecules (chain A, B and a symmetry related chain A in **Figure S35**), as reported, e.g., for *wt-Pd*PAL (PDB ID: 1W27²³). In the active site apparently complete post-translational modification of residues 202-204 to 4-methylideneimidazole-5-one (MIO), as characteristic for PAL active sites²⁶, was observed.

Conclusions

The combined results on the activity and selectivity of mutants of PAL from parsley, using a series of mono-substituted phenylalanine and cinnamic acid analogues, reveal the possible active site orientations of substrate aromatic substituents, as function of their *o*-, *m*-, *p*- substitution. In the case of *o*-substituted substrates *wt-PcPAL* proved to be an efficient biocatalyst, suggesting that, at least, for moderately-sized *o*-substituents there is sufficient space in the *wild-type* active site. However, substitution of L256 enabled an increase in activity, indicating spatial orientation of *o*-substituents towards this residue. Substituents in the *m*-position, and with an *o*-methoxy group, are likely orientated towards residue L134. In these cases mutations L134A, or of I460V in the case of electron-poor *m*-substituents, were necessary to provide high catalytic efficiency, suggesting that in these cases steric hindrance occurs within the active site of *wild-type* enzyme. For *p*-substituted substrates the catalytic properties of *wt-PcPAL* were significantly increased through substitution of residues F137 and I460, implying substituent interaction and presumably steric clashes with these active site residues.

In accord with the results of the mutational strategy, crystallographic analysis of the binding mode of *p*-methoxy cinnamic acid, at the active site of *PcPAL* I460V supports

the proposed orientation of methoxy group towards residue 460V and provides the first structure of an active PAL with a ring-substituted substrate-analogue bound at the active site.

The biocatalytic utility of the tailored PALs was demonstrated by the efficient preparative scale synthesis, of D- and L- phenylalanine analogues. The overall results thus provide evidence that structure based engineering of the PAL active site will be productive with respect to producing enzymes of industrial value.

Associated Content

Supporting Information. Experimental details, tables and figures related to site-directed mutagenesis, substrate synthesis, activity screens, HPLC methods, kinetic measurement and crystallization studies. This material is available free of charge on the ACS Publications website <http://pubs.acs.org>.

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Figure 1. PcPAL catalysis and active site residues a) Ammonia addition and elimination reactions catalyzed by PcPAL. b) An active site model of PcPAL15, (PDB ID: 1W27) with modeled trans-cinnamic acid indicating possible interactions with the hydrophobic binding pocket (orange). Predicted orientations of aryl ring substituents are blue (ortho-), red (meta-), and green (para-). The post-translationally produced 3,5-dihydro-5-methylene-4H-imidazol-4-one (MIO) prosthetic group and the polar region of active site is also shown (black). Residues placed in boxes were selected for alanine and valine scanning with the aim of accommodating of ring substituents. Side chains in dashed boxes are positioned below the plane of the substrate's aromatic ring, while those with normal boxes are positioned above this plane.

189x155mm (300 x 300 DPI)

Figure 2. Substrates with monosubstituted phenyl moieties bearing electron donating or electron withdrawing groups in all positions of the aromatic ring for probing the PcPAL mutant library.

190x72mm (300 x 300 DPI)

Figure 3: View of p-methoxy-cinnamic acid 1f at the active site of PcPAL I460V (PDB ID: 6RGS). Side chains of active site residues (white) involved in substrate binding, including those in the hydrophobic pocket are shown. The side chain of I460 as observed in the active site of wt-PcPAL (PDB ID: 1W27) is overlaid in magenta. The I460V substitution prevents the indicated steric clash of this side chain with the p-substituent of the substrate. See Figure S36 for full overlay of the two active sites.

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Figure 4. Overlay of active sites of I460V PcPAL – p-methoxy cinnamic acid 1f (cyan) complex and reported structures: a) overlay with structure of Y78F/C503S/C565S AvPAL with “cinnamate”, apparently refined into hydro-cinnamic acid (magenta) (PDB ID: 5LTM)30 b) overlay with the active site of TcPAM in complex with cinnamic acid (yellow) (PDB ID: 3NZ4)31, amino acids 76-96 are located in the active site and indicated as cartoon.

342x127mm (300 x 300 DPI)

Individual image for Figure 4a, if needed.

165x106mm (300 x 300 DPI)

Individual image for Figure 4b, if needed.

170x127mm (300 x 300 DPI)

Figure 5. Conversion and enantiomeric excess progression curves for a) the ammonia addition onto m-Br-cinnamic acid 1k using L134A PcPAL and for b) the ammonia elimination from rac-p-methyl-phenylalanine rac-2c using I460V PcPAL.

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