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

Alina Filip[^1], Emma Z. A. Nagy[^2], Souad D. Tork[^3], Gergely Bánóczi[^4], Monica I. Toșa[^5], Florin D. Irimie[^6], László Poppe[^7][^8][^9], Csaba Paizs[^10], and László C. Bencze[^11]

**Introduction**

The synthesis of natural and unnatural aromatic amino acids in the homochiral form is an important challenge in preparative chemistry, highlighted by the significant interest towards these building blocks in the development of therapeutic peptides and proteins[^1][^2][^3]. An attractive enzymatic route to enantiomerically pure *L*- or *D*-aromatic amino acids involves the use of aromatic ammonia-lyases (ALs) and 2,3-aminomutases (AMs),[^4] which act by the aid of a 3,5-dihydro-5-methylene-4H-imidazol-4-one (MIO) electrophilic prosthetic group formed autocatalytically. Among the so-called MIO enzymes, phenylalanine ammonia-lyases (EC 4.3.1.24/25) from *Petroselinum crispum* (PcPAL)[^5][^6][^7][^8] exhibit activity in ammonia addition onto bulky substrates. However, combined mutations that involve I460 besides the well-studied F137 led to mutants that exhibited activity in ammonia addition as well. The synergistic multiple mutations resulted in substantial substrate scope extension of PcPAL and opened up new biocatalytic routes for the synthesis of both enantiomers of valuable phenylalanine analogues, such as (4-methoxyphenyl)-, (napthalen-2-yl)-, (1,1′-biphenyl)-4-yl), (4-fluoro-1,1′-biphenyl)-4-yl), and (5-phenylthiophene-2-yl)alanines.

[^1]: A. Filip, E. Z. A. Nagy, S. D. Tork, G. Bánóczi, M. I. Toșa, F. D. Irimie, L. Poppe, C. Paizs, L. C. Bencze

[^2]: Biocatalysis and Biotransformation Research Centre, Babes–Bolyai University of Cluj-Napoca, Arany János Str. 11, 400028 Cluj-Napoca (Romania)

[^3]: E-mail: poppe@mail.bme.hu

[^4]: paizs@chem.ubbcluj.ro

[^5]: cslbencze@chem.ubbcluj.ro

[^6]: Biocatalysis and Biotransformation Research Centre, Babes–Bolyai University of Cluj-Napoca, Arany János Str. 11, 400028 Cluj-Napoca (Romania)

[^7]: E-mail: poppe@mail.bme.hu

[^8]: paizs@chem.ubbcluj.ro

[^9]: cslbencze@chem.ubbcluj.ro

[^10]: Prof. L. Poppe

Department of Organic Chemistry and Technology
Budapest University of Technology and Economics
Műegyetem Híd 3, 1111 Budapest (Hungary)

[^11]: These authors contributed equally to this work.

[^12]: Supporting information and the ORCID identification number(s) for the author(s) of this article can be found under https://doi.org/10.1002/ccct.201800258.

[^13]: © 2018 The Authors. Published by Wiley-VCH Verlag GmbH & Co. KGaA.

This is an open access article under the terms of the Creative Commons Attribution-NonCommercial License, which permits use, distribution and reproduction in any medium, provided the original work is properly cited and is not used for commercial purposes.
and cancer-related histone lysine demethylase KDM4A inhibitors. (Naphthalen-2-yl)alanine is used frequently as a phenylalanine analogue in the development of peptides, whereas (4-methoxyphenyl)alanine is a chiral intermediate in the synthesis of the antihypertensive drug tamsulosin. Amide derivatives of styrylalanines were identified as potent peptidyl-prolyl isomerase (PPIase) inhibitors at Pfizer. The origin of the potency was attributed to the (E)-ethene-1,2-diy linker that increases the distance between the aromatic moiety and the chiral alanine moiety.

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

Results and Discussion

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

To exclude the influence of mutation-induced improper folding on enzyme activity, the oligomerization state and thermal unfolding of the isolated and purified mutants were compared with those of the wt-PcPAL. Retention volumes obtained by using size-exclusion chromatography (Figure S2) revealed that all PcPAL variants were folded properly and existed in the native, tetrameric form, similar to the wild-type enzyme. The slight variations in the thermal unfolding temperatures ($T_m$), determined by using differential scanning fluorimetry, indicated that the mutations did not affect protein folding (Table S3). The only exception was the I460A mutation that decreased the $T_m$ value significantly (from 75:1.5 to 51:1.2°C; Table S4 and Figure S5) without affecting the tetrameric fold detected by using size-exclusion chromatography. As later tests showed that I460A-PcPAL was catalytically active (Tables S2–S19), we presumed that the mutation corrupted only the thermal stability without disrupting the main folding patterns related to enzyme activity.

Figure 1. Active site model of PcPAL with (E)-cinnamic acid as a model ligand and the surrounding residues within less than 5 Å distance. The colors of the amino acid side chains refer to their position with respect to the plane of the substrate: black—within, red—above, blue—below the plane. Hydrophobic binding pocket residues in boxes were exchanged individually or in combination with smaller hydrophobic amino acids V or A.
Next, the generated single-mutant PcPAL library (Table S1) was tested with the targeted substrate panel in ammonia elimination from arylalanines rac-1a–i (Scheme 1A) and in ammonia addition onto arylacrylates 2a–i (Scheme 1B). Results from the ammonia eliminations revealed that besides the known F137 to V or A mutations,13,17 the mutation of another highly conserved residue I460 (Figure S1) to V or A increased the activity significantly towards almost all substrates compared to the wild-type enzyme (Table 1 and Tables S5–S13).

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

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

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

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

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

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

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

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

### Table 2. Activity of wt-PcPAL compared to the best PcPAL mutants in the ammonia addition reaction of 2a–h.

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

[a] PcPAL variant: 50 μg, reaction volume: 500 μL, medium: 6 mM NH₄ buffer (pH 10, adjusted with CO₂), substrate concentration: 1 mM; assays were performed in 1.5 mL glass vials sealed with a PTFE septum at 30 °C, 200 rpm for 20 h; (b) conversion values [%].
Our mutational analysis revealed that in most cases single mutations of F137 and I460 in PcPAL were sufficient to perform ammonia elimination from bulky amino acids decently and additional mutations did not improve the conversions significantly. However, double mutants of PcPAL that involve F137 and I460 were required to achieve adequate ammonia addition activity with bulky arylacylates 2c–h. In the case of less bulky substrates 2a and b, active single mutants of PcPAL could be identified as well. These data demonstrated clearly that multiple mutations exhibited a strong, nonadditive cooperative effect on PcPAL activity in the ammonia addition reaction.

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

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

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

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

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

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

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

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

The maximal conversions in the kinetic resolutions (KRs) as well as the maximal ee of the unreacted α enantiomer (α-1a–h) were reached in all but one case in relatively short reaction times (14–40 h; Table 3). In ammonia elimination from (2-phe-
Table 4. Conversion of PcPAL-catalyzed ammonia additions onto 2a–h and yield and enantiomeric excess of the products 1a–h after 70 h reaction time.

| Substrate | PcPAL variant | c [%] | ee [%] | ee,1a [h] [%] |
|-----------|---------------|-------|--------|---------------|
| 2a        | F137V/I460V   | 74    | 65     | > 99          |
| 2b        | F137V         | 73    | 61     | > 99          |
| 2c        | F137V/I460V   | 23    | 19     | > 99          |
| 2d        | F137A/I460V   | 68    | 59     | 82            |
| 2e        | F137A/I460V   | 50    | 43     | 95            |
| 2f        | F137A         | 6     | nd     | nd            |
| 2g        | F137A/I460V   | 9     | nd     | nd            |
| 2h        | F137A/I460V   | 3     | nd     | nd            |

Table 5. Calculated relative binding energies (ΔΔE) of 1a–h in wt-PcPAL and in the most active PcPAL variants. The subscripts WT and MA correspond to the wild-type and most active mutant, respectively. Note, these quantities are not meant to determine actual binding energies computationally but to approximate them only.

| Substrate | Most active PcPAL variant | ΔΔEWT,a [kcal mol⁻¹] | ΔΔEEWT,a [kcal mol⁻¹] | ΔΔEMAI,a [kcal mol⁻¹] |
|-----------|---------------------------|----------------------|-----------------------|----------------------|
| 1a        | I460V                     | 2.9                  | 3.7                   | 0.8                  |
| 1b        | F137V                     | 4.9                  | -11.2                 | -16.1                |
| 1c        | F137V                     | 13.7                 | -4.0                  | -17.7                |
| 1d        | F137A                     | 29.8                 | -3.5                  | -33.3                |
| 1e        | F137A/I460V               | 35.5                 | 12.6                  | -22.9                |
| 1f        | F137A/I381                | 19.7                 | -5.3                  | -25.0                |
| 1g        | F137A                     | 32.1                 | 0.3                   | -31.8                |
| 1h        | F137A                     | 40.6                 | 7.5                   | -33.1                |

[a] Binding energies are related to the same property of L-Phe with wt-PcPAL in the form of ΔΔE = ΔΔEwt a - ΔΔEMa. For a detailed description of the calculation method and reasoning of the necessary relativization, see Supporting Information.
Comparisons of the N-MIO intermediates from the other L-arylalanines L-1b-d and f-h indicated similar situations. The only exception was L-1a, with a nearly unaffected affinity, which in turn suggested that the catalytic enhancement stemmed solely from the other presumed factor, a higher turnover number.

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

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

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

The fact that PALS are known to present difficulties in the transformation of substrates with electron-donating ring substituents and the recent efforts that focus on the discovery of new PALS with such activity$^{[16] [17]}$ highlights the excellent results obtained for the synthesis of both L- and d-4-methoxyphenylalanine (L- and d-1a).

Pharmaceutically important ([1,1′-biphenyl]-4-yl)alanines were the subject of recent AvPAL-mediated biotransformations in which the studied AvPAL variants showed no activity in ammonia addition onto 4-phenylcinnamic acid, thus a chemoenzymatic procedure was required that involves the AvPAL-mediated synthesis of L- and d-4-phenylcinnamylalanine followed by Pd-catalyzed Suzuki-coupling.$^{[18] [19]}$ In this frame, the tailored multiple PcPAL-based processes reported here represent the first direct enzymatic route towards both enantiomers of ([1,1′-biphenyl]-4-yl)alanines L- and d-1d and e.

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

Conclusions

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

The new PcPAL mutants enabled unprecedented PAL-mediated biocatalytic routes to the d and l enantiomers of (napthalen-2-yl)alanine 1a and 4-methoxyphenylalanine 1b. With the aid of tailored multiple mutations of PcPAL, direct biocatalytic routes could be developed towards d- and l-[1,1′-biphenyl]-4-
yl)alanines, d- and l-1d,e, valuable chiral intermediates for several drugs under development, as well as towards new non-natural amino acids d-(5-phenylthiophen-2-yl)alanines α-1f-h.

Experimental Section
For all experimental details see the Supporting Information.

Acknowledgements
Financial support for project PROMYS, (Grant Nr. IZ11Z0_166543) from the Swiss National Science Foundation (SNSF) and for project NEMSyB, ID P37_273, Cod MySMIS 131 (funded by National Authority for Scientific Research and Innovation (ANCSI) and European Regional Development Fund, Competitiveness Operational Program 2014–2020 (POC), Priority axis 1, Action 1.1) is gratefully acknowledged.

Conflict of interest
The authors declare no conflict of interest.

Keywords: amino acids · biocatalysis · phenylalanine ammonia-lyase · protein engineering · substrate scope extension

[1] J. A. Robinson, S. DeMarco, F. Gombert, K. Moehe, D. Obrecht, Drug Discovery Today 2008, 13, 944 – 951.
[2] K. Zerbe, K. Moehe, J. A. Robinson, Acc. Chem. Res. 2017, 50, 1323 – 1331.
[3] D. J. Craik, D. P. Fairlie, S. Liras, D. Price, Chem. Biol. Drug Des. 2013, 81, 136 – 147.
[4] F. Parmeggiani, N. J. Weise, S. T. Ahmed, N. J. Turner, Chem. Rev. 2018, 118, 73 – 118.
[5] A. Gloge, J. Zorl, Á. Kóvári, L. Poppe, J. Rétyey, Chem. Eur. J. 2000, 6, 3386 – 3390.
[6] C. Paizs, M. I. Toja, L. C. Bencze, J. Brem, F. D. Irimie, J. Rétyey, Heterocycles 2010, 82, 1217 – 1228.
[7] G. Renard, J. C. Guilleux, C. Bore, V. Malta-Vallette, D. A. Lerner, Biotechnol. Lett. 1992, 14, 673 – 678.
[8] W. Liu (Great Lakes Chemical Co.), US Pat 5,981,239, 1999, [Chem. Abstr. 1999, 131, 321632].
[9] I. Rowles, B. Groenenendaal, B. Binay, K. J. Malone, S. C. Willis, N. J. Turner, Tetrahedron 2016, 72, 7343 – 7347.
[10] I. Rowles, B. Groenenendaal, B. Binay, K. J. Malone, S. C. Willis, N. J. Turner, Bioorg. Med. Chem. 2010, ·43
[11] S. Lovelock, N. J. Turner, Bioorg. Med. Chem. 2014, 22, 5555 – 5557.
[12] N. J. Weise, S. T. Ahmed, F. Parmeggiani, E. Sirola, A. Pushpanath, U. Schell, N. J. Turner, Catal. Sci. Technol. 2016, 6, 4086 – 4089.
[13] S. Bartsch, U. T. Bomscheuer, Protein Eng. Des. Sel. 2010, 23, 929 – 933.
[14] S. T. Ahmed, F. Parmeggiani, N. J. Weise, S. L. Flitsch, N. J. Turner, ACS Catal. 2015, 5, 5410 – 5413.
[15] A. Dreisien, T. Hilberath, U. Mackfeld, A. Billmeier, J. Rudat, M. Pohl, J. Biotechnol. 2017, 258, 148 – 157.
[16] N. T. Weise, S. T. Ahmed, F. Parmeggiani, J. L. Galman, M. S. Dunstan, S. J. Charnok, D. Ley, N. J. Turner, Sci. Rep. 2017, 7, 13691.
[17] L. C. Bencze, A. Filip, G. Bánóczi, M. I. Toja, F. D. Irimie, Á. Gellért, L. Poppe, C. Paizs, Org. Biomol. Chem. 2017, 15, 3717 – 3727.
[18] J. E. Swedberg, C. I. Schroeder, J. M. Mitchell, T. Durek, D. P. Fairlie, D. J. Edmonds, D. A. Griffith, R. B. Ruggeri, D. R. Derksen, P. M. Loria, S. Liras, D. A. Price, D. J. Craik, Eur. J. Med. Chem. 2015, 103, 175 – 184.
[19] L. Moreira, M. Roatsch, M. C. D. Fürst, I. Hoffmann, J. Senger, M. Hau, H. Franz, R. Schüle, M. R. Heinrich, M. Jung, ChemMedChem 2016, 11, 2063 – 2083.
[20] L. Bai, Z. Li, J. Chen, N. N. Chung, B. C. Wilkes, T. Li, P. W. Schiller, Bioorg. Med. Chem. 2014, 22, 2333 – 2338.
[21] Z. Qian, X. Xu, J. F. Amacher, D. R. Madden, E. Cormet-Boyaka, D. Pei, Angew. Chem. Int. Ed. 2015, 54, 5874 – 5878; Angew. Chem. 2015, 127, 5972 – 5976.
[22] V. R. Arava, S. R. Amasa, B. K. G. Bhatthula, L. S. Kompella, V. P. Matta, M. C. Subba, Synth. Commun. 2013, 43, 2892 – 2897.
[23] C. A. Fink, F. Firozina, WO Patent 55723, 1999.
[24] A. M. M. Mjalli, D. R. Gohimmukkula, S. Tyagi, U.S. Patent 124654, 2009.
[25] L. Dong, J. Marakovits, X. Hou, C. Guo, S. Greasley, E. Dagostino, R. Ferre, M. C. Johnson, E. Kraynov, J. Thomson, V. Pathak, B. W. Murray, Bioorg. Med. Chem. Lett. 2010, 20, 2210 – 2214.
[26] M. T. Reetz, M. Boccola, J. D. Carballeira, D. Z. A. Vogel, Angew. Chem. 2005, 117, 4264 – 4268.
[27] M. T. Reetz, Angew. Chem. Int. Ed. 2013, 52, 2658 – 2666; Angew. Chem. 2013, 125, 2720 – 2729.
[28] L. Bungaruang, A. Gutmann, B. Niedetzky, Adv. Synth. Catal. 2016, 358, 486 – 493.
[29] S. L. Lovelock, R. C. Lloyd, N. J. Turner, Angew. Chem. Int. Ed. 2014, 53, 4652 – 4656; Angew. Chem. 2014, 126, 4740 – 4744.
[30] J. H. Bartha-Váró, M. I. Toja, F. D. Irimie, D. Weiser, Z. Boros, B. G. Vertessy, C. Paizs, L. Poppe, ChemCatChem 2015, 7, 1122 – 1128.
[31] F. Ender, D. Weiser, B. Nagy, L. C. Bencze, C. Palzs, P. Pálavics, L. Poppe, J. Flow Chem. 2016, 6, 43 – 52.
[32] D. Weiser, L. C. Bencze, G. Bánóczi, F. Ender, R. Kiss, E. Kökai, A. Szilágyi, B. G. Vétesy, O. Farkas, C. Palzs, L. Poppe, ChemBioChem 2015, 16, 2283 – 2288.

Manuscript received: February 13, 2018
Revised manuscript received: March 10, 2018
Accepted manuscript online: March 15, 2018
Version of record online: April 26, 2018