Tetrahedron xxx (2016) 1–5

Contents lists available at ScienceDirect

Tetrahedron

journal homepage: www.elsevier.com/locate/tet

Engineering of phenylalanine ammonia lyase from *Rhodotorula graminis* for the enhanced synthesis of unnatural L-amino acids

Ian Rowles [a], Bas Groenendaal [a], Baris Binay [b], Kirk J. Malone [a], Simon C. Willies [a], Nicholas J. Turner [a,*]

[a] School of Chemistry, University of Manchester, Manchester Institute of Biotechnology, 131 Princess Street, Manchester M1 7DN, UK
[b] Gebze Technical University, Department of Bioengineering, 41400, Çayırova, Gebze, Kocaeli, Turkey

ARTICLE INFO

Article history:
Received 16 October 2015
Received in revised form 20 May 2016
Accepted 8 June 2016
Available online xxx

Keywords:
Rhodotorula graminis Phenylalanine ammonia lyase
Unnatural L-amino acids
Directed evolution
Modeling

ABSTRACT

Phenylalanine ammonia lyase (PAL) catalyses the reversible non-oxidative deamination of phenylalanine to *trans*-cinnamic acid and ammonia. Analogues of L-phenylalanine are incorporated as pharmacophores in several peptidomimetic drug molecules and are therefore of particular interest to the fine chemical industry. PAL from *Rhodotorula graminis* (*Rgr*PAL) has shown an ability to accept analogues of L-phenylalanine. Our aim was to increase enzymatic activity with directed evolution towards a specific non-natural substrate through the cloning and over-production of PAL in *Escherichia coli*. The identified variants of *Rgr*PAL with significantly showed more catalytic efficient compared to the wild-type enzyme. These variants were used in a preparative scale biotransformation resulting in a 94% conversion to L-4-Br-phenylalanine (>99% ee).

1. Introduction

Phenylalanine ammonia lyase (PAL) enzymes catalyse the reversible amination of cinnamic acids to generate L-arylalanines in high enantiomeric purity.[1a,b] Under conditions of high ammonia concentration PALs can be employed as effective biocatalysts for the synthesis of a specific target products, in some cases on a scale suitable for manufacturing pharmaceutical intermediates.[2a,b] Although PALs have been isolated from different sources, *e.g.* from eukaryotes such as *Petroselinum crispum* (*Pc*PAL) and *Rhodotorula glutinis* (*Rg*PAL), as well as cyanobacteria such as *Anabaena variabilis* (*Av*PAL)[3a,b], the current narrow substrate specificity of these enzymes limits their wider application as preparative biocatalysts. Herein we report engineering of PAL from *Rhodotorula graminis* (*Rgr*PAL) and in particular the identification of variants with very high levels of activity towards a panel of substituted cinnamic acids including; 4-bromo, 3-bromo, 4-fluoro, 3-fluoro and 3-nitro cinnamic acid. We also report optimisation studies for use of one of these variants in the preparative synthesis of related variants of L-phenylalanine.

2. Results and discussion

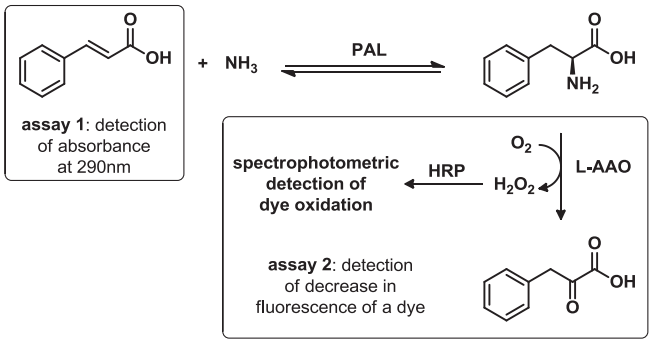
2.1. Assay development

In order to screen PAL variants for activity towards cinnamic acid derivatives two assays have been developed. The first assay takes advantage of the reversible nature of the PAL reaction and is a modification of the spectrometric assay for the detection of PAL activity reported by Khan and Vaidyanathan[4] and D'Cunha[5] whereby the activity of the PAL enzyme was determined by monitoring the deamination of L-phenylalanine to *trans*-cinnamic acid by measuring the absorbance at 290 nm (Assay 1, Scheme 1). However, this protocol needed to be modified for use in 96-well plate format, as the reported procedure was not applicable for screening libraries of protein variants. This screen can be used as an initial technique to identify PAL activity when screening libraries of protein variants and has the benefit of requiring no additional reporter systems for detection of PAL activity.

To enable screening of PAL enzyme activity in the synthetic direction a liquid phase assay was developed which uses both L-amino acid oxidase (L-AAO) and horseradish peroxidase (HRP) as reporter enzymes (Assay 2, Scheme 1).[3] Due to the 1:1 ratio of moles of hydrogen peroxide produced by the L-AAO for every mole of L-amino acid produced this assay can be used to directly quantify

* Corresponding author. Tel.: +44(0) 161 306 5173; fax: +44(0) 161 275 1311; e-mail address: nicholas.turner@manchester.ac.uk (N.J. Turner).

http://dx.doi.org/10.1016/j.tet.2016.06.026
0040-4020/



Scheme 1. Assays for detecting PAL activity for use in the screening of PAL variants.

the production of L-amino acids by the PAL enzyme variants and also to determine steady-state kinetics.

Using Assay 2 the relative activities of *Rgr*PAL towards a panel of cinnamic acid derivatives were measured (Table 1). These data reveal that 4-bromo cinnamic acid is a relatively poor substrate compared with cinnamic acid. Interestingly 4-fluoro cinnamic acid showed high activity and hence we speculated that the low activity of the 4-bromo compound was a result of steric issues. We therefore selected 4-bromo cinnamic acid as a useful target molecule to evolve PAL variants with higher activity.

Table 1
Relative activities of *Rgr*PAL towards a panel of different cinnamic acids

Substrate	Relative activity (%)
Cinnamic acid	100
4-Bromo cinnamic acid	17
3-Bromo cinnamic acid	112
4-Fluoro cinnamic acid	109
3-Fluoro cinnamic acid	128
3-Nitro cinnamic acid	54

2.2. Mutagenesis/library generation and screening

Site-directed mutagenesis of important first co-ordination sphere active-site residues was carried out in order to generate mutant libraries of the wild-type *Rgr*PAL enzyme. To guide selection of key active-site residues, a homology model of *Rgr*PAL was constructed. Using this homology model, five CASTing libraries[6,7] were designed at amino acid positions 143/144 (**A**), 272/276 (**B**), 369/372 (**C**), 473/477 (**D**) and 501/504 (**E**), (Fig. 1). Each library consisted of two active-site residues and these residues were randomised using the reduced amino acid library encoded by the degenerative codon NRT.[7] This codon only encodes eight amino acids and, therefore, considerably reduces the library size needed for screening >95% of all possible combinations of amino acids incorporated at each position.

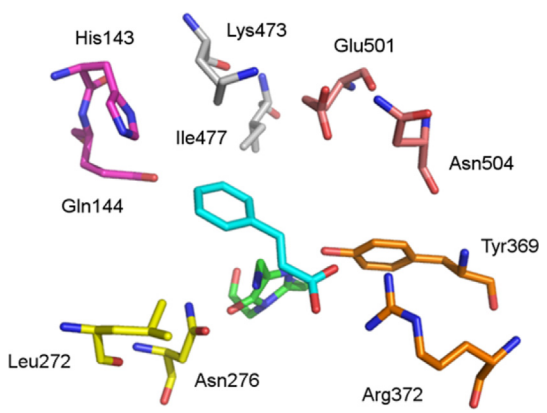


Fig. 1. Active site of PAL showing the 5 libraries and the residues targeted (Cinnamic acid in cyan, MIO cofactor in green).

The 5 libraries A–E (total=2400 colonies) were then initially screened for activity in the deamination direction (Assay 1, Scheme 1) with 4-bromo-L-phenylalanine as substrate. 43 positive hits were identified. All variants were purified and the specific activity of the deamination reaction measured. From this data four different variants (16D, 25E, 29B and 31E, Table 2) showed significantly increased activity towards this substrate with the 31E mutant performing best.

Table 2
Amino acid changes of the 4 PAL variants. (Specific activity is expressed in nmole/min/mg)

Mutant	Residue 143	Residue 144	Specific activity
WT	His	Gln	2.30±0.2
16B	Asn	Ser	168.34±16.8
25E	Ser	Asn	173.32±19.3
29B	Asn	Cys	176.20±28.6
31E	Cys	Asn	307.28±54.6

2.3. Modelling of active *Rgr*PAL variants

Interestingly all four mutants with improved activity came from the same library (Library A) containing mutations at positions His143 and Gln144. To investigate these mutations further we constructed models of the wild-type enzyme and for the best performing of these mutants (31E), and modelled substrates into the active sites. Modelling showed that cinnamic acid (not shown) interacts with Arg377 through its carboxylic acid moiety, with the phenyl group pointing towards His143 and Gln144. Docking of the 4-Bromo derivative (Fig. 2A) revealed that in the wild-type enzyme this substrate adopts a similar pose to cinnamic acid, with the phenyl group shifted towards Leu272 due to steric interaction of the substrate 4-bromo group with His143 and Gln144. However in the variant enzyme (31E) the mutations replace these residues with smaller residues (His143Cys/Gln144Asn) allowing 4-bromo cinnamic acid to adopt a binding mode closer to that of cinnamic acid (Fig. 2B). The modelling also demonstrated that whilst the wild-type enzyme can accept 4-bromo cinnamic acid as a substrate, the extra space afforded by the mutations present in 31E allow this substrate to occupy a much more favourable position leading to a much higher activity as observed experimentally.

2.4. Kinetics of PAL variants

The four PAL variants with enhanced activity were purified and then assayed against the initial panel of substrates in order to determine steady-state kinetic parameters (Table 3). The aim was to ascertain whether the mutations resulted in improved activity with substrates other than 4-bromo cinnamic acid.

The kinetic data shows an enhanced activity of the four variants towards the selected substrates. The results obtained also confirm the data obtained from the screen in the deamination reaction and shows that this assay can indeed be used for the selection of new improved variants. Interestingly, some of the variants also showed enhanced activities for other differently substituted substrates. In the case of 3-bromo cinnamic acid, the 29B variant showed a 3-fold higher activity than the wild-type and for the 3-fluoro substrate, all

Please cite this article in press as: Rowles, I.; et al., Tetrahedron (2016), http://dx.doi.org/10.1016/j.tet.2016.06.026

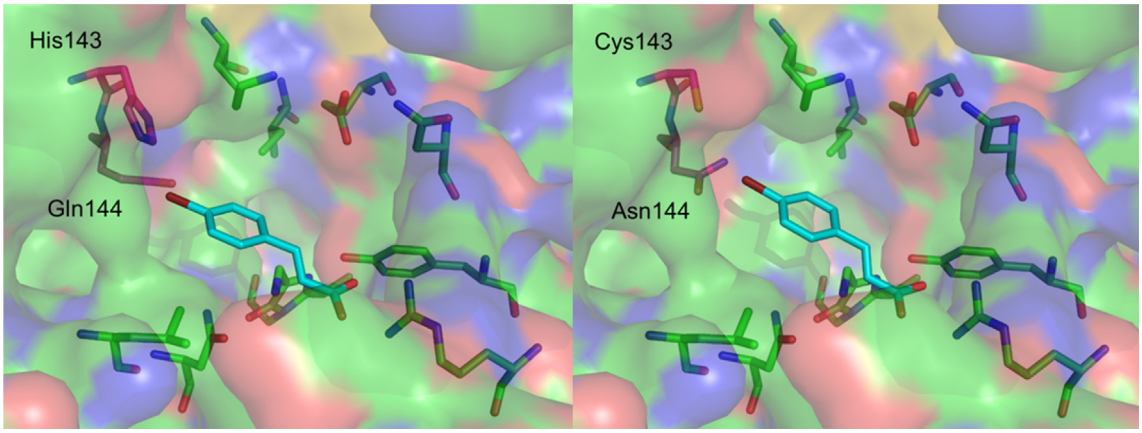
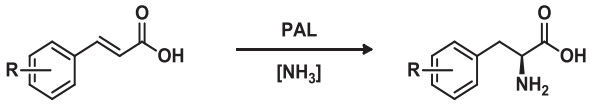


Fig. 2. 4-Br-cinnamic acid (cyan) docked in the actives sites of A) Wild-type *Rgr*PAL and B) *Rgr*PAL-31E.

Table 3
Kinetics of the WT and 4 variant PALs against a panel of substrates



Enzyme	Cinnamic acid		4-Bromo		3-Bromo	
	kcat s^{-1}	kcat/Km	kcat s^{-1}	kcat/Km	kcat s^{-1}	kcat/Km
WT	1.97±0.13	2.75±0.45	0.33±0.06	0.22±0.08	2.22±0.08	3.45±0.32
16D	nd	nd	3.53±0.20	5.76±0.81	nd	nd
25E	nd	nd	4.83±0.14	7.20±0.52	3.12±0.54	0.89±0.26
29B	3.46±0.96	1.27±0.61	5.99±0.14	29.38±2.60	6.70±0.40	5.04±0.61
31E	2.64±0.34	1.55±0.38	9.26±0.32	14.97±1.32	2.97±0.33	2.72±0.64
Enzyme	4-Fluoro		3-Fluoro		3-Nitro	
	kcat s^{-1}	kcat/Km	kcat s^{-1}	kcat/Km	kcat s^{-1}	kcat/Km
WT	2.14±0.38	1.26±0.43	2.52±0.24	2.81±0.61	1.06±0.07	1.24±0.20
16D	nd	nd	6.70±1.42	1.79±0.63	0.78±0.15	0.53±0.20
25E	nd	nd	4.66±0.34	3.38±0.49	1.31±0.24	0.55±0.18
29B	nd	nd	5.43±0.60	4.29±0.97	2.71±0.26	2.17±0.43
31E	nd	nd	8.63±0.87	8.68±1.92	2.76±0.49	4.19±1.86

four mutants showed a higher activity with the 31E being the best with a >3-fold increase.

2.5. Biotransformations

Having identified PAL variants with significantly improved activity, we decided to optimise the biotransformation conditions investigating reaction parameters such as substrate loading, ammonia source and concentration (Table 4). As can be seen, both ammonium carbamate and ammonium carbonate performed significantly better than the other sources tested reaching maximum conversion (~94%) after 2 h; reactions utilising the other ammonium sources only reached 45–50% conversion after the same time and still only reached a maximum conversion of 77% (in the case of ammonium sulfate) after 24 h.

A reason for the higher conversion with either ammonium carbamate or ammonium carbonate could be that they both can provide 2 molecules of ammonia whist maintaining an overall lower ionic strength in the reaction. Ammonium carbamate was selected for subsequent optimisation. Use of a lower concentration of ammonium carbamate in the reaction (1 M instead of 2.5 M) resulted in the yield of the reaction dropping significantly from 94% conversion after 4 h to 68%. Increasing the temperature of the reaction lowered the overall conversion slightly to 91% and 88% conversion at 37 °C and 45 °C, respectively although these conversions were reached after only 1 h at which point the 30 °C reaction was only at 79% conversion.

Table 4
Effect of different ammonia sources on PAL conversions, all assays conducted using 1 g/L substrate, 200 g/L ammonium carbamate pH 10, 5 g/L enzyme

Ammonia source	Time (h)	
	2	24
Carbamate	92	94
Carbonate	93	95
Acetate	46	61
Chloride	47	63
Hydroxide	51	59
Sulfate	51	77
Formate	46	63

3. Conclusions

In conclusion we have identified variants of *Rgr*PAL with significantly enhanced activity towards 4-bromo cinnamic acid. One of these variants (31E) showed a ca. 28-fold improvement in k_{cat}

Please cite this article in press as: Rowles, I.; et al., Tetrahedron (2016), http://dx.doi.org/10.1016/j.tet.2016.06.026

compared to the wild-type enzyme. This variant was used in a preparative scale biotransformation resulting in a 94% conversion to 4-bromo-L-phenylalanine (>99% ee).

4. Experimental

Competent cells (BL21 (DE3)) were purchased from Invitrogen or Agilent and were transformed according to the manufacturer's protocol. The empty vector (pET-16b) originates from Novagen. LB-Broth Miller and ampicillin were purchased from Formedium. All other chemicals and reagents were purchased from Alfa Aesar, Fisher or Sigma–Aldrich.

Reverse phase HPLC was performed on an Agilent system equipped with a G1379A degasser, G1312A binary pump, a G1329 autosampler unit, a G1315B diode array detector and a G1316A temperature controlled column compartment. Columns and conditions are indicated for each compound separately. Liquid phase assays for the determination of relative rates were recorded on a Spectramax M2 plate reader from Molecular Devices. Protein purification was performed on GE Healthcare ÄKTA Explorer 100 system using HiTrap Chelating HP columns (1 mL, GE Healthcare).

4.1. Production of PAL

A single colony of BL21 (DE3) pET 16b PAL was used to inoculate 8 mL of LB containing ampicillin (100 μg/mL), the OD_{600} was monitored and when a value of 0.6 was reached 6 mL of the starter culture was used to inoculate 600 mL LB medium in a 2 L baffled flask. The PAL protein was produced by incubating this culture for 18 h at 26 °C with 250 rpm continuous shaking.

After growth the cells were harvested by centrifugation at 7000 rpm for 20 min, the cell pellet was re-suspended in 15 mL 0.1 M potassium phosphate buffer pH 7.7, transferred to 50 mL falcon tubes and spun down at 4000 rpm for 20 min. The cell pellet was stored at −20 °C until use.

4.2. Protein purification

2.5 grams of cell paste was defrosted and resuspended in 6 mL of buffer A (100 mM KPi, pH 7.7, 300 mM NaCl). 6 mg of lysozyme was added and the suspension was incubated at 37 °C for 30 min, cooled on ice and sonicated to disrupt the cell walls (20 s pulse; 25 s pause; 20 cycles; 12–15 kHz). Cell debris was removed by centrifugation (20,000 rpm, 25 min), the supernatant was filtered (0.45 μM syringe filter) and loaded on a pre-prepared HiTrap column. Before loading of the supernatant the column was prepped by flushing it with the following: 5 mL of filtered water; 1 mL of 0.1 M $NiSO_4$; 5 mL of filtered water; 5 mL buffer A. Next the protein was eluted on the AKTA using a stepwise gradient of buffer A and buffer B (100 mM K-Pi, pH 7.7, 300 mM NaCl, 1 M imidazole) collecting 1 mL fractions. The stepwise gradient for elution of the PAL protein was: 1) 10 mL 100% Buffer A, 2) 10 mL 80:20 buffer A:B, 3) 15 mL 65:35 buffer A:B. The wells containing the purified enzyme were collected and concentrated to 1.5 mL using a Vivaspin column (30,000 MW cut-off), the volume was adjusted to 2.5 mL with 0.1 M KPi pH 7.7 and the solution was desalted on a PD10 column (GE Healthcare), equilibrated with 25 mL of 0.1 M KPi pH 7.7, and eluted with 3.5 mL of 0.1 M KPi pH 7.7).

The purity of combined and de-salted protein fractions was then checked by SDS-PAGE (Fig. 1), after purification between 20 and 25 mg of protein was obtained per litre culture. Protein concentration and yield was calculated using the BCA assay (Pierce) according to the manufacturer protocol.

4.3. Analysis of protein production and purity

Protein expression and purity was assessed using pre-cast 10–20 % Tris–HCl polyacrylamide gradient gels (Bio-Rad, Hercules, CA) and pre-stained broad range molecular weight marker (NEB, Ipswich, MA). Electrophoresis of samples was at 80 V for 1 h following the dye front and the gels stained with EZBlue™ Gel Staining Reagent.

Protein samples separated by SDS-PAGE were blotted onto a PVDF membrane using a BioRad semi-dry blotter at 150 mA for 1 h. The membrane was washed with PBS for 15 min, the buffer changed every 5 min, blocked with a milk solution (1 g milk powder in 20 mL PBS). The membrane was washed with PBS, and then incubated with an Anti-His-HRP conjugate antibody for 2 h. The membrane was washed again then stained with a DAB/H_2O_2 solution.

4.4. Homology model

A homology model of *Rgr*PAL was constructed, containing four monomers, based on the PAL enzyme from *R. glutinis* (PDB accession code 1T6J) which has 71.9% sequence identity. Loops for which there was no structural information and the three amino acids constituting the MIO cofactor (Ala-Ser-Gly) were excluded from the model building process. The MIO cofactor was built into the model by transferring the cofactor from the template, followed by energy minimisation of the cofactor. Homology models and docking were performed with Accelrys Discovery Studio 3.1.

4.5. Saturation mutagenesis

The homology model of PAL was used for the semi-rational redesign of the PAL active site in an attempt to modify the substrate profile of the enzyme. From the model potential sites for the mutations were selected and these sites were: 143/144 (**A**), 272/276 (**B**), 369/372 (**C**), 473/477 (**D**) and 501/504 (**E**). Saturation mutagenesis libraries were created at these positions and screened for activity towards the substrates. The primers used for this were as follows (see Table 5):

Table 5
Primer sequences 5′ to 3′, only the forward primer sequence is shown

Library	Sequence
A	GCTCATCGAG**NRTNRT**CTCTGCGGCGTGACG
B	CGAAGGAGGGT**NRT**GGTCTGGTC**NRT**GGAACGGCCGTC
C	CCAGGACCGC**NRT**CCGCTC**NRT**ACGTCGCCTCAGTTCC
D	GCTCAACTATCACGGC**NRT**GGCTTGGAC**NRT**CACATCGCTGCTTACGC
E	CGTCCAGCCCGCA**NRT**ATGGGT**NRT**CAGGCCGTCAACTCG

A QuikChange (Agilent) PCR reaction was carried out with pET 16b PAL as the template DNA using the primers stated above. PCR was carried out with an initial melting at 95 °C for 3 min then 21 cycles with a 95 °C melting temperature for 1.5 min, a 55 °C annealing temperature for 3 min and a 68 °C elongation temperature for 10 min and a final 68 °C elongation step of 12 min. After the PCR the parental strand of DNA was digested with 1 μL of DpnI for 3 h at 37 °C, then 1 μL of the digested PCR reaction mixture was used to transform *Escherichia coli* XL-1 Blue chemically competent cells (Agilent), according to the manufacturer's protocol.

Eight colonies were picked and the plasmid DNA purified and sequenced to confirm mutagenesis. The remaining colonies were taken up in 1 mL of LB Broth and the plasmid library purified, 1 μL of this purified plasmid library was used to transform BL21 (DE3) (Invitrogen).

Please cite this article in press as: Rowles, I.; et al., Tetrahedron (2016), http://dx.doi.org/10.1016/j.tet.2016.06.026

After transformation into BL21 (DE3), 480 colonies were picked and each one used to inoculate 800 μL of LB in a deep-well 96-well plate. They were grown overnight at 37 °C and the cells harvested by centrifugation at 4500 rpm for 15 min. The media was discarded before the pellet was resuspended in 100 μL of 10% (w/v) glycerol. The storage plates were then snap frozen on liquid nitrogen and stored at −80 °C.

4.6. Assay of PAL activity and library screening

4.6.1. Determination of specific activity of PAL. Substrate solutions of 4-bromo, 3-bromo, 4-fluoro, 3-fluoro and 3-nitro phenylalanine were made, 20 mM substrate in 100 mM Tris buffer pH 8.8. The screening was set up in a UV-Star Microplates (96 well, F-bottom, Greiner Bio-one) and each well contained the following: 140 μL Tris buffer 100 mM, pH 8.8, 10 μL of purified enzyme, 50 μL phenylalanine solution. Absorbance was measured using a plate reader at 290 nm for 1 h with intervals of 43 s and hits were identified by an increase in the absorbance.

4.6.2. Screening of libraries in the deamination reaction (Assay 1). 800 μL starter cultures (LB+ampicillin 100 μg/mL) were inoculated with a single PAL variant in a standard 96-deepwell plate. The cultures were grown overnight at 37 °C and 150 rpm 20 μL of the overnight cultures was then used to inoculate production cultures (2 mL of LB+ampicillin 100 μg/mL) in 48-deepwell plates and these cultures were grown overnight at 26 °C, 150 rpm. The remaining starter cultures were centrifuged (20 min, 4000 rpm), the media discarded and the cell pellets resuspended in 100 μL 10% glycerol then stored at −20 °C to be used as a reference once hits had been identified.

The cells from the production cultures were harvested by centrifugation (20 min, 4000 rpm) and the cell pellets resuspended in 100 μL of 100 mM Tris buffer pH 8.8. Substrate solutions of 4-bromo, 3-bromo, 4-fluoro, 3-fluoro and 3-nitro phenylalanine were made, 20 mM substrate in 100 mM Tris buffer pH 8.8. The screening was set up in a UV-Star Microplates (96 well, F-bottom, Greiner Bio-one) and each well contained the following: 140 μL Tris buffer 100 mM, pH 8.8, 10 μL of resuspended enzyme, 50 μL phenylalanine solution. Absorbance was measured using a plate reader at 290 nm for 1 h with intervals of 43 s and hits were identified by an increase in the absorbance.

4.6.3. Kinetics in the amination reaction (Assay 2). Solutions of the substrates under investigation were made up in 0.5 M ammonium carbamate/1M Tris, pH 9.0 and dilutions were made with the following concentrations: 3, 2, 1, 0.5, 0.2, 0.02, 0.002 and 0 mM of cinnamic acid.

Scopoletin solution − 288.25 mg of scopoletin was dissolved in 50 mL dH2O to make a stock solution. This stock solution was then diluted 1/5 to create a working solution that had a fluorescence of approximately 30,000 RFU.

The screening was set up in a 96-well plate and each well contained the following: 100 μL substrate solution, 10 μL HRP (1 mg/mL in dH_2O), 10 μL L-AAO (1 in 10 dilution of stock in dH_2O), 40 μL scopoletin solution, 40 μL purified PAL. Kinetic data was measured on a plate reader at 30 °C. Excitation wavelength 360 nm; emission wavelength 480 nm; 15 s interval.

4.7. Lab-scale biotransformation

10 mg of the cinnamic acid of choice was dissolved in 10 mL of ammonium carbamate (200 g/L, pH 10.0) and this solution was added to 50 mg of wet cell paste of the enzyme of choice in a 50 mL conical Falcon tube. The tube was shaking vigorously to homogenize the solution and the tube was put in a 30 °C shaking incubator (250 rpm). Samples were taken at the following time points: 1, 3, 8 and 24 h. Samples were taken in the following way: 500 μL of the biotransformation mixture, boil for 5 min at 95 °C, add appropriate amount of MeOH (depending on composition of mobile phase for HPLC analysis) and mix, centrifuge briefly (5 min, 13,200 rpm), take 500 μL and apply to a 0.22 μm filter vial (Thomson).

4.8. HPLC data for selected compounds

All cinnamic acid and phenylalanine derivatives were analysed using an Agilent Zorbax Extend-C18 column (50 mm, 4.6 mm, 5 μm) with mixtures of eluent A (0.1M NH_4OH pH 10.0) and B (methanol), isocratic, 1 mL/min, 40 °C. Retention times and mobile phase compositions are shown in Table 6.

Table 6
Retention times of selected compounds

Compound	Mobile phase (A:B)	Retention time (min)
4-Bromo cinnamic acid	60:40	2.85
4-Bromo-L-phenylalanine	60:40	1.50
4-Fluoro cinnamic acid	80:20	3.31
4-Fluoro-L-phenylalanine	80:20	1.39
3-Bromo cinnamic acid	60:40	2.51
3-Bromo-L-phenylalanine	60:40	1.43

Acknowledgements

We would like to acknowledge the Technology Strategy Board (TSB Grants BW053J and 4904-41146) for funding IR, BG and KJM. Also, we would like to acknowledge the The Scientific and Technological Research Council of Turkey (Grant code: TÜBİTAK, 2214) for funding BB.

References and notes

1. (a) Turner, N. J. *Curr. Opin. Chem. Biol.* **2011**, *15*, 234–240; (b) Heberling, M. M.; Wu, B.; Bartsch, S.; Janssen, D. B. *Curr. Opin. Chem. Biol.* **2013**, *17*, 250–260.
2. (a) Gloge, A.; Zoń, J.; Kövàri, A.; Poppe, L.; Rétey, J. *Chem.—Eur. J.* **2000**, *6*, 3386–3390; (b) Paisz, C.; Katona, A.; Rétey, J. *Chem.—Eur. J.* **2006**, *12*, 2739–2744; (c) De Lange, B.; Hyett, D. J.; Maas, P. J. D.; Mink, D.; van Assema, F. B. J.; Sereinig, N.; de Vries, A. H. M.; de Vries, J. G. *ChemCatChem.* **2011**, *3*, 289–292.
3. (a) Lovelock, S. L.; Lloyd, R. C.; Turner, N. J. *Angew. Chem., Int. Ed.* **2014**, *53*, 4652–4656; (b) Parmeggiani, F.; Lovelock, S. L.; Weise, N. J.; Ahmed, S. T.; Turner, N. *J. Angew. Chem. Int. Ed. Engl.* **2015**, *54*, 4608–4611.
4. Khan, N. U.; Vaidyanathan, C. S. *Curr. Sci.* **1986**, *55*, 391–393.
5. D'Cunha, G. B. *Enzyme Microb. Technol.* **2005**, *36*, 498–502.
6. Reetz, M. T.; Carballeira, J. D.; Peyralans, J.; Hobenreich, H.; Maichele, A.; Vogel, A. *Chem.—Eur. J.* **2006**, *12*, 6031–6038.
7. Reetz, M. T.; Carballeira, J. D. *Nat. Protoc.* **2007**, *2*, 891–903.