Bi-enzymatic Conversion of Cinnamic Acids to 2-Arylethylamines

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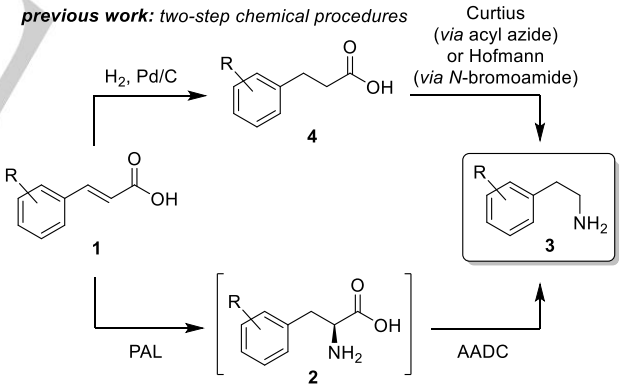
Abstract: The conversion of carboxylic acids, such as acrylic acids, to amines is a transformation that remains challenging in synthetic organic chemistry. Despite the ubiquity of similar moieties in natural metabolic pathways, biocatalytic routes seem to have been overlooked for this purpose. Herein we present the conception and optimisation of a two-enzyme system, allowing the synthesis of β-phenylethylamine derivatives from readily-available ring-substituted cinnamic acids. After characterisation of both parts of the reaction in a two-step approach, a set of conditions allowing the one-pot biotransformation was optimised. This combination of a reversible deaminating and irreversible decarboxylating enzyme, both specific for the amino acid intermediate in tandem, represents a general method by which new strategies for the conversion of carboxylic acids to amines could be designed.

Research into methods allowing the interconversion of simple functional groups has been a focus in synthetic organic chemistry over the last century. Despite sustained efforts in this area, there are still a number of such transformations which feature unstable intermediates, lengthy multi-step syntheses and multiple purification steps, resulting in elevated energy consumption, waste generation and solvent use. Examples of this are heteroatom migrations (including the Curtius rearrangement and modifications of Hofmann, Lossen and Schmidt degradations), which remain some of the most facile methods to produce primary amines from a carboxylic acid starting material (Scheme 1).[1] In spite of the utility of such approaches, chemical routes to these high value compounds require multi-step synthesis and several isolation steps. The harsh conditions required for many of the latter steps may prove incompatible with other moieties in the starting material and the need for chromatographic purification steps using environmentally-damaging solvents is usually undesirable. Furthermore, the use of potentially explosive and highly toxic azide derivatives (in Curtius-type procedures) is of particular concern, mainly in an industrial setting.

Biocatalytic strategies have gained much attention in the areas of synthetic chemistry and pharmaceutical science, due to the ever increasing demand for concise, sustainable and mild approaches to chemical synthesis. The ability to combine renewably-sourced and highly selective biological catalysts under ambient and compatible conditions, without the need for specialised work up of intermediates, presents great advantages over current synthetic methods.[2–4] The range of enzymes available in nature and near infinite combination that could be imagined from these poses the possibility of designing new strategies for classical chemical transformations, complementary to established synthetic methods and even to other enzymatic routes.

By performing a retrosynthetic analysis, a two-enzyme pathway was envisaged to obtain β-phenylethylamine derivatives from easily accessible cinnamic acids. This involved placing the amination of the candidate unsaturated carboxylic acid as the first step in the process, followed by an irreversible release of CO_2. Cinnamic acid derivatives were chosen due to the additional alkene moiety conjugated to the carboxyl group to be converted, thus allowing additional and orthogonal transformations in a stepwise fashion. It was reasoned that, despite the potentially unfavourable equilibrium of the initial enzymatic step, the subsequent decarboxylation would serve to remove the amination product and thus drive overall conversion, much in the same way as has been shown with the use of aldolases and oxidases to produce a variety of amino alcohols.[5] To this end, a combination of phenylalanine ammonia lyase (PAL) and an aromatic amino acid decarboxylase (AADC) was considered (Scheme 1). This was due to the widely reported use of ammonia lyase enzymes for synthetic purposes[6–10] and the decarboxylation chemistry of AADCs uniquely enabled by the α-amino moiety,[5,11] avoiding possible siphoning of the PAL carboxylic acid substrate into a decarboxylated by-product. Owing to the use of truly catalytic chemical groups by both enzymes - 4-methylideneimidazole-5-one (MIO) for PAL[8,9,12] and pyridoxal-5'-phosphate (PLP) for AADC[5,11] - it was predicted that no additional supplementations or recycling systems would be required, with each holoenzyme being regenerated during their respective catalytic cycles.



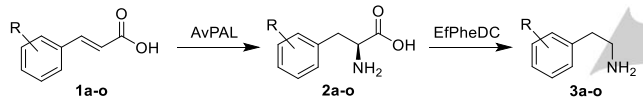
Scheme 1. Methods allowing the conversion of 2-arylacrylic acids to 2-arylethylamines.

Substituted β-arylethylamines **3** are key building blocks of pharmaceuticals and bioactive molecules, and the parent compound β-phenylethylamine **3a** is known to function as a neurotransmitter and antidepressant.[11] There is evidence in the literature of the conversion of various cinnamic acids **1** to the corresponding amines **3**, by a two-step procedure based on hydrogenation followed by Curtius or Hofmann rearrangements, *e.g.* in the synthesis of peptidomimetic probes and small molecule pharmaceutical candidates.[13–16] For example, conversion of 3-bromocinnamic acid required a five step

synthesis with an overall yield of 24%, a method that would still require a de-protection step to afford the free amine.[14] To complement existing strategies, a biocatalytic route requiring mild aqueous conditions was sought, using two enzymes: the cyanobacterial phenylalanine ammonia lyase AvPAL from *Anabaena variabilis*[8,9,17] and a decarboxylase from *Enterococcus faecium*, referred to from hereon as EfPheDC.[11]

Production of both enzymes as either wet (EfPheDC) or dry (AvPAL) whole cell biocatalysts allowed simple investigation of their pH optima. The enzymes were shown to operate under very different conditions in this respect, as consistent with previous studies of PAL enzymes[18,19] and the potential involvement of AADCs with bacterial acid stress responses.[20] As such, initial experiments focussed on the development of a sequential reaction whereby cells, water and ammonium carbamate (required for PAL-mediated amination) were removed via centrifugation and evaporation, leaving a crude isolate[21] for conversion by EfPheDC under separate conditions. Following initial test with temperatures ranging from 20 to 60°C, the optimum incubation was found to be at 30°C (see Supporting Information). Using this method, a panel of 15 cinnamic acids **1a-o** known to be good PAL substrates was tested (Table 1).

Table 1. Telescopic amination-decarboxylation of various cinnamic acids to yield the corresponding 2-arylethylamines as catalysed by AvPAL (step 1) and EfPheDC (step 2).[a]



1a-o **2a-o** **3a-o**

Subs.	R	Conv. (1 to 2)[b] [%]	Conv. (2 to 3)[b] [%]	Overall conv.[b] [%]
1a	H	99	93	92
1b	*o*-Br	98	2	2
1c	*m*-Br	94	81	75
1d	*p*-Br	94	37	34
1e	*o*-Cl	97	7	7
1f	*m*-Cl	93	96	86
1g	*p*-Cl	95	48	45
1h	*o*-F	>99	98	98
1i	*m*-F	92	87	78
1j	*p*-F	96	89	84
1k	*o*-Me	85	2	<1
1l	*p*-Me	73	51	35
1m	*o*-NO_2	99	<1	<1
1n	*m*-NO_2	98	22	21
1o	*p*-NO_2	91	2	2

[a] Reaction conditions: step 1. 32 mM **1**. 10 mg mL^{-1} AvPAL *E. coli* dry cells, 4 M ammonium carbamate, pH 9.9, 30°C, 250 rpm, 22h; step 2. 10 mg mL^{-1} EfPheDC *E. coli* wet cells, 100 mM sodium citrate pH 4.4, 30°C, 250 rpm, 22 h. [b] Determined by reverse phase HPLC analysis on a non-chiral phase.

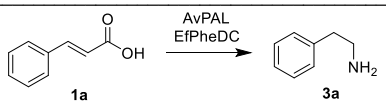
The decarboxylation step was found to give variable conversions, with the unsubstituted, fluoro- and 3-chloro/bromo-compounds giving the highest values. Interestingly, any substrates with *ortho*-groups larger than a fluorine atom gave extremely low conversions (<10%) with EfPheDC. Increasing the size of substituents at the 4-position had a less dramatic but still noticeable effect, with the *para*-bromo-, *para*-methyl- and *para*-chloro- giving moderate conversions. For the nitro substituents, low conversions were seen all around, but with a preference for the 3-substituents, as seen with chloro- and bromoarene isomers. To add evidence to the assertion that the decarboxylase displayed arene substitution preference, a smaller panel of various phenylalanine derivatives was tested with this enzyme, revealing low conversions for D-phenylalanine, *rac*-2-bromo- and L-4-nitro- compounds (see Supporting Information). However, investigations of the enantioselectivity of AvPAL showed low enantiomeric excess values only for the nitrophenylalanines, as observed previously.[8,9] This revealed that, although EfPheDC displayed a clear preference for L-amino acids, production of racemic reaction intermediates by AvPAL was unlikely to be the reason for low overall conversion in most cases (see Supporting Information).

Having demonstrated the effectiveness of AvPAL-EfPheDC tandem reactions for the synthesis of various 2-arylethylamines, the compatibility of the two steps for construction of a biocatalytic cascade was investigated. It was hypothesised that the catalytic activity of the PLP-dependent decarboxylase may be affected detrimentally by the high concentrations of ammonia associated with the initial amination step. Biotransformations of L-phenylalanine in ammonium chloride buffer revealed this to be the case, with conversions of ~20% only observed at ammonia concentrations 8-fold to 20-fold lower than commonly required for PAL reactions (see Supporting Information). Interestingly, the use of the diluted reaction buffer in the first cascade attempt gave just 14% conversion by AvPAL and no traces of β-phenylethylamine, possibly due to the short-lived activity of EfPheDC and slow amination rate under these conditions. In an attempt to rebalance the ratio of cinnamate to ammonia as the primary driving force for the initial amination reaction, various substrate concentrations were tested, and an increasing amount of β-phenylethylamine could be observed with decreasing substrate concentration (see Supporting Information).

In spite of the improvements seen (conversion up to 34% of 2 mM starting material after 22 h) by increasing the rate of amination, the focus was shifted towards further optimisations designed to increase the efficiency of the decarboxylation step. It was reasoned that this would increase removal rate of the intermediate amino acid and increase the rate of the PAL reaction to maintain the equilibrium. Initially, changing the ammonia concentration for the cascade reaction to 1000 mM (to favour the first step) and 250 mM (to favour the second step) both resulted in lower levels of the amine (Table 2, entries 1-3). As such, 500 mM was used as a compromise with the pH either increased or decreased to favour each step in turn (Table 2, entries 4-6). This resulted in a slight improvement at the more acidic pH and extremely poor yield of **3a** at the more basic pH. As this reinforced the idea that the activity of the decarboxylase was the bottleneck of the cascade, the loading of EfPheDC-containing whole cells was varied. As expected, there was a positive correlation between the amount of catalyst and the effective siphoning of the PAL-product to amine (Table 2, entries 7-10). This also resulted in greater overall conversion with

increasing decarboxylase loading, as high as 88% with 40 mg mL^{-1} cells, but with the relative ratio of **1a** to **2a** remaining constant. This proves that, as the thermodynamic equilibrium of the amination reaction remains unaltered by the removal of substrate, the method can serve to increase the overall productivity of the system and approach higher conversions to amine.

Table 2. Effect of varying ammonia concentration, buffer pH and EfPheDC whole cell catalyst loading on the product distribution in the AvPAL-EfPheDC cascade.[a]



1a 3a

Ammonium carbamate [mM]	pH	EfPheDC *E. coli* wet cells [mg mL^{-1}]	**1a** [%][b]	**2a** [%][b]	**3a** [%][b]
1000	7.7	20	56	29	15
500	7.7	20	53	13	34
250	7.7	20	16	56	28
500	8.8	20	72	23	5
500	7.7	20	53	13	34
500	6.6	20	19	43	39
500	6.6	0	28	72	0
500	6.6	10	20	47	33
500	6.6	20	19	43	39
500	6.6	40	12	27	61

[a] Reaction conditions: 2 mM **1a**, 10 mg mL^{-1} AvPAL *E. coli* dry cells, 30°C, 250 rpm, 22 h. [b] Determined by reverse phase HPLC analysis on a non-chiral phase.

This work has demonstrated the biocatalysed conversion of easily accessible arylacrylic acids to β-arylethylamines using a combination of PAL and AADC enzymes. The integration of the well-characterised PAL biocatalyst with an AADC not widely used for biotransformations prompted a substrate screen of the latter enzyme, showing it to have a strict enantiopreference for L-amino acids, broad substrate scope (particularly *meta*-substituted) and possibly mild inhibition of activity in the presence of D-enantiomers. The two biocatalytic steps could be easily run sequentially in the same vessel, without chromatographic purifications or complex separation steps in between. This was achieved through simple removal of whole cells and sublimation of the volatile reaction buffer following the first step, allowing up to 98% conversion overall at ~30 mM substrate concentration. The different pH optima and ammonia tolerance exhibited by each enzyme warranted optimisation of a cascade approach for the one-pot conversion of cinnamic acid to its corresponding amine. By focussing on the requirements of the second, irreversible decarboxylation step (i.e., low pH, low ammonia concentration and increased cell loading) it was found that conversion up to 61% to β-phenylethylamine could be achieved, with the AADC acting to remove the initial amination product thus enabling further consumption of starting material to redress the equilibrium of the PAL reaction. This strategy, combining an amino acid forming enzyme with an orthogonal decarboxylase, could potentially be applied to different classes of substrates, such as to allow formal biocatalytic NH_3/CO_2 exchange with a variety of acrylic acids.

Acknowledgements

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Keywords: arylethylamines • biocatalysis • enzyme cascades • ammonia lyases • decarboxylases

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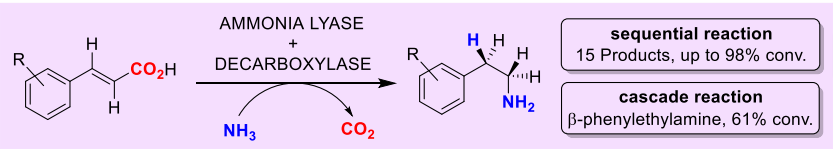

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Page No. – Page No.

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