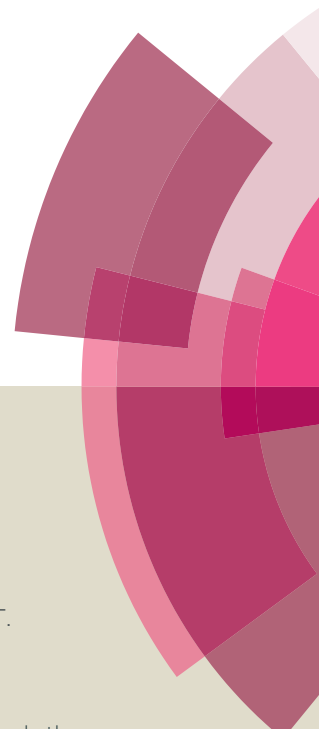


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COMMUNICATION

Intensified Biocatalytic Production of Enantiomerically Pure Halophenylalanines from Acrylic Acids Using Ammonium Carbamate as the Ammonia Source[†]

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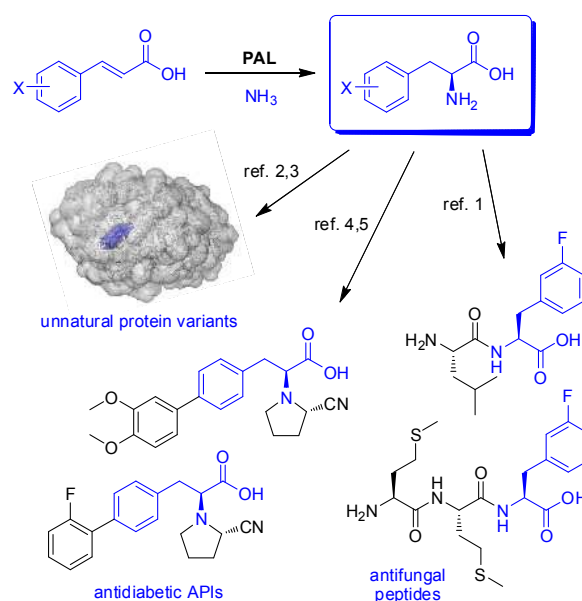
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An intensified, industrially-relevant strategy for the production of enantiopure halophenylalanines has been developed using the novel combination of a cyanobacterial phenylalanine ammonia lyase (PAL) and ammonium carbamate reaction buffer. The process boasts STYs up to >200g L⁻¹ d⁻¹, ees ≥98% and simplified catalyst / reaction buffer preparation and work up.

Ring-halogenated phenylalanine analogues are a class of unnatural amino acids used in variety of research endeavours in medicinal chemistry and chemical biology. Compounds such as 3-fluoro-L-phenylalanine, for example, have been identified as key components of antifungal di- and tripeptides, effective against multidrug-resistant strains of the opportunistic human pathogen *Candida albicans*.¹ This same molecule, along with its 4-fluoro regioisomer, has been shown to improve the catalytic properties of lipase under industrially-relevant conditions via incorporation into the polypeptide in lieu of natural proteinogenic amino acids.² In a similar vein, genetic incorporation of *para*-halo-L-phenylalanines into a maltose binding protein is reported to enable site-specific tagging following Suzuki-Miyaura coupling with a furanylboronic acid.³ This coupling method can also be used to functionalise bromo- and chloro- phenylalanine derivatives to create drug molecules such as the biaryl dipeptidyl peptidase IV inhibitors, novel candidates for the management of type II diabetes.^{4,5}

The use of enzymes to carry out highly chemo-, regio- and enantioselective syntheses is an attractive strategy in terms of minimising the environmental impact (e.g. from side product generation, protection/deprotection steps) of chemical processes. This is due to their ability to mediate challenging reactions under mild aqueous conditions, reducing both energy input and organic solvent waste. Enzyme catalysts are built from natural primary



Scheme 1. Phenylalanine ammonia lyase (PAL) as a strategy for the production of halophenylalanines for use in the creation of tailored proteins and pharmaceuticals.

metabolites in a renewable manner using facile recombinant DNA technologies. Biocatalysts with a well characterised ability to produce halogenated arylalanines are the phenylalanine ammonia lyase (PAL) enzymes, which allow the selective addition of free ammonia to inexpensive and readily-available cinnamic acid derivatives.^{4,6-11} This process is 100% atom efficient, relying solely on a catalytic tyrosine and the 4-methylideneimidazole-5-one (MIO) prosthesis, thus mitigating the need for expensive and wasteful cofactor supplementation / regeneration. Due to the unique chemistry, difficult to mimic with traditional synthetic methods, PALs have been used in the synthesis of the pharmaceutical intermediates 4-bromo-⁴ and 2-chloro-L-phenylalanine.^{9,12} The latter process - used in the industrial-scale production of the antihypertension drug perindopril - is a rare example of an intensified reaction, with a space-time yield (STY) of 51.2 g L⁻¹ d⁻¹.¹²

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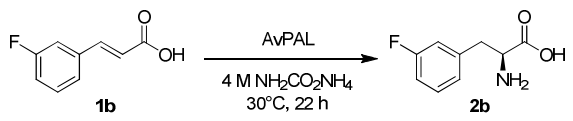
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Despite the utility of halophenylalanines and the demonstrated efficiency of PAL-catalysed reactions,^{6,7,10} there exist very few examples of large scale syntheses of these compounds using this method. Herein we report a superior intensified method for the preparative scale amination of readily-accessible halocinnamates to give enantiopure amino acids in good purity with a chromatography-free workup, employing a novel ammonia source. Previous studies with ammonia lyase biocatalysts revealed that they suffer from inactivation and can require lengthy formulation procedures for effective use.^{13,14} In the interests of simplifying catalyst preparation, the previously characterised enzyme from the cyanobacterium *Anabaena variabilis* (AvPAL)¹⁵ was used due to its demonstrated thermostability¹⁶ and conversion of diverse cinnamate derivatives.⁸⁻¹⁰ Through various optimisation steps, a method was arrived at by which 150 mM 3-fluorocinnamic acid **1b** could be converted to 93% (details of the optimisation steps are reported in the ESI). These reaction parameters were: 20 mg mL⁻¹ lyophilised *Escherichia coli* whole cells harbouring the AvPAL biocatalyst, 13% w/v ammonia solution pH adjusted with carbon dioxide (pH 9.5), 30°C, 200 rpm, 22 h. Despite the improvements in reaction parameters, the method did, however, require a large quantity of CO₂ to form an ammonium carbonate solution of sufficient concentration and pH. As this would constitute a major problem and increase costs in an industrial setting, an alternative ammonia source was sought for further investigations.

Use of ammonium carbamate was considered due to its high solubility, volatility, low cost and unbuffered pH ~10. As an initial test, a 150 mM amination reaction of 3-fluorocinnamic acid **1b** was conducted with 4 M ammonium carbamate, affording 98% conversion within a 22 h time period indicating both an increase in reaction productivity and a shift in equilibrium. Similar improvements have been observed with different biocatalytic systems¹⁷ and may be due to the potential of the carbamate counter-ion to act as a slow release source of additional ammonia, providing more of this reactant. The effect of substrate loading with this new system was tested with solutions of 50-350 mM substrate (Table 1) and it was found that, even up to 300 mM substrate concentration, ≥97% conversion could be obtained (dropping to 81% at 350 mM). All conversions were calculated from combined soluble and insoluble fractions of the reaction mixture, since the substrate was found not to be fully soluble at concentrations >200 mM.

In light of the efficiency conferred by the new reaction buffer, preparative scale (4 mL) reactions were set up with a variety of cinnamic acid derivatives (**1a-i**, Table 2), previously reported to be substrates for AvPAL. In each case, reaction parameters were altered so as to allow >95% conversion with the 4 M ammonium carbamate and 20 mg mL⁻¹ catalyst at 30°C. It was observed that, for fluorinated compounds (**1a-c**, **g** and **h**) 100 mM substrate concentration was sufficient, while for the less soluble substrates (such as the chloro-substituted **1d-f** and **i**), concentrations of 50 mM were more appropriate. In each set of compounds the least soluble *para*-isomer seemed to give less than the desired conversion after 1 day - a situation remedied by additional

Table 1. Effect of varying substrate concentration on intensified, small-scale aminations of **1b** by AvPAL.

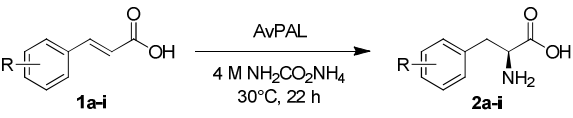


Substrate conc. [mM]	Conv. [%]	ee of 2b [%]	Theoretical STY [g L ⁻¹ d ⁻¹]
50	>99	>99 (S)	~9
100	99	>99 (S)	~18
150	98	99 (S)	~26
200	98	99 (S)	~34
250	97	98 (S)	~43
300	97	98 (S)	~51
350	81	98 (S)	~50

incubation time to achieve >95% conversion. The apparent exception to this observation was the 3,4-difluorocinnamate **1g** which, despite having a *para*-fluoro group, was already found to give 97% conversion within 24 hours. In all cases the (*S*)-enantiomer of **2** was produced with excellent stereoselectivity, with ee values of 98% or above being achieved. Given the reasonable STY values achieved (4.7-19.5 g L⁻¹ d⁻¹) all products could be isolated by centrifugation to remove the whole cell catalyst and evaporation of the volatile reaction buffer, with no need for further purification. NMR analyses of the remaining quantitative crude isolate (see ESI) reflected the purity of the product as previously estimated by HPLC.

As a demonstration of the industrial viability of our method, a gram-scale synthesis of (*S*)-**2b** as a model target product was

Table 2. Optimisation of reaction parameters to give >95% conversion of a panel of halocinnamates **1a-i** in AvPAL-catalysed biotransformations.



1	R	Subs. conc. [mM]	Time [h]	Conv. [%]	ee of 2b [%]	STY [g L ⁻¹ d ⁻¹]
1a	2-F	100	24	>99	>99 (S)	18.3
1b	3-F	100	24	99	>99 (S)	18.1
1c	4-F	100	48	96	99 (S)	8.8
1d	2-Cl	50	24	>99	>99 (S)	10.0
1e	3-Cl	50	48	96	98 (S)	4.8
1f	4-Cl	50	48	95	98 (S)	4.7
1g	3,4-F ₂	100	24	97	99 (S)	19.5
1h	2-Cl-4-F	100	48	96	99 (S)	10.4
1i	2,6-Cl ₂	50	24	96	>99 (S)	11.2

optimised, in order to find a balance between high conversion and space-time yield (see ESI). The final reaction procedure simply required addition of 5.3 g ammonium carbamate, 1 g 3-fluorocinnamate and 1 g dried whole cell catalyst powder to 17 mL deionized water under stirring (200 rpm) at 30°C. After a 6 hour reaction time a sample of the reaction was taken and analysed, revealing a conversion of 92% to (S)-**2b** (ee 99%). This value equated to approximately 1 g amino acid being produced, giving a theoretical space-time yield of 237.6 g L⁻¹ d⁻¹ - a value much higher than existing industrial scale synthesis of a pharmaceutical intermediate using a related PAL.¹² The process presented here also features higher catalyst productivity, a lack of requirement for batch addition of substrate or catalyst and ready availability of the volatile ammonium donor salt, without the need for pH adjustment or lengthy ion-exchange work-up methods. The environmental factor (E factor) of the reaction is also calculated to be ~5, which is far lower than the typical values for fine chemical synthesis, an improvement on other PAL-mediated reactions and borderline for bulk chemical production.¹⁸

This improved protocol proved also to be compatible with our recently reported telescopic combination of cinnamic acid synthesis and PAL hydroamination.¹⁹ The Knoevenagel-Doebner condensation of 3-fluorobenzaldehyde **3** and malonic acid was performed under optimised conditions. The crude reaction mixture (~1 M concentration of **1b**) was then used directly as starting material for subsequent biotransformation reactions. Different volume ratios of the reaction mixture and ammonium carbamate solution were

Table 3. Comparison of our intensified PAL-mediated gram-scale synthesis of **2b** with the best reported industrial method.

Method	DSM ¹²	This work
Catalyst	undisclosed PAL	PAL from <i>Anabaena variabilis</i>
Catalyst productivity	0.14 g g ⁻¹ (wet whole cells)	1 g g ⁻¹ (dry whole cells)
Substrate conc.	100 mM (20 × 5 mM batch additions)	354 mM
Reaction conditions	1 L, 30°C, pH 11.0, 200 rpm	17 mL, 30°C, pH 9.9, 200 rpm
Time	8.5 h	6 h
Conv. (Product conc.)	91% (91 mM)	92% (326 mM)
STY	51.2 g L ⁻¹ d ⁻¹	237.6 g L ⁻¹ d ⁻¹
E factor ^a	7.14 (54.0)	5.30 (22.3)

^a The E factor values in brackets include waste water in the calculation.

combined before addition of 20 mg mL⁻¹ catalyst (Table 4). Conversions to (S)-**2b** were found to be slightly lower than (but comparable to) the corresponding reactions with pure **1b**, with equally excellent ee values. The lowered conversions are likely due to the increased concentration of DMSO from the initial chemical

reaction, shown previously to be mildly inhibitory to the AvPAL reaction.¹⁹ This result constitutes an improvement of this extended approach to amino acid synthesis, allowing greater telescopic loading of the first reaction mixture into a more intensified hydroamination to yield the final product, thus incurring less overall waste from the biocatalytic step.

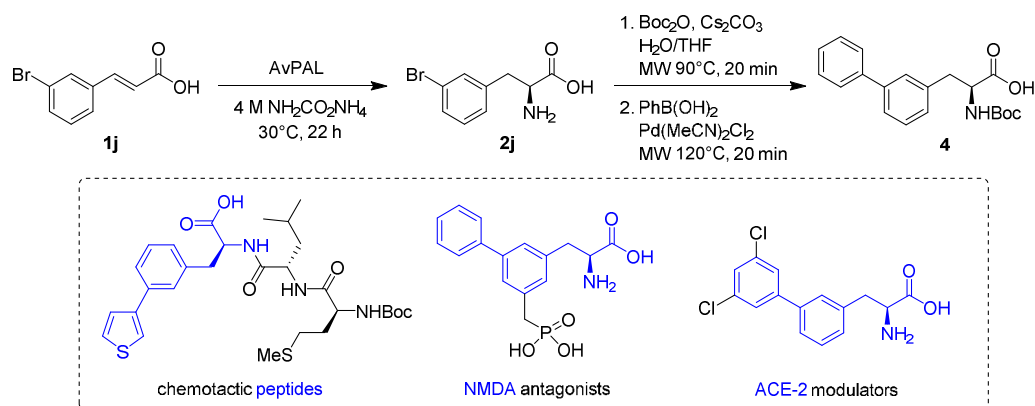
Table 4. Telescopic synthesis of **2b** from the corresponding benzaldehyde **3** via Knoevenagel-Doebner condensation and subsequent PAL-mediated ammonia addition.

Volume ratio ^a [% v/v]	Substrate conc. [mM]	Overall conv. [%]	ee of 2b [%]
10	~100	95	99 (S)
20	~200	93	99 (S)
30	~300	83	>99 (S)

^a Percentage by volume of the crude condensation mixture added to the carbamate solution containing PAL.

Furthermore, our intensified biotransformation process was also applied to the synthesis of the pharmaceutical building block biarylalanine **4** starting from 3-bromocinnamic acid **1j** (Scheme 2). The chemo-enzymatic approach combining PAL-mediated hydroamination with Suzuki-Miyaura coupling was previously employed for the synthesis of 4-aryl substituted phenylalanines,⁴ but this process required an ion exchange chromatography step to remove the high concentration of ammonium sulphate and unreacted cinnamic acid substrate. The downstream process could be considerably mitigated by adopting ammonium carbamate under optimised conditions: almost full conversion (96%) of **1j** to (S)-**2j** was achieved, leaving a negligible amount of starting material (ESI). The crude product obtained after removal of ammonium carbamate by sublimation could be used without further treatment for *N*-protection followed by Suzuki-Miyaura coupling,⁴ affording **4** in 24% overall isolated yield from **1j** (Scheme 2). The structural moiety of 3-aryl substituted phenylalanine is present in several pharmaceutical ingredients and building blocks, such as selective *N*-methyl-D-aspartate receptor (NMDA) antagonists for treatment of Parkinson's disease,^{20,21} angiotensin-converting enzyme 2 (ACE-2) modulators for weight disorders²² and chemotactic peptides.²³

In conclusion, an improved and simplified method for the production of various halogenated amino acids in excellent enantiomeric excess has been reported. The catalyst of choice is a recombinant, stable wild-type PAL produced from renewable feedstocks, harboured within lyophilised whole cells to mitigate lengthy catalyst purification and/or immobilisation steps.



Scheme 2. Chemo-enzymatic strategy for the synthesis of biarylalanine **4** from **1j**, consisting of PAL amination, *N*-protection and Suzuki-Miyaura coupling. Examples of pharmaceutical ingredients or candidates containing the substructure of **4** are shown in blue.

The 100% atom efficient synthesis has been achieved on a commercially-relevant scale - a considerable improvement on current industrial methods. The use of cheap, raw ammonium carbamate as a high-concentration ammonia donor salt lessens the work associated with producing high molarity, pH adjusted buffers and allows the equilibrium of the reaction to be driven towards product formation, due to its high solubility and ammonia content. The nature of the reaction mixture, combined with the optimised conversions (>95%) allow easy crude isolation of the amino acid products to a good level of purity - sufficient to enable easy modular integration with further chemical steps.

Acknowledgements

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