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Biocatalytic transamination with near-stoichiometric inexpensive amine donors mediated by bifunctional mono- and di-amine transaminases

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James L. Galman,[a] Iustina Slabu,[a] Nicholas J. Weise,[a] Cesar Iglesias,[b] Fabio Parmeggiani,[a] Richard C. Lloyd,[c] Nicholas J. Turner*,[a]

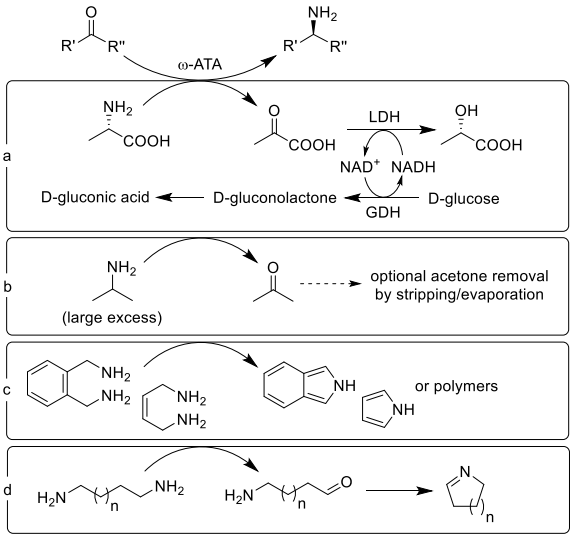
The discovery and characterisation of enzymes with both monoamine and diamine transaminase activity is reported, allowing conversion of a wide range of target ketone substrates with just a small excess of amine donor. The diamine co-substrates (putrescine, cadaverine or spermidine) are bio-derived and the enzyme system results in very little waste, making it a greener strategy for the production of valuable amine fine chemicals and pharmaceuticals.

Biocatalytic strategies for the synthesis of high added-value optically pure chiral intermediates provide extraordinary benefits to the pharmaceutical and agrochemical industries, due to the excellent activity, stereo-/regioselectivity and stability of many biocatalysts, often unmatched by their corresponding chemical equivalents.[1] There are obvious sustainability benefits to such approaches, including renewable resourcing of recombinant biocatalysts, solvent- and metal-free chemical transformations and ambient temperature/pressure of operation. In spite of this highly cited "greenness", the concept of any given biocatalytic transformation being effectively environmentally benign has been questioned in several instances.[2] Many widely used biotransformation protocols require, for example, expensive cofactor supplementation and/or large excesses of co-substrate to combat poor atom efficiency and unfavourable equilibria.

The asymmetric amination of ketones to enantiomerically enriched amines mediated by ω-amine transaminases (ω-ATAs, E.C. 2.6.1.18) is undoubtedly one of the most studied and exploited biotransformations which has become integrated into modern industrial practice. The broad substrate tolerance of this class of enzymes is one of their most appealing features in synthetic applications. For example of sitagliptin manufacturing is one of the most cited success stories of industrial biocatalysis.[3]

For these reactions, the thermodynamic equilibria are often unfavourable and hence different methods for shifting the equilibrium have been sought to address this long-standing challenge (Scheme 1).[4] One of the earliest approaches, still heavily relied on, was the coupling of the enzymatic reaction with a secondary concomitant irreversible reaction (e.g., conversion of pyruvate by-product to L-lactate with lactate dehydrogenase, paired with an oxidoreductase to recycle NAD(P)H, Scheme 1a).[5,6] Whilst producing naturally-degradable biochemicals as by-products, this method has the obvious disadvantage of requiring two additional enzymes and an expensive cofactor to achieve high conversion of the starting ketone.

On the other hand, for industrial scale application, the approach of choice is the use of a sacrificial amine donor in large excess to drive the equilibrium towards the desired



Scheme 1. Different approaches to shifting the thermodynamic equilibrium of ω-ATA transaminations.

[a] *Manchester Institute of Biotechnology (MIB), School of Chemistry, The University of Manchester, Manchester, United Kingdom.*
[b] *Facultad de Quimica, Universidad de la República, Montevideo, Uruguay.*
[c] *Dr. Reddy's Laboratories, Chirotech Technology Centre, Cambridge, United Kingdom.*
* *Corresponding author: nicholas.turner@manchester.ac.uk*

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product. Isopropylamine is widely used for this purpose, because of its effectiveness and availability.[7] This approach has proven very cost-efficient in many cases, however, a 100-fold excess (or higher) is required to drive equilibrium. Also, in order to improve the yields, the removal of the volatile acetone waste product by evaporation or stripping needs to be implemented using specialised temperature platforms and heat-stable enzymes.[8]

More recently, the use of synthetic diamines such as *o*-xylylenediamine[9] and but-2-ene-1,4-diamine[10] has been the focal point to displace the equilibria of ω-ATA reactions by spontaneous cyclisation and subsequent ring aromatisation of the aminocarbonyl by-product (Scheme 1c). However, these donors are usually expensive, highly toxic and often form difficult to remove polymers following aromatisation, adding to downstream processing costs.

In contrast, biogenic terminal diamines present themselves as renewably-sourced alternative amino donors. In addition to their simple bio-based production and relatively low cost, upon transamination they are converted into reactive amino aldehydes which spontaneously convert to cyclic imines, thus driving the equilibrium towards amination of ketones. Enzymes active on such compounds, known as α,ω-diamine transaminases (α,ω-DTAs), have exploited these advantages for the synthesis of *N*-heterocycles precursors.[11] However, these enzymes were found to have strict preference for pyruvate over other ketones, making them unsuitable for the synthesis of a broad range of amines. Nevertheless, if the substrate specificity could be addressed, biogenic diamines would offer a distinct approach to equilibrium issues (Scheme 1d), intrinsically "greener" than previous ones, since near-stoichiometric loadings of sacrificial diamines could be applied, in principle.

Herein, we report the discovery and characterisation of a panel of bifunctional α,ω-DTA/ω-ATA enzymes that readily accept cheap and easily-accessible mono-/diamine donors as well as possessing broad ketone acceptor scope, allowing an equilibrium shift to reach theoretical yield of 100% aminated product with almost stoichiometric donor loadings.

Results and discussion

The lack of activity of commonly used ω-ATAs towards simple aliphatic diamines such as putrescine (1,4-diaminobutane, **1a**), cadaverine (1,5-diaminopentane, **1b**) and spermidine (1,8-diamino-4-azaoctane, **1c**) has been previously reported.[10,11] Indeed, the only relevant example is a single commercial transaminase (ATA256) recently found through extensive screening of a large biocatalyst panel from Codexis, highlighting the rarity of such activity.[12] This transaminase was shown to have activity with a narrow range of ketone acceptors, and its commercial nature precludes evolutionary, sequence and structural insight into its substrate scope or optimisation through engineering. To address this gap in the transaminase toolbox, we undertook a search for new candidate enzymes able to accept diamine donors.

As a promising starting point for the development of an enzyme with broader substrate range we identified the putrescine transaminase gene *spuC*, part of the polyamine uptake and utilization pathway in *Pseudomonas aeruginosa* PAO1.[13] The *spu* (spermidine utilisation) operon, consisting of 9 genes, was previously characterised as responsible for spermidine transport and the catabolic route of putrescine in the arginine decarboxylase pathway. Genes with analogous functions are found in *E. coli* such as the complementary *ygjG* gene encoding for putrescine transaminase that suggests a common pathway between the two microorganisms.[14]

To probe the evolutionary relationships between known putrescine transaminases and well-characterised ω-TAs, a multiple sequence alignment was performed, from which a cladogram could be constructed to infer the order of divergence events (Figure 1). Interestingly, it was found that sequences with reported diamine transaminase activity clustered within the ω-ATA sequences as polyphyletic groups. SpuC from *P. aeruginosa* (PA-SpuC) was revealed to have high sequence identity with the well-studied Cv-ATA from *Chromobacterium violaceum* (55%)[15,16] and only 24% identity with the more functionally similar *E. coli* YgjG protein.[14] This indicated two separate acquisitions of such activity in this family, with one convergence (Pa-SpuC) occurring relatively recently in evolutionary history compared to the other. As previous reports with the more ancient putrescine transaminases (Ec-, Bme- and Bmy-YgjG) revealed minimal acceptance of monoamine substrates,[11] it is probable that any ancestral ω-TA function of these enzyme has been lost. Due to the higher sequence similarity between the Pa-SpuC and characterised ω-ATA biocatalysts, this enzyme was chosen for investigation of potential latent ω-ATA side-activity. Previous experiments carried out with Pa-SpuC revealed that, although it was likely to be involved in the catabolism of diamines,[13] minimal activity was observed with putrescine and derived amides.[17] In light of this, a selection of predicted orthologues were mined from publically-available sequence data, cloned and expressed to access the natural diversity of SpuC enzymes with respect to activity and potential monoamine/diamine acceptance.

A Basic Local Alignment Search Tool (BLAST) analysis of the characterised *spuC* gene from the pathogenic *P. aeruginosa* PAO1 strain revealed other homologous genes from

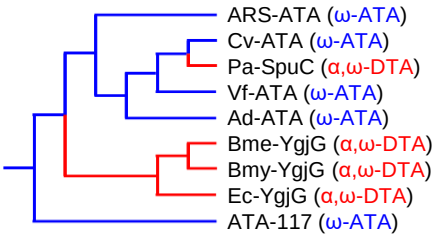
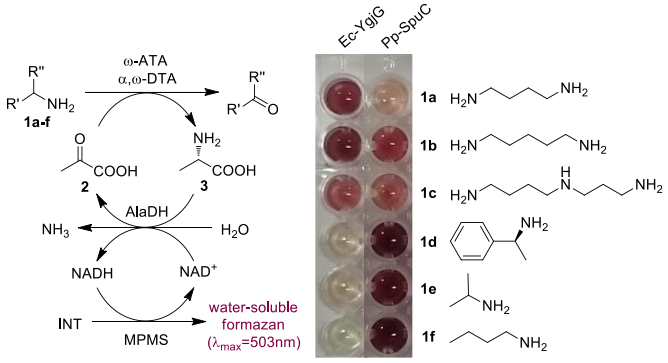


Figure 1. Cladogram showing evolutionary relationships between various wild-type transaminase enzymes as inferred from amino acid sequence identity.

Pseudomonas species, in particular *P. putida* (Pp-*spuC*), *P. chlororaphis* subsp. *aureofaciens* (Pc-*spuC*) and *P. fluorescens* (Pf-*spuC*) all with an aligned protein sequence identity of 69% (UNIPROT). The three putative *spuC* genes were cloned from our in-house NCIMB culture collection and subcloned into apET-28b vector followed by overexpression in *E. coli* BL21(DE3) cells. All three enzymes were successfully overproduced and purified as recombinant N-terminal His_6-tagged protein for screening against a representative panel of diamine and monoamine donors.

In order to determine the activity of the SpuC enzymes, an adapted version of the previously described L-amino acid dehydrogenase colorimetric assay[18] was employed, using pyruvate as the amino acceptor, L-alanine dehydrogenase from *Bacillus megaterium* to regenerate pyruvate with concomitant reduction of NAD^+, and the phenazine-tetrazolium system (MPMS+INT) for colour development (Scheme 2). The results for amines **1a-f** are summarised in Table 1 (additional mono- and diamines were tested, and kinetic constants were determined, see ESI).



Scheme 2. Activity assay used for the characterisation of ω-ATAs and α,ω-DTAs.

Table 1. Specific activities of the novel SpuC enzymes against amine donors **1a-f**, compared with Ec-YgjG and Cv-ATA.

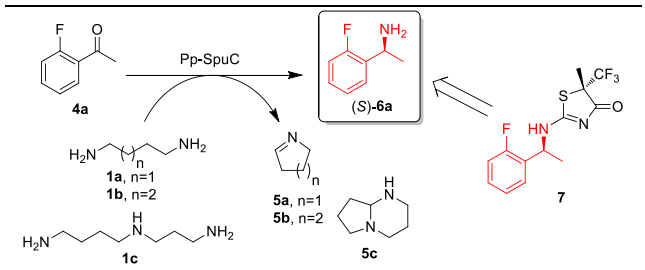
	Specific activity (U/mg)[a]				
Subs.	Pp-SpuC	Pf-SpuC	Pc-SpuC	Ec-YgjG	Cv-ATA
1a	1.3	0.4	1.4	2.7	n.d.
1b	27.3	16.5	15.1	2.7	7.7
1c	15.7	16.5	21.5	2.1	n.d.
1d	23.2	25.7	30.4	n.d.	28.9
1e	20.4	18.5	18.7	0.05	5.6
1f	16.5	19.3	13.5	n.d.	14.1

Expt. Cond.: 5 mM **1a-1f**, 1 mM **2**, 1 mM PLP, 0.2 mg mL^{-1} purified ATA, 100 mM sodium phosphate buffer, pH 8.0, 30°C. a: one unit of enzyme activity was defined as the amount of enzyme that produced 1 µmol of alanine per min at 30°C. n.d.: not determined (too low activity).

Our initial findings showed that all the recombinant SpuC proteins displayed high activity with the biogenic diamine donors **1a-1c** of interest with comparable specific activities within the different *Pseudomonas* species. Despite a 2-fold increase in activity of Ec-YgjG with putrescine **1a**, Pp-SpuC exhibited a 10-fold greater activity with cadaverine **1b**, Pc-SpuC with spermidine **1c**, with little or no activity displayed with Cv-ATA. Surprisingly, the SpuC enzymes also gave promiscuous activity with industrially relevant monoamines (*S*)-methylbenzylamine **1d**, isopropylamine **1e** and butylamine **1f**, which have been extensively studied with ω-ATAs, but none has been reported to have high activity with biogenic amines. Further studies on the diaminoalkane series was in close agreement with Cv-ATA as the carbon backbone increased in size (>C_6), in contrast to the diminished activity presented in Ec-YgjG (see ESI). This pattern is in part due to the narrow hydrophobic channel approaching the enzyme active site elucidated from recent crystal structures.[19]

Encouraged by these preliminary studies, we turned to the investigation of non-keto acid prochiral acceptors for preparative applications. As a model keto acceptor for reaction optimisation we chose *o*-fluoroacetophenone **4a**, since the chiral amine (*S*)-**6a** produced after transamination can be coupled to rhodanine scaffolds resulting in heterocycle analogues (e.g., **7**) with a range of pharmacological activities such as the clinical treatment of type II diabetes mellitus. [20,21] Biotransformations were conducted using our SpuC enzymes using different amounts of the diamine donors **1a-c**. The best conversions (Table 2) were obtained with Pp-SpuC (that also showed the highest specific activity for **1a-b** before), although the values for Pf-SpuC and Pc-SpuC were very similar.

Table 2. Testing of different diamine loadings in the transamination of **4a** mediated by Pp-SpuC.



Diamine	**1a**		**1b**		**1c**	
Equiv.	C (%)[a]	ee (%)[a]	C (%)[a]	ee (%)[a]	c (%)[a]	ee (%)[a]
1.5	84	>99	95	>99	83	>99
5	91	>99	96	>99	78	>99
10	87	>99	95	>99	73	>99
25	77	>99	83	>99	63	86

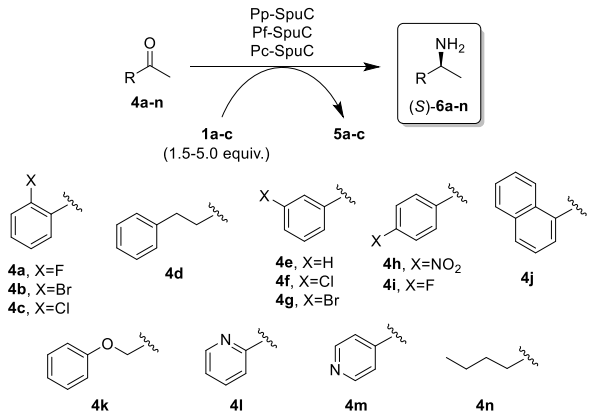
Expt. Cond.: 5 mM **4a**, 1 mM PLP, 1% v/v DMSO, 2 mg mL^{-1} purified SpuC, 50 mM HEPES buffer, pH 9.0, 40°C, 250 rpm, 24 h. a: measured by HPLC on a chiral stationary phase.

Remarkably, high conversions were observed at near-stoichiometric amounts of diamine donors **1a-1c**, indicating an efficient displacement of the equilibrium via *in situ* cyclisation, providing a greener substitute than 50 equivalents of volatile isopropylamine. The transamination of **1a-b** gave ω-aminoaldehydes which underwent spontaneous cyclisation to 1-pyrroline **5a** and 1-piperideine **5b**, respectively (as confirmed by GC-MS). Biotransformations were conducted at pH 9.0 and it was previously shown via ^1H NMR that the imine monomers are stable at conditions between pH 7-13.[22] Amino donor **1c**, instead, afforded the corresponding amino-imine that spontaneously cyclises a second time to yield the fused bicyclic structure **5c** under biotransformation conditions. Interestingly, increasing the concentration of **1c** not only did not improve

the conversion to **6a**, it was even detrimental, presumably due to side-product inhibition. The optical purity of the product was almost invariably >99% with all three enzymes tested.

In order to test the feasibility of larger scale processes we also performed a preparative biotransformation with **4a** and 2 equivalents of **1b**, purifying the product by column chromatography after extraction. The procedure afforded (*S*)-**6a** with an isolated yield of 79% and >99% ee.

Finally, to prove the wide applicability of this procedure, we also tested a panel of prochiral ketones **4a-n** that afford synthetically useful chiral amines of interest for the production of fine chemicals and pharmaceuticals (Scheme 3). Even though the monomeric imines **5a** and **5b** are known to oligomerise in aqueous environment,[12] the corresponding products (as well as the bicyclic product **5c**) are water soluble and do not interfere with the standard work-up. Therefore, the chiral amine products of the transamination **6a-n** can be easily recovered by extraction (together with residual ketone) for HPLC analysis. The highest conversions obtained for each substrate among the three SpuC enzymes are listed in Table 3. The (*S*)-enantiomer of the product was formed with all substrate/enzymes pairs, most often with perfect enantioselectivity. Only substrates **4d**, **i**, **k** and **n** afforded in some cases slightly lower ee values (56-96%), however, at least one of the three SpuC enzymes tested provided >99% ee. This emphasises the importance of screening several different members of the family for each application.



Scheme 3. Panel of aromatic and aliphatic ketones tested with SpuC enzymes.

The sterically hindered *ortho*-substituted ketone substrates **4a-c** afforded high conversions with high enantioselectivities (>99%). This high conversion is presumably due to a stabilising interaction between the amine formed and the halogen atom at the *ortho*-position, that drives the transamination reaction more readily,[23] than solely based on the electrostatic activation of the halogen substituent (as evident from the contrasting low conversion attained for the *para*-fluoro derivative **4i** and the high conversion for the *para*-nitro derivative **4h**). Likewise, a similar stabilising effect of the intramolecular *H*-bond with the newly formed amine and vicinal oxygen atom of **4k** has been suggested,[24] that would account for the good conversion (41-59%) of this substrate.

Table 3. Amination of ketones **4a-n** with diamine donors **1a-c** (only the best conversion obtained among the three SpuC enzymes is shown).

	Diamine **1a**			Diamine **1b**			Diamine **1c**		
Subs.	SpuC	c (%)[a]	ee (%)[a]	SpuC	c (%)[a]	ee (%)[a]	SpuC	c (%)[a]	ee (%)[a]
4a	Pf	92	>99	Pc	97	>99	Pf	92	>99
4b	Pp	80	>99	Pc	93	>99	Pp	67	>99
4c	Pf	75	>99	Pc	91	>99	Pf	81	>99
4d	Pp	37[b]	69	Pc	72[b]	74	Pp	47	>99
4e	Pf	12[b]	>99	Pp	42[b]	>99	Pp	13	>99
4f	Pf	33[b]	>99	Pc	62[b]	>99	Pf	27	>99
4g	Pp	63[b]	>99	Pp	80[b]	>99	Pp	62	>99
4h	Pf	57	>99	Pc	93	>99	Pf	63	>99
4i	Pp	12[b]	>99	Pc	35[b]	80	Pp	13	>99
4j	Pp	32[b]	>99	Pp	59[b]	>99	Pp	28	>99
4k	Pp	92	>99	Pp	99	96	Pp	88	56
4l	Pp	79	>99	Pp	96	>99	Pf	91	>99
4m	Pp	58	>99	Pp	95	>99	Pp	55	>99
4n	Pp	99[b]	92	Pf	99[b]	>99	Pp	91	95

Expt. Cond.: 5 mM **4a-n**, 1.5 equiv. **1a-c**, 1mM PLP, 1% v/v DMSO, 2 mg mL^{-1} purified SpuC, 50 mM HEPES buffer, pH 9.0, 40°C, 250 rpm, 24 h. a: determined by HPLC on a chiral stationary phase. b: in these cases 5. equiv. of amine donor were used to increase the conversion values.

For non-activated ketone substrates (**4d-g**, **j** and **n**) the conversions appeared considerably lower, therefore we tested also a slightly higher loading of amine donors **1a-b** (5 equiv.), leading to modest to excellent conversions. Interestingly, however, with spermidine **1c** comparable conversions were reliably obtained at near-stoichiometric amounts (1.5 equiv.) compared to the better amino donor **1b** (5 equiv.). This effect can be rationalised by the higher stability of the bicyclic amine product **5c**.

A few examples of the pharmaceutical relevance of the chiral amine products thus obtained are several *N*-Methyl-D-aspartate (NMDA) glycine-site antagonists used in the treatment neuropathic pain (*i.e.*, Parkinson's disease), synthesised from (*S*)-**6l-m**,[25] or the analogues of the antiarrythmic mexiletine obtained from (*S*)-**6k**.[26] All these compounds were obtained in almost quantitative conversion and excellent enantioselectivity (Table 3).

Conclusions

In summary, we report the identification, heterologous production and characterisation of SpuC orthologues, a class of transaminases that uniquely allow the conversion of numerous ketone substrates at the expense of natural diamines. The enzymes were found to afford high conversion of a broad range of substrates with a modest excess of amine donor, giving good to excellent enantiomeric excess values for pharmaceutically relevant chiral amines.

The application of these bifunctional SpuC enzymes effectively combines the appealing substrate breadth of traditional ω-ATAs with the equilibrium shifting potential of diamine donor acceptance by α,ω-DTAs. This shift, requiring just 1.5-5.0 equivalents of bio-derived putrescine or cadaverine, represents a clean, 'green' and effective synthetic strategy, as opposed to the widespread use of a 50-fold excess of conventional, often poorly-accepted monoamines. The method also mitigates the need for expensive cofactor

supplementation and complex regeneration systems often employed in conjunction.

The approaches developed in this work are particularly relevant to recent advances in metabolic engineering of host strains for enhanced fermentation of putrescine,[27] potentially enabling the creation of an integrated microbial cell catalyst for chiral amine synthesis.

Acknowledgements

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