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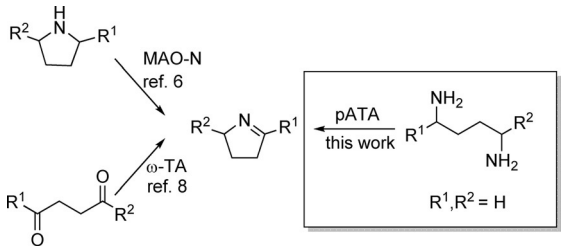
# Putrescine Transaminases for the Synthesis of Saturated Nitrogen Heterocycles from Polyamines

Iustina Slabu,[a] James L. Galman,[a] Nicholas J. Weise,[a] Richard C. Lloyd,[b] and Nicholas J. Turner*[a]

Putrescine transaminase (pATA; EC 2.6.1.82) catalyzes the transfer of an amino group from terminal diamine donor molecules to keto acid acceptors by using pyridoxal-5′-phosphate as a cofactor. The *ygjG* genes from *Escherichia coli* K12, *Bacillus megaterium*, and *Bacillus mycoides* were successfully cloned and expressed in *E. coli* BL21(DE3) cells. The three putrescine transaminases were all shown to prefer diaminoalkanes as substrates and thereby generated cyclic imines from the ω-amino aldehyde intermediates. The addition of a mild chemical reducing agent rapidly reduced the imine intermediate in situ to furnish a range of *N*-heterocycle products. We applied pATA in a biomimetic synthesis of 2,3-dihydro-1*H*-indolizinium-containing targets, notably the bioactive alkaloid ficuseptine.

Saturated nitrogen-containing heterocycles are prevalent in organic chemistry; they are frequently found in natural products[1] and are used as intermediates for target molecule synthesis. In particular, substituted pyrrolidines, piperidines, and azabicyclic motifs are found in bioactive natural products[2,3] as well as in currently marketed agrochemicals, fine chemicals, and pharmaceuticals. A number of synthetic strategies have been developed for the preparation of *N*-heterocyclic compounds including hydroamination, C–H amination, and cyclization reactions.[4] During the last decade, microbial and enzymatic catalysis have gained considerable interest for the production of enantiopure amine-containing building blocks.[5] Biocatalysts are typically highly regio- and stereoselective and can be used under mild conditions. Successful biocatalytic strategies have been reported for the synthesis of chiral cyclic amines from different enzyme classes, including monoamine oxidases,[6] imine reductases,[7] and ω-transaminases.[8]

Polyamines such as putrescine and cadaverine are biogenic diamines present in almost all living cells, in which they modulate cellular proliferation and protein synthesis.[9] These polyamines are also used industrially for the production of commercial products such as plastics, fabric softeners, and petroleum additives.[10] Recently, researchers have elegantly shown the potential to engineer overproducing strains of bacteria such as *Corynebacterium glutamicum* and *E. coli* to provide an alternative to polyamine production,[11] which at present is largely achieved by chemical synthesis. Three catabolic routes for the degradation of putrescine have been proposed, namely, oxidative deamination by amine oxidases, transamination by transaminases, and glutamylation of putrescine with subsequent oxidation steps to γ-aminobutyric acid (GABA).[12] Amongst these, the transaminase pathway for catabolism of putrescine has not previously been investigated for potential application in biocatalysis. Transaminases are pyridoxal-5′-phosphate(PLP)-dependent enzymes that have shown remarkable versatility in the asymmetric synthesis of chiral amino building blocks.[8] Terminal diamines present themselves as attractive alternative substrates for transaminases. In addition to their relatively low cost and high availability, upon transamination diamines are converted into reactive amino aldehydes[13] (Scheme 1), which spontaneously cyclize to cyclic imines thus driving the reaction towards product formation. We envisaged using putrescine transaminases (pATAs) to generate imines by this process for further application in the synthesis of *N*-heterocycles as described herein.




**Scheme 1.** Selected biocatalytic strategies for the synthesis of cyclic imines. MAO-N: monoamine oxidase, ω-TA: ω-transaminase.

To identify suitable candidate transaminases with activity towards diamines, we initially screened ω-transaminases from *Chromobacterium violaceum*,[14] *Alcaligenes denitrificans*,[15] *Arthrobacter citreus*,[16] commercially available ATA-113, and *Arthrobacter* sp. (ATA-117)[17] against a panel of polyamines by using our well-developed L/D-amino acid oxidase colorimetric assay.[18] However, none of these ω-transaminases showed any activity towards either putrescine (**1c**) or cadaverine (**1d**) as the amino donor with pyruvate as the keto acceptor. We there-

[a] *I. Slabu, Dr. J. L. Galman, N. J. Weise, Prof. N. J. Turner*
*School of Chemistry*
*University of Manchester, Manchester Institute of Biotechnology*
*131 Princess Street, Manchester, M1 7DN (UK)*
*Fax: (+44) 161-445-0093*
*E-mail: Nicholas.Turner@manchester.ac.uk*

[b] *Dr. R. C. Lloyd*
*Dr. Reddy's Laboratories, Chirotech Technology Centre*
*410, Cambridge Science Park, Milton Road, Cambridge, CB4 0PE (UK)*

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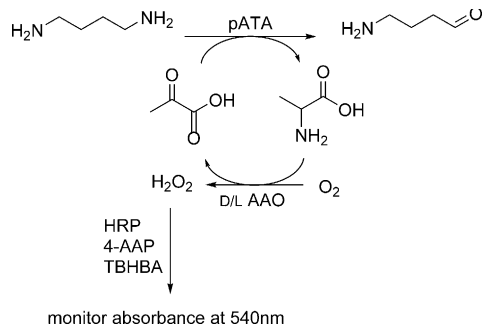
fore targeted the *ygjG* gene coding for putrescine transaminase from *E. coli* K12 (*Ec-ygjG*),[19,20] for which little data has been reported regarding substrate scope. A basic local alignment search tool (BLAST) analysis of the *ygjG* gene revealed two other putative transaminase genes from *Bacillus megaterium* (*BM-ygjG*) and *Bacillus mycoides* (*BMy-ygjG*) with protein sequence identities of 57 and 58%, respectively. The *ygjG* genes were subcloned into a pET-28b vector followed by overexpression in *E. coli* BL21(DE3) cells. Purified recombinant $His_6$-tag protein was obtained and employed in specific activity experiments. In parallel reactions, either L-amino acid oxidase (AAO) from *Crotalus adamanteus* or D-AAO from porcine kidney was added to reactions containing the diamine and *Ec-ygjG* with pyruvate as the amine acceptor (Scheme 2). In the

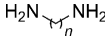
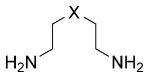
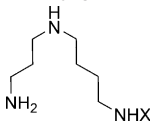
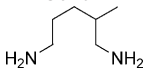
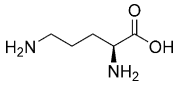
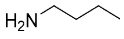



**Scheme 2.** Putrescine transaminase (pATA)/amino acid oxidase (AAO) liquid-phase colorimetric assay with the use of amino acid oxidase (L-AAO), horseradish peroxidase (HRP), 4-aminoantipyrine (4-AAP), and 2,4,6-tribromo-3-hydroxybenzoic acid (TBHBA). Assay conditions: diamine 10 mM, pyruvate 10 mM, TBHBA 4.5 mM, 4-AAP 1.5 mM, PLP 0.1 mg mL$^{-1}$, HRP 0.1 mg mL$^{-1}$, L-AAO 4.5 U mL$^{-1}$, pATA (purified enzyme) 5 mg mL$^{-1}$.

L-AAO experiment, the generation of L-alanine was confirmed as the product of transamination, whereas the use of D-AAO did not lead to the production of D-alanine. We subsequently used this method to screen all three putrescine transaminases, *Ec-ygjG*, *BMy-ygjG*, and *BM-ygjG*, against a broad range of diamines and polyamines to assess substrate scope (see Table 1 and Table S2 in the Supporting Information).

In the diaminoalkane series **1a–l**, the length of the alkyl chain was found to be an important parameter in determining the relative activities of the substrates (Table 1). No activity was observed with diamines possessing alkyl chains with two or three C atoms, that is, **1a** and **1b**, possibly as a result of the in situ generation of a bridged bicyclic complex with PLP, which would thereby effectively inhibit the catalytic role of the cofactor.[21] The highest activity was observed with putrescine (**1c**, set to 100%), and thereafter, the activity subsequently decreased in a stepwise manner with increasing chain length from **1d** to **1f**. Diamines containing greater than eight C atoms, that is, **1g–i**, had correspondingly lower measureable activity. We also examined diamines **2a–c**, **3a**, and **3b** containing heteroatoms in addition to branched diamine **4**. All of these diamines were shown to be active, although the lower activities of the bulky substrates can possibly be rationalized by the presence of a small substrate binding-site entrance with

**Table 1.** Specific activities of *Ec-ygjG* pATA on polyamines **1–6**.[a]

| | Substrate | Specific activity [mU mg$^{-1}$][b] |
|---|---|---|
|  | **1a**, $n=2$ | 0.62 |
| **1a–i** | **1b**, $n=3$ | 0.83 |
| | **1c**, $n=4$ | 270.04 |
| | **1d**, $n=5$ | 251.13 |
| | **1e**, $n=6$ | 164.42 |
| | **1f**, $n=7$ | 57.70 |
| | **1g**, $n=8$ | 27.04 |
| | **1h**, $n=9$ | 18.90 |
| | **1i**, $n=10$ | 8.10 |
|  | **2a**, X=N | 62.10 |
| | **2b**, X=O | 124.21 |
| **2a–c** | **2c**, X=S | 13.05 |
|  | **3a**, X=H | 121.51 |
| | **3b**, X=$(CH_2)_2NH_2$ | 16.20 |
| **3a–b** | | |
|  | **4** | 40.50 |
|  | **5** | 0.04 |
|  | **6** | 0.04 |

[a] The rates were determined by using a horse radish peroxidase/L-AAO colorimetric assay with purified enzyme. [b] Assay conditions: 10 mM amine, pATA (purified enzyme) 0.2 mg mL$^{-1}$, pH 8.0, 30 °C.

a highly hydrophobic channel to the active-site cavity relative to that of other class III transaminases.[22]

Interestingly, putrescine transaminase was found to exhibit higher selectivity for diamines than for monoamines of comparable size; for example, putrescine (**1c**) showed high activity with *Ec-ygjG* pATA, whereas corresponding monoamine butyl amine **6** showed no detectible activity. Ornithine **5**, mono *N*-*tert*-butoxycarbonyl (Boc)-protected derivatives of **1a** and **1b** (amines **10** and **11**, respectively), and diamines **7a**, **7b**, **8**, and **9** were also found to be inactive substrates, which suggests that the *ygjG* enzyme is strictly involved in the degradation of linear diamines (see Table S2).

A recent report suggested that the N2 atom of **1c** is stabilized by a hydrogen bond to a conserved Q119 residue (Q=glutamine),[22] which is also found in a range of GABA/α-ketoglutarate aminotransferases with low putrescine amino donor specificity.[23] The catalytic role of Q119 was therefore examined by mutating this residue to alanine, which resulted in a 60% loss of activity with **1c** as the substrate (Figure 1). This reduced activity is consistent with the role of Q119 in stabilizing the orientation of putrescine bound as the PLP–substrate imine, although it is clearly not crucial for catalysis. Furthermore, for substrates with increasing chain lengths from $C_5$ to $C_8$, the effect of the mutation on activity was less pronounced.

Further insight into the factors affecting substrate recognition was gained by mutation of other active-site residues of

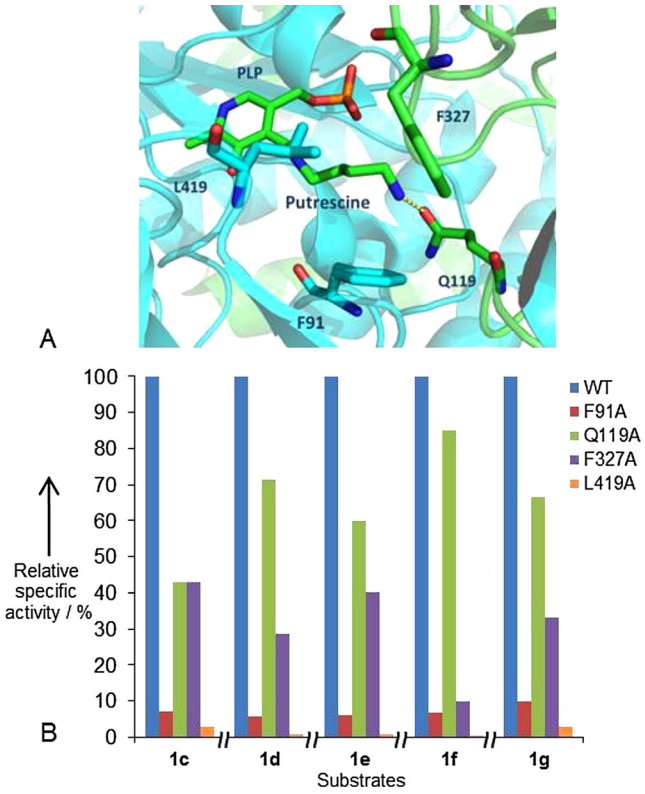


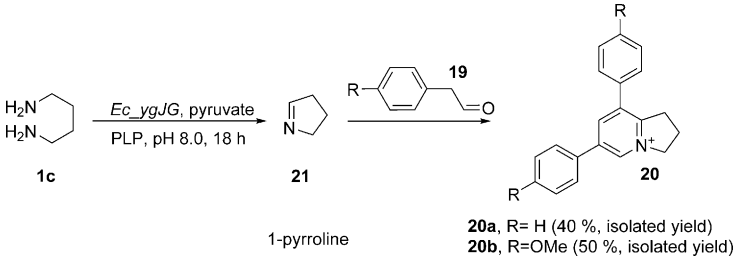

**Figure 1.** a) PyMol representation of the pATA enzyme (PDB: 4UOX), highlighting the important residues in the enzyme substrate cleft. b) The effect of mutation to alanine of substrate-recognition residues on the specific activities of the *Ec-ygjG* transaminase in the **1c–g** series. Substrates were normalized with respect to activity of the wild-type (WT) enzyme.

the *Ec-ygjG* transaminase. According to the published crystal structure of this enzyme (PDB: 4UOX), the substrate-binding residues are not conserved between *Ec-ygjG* and its structural homologues,[22] unlike the PLP-binding residues. A combination of bulky residues that protrude into the active-site cleft (i.e., F327, F91, and L419; F = phenylalanine, L = leucine) ensures that the entrance to the active site is hydrophobic and narrow, and this allows the enzyme to discriminate between diamines **1c** and **5**, which only differ by the presence of a carboxylate group. These residues are unique to this transaminase, and it has been suggested that they are responsible for substrate binding[24] (Figure 1a).

A series of point mutations to alanine were performed to examine the effect of each of these residues (i.e., F91A, F327A, L419A; A = alanine) on the transamination of diaminoalkanes **1c–g**. Interestingly, mutation of F to A at position 91 resulted in a complete loss of activity, and similarly, this effect was also observed for L419A. Substitution of F with A at position 327 resulted in an approximately 50% loss of activity for shorter-chain diamines **1c–e**; the decrease was more pronounced for longer-chain diamines **1f** and **1g**. These results indicate that the F91 and L419 residues are important in catalysis; however, further mutagenesis studies are required to elucidate completely the factors responsible for substrate binding and catalysis (Figure 1b).

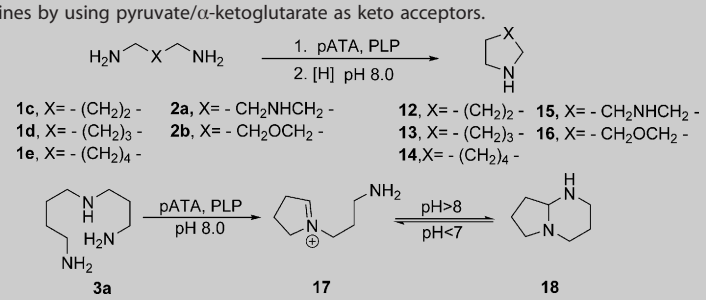
To develop preparative-scale reactions by using putrescine transaminase, we initially focused on using **1d** as a model substrate. Transamination of **1d** yielded 1-amino-5-pentanal, which underwent cyclization to 1-piperideine followed by rapid pH-dependent polymerization resulting in a mixture of oligomers that was unable to revert back to the imine monomer.[25,26] The imine is only stable at low pH values, which are not compatible with the transamination reaction. An alternative approach was therefore pursued, involving in situ reduction of the imine intermediate to prevent polymerization. Several chemical reducing agents were tested, including $NH_3BH_3$, $NaCNBH_3$, and $Na(AcO)_3BH$ with pyruvate and α-ketoglutarate as the keto acceptors (see Table 2 and Table S3). In all cases, GC analysis revealed clean monomeric product peaks. Both $Na(AcO)_3BH$ and $NaCNBH_3$ reduced the imines with high conversions with no loss of transaminase activity. Using $Na(AcO)_3BH$ (10 equiv.), the highest conversions were observed for **1c–e**, whereas the presence of a heteroatom in similar substrates **2a** and **2b** resulted in lower conversion values (Table 2). Interestingly, substrate **3a** gave bicyclic product **18**. Preparative-scale studies resulted in isolation of open-chain *N*-substituted pyrroline **17** from acidic workup, which confirmed that transamination occurred at the amine of the C4 terminus as expected. Conjugate addition of the C3 terminal amine of **17** was pH dependent and generated fused bicyclic structure **18** in 60% yield under basic conditions.

As an alternative to chemical reduction of the imine, we examined the use of imine reductases. One-pot enzyme cascades with **1c** and **1d** with the use of *Ec-ygjG* with (*R*)- or (*S*)-imine reductase (IRED) from *Streptomyces* sp. GF3587[27] and *Streptomyces* sp. GF3546[28] were separately examined. Interestingly, both (*S*)-IRED and (*R*)-IRED showed good to excellent conversions (up to 99%), highlighting the potential for combining these two enzymes in vivo for the conversion of polyamines into *N*-heterocycles (see Table S3). Finally, to demonstrate the utility of putrescine transaminase in synthesis, we developed a biomimetic one-pot route to 2,3-dihydro-1*H*-indolizinium motifs, notably the antibacterial and antifungal alkaloid ficuseptine (Scheme 3).[29] Starting from **1c**, we prepared **20a** (40% yield) and analogue **20b** (50%), both of which were previously reported to require a five-step chemical synthesis[29] or a similar biomimetic route under harsh acidic conditions.[30]




**Scheme 3.** Biomimetic formation of 2,3-dihydro-1*H*-indolizinium motifs by using two molecules of aldehyde **19** and in situ product 1-pyrroline (**21**) to generate **20a** and **20b**.

**Table 2.** Transamination of polyamines by using pyruvate/α-ketoglutarate as keto acceptors.




| Substrate | Product | Conversion [%] | | | | | |
|---|---|---|---|---|---|---|---|
| | | *Ec-ygjG* | | *BM-ygjG* | | *BMy-ygjG* | |
| | | Acceptor A[a] | Acceptor B[b] | Acceptor A[a] | Acceptor B[b] | Acceptor A[a] | Acceptor B[b] |
| **1c** |  **12** | 98 | 98 | 97 | 95 | 97 | 95 |
| **1d** |  **13** | 99 | 99 | 97 | 95 | 97 | 95 |
| **1e** |  **14** | 97 | 93 | 97 | 95 | 97 | 95 |
| **2a** |  **15** | 15 | 15 | 10 | 14 | 15 | 12 |
| **2b** |  **16** | 60 | 53 | 72 | 72 | 65 | 64 |
| **3a**[c] |  **18** | 51 | 72 | 56 | 58 | 39 | 25 |

[a] Keto acceptor: pyruvate. [b] Keto acceptor: α-ketoglutarate. Reaction conditions: 400 μL reaction, 5 mM diamine; 10 mM keto acceptor, buffer: 50 mM TRIS, 300 mM NaCl, 1 mM PLP; 10 equiv. $Na(AcO)_3BH$; cell-free lysate. [c] Reducing reagent was not added. Conversions were measured by GC-FID by comparison with commercially available standards by using an empty vector control as baseline.

In summary, three putrescine transaminases, with broad specificity for terminal aliphatic diamines, were characterized, and their potential for biocatalysis was subsequently investigated. These transaminases used pyruvate and α-ketoglutarate as keto acceptors and were combined with a chemical reducing reagent to generate a range of different *N*-heterocycles in good yields. Further engineering of these enzymes should lead to new biocatalysts for the synthesis of precursors for pyrrolidine- and piperidine-derived alkaloids.

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**Keywords:** alkaloids · amino aldehydes · biocatalysis · diamine transaminase · enzymes

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