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Journal Name

ARTICLE

A metagenomics approach for new biocatalyst discovery: Application to transaminases and the synthesis of allylic amines

Damien Baud,^{a§} Jack W. E. Jeffries,^{b§} Thomas S. Moody,^c John M. Ward,^{b*} and Helen C. Hailes^{a*}

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Transaminase enzymes have significant potential for the sustainable synthesis of amines using mild aqueous reaction conditions. Here a metagenomics mining strategy has been used for new transaminase enzyme discovery. Starting from oral cavity microbiome samples, DNA sequencing and bioinformatics analyses were performed. Subsequent *in silico* mining of a library of contiguous reads built from the sequencing data identified 11 putative Class III transaminases which were cloned and overexpressed. Several screening protocols were used and three enzymes selected of interest due to activities towards substrates covering a wide structural diversity. Transamination of functionalized cinnamaldehydes were then investigated for the production of valuable amine building blocks.

Introduction

The use of biocatalytic strategies holds significant potential for the sustainable synthesis of fine chemicals and pharmaceuticals. One important class of enzymes is the transaminases (TAMs, EC 2.6.1) that can convert ketones and aldehydes into the corresponding amines in high yields and selectivities under mild aqueous reaction conditions.^{1,2} TAMs catalyse the amino group transfer from an amine donor to a ketone, or aldehyde amine acceptor, using pyridoxal-5'-phosphate (PLP) as co-factor.³ The first step of the reaction is the transfer of the amino group of the amine donor resulting in the formation of pyridoxamine-5'-phosphate and release of the amine donor as a ketone or aldehyde. The second step is the regeneration of PLP with the transfer of the amino group to the amine acceptor which is released as an amine.^{1,4} Notably, TAMs are an interesting alternative to chemical synthetic methods from an environmental and economic point of view and have been reported in the synthesis of a number of (*R*)- or (*S*)-pharmaceutical ingredients or biologically active compounds.⁵

Currently TAMs have significant potential for the synthesis of an increasing number of amine products. Although a number of TAMs have been reported and are now available commercially there is a need to identify new TAMs with variation in the primary sequence for future applications including enzyme engineering projects: a

unique starting point has the potential to ensure more diverse enzymes are available. In addition new TAMs not encumbered by the current patent space would be very valuable. Here our approach has been to use a metagenomics mining strategy for new TAM discovery.

Metagenomics is the concept of processing and analysing DNA extracted from an environment as if it were a single large genome.⁶ A frequently quoted statistic suggests that on average only 0.1-1% of bacteria are culturable from any given niche, depending on the complexity and the understanding of said niche.^{7,8} Attempts have been made to address this shortfall between cultivatable bacteria and the diversity known to be present. Extracted DNA can be fragmented and ligated into large Bacterial Artificial Chromosome (BAC) clone libraries. Once the environmental DNA is in a suitable host such as *E. coli* the BACs can be screened for desired activity using plate screens or amplified to a level compatible with Sanger sequencing methods of analysis. Such BAC and Fosmid libraries are currently used to interrogate metagenomic samples to discover new enzyme activities or new examples of enzymes with existing activities, although this is a strategy that requires suitable high-throughput screens.^{9,10}

With the advent of high throughput sequencing technologies, raw DNA extracted from the environment can be used for sequence based analysis without the need for amplification in BAC libraries. Most of the research using such sequence based analysis has been aimed towards using microbial 16S based taxonomy to understand the range and diversity in a microbiome. More recently it has been used to observe how the microbiome changes with different inputs or host alterations, interactions between organisms and between organisms and the host, with protein annotations being used primarily to infer nutrient use and nutrient flow through the niche.¹¹⁻¹³

^a Department of Chemistry, UCL, 20 Gordon Street, London, WC1H 0AJ, UK

^b Department of Biochemical Engineering, UCL, Bernard Katz Building, London, WC1E 0AH, UK

^c Almac, Department of Biocatalysis & Isotope Chemistry, 20 Seagoe Industrial Estate, Craigavon, BT63 5QD, N. Ireland, UK

[§] Joint first authors

* Corresponding authors

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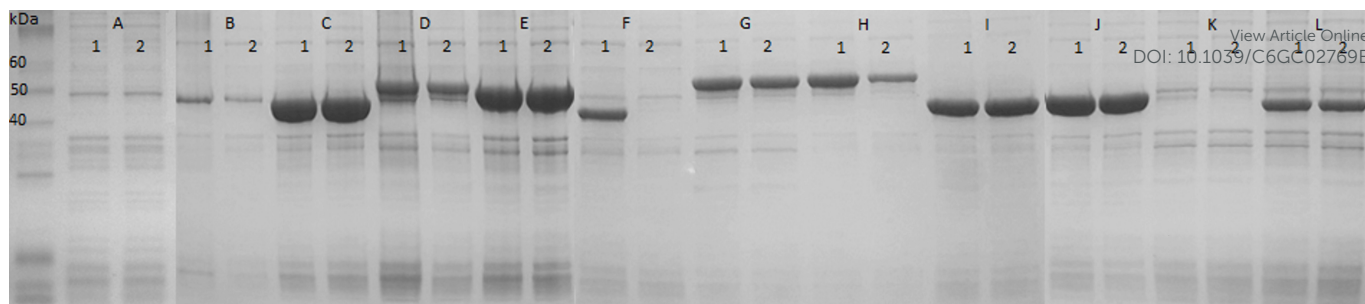


Figure 1. SDS gels of induced TAMs. Two samples for each enzyme was loaded onto the gels, total protein fraction and soluble fraction respectively (1= total protein fraction and 2= soluble fraction). 5 μ L of sample was loaded into each well (from a mixture of 10 μ L of protein sample and 30 μ L of loading dye). Protein markers 10–250 kDa (broad range ladder NEB). Enzyme molecular weights were calculated using the online ExpPASy ProtParam tool and are given in kDa after the plasmid name. **A)** Empty vector **B)** pQR1108 – 52; **C)** pQR1109 – 46.2; **D)** pQR1110 – 50.4; **E)** pQR1111 – 47.5; **F)** pQR1112 – 44.6; **G)** pQR1113 – 52.2; **H)** pQR1114 – 54; **I)** pQR1115 – 47.4; **J)** pQR1116 – 47.9; **K)** pQR1117 – 48.6; **L)** pQR1118 – 48.4.

More recently it has been used to observe how the microbiome changes with different inputs or host alterations, interactions between organisms and between organisms and the host, with protein annotations being used primarily to infer nutrient use and nutrient flow through the niche.^{11–13}

Sequence data is used less frequently as an aid to enzyme discovery, most often by providing consensus sequences for primer design.^{14,15} The strategy applied here uses a recently reported sequence-directed enzyme retrieval method involving the identification of individual open reading frames (ORFs) with subsequent specific primer design to each non redundant example of a desired enzymes class.¹⁶ From amino acid sequence alignment there are six groups of TAMs.¹ The Class III TAMs are characterized by broad substrate acceptance and high regio- and stereoselectivities and have been used in a wide range of applications for the synthesis of single isomer chiral amines.^{1,2} Here, the interrogation of an *in silico* metagenomic library identified 15 full length non redundant Class III transaminases of which 11 were successfully expressed and used in screens to identify substrate profiles.

Results and discussion

Protein selection and production

DNA, extracted from combined oral tongue scrapings from nine volunteers, was sequenced using Roche 454 Titanium platform.¹⁶ Sequencing returned 1.182 million reads with a mean sequence length of 385 base pairs (bp). This raw data was submitted to bioinformatics analysis for quality control to remove corrupted sequences, trim corrupted ends and filter out human sequences. After this the data set contained 1,048 million reads with a mean length of 410 bp. This reduced data set was assembled using MIRA for the creation of a contiguous (contig) read library, where individual reads were joined into longer stretches of DNA with Spoiler detection in MIRA to prevent chimeric contigs from joining. Sequencing of the DNA sample was of

sufficient depth to create 39,971 contigs containing 76.8 Mbase pairs with the largest contig being 42 kbp and 2,347 contigs were over 5 kb giving a large library of contigs containing several full length open reading frames (ORFs).¹¹ The contig library was analysed with the Genemark software to identify and mark ORFs. These putative protein-coding stretches of DNA were then scanned by the Pfam standalone tool. This process marked the ORFs with a Pfam ID linked to a specific protein family or enzyme class. Because of this all ORFs of a desired enzyme class, provided they have a Pfam ID, within the contig library could be extracted.

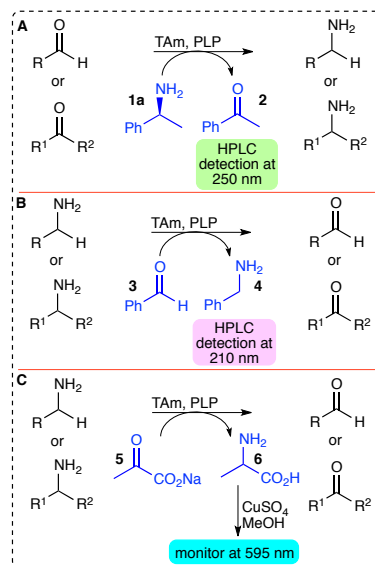
The Pfam ID for Class III TAMs was used to extract all ORFs marked from the contig library. 53 ORFs were identified of which 15 were full length and non-redundant. Enzymes identified from the metagenome were to be cloned using conventional restriction cloning techniques. To this end the sequences for retrieval were scanned for internal restriction sites. Primer pairs were designed for enzymes including an NdeI restriction site in the forward primer and various restriction enzyme sites in the reverse primer depending on any endogenous restriction sites. 11 out of 15 sequences were successfully retrieved from the metagenome. Blunt end PCR product was ligated into the PCR capture vector PCR-Blunt and through a series of restrictions and ligation was cloned into the pET29a expression vector. The pET29a vector containing the enzyme DNA sequences was transformed into the BL21*DE3 pLysS strain of *E. coli* for expression. The 11 putative enzymes were named with pQR numbers (See SI) and analysed by SDS-PAGE. All of the enzymes had a good level of expression except pQR1108 with a lower level of total expression of soluble and insoluble protein (figure 1, **B**) and pQR1117 (figure 1, **K**) with very little total expression. pQR1112 was completely insoluble (Figure 1, **F1** (total protein), **F2** (soluble fraction)) and pQR1114 was partially insoluble (Figure 1, **H1** (total protein), **H2** (soluble fraction)).

Screening of 11 potential TAMs

The 11 potential TAm enzymes were then screened as crude cell lysates using three different methods (Scheme 1): an (*S*)- α -methylbenzylamine ((*S*)-MBA **1**) amine donor assay against 21 aldehydes and ketones (Figure 2A) with detection of acetophenone **2** formation by HPLC at 250 nm (Scheme 1A); a benzaldehyde **3** amine acceptor screening method based on benzylamine **4** formation detected by HPLC at 210 nm (Scheme 1B) with five classical amine donors (Figure 2B), and a copper sulfate assay based on the detection of alanine **6** by formation of a complex with copper,¹⁷ monitored with a spectrophotometer at 595 nm using sodium pyruvate **5** as amine acceptor (Scheme 1C) and nine amine donors (Figure 2C).

Three of the 11 TAm enzymes, pQR1108, pQR1113 and pQR1114 were found to accept a wide range of structurally diverse aldehydes and ketones **7–25** from the (*S*)-MBA screening, and selected results are shown in Figure 3. pQR1108 showed the highest activity towards both linear and cyclic substrates. For the cyclic substrates, the best conversion yields were obtained with benzaldehyde **3** (61%) and cinnamaldehyde **15** (44%). Modification or absence of the aromatic ring led to significantly lower conversions with for example 4-hydroxybenzaldehyde **13** and cyclohexanecarboxaldehyde **10** (22% and 24% conversion yields, respectively). More hindered aromatic substrates like 1-indanone **14** resulted in lower conversion yields (8%). Cyclohexanone **7** was also well accepted by pQR1108 (27%) and modification of the six-membered ring led to smaller activity for 2-methylcyclohexanone **8** (4%) or no activity for the enone cyclohex-2-en-1-one **9**. When using linear substrates, the length of the carbon chain seemed to be important: a 42% conversion yield was obtained with pQR1108 and butanal **25** but 11% for octanal **24**. Functionalized aldehydes or ketones were also accepted, for example when using L-erythrose **20** (9% yield) and glycoaldehyde **21** (9% yield) (Figure 3). Linear ketones such as 2-heptanone **22** and 2-butanone **23** were not accepted. In order to confirm that the activity was due to the overexpressed TAm an assay for each (positive) substrate was performed in triplicate with *E. coli* cells containing an empty vector: no activity was observed. The benzaldehyde screening with five classical amine donors ((*S*)-MBA **1a**, (*R*)-MBA **1b**, (*S*)-alanine **6a**, (*R*)-alanine **6b** and isopropylamine **26**) did not give rise to any new activities: benzylamine formation was only observed when using (*S*)-MBA. This highlighted that all three enzymes exhibited (*S*)-stereoselectivity. For the copper sulfate screening, with sodium pyruvate as amine acceptor and nine different amine donors **4**, **27–34**, no activity was observed, consistent with the (*S*)-MBA screen where pyruvate **5** was not accepted as a substrate. The remaining 8 enzymes showed negligible activity against the substrates screened. This reflected for pQR1117 poor enzyme expression, pQR1112 low levels of soluble protein formed, and for pQR1109, pQR1110, pQR1111, pQR1115, pQR1116, pQR1118 most likely that preferred donors/acceptors were not screened. Using a combination of the Bradford assay and SDS page

densitometry to calculate protein loading, specific activities for pQR1108, pQR1113 and pQR1114 were investigated for two substrates **3** and **10**.



Scheme 1. Screening assays performed with the TAm enzymes. **A** (*S*)-MBA **1a** screening reaction conditions: 5 mM aldehyde or ketone, 25 mM (*S*)-MBA **1a**, 1 mM, KPi pH 7.5 100 mM, enzyme as crude cell lysate 0.2–0.4 mg/mL, 18 h, 30 °C, 500 rpm. **B** Benzaldehyde screening reaction conditions: 5 mM benzaldehyde **3**, 25 mM amine donor, 1 mM, KPi pH 7.5 100 mM, enzyme as crude cell lysate 0.2–0.4 mg/mL, 18 h, 30 °C, 500 rpm. **C** Copper sulfate screening reaction conditions: 15 mM sodium pyruvate **5**, 20 mM amine donor, 1 mM KPi pH 7.5 100 mM, enzyme as crude lysate 0.2–0.4 mg/mL, 1 h, 30 °C, 500 rpm.

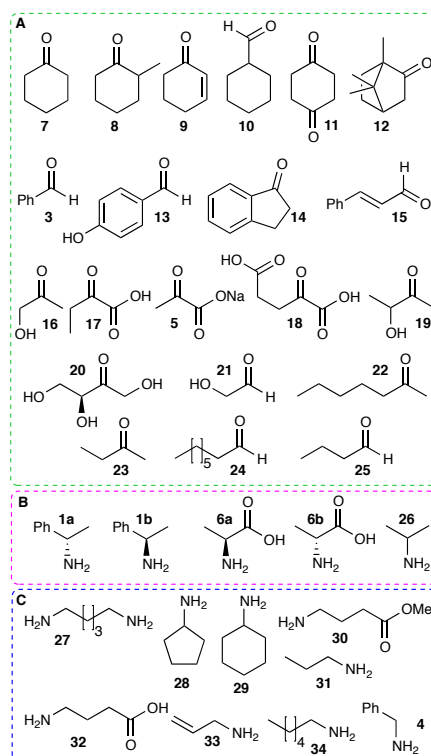


Figure 2. Substrates screened with assays **A** ((*S*)-MBA **1** and HPLC detection of **2**), **B** (benzaldehyde **3** and HPLC detection of **4**), and **C** (pyruvate **5** and spectrophotometric detection of alanine **6** after treatment with CuSO₄).

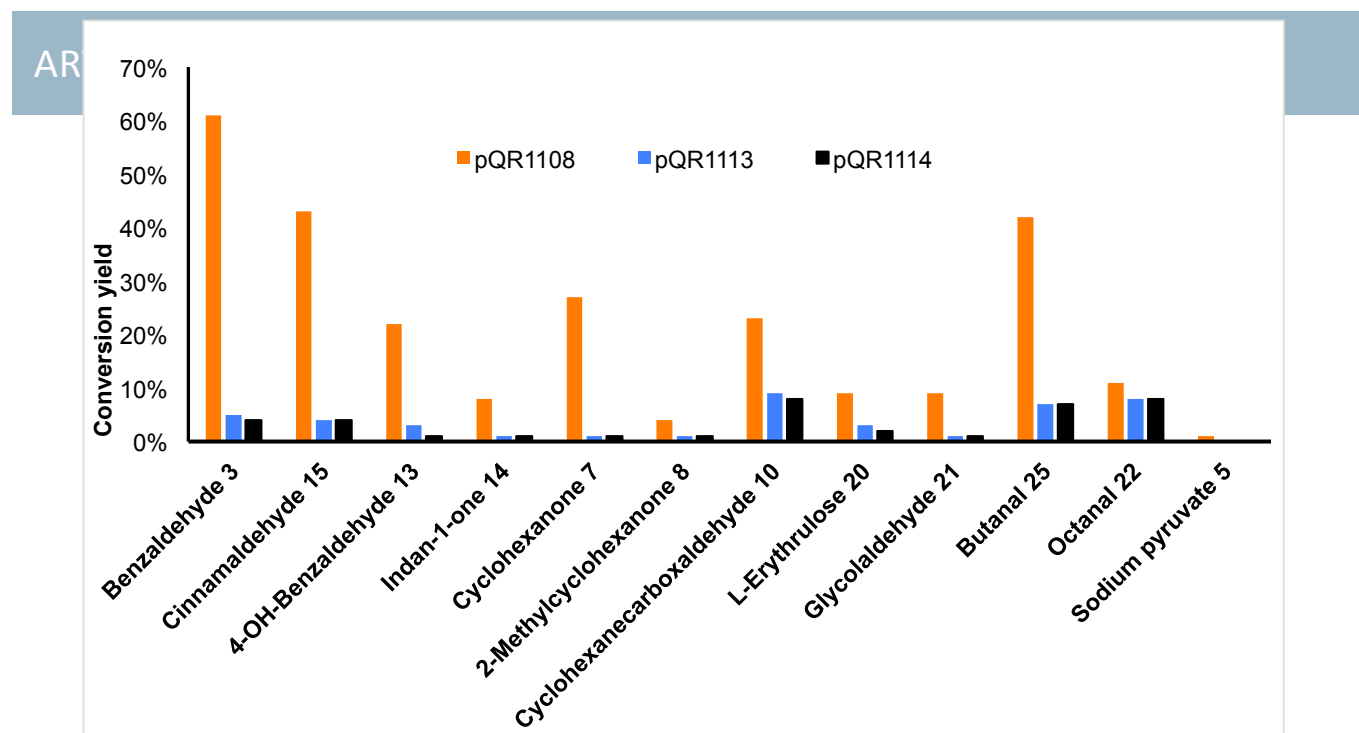


Figure 3. Screening results of three of the TMs against 12 selected substrates. Screening conditions: 5 mM aldehyde or ketone, 25 mM (S)-MBA **1a**, 1 mM KPi (pH 7.5, 100 mM), enzyme as crude cell lysate 0.2–0.4 mg mL⁻¹, 18 h, 30 °C, 500 rpm. Conversion yields (Scheme 1A assay via detection of **2**) were obtained from three independent experiments and varied by $\leq 4\%$.

pQR1108 had the highest specific activities of 1.7 $\mu\text{mol min}^{-1} \text{mg}^{-1}$, and 0.4 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for **3** and **10** respectively. pQR1113 had an activity of 0.1 $\mu\text{mol min}^{-1} \text{mg}^{-1}$ for **10**. It was not possible to calculate specific activities for pQR1113 with **3** and pQR1114 with **3** and **10** due to low activity over the course of the reaction.

Sequence homology of the 11 potential TMs was compared^{18,19} against 4 reported TMs from *Vibrio fluvialis* (Vf-TAM)²⁰, *Chromobacterium violaceum* DSM30191 (Cv-TAM)²¹, *Pseudomonas putida* KT2440 (Pp-TAM)²² and *Klebsiella pneumonia* JS2F (Kp-TAM)²³. Interestingly, pQR1113 and pQR1114 were found to have high sequence homology (93% identity) in agreement with the screening results. pQR1108 was found to have reasonably high sequence homology with Kp-TAM (55% identity plus 23% similarity) (Figure 4). The three enzymes had less than 30% identity to Cv-TAM and Vf-TAM. The remaining 8 enzymes which had shown negligible activity had less than 30% identity with the 4 reported TMs (see Table 1 SI for sequence homology comparison to Cv-TAM). pQR1109, pQR1110, pQR1111, pQR1115, pQR1116 and pQR1118 had between 70–95% homology with each other and it is likely that these TMs require a different amine donor. Similarly pQR1113, pQR1114 and pQR1117 form a cluster of TMs

that are closely related to each other and are likely to have other donor specificity.

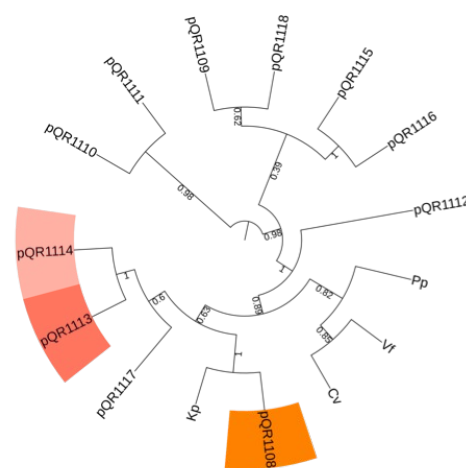


Figure 4. Amino acid sequences of the metagenomics TMs and 4 TMs from identified species (Kp-TAM, Pp-TAM, Cv-TAM and Vf-TAM) were aligned and used to generate a maximum likelihood phylogenetic tree (bootstrap values calculated from 1000 replicates).^{19,24}

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-MASMTGGQGMGRGSMNKPQSEARAETYSLYGTFDMPSLHQRTGVTVVTHGEGPIYVDVNGRRYLDANSGLWNMVAGFDHKGLIDAQAQYERFPYGHAFGRMSDQTVMLSEKLVESVP
Cv -----MQKQRTSQWRELDAAHHLHPTDTASLNQAGRVMTARGEGVYLWDSGNGKIIDGMAAGLCVNVNGYGRKDFABEAARKQMEELPFYNTFFKTHPAVBLESSLLAEVTP
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pQR1108 MLYNNENCQSVFKKNMSEFSSLLDFDRHLLHPTYSMTDPLFPVYVPRRA---EGVHIELADGTRLIDGMSWWCAIHGYNHPLVQAVEAQIKQMS-HVMFGGLTHESAVELGKLLVGLIP
Kp -----MTLDDLAFLDRRHWHPTYSMTSPLFPVYVPSA---HCEGLSLAGGQLVDGMSWWAAIHGYNHPLRNAALQKQIDQMS-HVMFGGITHPPAVALCRQLVAMTP
          *:          *:          *:          *:          *:          *:          *:          *:          *:          *:          *:          *:
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Pp GNLNHVFETSSGESCALTAVKMVRVAYRWLKQGAATKTKMIRGRARYHGHVNIAGTISLGGVY-NRKLEPGQMQDVDHLPHLTLHSLHDLHDSANI-----MPK-EGGI-ALADELLKLTELHDASNI
pQR1113 EGLNKFVLSDDGSTALEVAIKMAYQYRQLVGQTKTKTFIALNAGYHGDITGTVSVGGIQL-FHQVFHNLFKPLTLP-----SPGVYR-DLADRDKAF-DESLAEELERILNE-EGNEI
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pQR1108 AAFLEIPVVGAGGMYFYHPQYLRGLRDLCDFFIVLIDFELATGFGRTGRFFACEHAEVVDIMCIGKLSGGYMTLAAATITLQVETIS---RGEAGVMFHGFTFMANPLACAVAC
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Cv ANVAALRDEGIVQRVKDILGIPMYQRKWRFTFSREHVDVDRVGLWQAFITLVNKNAREKLPDFDEIGITLRCITIFRNNLMIRACGGDITVASAPPLVMEVDEMLAVERCEERFEPQTL
Pp AALCLLQKNIQVGSVAEV-APHEFKALH-IGKANVNDIDRNGFLAGAIQIAPRDGA-IVRPE-----AGMALWKAAGFYVRGGDITQGFQTFNSKPDQLDRFDAGEVKNLKD-
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pQR1114 ASLKVFEKRYEGLPKK-IEATFNALKP-IEQILKHVEKVRQGLVIGLEKDVATHEAYRPNDVAKAVILAREGLICRPIGDVIMPLPLASTVEQLEDMVRIVGESIKRATTEE
pQR1108 ASVKLLSGDWQANICRI-ESILKRLKA-AWDIRGVKDVRLVIGAVIGLEKGVMA-----RFQADCVAGIWRPFGRLVYLMPPYIISDILLTELADKTVQLLKEHSPW-
Kp ESLRLLSEGEWQPVQAAI-EAQLQAEIAP-ARGSALVADVRLVIGAVVETRRPVNMA-----ALQRFVEQGVWRPFGRLVYLMPPYIITPEQLTRITRAVNQAVQDETFFS
          *:          *:          *:          *:          *:          *:          *:          *:          *:          *:          *:          *:
VF AAA-ALE-----
Cv KAR-GLA-----
Pp -----
pQR1113 GLEDDRPILIE
pQR1114 GWLEDLKPIIFW
pQR1108 -----
Kp E-----

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Figure 5. Multiple alignment of the active metagenomics enzymes with Kp-TAM, Pp-TAM, Cv-TAM and Vf-TAM. Residues important for substrate and PLP binding in the Cv-TAM dimer are shown in red for the key residues in the first monomer and in green for the key residues in the second monomer.²⁶ Lysine 288 crucial for Schiff base formation is represented with a red star.

The important active site residues of pQR1108, pQR1113 and pQR1114 were also compared with the same 4 reported TAMs. Nine residues are considered as very important in the Cv-TAM dimer and are colored in red for the key residues of the first monomer and in green for the key residues of the second monomer (Figure 5).²⁵ Most residues were conserved in the 3 new enzymes. For example, Lys288 (using numbering of amino acids based on the sequence of Cv-TAM) is involved in the formation of a covalent bond with the PLP. Asp259, Ser121 and Tyr153 which are known to be important for the coordination of the PLP in the active site are also all fully conserved in our 3 enzymes. The residue Asp259 is involved in hydrogen bonding with the pyridine ring of the PLP and Ser121 and Tyr153 have roles in the phosphate group coordination of the PLP.

Reaction optimisation

The main parameters influencing the rate of a biocatalytic transamination (temperature, buffer, substrate and co-

factor concentrations, pH, co-solvents,) were studied with enzyme pQR1108, pQR1113 and pQR1114 using (S)-MBA **1a** with cyclohexanecarboxaldehyde **10** (5 mM) as amine acceptor in order to identify the preferred reaction conditions. The results are shown in Figure 6. Regarding the buffer, a slightly higher conversion was obtained with pQR1108 and PIPES, while HEPES gave a much lower conversion: due to the low cost of potassium phosphate buffer this was used in subsequent experiments. For (S)-MBA **1a** concentration, with pQR1108 no inhibition was observed when using up to 50 mM, and for the 3 enzymes 25 mM of **1a** was preferred so was used. A slight increase in conversion yield was also observed for pQR1108 when lowering the potassium phosphate (KPi) buffer concentration to 50 mM (27% yield compared to 24% yield at 100 mM) and by operating at pH 8.0 (30% yield against 24% yield at pH 7.5). For pQR1113 and pQR1114 a pH of 7.5 and 50 mM KPi was preferred.

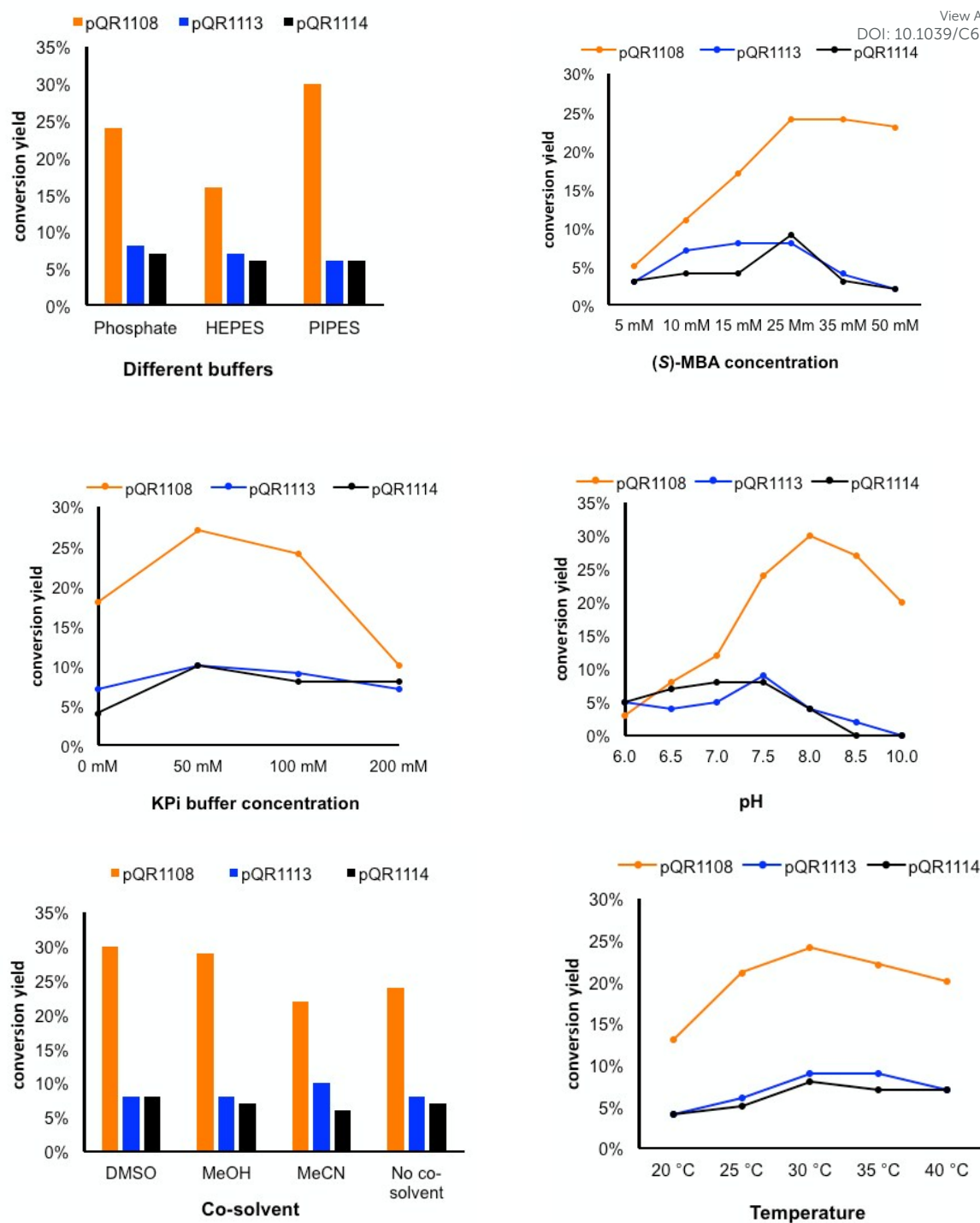


Figure 6. Effect of various parameters for the transamination of cyclohexanecarboxaldehyde **10**. Conversion yields (Scheme 1A assay via detection of **2**), were obtained from experiments in triplicate and varied by $\leq \pm 4\%$.

Using these preferred conditions it was established that the 3 enzymes were not affected by addition of a co-solvent (10% v/v). Interestingly, the conversion with pQR1108 increased with addition of co-solvents DMSO or MeOH (30% and 29% yields compared to 24% without cosolvent)

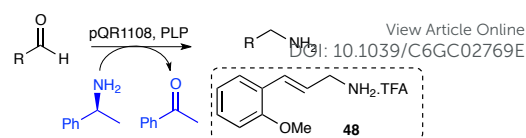
perhaps reflecting increased substrate solubility. The best reaction temperature observed was 30 °C for the 3 enzymes, and a PLP concentration in the range of 1-1.5 mM was required (data not shown). With the following improved conditions, (S)-MBA **1a** 25 mM, amine acceptor **10** 5 mM, PLP 1 mM, KPi buffer pH 8 and 50 mM, and

DMSO as co-solvent (10% v/v) and pQR1108 as cell lysate (0.3 mg/mL) for 18 hours gave a 41% conversion yield.

Synthesis of allylic amines

The transamination of conjugated aldehydes including cinnamaldehyde and functionalised derivatives could give access to allylamine building blocks for the synthesis of analogues of drugs such as the antifungal Naftin® (naftifine).^{26,27} They could also be used for the synthesis of cinnamylamine derivatives: indeed several related compounds such as cinnamamides have shown promising activities against breast cancer cells.^{28,29}

Transaminases have been reported for the synthesis of a wide range of amines, however little has been described on the acceptance of conjugated aldehydes/ketones other than the use of cinnamaldehyde **15** as an amine acceptor with Cv-TAm and Vf-TAm.^{21,30,31} Since pQR1108 demonstrated good activity towards cinnamaldehyde **15** in initial screens it was used with a range of conjugated aromatic aldehydes **35-41**, 3-phenylpropanal **42** to investigate the acceptance of a non-conjugated analogues, and several conjugated aliphatic analogues **43-47** to generate allylic amines (Table 1). The previously optimized reaction conditions were used with (S)-MBA **1a** as the amine donor. Cinnamaldehyde **15** was most readily accepted in comparable conversion yields to those reported with Cv-TAm.²¹ Other cinnamaldehyde analogues were accepted other than (E)-3-(4-(dimethylamino)phenyl)acrylaldehyde **38** probably due the dimethylamine electron donating group reducing the electrophilicity of the aldehyde. Modification to the aromatic ring with other electron donating groups including *ortho*- and *para*-methoxy and *para*-methyl moieties, **35**, **39** and **37** respectively, gave rise to lower yields (39%-58%) than with cinnamaldehyde again reflecting the lower electrophilicity of the aldehyde. (E)-3-(4-Bromophenyl)acrylaldehyde **36** was also readily accepted in 48% conversion yield. Increasing the steric bulk of the amine acceptor and addition of a methyl group or bromine at the alkene C-2 position led to a slight decrease in conversion yield compared to cinnamaldehyde: (2E)-2-methyl-3-phenylpropenal **40** and (2Z)-2-bromo-3-phenylpropenal **41** were accepted in 57% and 54% yield. The non-conjugated aldehyde **42** gave a conversion yield similar to the substituted cinnamaldehydes (56%). For the linear conjugated aldehydes **43-47**, acrolein **43** was accepted although the use of crotonaldehyde **44** gave rise to higher yields. 3-Methylcrotonaldehyde **45**, 2-pentenal **46** and 2-methyl-2-pentenal **47** were also accepted but in slightly lower conversion yields than for crotonaldehyde **44**. The ease of generating such allylic amines using transaminases is extremely useful and has not been reported to date: it provides a sustainable synthetic approach avoiding the use of metal catalysts or toxic reducing agents.



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Table 1. Conjugated aldehydes screened.

Aldehyde	Conversion ^a
15 	72% 75% ^b
35 	39%
36 	48%
37 	49%
38 	0%
39 	58% 57% ^b 84% ^c of 48
40 	57%
41 	54%
42 	56% 58% ^b
43 	35%
44 	49%
45 	45%
46 	37%
47 	37%

Reaction conditions: (S)-MBA 25 mM, amine acceptor 5 mM, PLP 1 mM, KPi buffer 50 mM at pH 8, DMSO as co-solvent (10% v/v) and pQR1108 cell lysate (1.2 mg/mL), 500 rpm for 18 hours. ^aConversions (Scheme 1A assay via detection of **2**) were obtained from three independent experiments and varied by $\pm 4\%$; ^bQuantified using the amine product and HPLC; ^cIsolated yield after scale-up.

To demonstrate the applicability of using pQR1108 further a preparative scale reaction was carried out with the optimised reaction conditions and 2-methoxycinnamaldehyde **39**. After 18 hours a quantitative

conversion based on acetophenone formation was observed possibly due to an improved reaction when stirring on the larger scale. After purification, amine **48** was isolated in 84% yield.

Conclusions

The present study demonstrates the efficiency of combining metagenomics for new enzyme discovery and screening to identify new transaminases. Metagenomics allows the mining of enzymes based on their protein sequence from *in silico* libraries of enzymes. It can thus uncover diverse enzymes that may have not been detected in microwell or agar plate screens. Eleven putative transaminases have been screened against several substrates and three new transaminases were identified as being active towards a broad range substrates. The biocatalytic potential of the new TAmS was illustrated with the sustainable synthesis of allylic amines using pQR1108: such amine building blocks can be used as precursors for the synthesis of analogues of several drugs. We are currently expanding the enzyme discovery approach using other metagenomic samples and a range of important biocatalytic enzyme families.

Experimental

General information. The solvents and chemicals were purchased from Sigma Aldrich or Fluorochem and were used as supplied. NMR spectra were recorded on a Bruker Spectrometer AMX300. Chemical shifts (in ppm) are quoted relative to tetramethylsilane and referenced to residual protonated solvent. Coupling constants (*J*) are measured in Hertz (Hz) and multiplicities for ¹H NMR coupling are shown as s (singlet), d (doublet), t (triplet), and m (multiplet). HPLC analyses were performed on an Agilent 1260 Infinity provided with a 1260 VWD detector. Mass spectrometry analyses were performed at the UCL Chemistry Mass Spectrometry Facility using a Finnigan MAT 900XP mass spectrometer. Accurate mass determination was performed by the peak-matching method.

Enzyme selection and production. Sequences generated by Roche 454 titanium platform were passed to the online platform MG-Rast, redundant sequences were removed, sequences filtered against human reference sets and dereplicated. The filtered output of MG-Rast was further analysed using the Galaxy platform to assign a Phred quality score to each nucleotide base. All sequence fragments that had a minimum score of 20 and length of 50 nucleotides were carried on for further analysis. The MIRA assembler software was used to take these individual sequences reads and assemble them into larger contiguous reads. These ORFS within these contigs were marked using the Genemark software and subsequently marked with a Pfam ID using the Pfam standalone tool.

Contigs that contained ORFs marked with the Pfam identifier for Class III TAmS were collected and visualised using the Artemis DNA viewer. ORFS that were truncated by being at the start or end of the contig were discarded. DNA corresponding to full length ORFs was excised. Due to inaccuracies in the *in silico* sequences stemming from the sequencing technology, ORFs comprising the whole sequence were often fragmented across the three reading frames. Due to this DNA sequence downstream of the suspected stop codon was collected to ensure the full length of the coding sequence was taken forward for analysis. Collected DNA for the ORFs was aligned and visualised using the MEGA software. Multiple sequences were highly similar at the amino acid level, for these matching sequences one example was chosen to take forward for primer design.

PCR primers were designed for the 15 identified enzymes. Due to inconsistencies in the *in silico* DNA sequences, terminal primers were designed beyond the suspected 3' end of the enzyme. Primers were designed to contain an *Nde*I restriction site in the forward primer and an *Nco*I, *Xho*I or *Bgl*II site in the terminal primer depending on restriction sites within the coding sequence. PCR was carried out on the metagenomics DNA sample using NEB Phusion PCR kit. All reactions were carried out following the standard protocol given in the kit with 52 ng of metagenomic DNA per reaction and a primer concentration of 5 pmol per reaction. Successful PCR was followed by gel electrophoresis with PCR products cut out of the gel and DNA recovered using Qiagen Gel extraction columns. Blunt ended PCR products were ligated into a PCR-Blunt capture vector and transformed into Top10 cloning cells. Top10 cells harbouring the plasmid were grown overnight in 5 mL of Terrific Broth with kanamycin 50 mg/mL. Plasmids were extracted using Qiagen miniprep kits and sent for sequencing to confirm the 3' end of the coding sequence of the enzymes with confidence. A second round of terminal primers were designed to remove the stop codon and introduce a restriction site. PCR was carried out using these primers and the PCR capture vector containing the PCR product from the first round of PCR. The same process of cloning as described above was carried out on the 2nd round of PCR products. Plasmid carrying the PCR product from this second round of cloning was purified and digested with *Nde*I and the requisite restriction enzymes to generate cohesive ends. The digested plasmid was run on a gel and the cohesive ended insert cut out and extracted from the gel. The digestion fragments were ligated into pET29a, digested to generate matching cohesive ends, and transformed into BL21* DE3 pLysS cells.

Small scale enzyme expression and preparation. The TAmS in *E. coli* BL21(DE3)pLysS were added to terrific broth medium (5 mL) containing kanamycin (50 mg/mL) and chloramphenicol (25 mg/mL) and incubated overnight at 37 °C: 1 mL of the overnight culture was then used to inoculate terrific broth (100 mL containing the same concentration of

antibiotic) which was grown at 37 °C until an OD₆₀₀ of 0.6 was reached at which point they were induced with 1 mM IPTG and grown for a further 5 h at 25 °C. The cells were harvested by centrifugation, the supernatant removed, and the cell pellet resuspended in potassium phosphate buffer (KPi; 5 mL of 0.1 M KPi pH 7.5, 1 mM PLP) and flash frozen in liquid nitrogen. The frozen pellet was placed on a freeze dryer. 25 mg of freeze dried cell was resuspended in the same 0.1 M KPi buffer pH 7.5 (1 mL) as before and sonicated. The lysed freeze dried cells were spun down and the enzyme concentration was determined using the Bradford method and then diluted if necessary to obtain an enzyme concentration of between 2.0 and 4.0 mg.mL⁻¹.

Initial (S)-MBA screening method. (S)-MBA **1a** (25 mM) and PLP (1 mM) in KPi buffer (pH 7.5, 100 mM), and the substrate (5 mM in water other than for cinnamaldehyde **15** and 1-indanone **14** when DMSO was used) were added in a 96 well-plate to give a total volume of 180 µL. The reaction was started by the addition of 20 µL of clarified cell extract (final concentration of 0.2-0.4 mg.mL⁻¹). After incubation for 18 h at 30 °C and 500 rpm, the reaction was quenched with 1 µL of TFA and denatured protein was removed by centrifugation. The supernatant (180 µL) was diluted with water (540 µL) and analysed by HPLC using an ACE 5-C18 300 column (150 x 4.6 mm) with UV detection at 250 nm. The concentration of acetophenone produced was determined using a linear gradient 15% - 72% over 10 minutes at 1 mL.min⁻¹ (A = water with 0.1% of TFA and B = acetonitrile) with subtraction of a negative control without amine acceptor for all substrates. The acetophenone produced eluted at a retention time of 8.8 minutes. Results were verified in triplicate against a negative control without amine acceptor following the same procedure.

Benzaldehyde screening method. The amine donor (25 mM) and PLP (1 mM) in KPi buffer (pH 7.5, 100 mM), and benzaldehyde (5 mM in water) were added in a 96 well plate to give a total volume of 180 µL. The reaction was started by the addition of 20 µL of clarified cell extract (final concentration of 0.2-0.4 mg.mL⁻¹). After incubation for 18 h at 30 °C and 500 rpm, the reaction was quenched with 1 µL of TFA and denatured protein was removed by centrifugation. The supernatant (180 µL) was diluted with water (540 µL) and analysed by HPLC using a ACE 5-C18 300 column (150 x 4.6 mm) with UV detection at 210 nm. The concentration of benzylamine was determined using a gradient 15% - 72% B over 10 minutes at 1 mL.min⁻¹ (A = water with 0.1% of TFA and B = acetonitrile). The benzylamine produced eluted at a retention time of 3.4 minutes.

CuSO₄/MeOH screening method. Preparation of the CuSO₄/MeOH solution: 300 mg of copper(II) sulfate pentahydrate was dissolved in 500 µL of water and 30 mL of MeOH. Sodium pyruvate (25 mM) and PLP (1 mM) in KPi

buffer (pH 7.5, 100 mM) and the substrate (5 mM in water) were added in a 96 well plate to give a total volume of 180 µL. The reaction was started by the addition of 20 µL of clarified cell extract (final concentration of 0.2-0.4 mg.mL⁻¹). After incubation for 1 h at 30 °C and 500 rpm, 40 µL of the prepared staining solution was added and the reaction mixture was then centrifuged (1 minute at 9000 rpm) to remove the insoluble phosphate-copper complex. The supernatant was then added to a 96 well-plate and absorbance was measured at 595 nm with a spectrometer SpectraMax Plus 384 from Molecular Devices. DOD was then calculated between the assay and a negative control without amine acceptor. This value was compared with DOD calculated for CV2025 and benzylamine as an amine donor and used as a reference (for 100% of relative activity). The limit of sensitivity was established to be 10% of relative activity.

(S)-MBA screening with preferred conditions for conjugated aldehydes. All substrates were solubilized in DMSO to give a final concentration of 10% v/v. (S)-MBA (25 mM) and PLP (1 mM) in KPi (pH 8.0, 50 mM) and the aldehyde (5 mM in water) were added to a 96 well plate to give a total volume of 140 µL. The reaction was started by the addition of 60 µL of clarified cell extract of pQR1108 (final concentration of 1.2 mg.mL⁻¹). After an incubation for 18 h at 30 °C and 500 rpm, the reaction was quenched with TFA (1 µL, 0.5% v/v) and denatured protein was removed by centrifugation. The supernatant (180 µL) was diluted with water (540 µL) and analysed by HPLC using a ACE 5-C18 300 column (150 x 4.6 mm) with UV detection at 250 nm. Concentrations of acetophenone were determined using a linear gradient 15% - 72% B over 10 minutes at 1 mL.min⁻¹ (A = water with 0.1% of TFA and B = acetonitrile) with subtraction of a negative control without amine acceptor for all the substrates. The acetophenone produced eluted at a retention time of 8.8 minutes.

Preparative scale reaction. The enzymatic reaction was performed using a total volume of 100 mL, containing (S)-MBA **1** (25 mM, 2.5 mmol, 302 mg) as amine donor, (E)-3-(2-methoxyphenyl)acrylaldehyde **39** (5 mM, 0.5 mmol, 82 mg) as amine acceptor, PLP (1 mM), KPi buffer pH 8.0 (50 mM), cell lysate of pQR1108 (1.2 mg.mL⁻¹) and DMSO as co-solvent (10% v/v) at 30 °C and 500 rpm for 18 h. The reaction mixture was acidified and centrifuged. The supernatant was extracted with Et₂O (3 x 100 mL) then basified with NaOH (2 M) and re-extracted with Et₂O (3 x 100 mL). The combined organic extracts were concentrated *in vacuo*. The product was added to water with 0.1% TFA (8 mL) and purified by preparative HPLC (Varian ProStar model 210) using a Dr. Maisch GmbH C8 Reprosil Gold 200 column (150 x 10 mm, 5µm) with UV detection at 210 nm with a linear gradient 5% - 50% B over 10 minutes at 6 mL.min⁻¹ (A = water with 0.1% of TFA and B = acetonitrile with 0.1% of TFA). Compound **48** eluted with a retention time of 5.4 minutes to afford compound **48** as a white solid (116 mg,

84%). M.p. 217 °C (decomposed); ^1H NMR (300 MHz; D_2O) δ 7.52 (1H, d, J = 7.5 Hz, 6-H), 7.32–7.43 (1H, m, 4-H), 6.98–7.09 (3H, m, 3-H, 5-H, =CHPh), 6.30 (1H, dt, J = 15.6 and 6.9 Hz, CH=CHPh), 3.86 (s, 3H, OCH_3), 3.76 (2H, d, J = 6.9 Hz, CH_2N); ^{13}C (75 MHz; D_2O with MeOH as a standard, TFA salt signals not described) δ 157.0, 131.5, 130.6, 127.9, 125.0, 121.8 (signals superimposed), 112.6 (CH), 56.2 (CH_3), 42.2 (CH_2); m/z (CI+) 164 ($[\text{M}]^+$, 60%), 147 (100); HRMS (CI+) found $[\text{M}]^+$ 164.1071; $\text{C}_{16}\text{H}_{15}\text{N}_3\text{O}_4$ requires 164.1070.

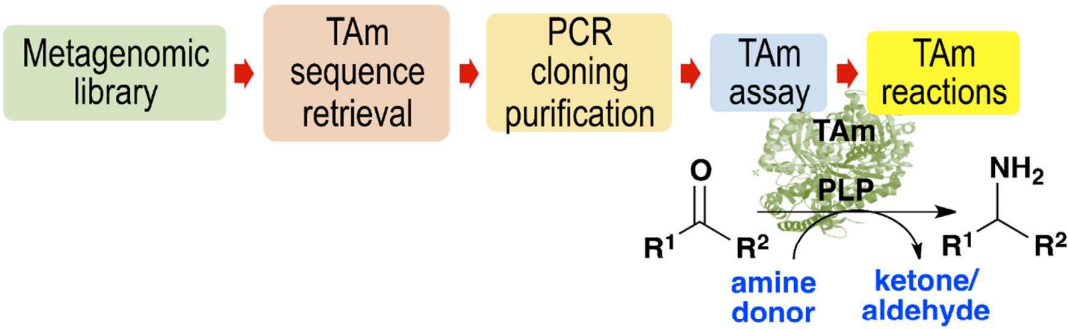
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ToC Entry



A metagenomics mining strategy was used to identify new transaminases that were utilised in the synthesis of allylic amines