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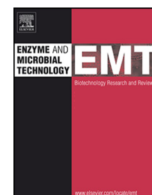

Multi-step biocatalytic strategies for chiral amino alcohol synthesis

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ABSTRACT

Chiral amino alcohols are structural motifs present in sphingolipids, antibiotics, and antiviral glycosidase inhibitors. Their chemical synthesis presents several challenges in establishing at least two chiral centres. Here a *de novo* metabolic pathway using a transketolase enzyme coupled with a transaminase enzyme has been assembled. To synthesise this motif one of the strategies to obtain high conversions from the transaminase/transketolase cascade is the use of hydroxypyruvate (HPA) as a two-carbon donor for the transketolase reaction; although commercially available it is relatively expensive limiting application of the pathway on an industrial scale. Alternately, HPA can be synthesised but this introduces a further synthetic step. In this study two different biocatalytic strategies were developed for the synthesis of (2*S*,3*R*)-2-amino-1,3,4-butanetriol (ABT) without adding HPA into the reaction. Firstly, a sequential cascade of three enzymatic steps (two transaminases and one transketolase) for the synthesis of ABT from serine, pyruvate and glycolaldehyde as substrates. Secondly, a two-step recycling cascade where serine is used as donor to aminate erythrulose (catalysed by a transketolase) for the simultaneous synthesis of ABT and HPA. In order to test the novel pathways, three new transaminases are described, two ω-transaminases able to accept a broad range of amine acceptors with serine as amine donor; and an α-transaminase, which showed high affinity towards serine (K_M: 18 mM) using pyruvate as amine acceptor. After implementation of the above enzymes in the biocatalytic pathways proposed in this paper, the two-step recycling pathway was found to be the most promising for its integration with *E. coli* metabolism. It was more efficient (10-fold higher conversion), more sustainable and cost-effective (use of low cost natural substrates and only two enzymes), and the reaction could be performed in a one-pot system.
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1. Introduction

Chiral amino alcohols are key functionalities in several sphingolipids, antibiotics such as chloramphenicol and thiamphenicol, and antiviral glycosidase inhibitors such as the deoxynojirimycin family among others [1]. These compounds can be synthesized by either chemical or biocatalytic methods. Among the various chemical methods available, diastereoisometric crystallisation [2] and asymmetric hydrogenation [3] have been established. However, the low maximum yield (up to 50%) and the pre-activation of the substrate as well as the high price of the transition metal and recycling/disposal, respectively, are their main disadvantages [2]. Biocatalysis on the other hand allows the development of sustainable manufacturing processes and the production of highly enantiopure compounds in high yields due to the enzymes chemo-, regio-, and enantioselectivity [4]. Monoamine oxidases, lyases, transaminases [2] and the recently described imine reductases [5,6] and norcoclaurine synthase [7] have been implemented in the synthesis of chiral amino alcohols. Among them, transaminases are industrially desirable as they have broad substrate specificity and high levels of regio- and stereoselectivity, in spite of their unfavourable equilibrium and substrate and/or product inhibition [8–10]. They have been successfully used at an industrial scale replacing chemical synthesis, *e.g.*, in sitagliptin manufacture. Sitagliptin is an antidiabetic compound, which has been chemically synthesised by rhodium-catalysed asymmetric enamine hydrogenation at 250 psi. Following various rounds of substrate walking

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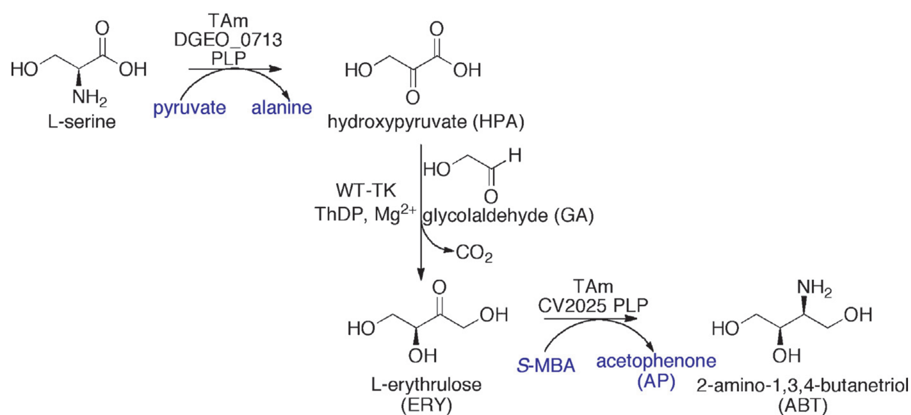

Fig. 1. Reaction scheme for the synthesis of ABT by a three-step sequential cascade. An α-transaminase (DGEO_0713) catalyses the amination of pyruvate with serine as amine donor, producing alanine and HPA. Then, a transketolase enzyme (TK) uses HPA and GA as substrates for the synthesis of ERY with release of CO_2. Finally, an ω-transaminase (CV2025) catalyses the amination of the formed ERY with MBA, synthesizing ABT and acetophenone.

and directed evolution, an ω-transaminase was engineered and successfully employed in the synthesis of sitagliptin at higher yields and productivity, and lower waste compared to the chemical method [11]. Another example is for the synthesis of valinol, a 1,2 amino alcohol previously synthesised by valine reduction employing agents like $LiAlH_4$. Recently a sustainable biocatalytic process was described using several ω-transaminases leading to optically pure enantiomers and 99% yield when the *R*-configuration was produced and 94% conversion when the *S*-isomer was synthesised [12].

In addition to single step biotransformations, transaminases have been coupled to additional enzymatic steps broadening the scope of the chemicals produced from inexpensive substrates. For instance, the synthesis of L-norephedrine was performed by coupling an *R*-selective pyruvate decarboxylase from *Saccharomyces cerevisiae* [13] or an acetohydroxyacid synthase I from *Escherichia coli* [14] with an ω-transaminase. In the first step benzaldehyde and pyruvate were used as substrates for the production of L-phenylacetylcarbinol, which was then aminated by the transaminase synthesising L-norephedrine. The latter step used alanine as amine donor releasing pyruvate as co-product, which was further employed as a substrate for the first reaction generating a recycling mechanism able to shift the equilibrium of the cascade [13,14].

In our group we have been studying *de novo* pathways coupling a transketolase enzyme with a transaminase enzyme. In an early study, a one-pot *in vivo* approach was performed for the production of 2-amino-1,3,4-butanetriol (ABT), using glycolaldehyde (GA) and β-hydroxypyruvate (HPA) as substrates for the transketolase step, followed by transamination of the erythrulose product with *S*-(α)-methylbenzylamine (MBA) as amine donor, employing a transaminase from *Pseudomonas putida*. In spite of the successful synthesis of the amino alcohol using *E. coli* as whole cell biocatalyst, the resulting yield (∼21% mol/mol) was low due to a long reaction time for the transaminase step, possibly because of its low affinity towards the substrates, or side reactions consuming erythrulose [15]. Following the description of the CV2025 transaminase from *Chromobacterium violaceum* [16], this enzyme was implemented in the above transketolase/transaminase pathway, and with the replacement of MBA with isopropylamine (IPA) as amine donor, a 4-fold increase in the percentage of conversion (from 21% to 87%) for the synthesis of ABT was achieved [17].

In both studies, the reaction was performed in resting cells without any host optimisation, requiring the supply of all substrates involved, namely MBA or IPA, GA and HPA. Both MBA and IPA are non-natural compounds; HPA is available within the cell in the phosphorylated form; and GA is found in the biosynthetic pathway of folic acid (in pentose and glucuronate interconversions) and vitamin B6, glyoxylate and dicarboxylate metabolism [18]. Therefore, to perform *de novo* pathway integration, both amine donor and keto donor for the transaminase and transketolase reactions would have to be provided by either an additional enzymatic step or an alternative donor than the ones previously employed.

In this work, we have *in vitro* studied different strategies to supply non-phosphorylated HPA to the previously described enzymatic pathway, as well as the possibilities of using alternative amine donors for the transaminase step. The first system (Fig. 1) involved the addition of a serine transaminase that can produce HPA by the amination of pyruvate using serine as amine donor followed by the previously described cascade [15,17], generating a three step pathway. The second reaction system (Fig. 2) corresponds to a two-step recycling system of the co-product. Thus by aminating erythrulose with serine, ABT and HPA could be synthesised simultaneously, the latter can be further consumed during the transketolase reaction recycling this substrate (HPA) in a two-step pathway. The conversion of HPA by transketolase and the release of CO_2 in the reaction should effectively drive the enzyme pathway to the formation of ABT.

To achieve the synthesis of ABT as a case study using the proposed alternative pathways, three new transaminases were identified and characterised in terms of their kinetic parameters and amine acceptor profile. The best candidates were tested *in vitro* as possible biocatalysts of the novel cascades described in this work.

2. Materials and methods

2.1. Strains and plasmids

Strains and plasmids used in this study are described in Table 1.

2.2. pQR plasmids construction

Each of the transaminase genes, described in Table 1, was amplified by PCR implementing the primers illustrated in Table 2. PCR products were incorporated into pCR4-TOPO (Life Technologies, USA) according to the manufacturer recommendations. Before transferring the gene to pET29a+ (Novagen, USA) expression vector, each sequence was confirmed by gene sequencing performed by the Wolfson Institute at UCL. pQR plasmids were finally constructed by ligating the plasmid pET29a+, previously restricted with *Nde*I and *Xho*I (New England BioLabs, UK), with the corresponding fragment obtained from the restriction of pCR4-TOPO::PCR with the same restriction enzymes.

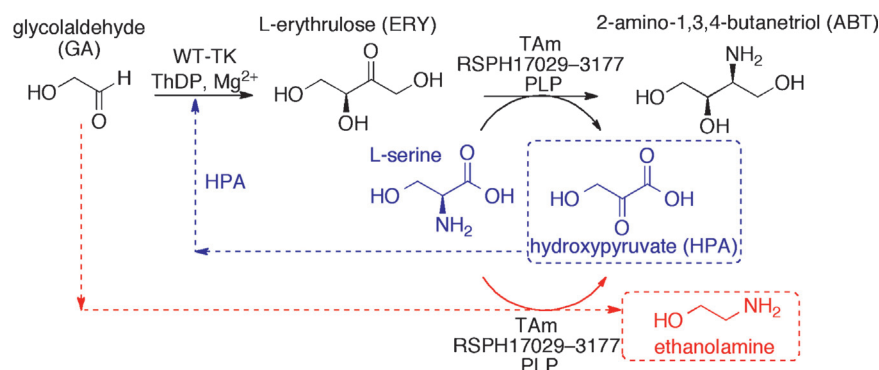

Fig. 2. Reaction scheme for the synthesis of ABT by a recycling *de novo* pathway. A novel ω-transaminase (RSPH17019_3177) catalyses the amination of erythrulose using serine as amine donor, synthesising ABT and HPA. Then, HPA is employed as substrate with GA for the synthesis of erythrulose catalysed by transketolase (TK). The side reaction catalysed by RSPH17029_3177 where GA is aminated to synthesise ethanolamine and HPA is highlighted in red, and the recycling reaction is highlighted in blue.

Table 1
Strains and plasmids used in this study.

Strains/plasmids[a]	Locus tag (enzyme)	Gene source and GenBank number	Transaminase type
E. coli BL21-Gold (DE3)[b]			
E. coli Rossetta-2 (DE3)[c]			
pQR801	CV2025	*Chromobacterium violaceum* DSM30191 AAQ59697.1	MBA:pyruvate transaminase
pQR958	PP_3718	*Pseudomonas putida* DSM6125 AAN69315.1	Acetylornithine aminotransferase
pQR977	DGEO_0713	*Deinococcus geothermalis* DSM11300 ABF45015.1	
pQR980	DGEO_1416	*Deinococcus geothermalis* DSM11300 ABF45711.1	Acetylornithine aminotransferase
pQR1005	KPN_00255	*Klebsiella pneumonia*[d] ABR75708.1	4-aminobutyrate aminotransferase
pQR1008	KPN_00955	*Klebsiella pneumonia*[d] ABR76391.1	Aspartate aminotransferase
pQR1010	KPN_01493	*Klebsiella pneumonia*[d] ABR76925.1	Succinylornithine transaminase
pQR1011	KPN_03745	*Klebsiella pneumonia*[d] ABR79132.1	Acetylornithine aminotransferase
pQR1018	PPUTGB1_2189	*Pseudomonas putida* DSM6125 ABY98090.1	Acetylornithine aminotransferase
pQR1019	RSPH17025_2835	*Rhodobacter sphaeroides* ATCC17025 ABP71721.1	
pQR1020	PPUT_2046	*Pseudomonas putida* DSM6899 ABQ78186.1	Acetylornithine aminotransferase
pQR1021	RSPH17029_3177	*Rhodobacter sphaeroides* ATCC17025 ABN78277.1	

[a] Construction of the pQR expression plasmids is described in Section 2.
[b] Agilent Technologies, USA55.
[c] Novagen, USA
[d] J. Ward collection. Equivalent strain to *Klebsiella pneumonia* DSM 30,104.

Table 2
Primers used for the transaminase cloning.

Plasmid	Forward primer	Reverse primer
pQR801	5-CATATGCAGAAGCAACGTACGACCAGCC-3	5-CTCGAG CTAAGCCAGCCCGCGCGCCTTCAG-3
pQR958	5-CATATGGCCACCCCAAGCAAAGCATTCG-3	5-CTCGAGGCCTTGGTACAGGCCAAGCGCCGT-3
pQR977	5-CAT ATGAGTGAGCGCCCACTGCC-3	5-CTCGAGAGCCTCGCACCCAGCCATTTCCCG-3
pQR980	5-CATATGACGGGCACGAAGACCAA-3	5-CTCGAGCGTCTGCTTGTCTTCCCGCAGCTC-3
pQR1005	5-CATATGAACAGCAATAAAGCAAT-3	5-CTCGAG CTGTTTCGCCTCGCTAAAGC-3
pQR1008	5-CATATGTTTGAGAACATTACCG-3	5-CTCGAGTACGGCGACGATGGCTTCGCACAG-3
pQR1010	5-CATATGTCAGGCAGTATGACTCG-3	5-CTCGAG GGTTAAGCGGGCAACGGCGCGATC-3
pQR1011	5-CATATGGCAACTGAACAACCGGC-3	5-CTCGAGGTTAATCACCTTTGCCACCGCCTG-3
pQR1018	5-CATATGGCCACCCCAAGCAAAGC-3	5-CTCGAGGCCCTGGTACAGGCCAAGCGCAGT-3
pQR1019	5-CATATGGCGCTGAATGACGCGGC-3	5-CTCGAGGGCGGCCGGTTCTTTCGCGGTCAG-3
pQR1020	5-CATATGGCCACCCCAAGCACAGC-3	5-CTCGAGGCCTTGGTACAGGCCAAGCGCCGT-3
pQR1021	5-CATATGACGCGGAATGACGCGAC-3	5-CTCGAGGGCGGCCGGTTCTTTCGCGGTCAG-3

2.3. Growth media, culture conditions and enzyme purification

Glycerol stocks of all cultures were stored at −80 °C in 2xYT broth (16 g/l tryptone, 10 g/l yeast extract, 5 g/l NaCl) containing 20% glycerol. Overnight cultures were prepared in 2xYT broth supplemented with appropriate antibiotics. Cells were subcultured using 1% v/v inoculum in 2l shake flasks containing 500 ml of the same supplemented broth at 37 °C and 250 rpm. Transaminases were induced with 1 mM of IPTG when growing in early exponential phase (OD_{600} = 0.7), and temperature was dropped to 30 °C until harvesting. Cells were harvested by centrifugation after 5 h of induction, and stored at −20 °C. 15 min before harvesting pyridoxal-5′-phosphate (PLP) was added to the cells expressing the transaminases at a final concentration of 400 μM to improve enzyme activity.

The cell pellet after induction was resuspended in 50 mM Tris–HCl pH: 8.0 and 400 μM PLP at a 1:25 volume ratio (1 ml of the resuspension buffer per 25 ml of cell suspension); and sonicated (Soniprep 150 sonicator, MSE Sanyo Japan) on ice using 10 cycles of 10 s On, 10 s Off at 10 μ. The sonicated suspension was centrifuged at 13.000 rpm at 4 °C for 60 min. Purification was carried out by nickel affinity using His-Select®Nickel Affinity Gel (Sigma–Aldrich, USA), following manufacturer recommendations, and supplementing each of the buffers with 400 μM PLP. 250 mM of imidazole was added to the eluted enzyme and was removed using PD-10 columns (General Electric, USA) with 50 mM Tris–HCl pH: 8.0 and 400 μM

PLP as desalting buffer. Bradford reagent (Sigma–Aldrich, USA) was use for total protein concentration quantification [19] with bovine serum albumin (BSA) (Sigma–Aldrich, USA) as standard. Enzyme purity was confirmed by SDS-PAGE using 12% Tris-Glycine Precast Gels (Invitrogen, USA).

2.4. Enzyme reactions

Commercially available substrates were purchased from Sigma–Aldrich (unless otherwise stated). ABT was chemically synthesised following the method by Dequeker et al. [20] and [15]. The 1,2-acetonide benzylamine diastereomeric intermediates were separated by silica flash column chromatography (particle size 40–63 μm) to give the (2*R*,3*S*)-ABT after subsequent deprotections. Racemic ketodiols 1,3-dihydroxypentan-2-one, 1,3-dihydroxyhexan-2-one, 1,3-dihydroxyheptan-2-one and 1,3-dihydroxyoctan-2-one were synthesised as described previously using an *N*-methylmorpholine-catalysed biomimetic reaction in water [21–23]. 6-Aminoquinolyl-*N*-hydroxysuccinimidyl carbamate (AQC) was synthesised according to Cohen and Michaud [24]. All reactions were performed in 2 ml glass vials at 30 °C and 350 rpm in 50 mM Tris–HCl pH 8.0, containing: 400 μM PLP; substrates which varied according to the experiment performed; 0.3 mg/ml of transaminase; and 0.1 mg/ml of transketolase enzyme or otherwise stated, 9 mM $MgCl_2$ and 2.4 mM TPP when studying the coupled systems. Prior to the addition of the enzyme(s), substrates were pre-incubated for 15 min at 30 °C and 350 rpm in a Thermomixer Comfort shaker (Cambridge, UK). Transketolase enzyme was also pre-incubated with 9 mM $MgCl_2$ and 2.4 mM TPP at 25 °C for 20 min, when required. After the reaction, 80 μl aliquots were quenched with 80 μl of 0.2% TFA. All samples were analysed by HPLC.

2.5. HPLC detection method and enantiopurity determination

HPA, pyruvate and erythrulose detection, no higher than 10 mM, was carried out by injecting 20 μl samples into a Dionex X500HPLC (Camberley, UK) comprising a Famos 120 autosampler, an Ultimate 3000 generation II isocratic pump, a LC30Chromatography column oven, and an AD20 Absorbance Detector. The system was equipped with an Aminex HPX-87H Ion exchange column (300 mm × 7.8 mm; Bio-Rad, Hemel Hempstead, UK) run at 60 °C with 0.6 ml/min isocratic flow of 0.1% TFA as mobile phase. Detection was performed by UV absorbance at 210 nm and peak identification and quantification were carried out using Chromeleon client 6.80 software. Commercially available standard compounds were analysed in parallel with the samples. The retention time of detected compounds are as follows: HPA: 8.3 min; pyruvate: 9.9 min; erythrulose: 11.5 min. Serine and ABT detection was performed by derivatising 75 μl samples with 150 μl of 10 mg/ml AQC in dry acetonitrile; followed by the addition of 225 μl of 0.2 M borate buffer pH: 5. 150 μl derivatised samples were injected into a Dionex Ultimate 3000HPLC (Camberley, UK) with a ACE 5C18 reverse phase column (150 mm × 4.6 mm, 5 μm particle size; Advance Chromatography Technologies, Aberdeen, UK) controlled by Chromeleon client 6.80 software, implementing the method developed by Ingram et al.

For analysis of the enantiopurity of ABT formed in the biocatalytic reaction, it was isolated and analysed by NMR spectroscopy (Bruker Avance-600 MHz spectrometer recorded at 296 K). Comparison of NMR spectra of the ABT generated in this work to ABT synthesised chemically (using an identical method to that previously reported by Ingram and colleagues [15]) indicated that only one isomer was formed (within detection levels) in the enzymatic cascade.

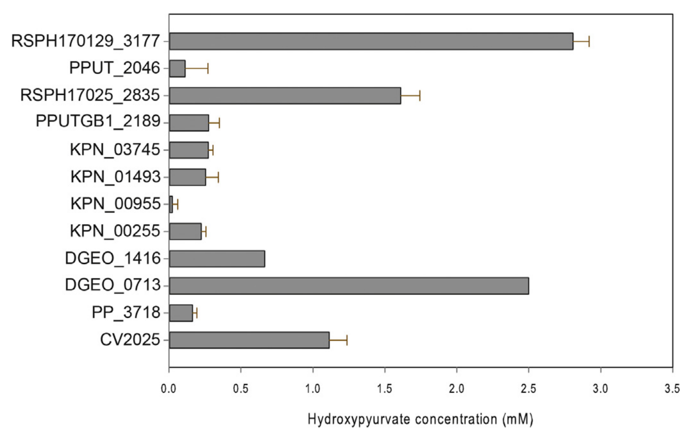

Fig. 3. Conversion of HPA obtained from bioconversions using 12 different transaminases. Reactions were carried out with 10 mM pyruvic acid and L-serine. HPA concentration was quantified after a 2 h reaction by HPLC (see Section 2 for details). Data are the means of three independent reactions ± SE.

3. Results

3.1. Serine:pyruvate transaminase identification and selection

All the proposed cascades in this work depend on a transaminase able to either aminate pyruvate or erythrulose using serine as an amine donor (Figs. 1 and 2). It has been reported that ω-transaminases are active with serine at a very low rate [9,16]; but there is not a clear description of an α-transaminase that employs serine as amine donor. As a result, from previous studies in our group 12 transaminases from different organisms (Table 1) were identified as possible catalytic candidates for the synthesis of HPA from serine by colorimetric screening assays. To corroborate the initial results and identify the enzymes with highest conversions under our reaction conditions, a quantitative assay was employed to determine the amount of HPA generated when using 10 mM of both pyruvate and L-serine as substrates (Fig. 3). After 2 h of reaction, DGEO_0713 (*Deinococcus geothermalis*), RSPH17025-2835 (*Rhodobacter sphaeroides*) and RSPH17029-3177 (*Rhodobacter sphaeroides*) synthesised 2.5 mM, 1.6 mM and 2.8 mM of HPA, respectively. As a comparison the conversion achieved by the CV2025 transaminase was 1.1 mM of HPA, which had been previously reported as able to use serine as an amine donor [16]. The above enzymes were therefore selected for further kinetic analysis and amine acceptors profile studies.

3.2. Enzyme characterisation: kinetic parameters and amine acceptors profile

In Table 3 the kinetic parameters are shown when using L-serine as an amine donor and pyruvate as the amine acceptor for CV2025, DGEO_0713, RSPH17025_2835 and RSPH17029_3177 transaminases. DGEO_0713 was the most efficient enzyme using serine as amine donor, as it had the highest affinity towards the compound in spite of its low turnover number, and was the only enzyme not inhibited by pyruvate. Conversely, RSPH17029_3177 showed the lowest affinity towards serine, while the catalytic constant was the highest among the four enzymes tested.

From the amino acid sequences CV2025, RSPH17025_2835 and RSPH17029_3177 were classified as ω-transaminases indicating a potential broad substrate tolerance with the ability to act on aldehydes, ketones and keto acids [10,16,25]. Alternatively, DGEO_0713 was classified as an α-transaminase, and thus it would be expected to be more restricted with respect to the range of substrates accepted [26,10].

Table 3
Kinetic parameters of the transaminases CV2025, DGEO_0713, RSPH17025_2835, RSPH17029_3177.

Transaminase	Substrate	K_M (mM)	k_{cat} (min^{-1})	k_{cat}/K_M($mM^{-1} \times min^{-1}$)
CV2025	Serine[a]	148	64	0.4
	Erythrulose[b]	37.7	2.9	0.08
DGEO_0713	Serine	18	28	1.55
	Pyruvate[c]	9.6	56	5.9
RSPH17025_2835	Serine	300.4	91	0.3
	Erythrulose	75.4	11	0.15
RSPH17029_3177	Serine	367	147.5	0.4
	Erythrulose	65.4	17.6	0.27

[a] 10 mM of pyruvic acid and varying L-serine concentrations from 5 mM to 200 mM were used.
[b] 80 mM of L-serine and varying erythrulose concentrations between 5 mM and 200 mM were used.
[c] 80 mM of L-serine and varying pyruvic acid concentrations between 5 mM and 200 mM were used.

Table 4
Amine acceptors profile of the transaminases CV2025, DGEO_0713, RSPH17025_2835, RSPH17029_3177.

Amine acceptor	CV2025	DGEO_0713	RSPH17025_2835RSPH17029_3177					
	Conv.[a] (mM)	Initial rate[a] (mM/min)	Conv. (mM)	Initial rate (mM/min)	Conv. (mM)	Initial rate (mM/min)	Conv. (mM)	Initial rate (mM/min)
Aldehydes								
Glycolaldehyde	5.9	0.02	n.r.[b]	n.r.	7.8	0.04	7.2	0.04
Propanaldehyde	4.2	0.03	n.r.	n.r.	5.6	0.07	5.7	0.07
Butyraldehyde	6.3	0.04	n.r.	n.r.	6.7	0.1	6.8	0.1
Valeraldehyde	7.2	0.02	n.r.	n.r.	8.3	0.05	8.4	0.05
Hexanaldehyde	5.4	0.01	n.r.	n.r.	7.0	0.09	7.0	0.1
Ketones								
Erythrulose	0.7	0.001	n.r.	n.r.	1.9	0.005	2.4	0.01
1,3-Dihydroxypentan-2-one	2.0	0.008	0.8	0.002	1.4	0.008	1.6	0.008
1,3-Dihydroxyhexan-2-one	1.6	0.007	n.r.	n.d	2.2	0.01	2.1	0.01
1,3-Dihydroxyheptan-2-one	1.0	0.006	n.r.	n.d	1.4	0.007	1.5	0.008
1,3-Dihydroxyoctan-2-one	0.6	0.001	n.r.	n.r.	1.1	0.0009	1.1	0.008
Keto acids								
Pyruvic acid	7.4	0.03	7.5	0.13	10.4	0.1	10.8	0.1

[a] 80 mM of L-serine and 10 mM of the corresponding amine acceptor were used as substrates and both conversions (Conv.) and initial reaction rates were measured by HPA production from serine in a HPLC.
[b] Not reactive: HPA levels below the detection limit 0.005 mM.

To confirm the above classifications and test whether the enzymes were able to synthesise ABT and additional analogues for future applications, other aliphatic aldehydes were selected that are accepted by transketolase wild type or described mutants, and the ketodiols produced from the corresponding reactions [23].

The data obtained (Table 4) confirmed the above classification when using serine as an amine donor: all ω-transaminases reacted with the aliphatic aldehydes, ketones and keto acids tested with conversions in the 1–9 mM range; whereas DGEO_0713 was only active with pyruvate. Regarding the use of aldehydes as amine acceptors both RSPH17025_2835 and RSPH17029_3177 were approximately 2-times faster CV2025, and a higher conversion of *circa* 10% was noted compared to CV2025. All ω-transaminases improved their activity when the length of aliphatic side chain increased except CV2025, which decreased for chain lengths longer than butanal with an increasing number of carbon residues. With dihydroxyketones as amine acceptors, both RSPH17025_2835 and RSPH17029_3177 were approximately 3-times faster than CV2025. However, while RSPH17025_2835 decreased its activity gradually upon increasing the length of the alkyl chain, RSPH17029_3177 had a constant activity as the chain length increased. The transaminases had negligible activity towards 1,3-dihydroxyoctan-2-one, the ketone tested possessing the longest alkyl chain.

As shown in Fig. 1, the sequential pathway requires a transaminase able to specifically use serine and pyruvate as substrates for the first step, as well as the CV2025 transaminase previously described for the final step of the cascade [16,17]. Based on the characteristics described above, the best choice was DGEO_0713 because it was not inhibited by pyruvate and was not active with any of the other compounds present. Alternatively, the two-step recycling cascade (Fig. 2) depends on a transaminase able to aminate ketones using serine as amine donor. From the data shown in Table 4, the best candidates were RSPH17025_2835 and RSPH17029_3177, as both were active towards a broad range of amine acceptors, synthesising ABT, 2-aminopentane-1,3-diol, 2-aminohexane-1,3-diol, 2-aminoheptane-1,3-diol and 2-aminooctane-1,3-diol. Also, they were 2-times faster than CV2025, previously used for ABT synthesis [17]. Based on the kinetic data (Table 3) for erythrulose, the most efficient enzyme was RSPH17029_3177, showing the highest catalytic constant and affinity for this particular ketone; therefore this enzyme was employed for the transamination step.

3.3 ABT synthesis using two different de novo *pathways*

To test whether each of the cascades could be implemented in the synthesis of chiral amino alcohols ABT was used as a case study, because it could be compared with previous reports [15,17], and the activity of the transketolase enzyme has been widely characterised for the synthesis of erythrulose where it shows a strong activity towards the substrates involved [27–31].

The sequential strategy shown in Fig. 1 involved a transamination step, performed by the enzyme DGEO_0713 using serine and pyruvate as substrates, followed by the previously described *de novo* pathway implementing CV2025 transaminase [15,17]. This enzyme is an ω-transaminase active with ketones, aldehydes and keto acids [26,10,16], such as erythrulose, GA and pyruvate; therefore, the effectiveness of the system was tested in two sequential steps to avoid side reactions. The first step is a one-pot pathway of two enzymatic steps where DGEO_0713 transaminase catalysed the production of HPA, the substrate for the subsequent transketolase step required for the synthesis of erythrulose; and the second

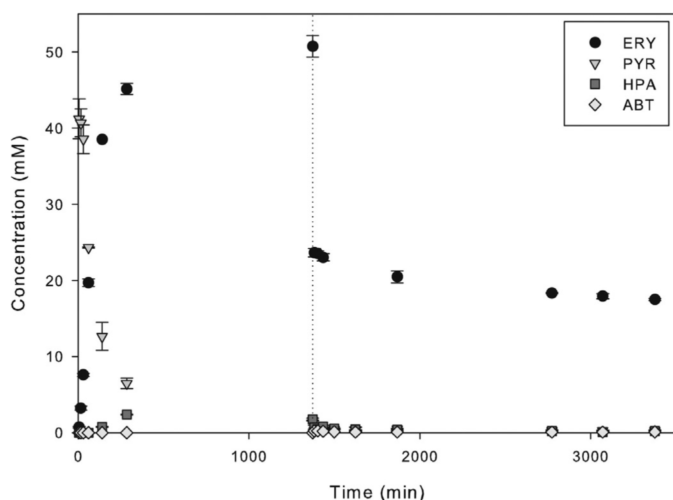

Fig. 4. Reaction profile for the synthesis of ABT via the three-step sequential pathway using 0.5 mg/ml pure DGEO_0713, 0.3 mg/ml pure transketolase, and 1 mg/ml pure CV2025. Reactions were carried out with 100 mM L-serine, 50 mM pyruvate, 50 mM GA and 10 mM MBA. Erythrulose, pyruvate, HPA and ABT were quantified over time for 55 h by HPLC (see Section 2 for details). The dotted line represents the time of CV2025 and MBA addition causing 1:2 dilution of the synthesised erythulose. Data are the means of three independent reactions ± SE.

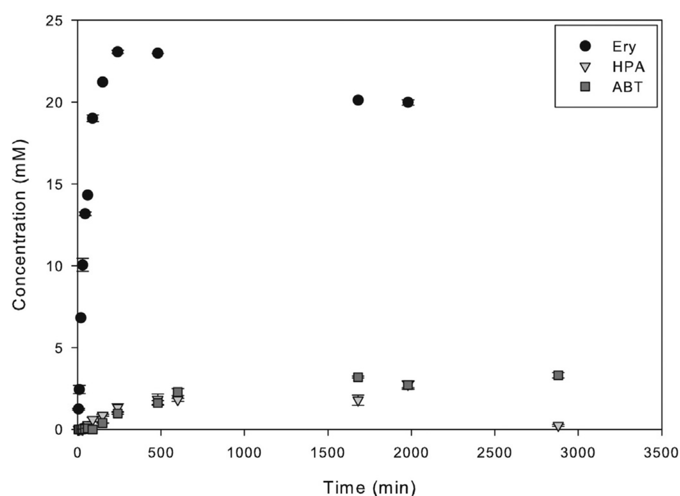

Fig. 5. Reaction profiles for the synthesis of ABT through the two-step recycling cascade using 1 mg/ml pure RSPH17029_3177 and 0.1 mg/ml pure transketolase, 100 mM L-serine and 40 mM GA. Erythrulose, HPA and ABT were quantified over time for 50 h by HPLC (see Section 2 for details). Data are the means of three independent reactions ± SE.

step is the transamination of erythrulose by CV2025 [16,17]. We implemented a higher serine concentration in order to shift the equilibrium of the first step towards the product HPA and to ensure that the pyruvate and the GA were fully consumed. Fig. 4 shows the reaction profile of the system over approximately 55 h. The first 24 h correspond to the synthesis of erythrulose giving a final conversion of 43.7 mM of erythrulose and 3.5 mM of HPA as intermediate products, leaving 3 mM of unreacted pyruvate. The following 31 h of reaction corresponds to the final transamination step that resulted in 3% conversion of ABT based on the concentration of MBA added, and the consumption of the remaining pyruvate and HPA, which are preferable substrates for the CV2025 transaminase [16]. A concentration of 10 mM of MBA was used to avoid inhibition by this substrate [8,17]. Isopropylamine (IPA) was also tested as an alternative amine donor, as a higher conversion of ABT was previously reported when using IPA rather than MBA in a two-step coupled reaction [17]. In contrast to this result, when implemented in a three-step coupled reaction, ABT was not detected after 50 h reaction (see Supplementary information, Fig. 2) (Fig. 5).

The two-step recycling cascade shown in Fig. 2 is a co-product recycling mechanism, in which a transaminase catalyses the amination of erythrulose using serine as amine donor for the synthesis of ABT and HPA. HPA is then used as 2-carbon donor by a transketolase-catalysed reaction with GA as keto acceptor for the irreversible synthesis of erythrulose and the release of carbon dioxide. Because of the recycling nature of the system the reaction required an initial input of HPA, either manually or from an alternative reaction, such as the amination of GA with serine, resulting in the production of the required HPA and ethanolamine as a side product (Fig. 2).

Based on the kinetic constants previously determined it was clear that in order to synthesise ABT using the two-step recycling system (Fig. 2), a high serine concentration will be required to drive the reaction forward, and it has been reported that concentrations above 40 mM of GA inhibits the TK reaction [30]. Based on our previous work [17] a 3:1 ratio of transaminase to transketolase was implemented, achieving 50% conversion of erythrulose (20 mM) from GA, as half of the GA is probably consumed in the side reaction with serine. Thus based on the concentration of erythrulose synthesised, 18% ABT conversion was obtained or 9% overall ABT conversion based on the GA initial concentration. It was not expected to achieve a 100% conversion of erythrulose due to the higher affinity (K_M: 7.9 mM) and higher turnover (k_{cat}: 21.2 min^{-1}) of the transaminase towards GA compared to erythrulose (Table 3) when using serine as amine donor. Finally, after 55 h reaction there was an evident depletion of HPA over time, which could be attributed to the chemical degradation as reported by Rios-Solis et al.

One of the main benefits of using enzymes as catalysts for chemical synthesis is their enantioselectivity and enantiospecificity that simplifies the downstream processing. An *ee* > 95% was determined for the ABT synthesised using RSPH17029_3177 with L-serine and erythrulose as substrates. A 95% *ee* was also reported by Rios-Solis and colleagues [17] when synthesising ABT from MBA and erythrulose using CV2025.

4. Discussion

The sequential pathway (Fig. 1) is an interesting approach for chiral amino alcohol synthesis as it is not restricted by an ω-transaminase able to aminate ketones using serine as amine donor. Therefore, this pathway could be used to produce a more diverse set of chiral amino alcohols than the two-step recycling cascade. However, when tested for ABT synthesis it was clearly compromised by the presence of residual substrates (pyruvate, GA and HPA) from the previous enzymatic steps, as they are preferable substrates for the CV2025 enzyme, despite the surplus of serine, pushing the reaction towards the product side [16]. In addition, the increase in ABT concentration did not correlate with the consumption of erythrulose, which can be attributed to ABT or erythrulose degradation either caused by the components in the reaction or by the enzymes. Finally, the implementation of an alternative amine donor that has shown better conversions in other studies [17] had a detrimental effect on this system. A result, in spite of its potential to produce a wide range of chiral compounds, it has difficult biological and engineering challenges that need to be addressed.

On the other hand, the two-step recycling cascade system (Fig. 2) described in this work is a novel approach for the synthesis of chiral amino alcohols. The recycling of the co-product is an attractive *de novo* pathway to integrate with the host metabolism as only two natural substrates are required: serine which is naturally synthesised and has been overproduced [32–34]; and GA is

found in the biosynthetic pathway of folic acid, in the pentose and glucuronate interconversions, and vitamin B6, glyoxylate and dicarboxylate metabolism [18]. This pathway only consists of two enzymatic steps, thereby decreasing the metabolic burden generated by their overexpression and simplifying the expression system required to achieve the necessary enzyme levels within the host. The side reaction catalysed by the transaminase is required for the cascade to take place, having a relatively positive effect on the ABT synthesis, and avoiding the sequential addition of enzymes to increase final yields as described elsewhere [14]. In conclusion, it is not only more efficient than the sequential pathway (6-fold higher conversion), but it is more sustainable and cost-effective.

The two-step recycling cascade requires an enzyme able to aminate both aldehydes and ketones with serine as the amine donor, which has not been reported in the literature to date, as the available transaminases have no or very low activity with the above substrates [35,9,16]. Consequently, several new transaminases were identified and characterised: two new ω-transaminases (RSPH17025_2835 and RSPH17029_3177) with better activity than the transaminase from *C. violaceum* [16], when using serine as amine donor; and a highly selective serine transaminase (DGEO_0713) with high activity towards its substrates serine and pyruvate. The two ω-transaminases are very similar in both DNA sequence and substrate profile, but RSPH17029_3177 possess a higher affinity towards serine and has larger turnover number than RSPH17025_2835.

The implementation of the enzyme RSPH17029_3177 in the cascade proved the system's feasibility for the synthesis of ABT by supplying GA and serine, and its potential for metabolic integration. In addition, according to the substrates profile RSPH17029_3177 is capable of aminating various aliphatic ketones, and so different aliphatic chiral amino alcohols could be potentially produced using the two-step recycling cascade, such as 2-aminopentane-1,3-diol, 2-aminohexane-1,3-diol, 2-aminoheptane-1,3-diol and 2-aminooctane-1,3-diol.

It is important to take into account that the two-step recycling cascade has two main limitations that restrict the possible maximum conversion. One is the differential activity of the transaminase towards GA and erythrulose, whereby GA is preferred by the enzyme; resulting in a large consumption of the GA by the side reaction. The other constraint is the equilibrium of the transamination reaction, which cannot be shifted by the transketolase reaction. This is because the HPA produced by the amination of erythrulose cannot be fully consumed because GA has been fully depleted by the transaminase side reaction with serine and GA as substrates. To fully overcome the system's limitations the transaminase could be engineered to improve its affinity to ketones, and reduce it to aldehydes.

In a previous study, an acetohydroxyacid synthase was coupled to CV2025 for the synthesis of (1R,2S)-norephedrine (NE) from benzaldehyde, pyruvate and alanine as substrates [14], following a similar reaction scheme to the two-step recycling cascade described in this work. The above cascade recycles the pyruvate substrate using two enzymes but requiring three substrates rather than two. When the cascade was performed in one-pot a 2% conversion was achieved based on the pyruvate concentration (10 mM). The final conversion was further 40-times improved by adding the enzymes sequentially to the reaction. In spite of the higher conversion, when implemented in a whole cell system the synthesis of NE presents more limitations than the two-step recycling system described here. It requires the addition of three different substrates and, the enzymes must be finely expressed in a sequential manner to achieve high conversions. As a result its metabolic integration is more complex than the system described in this work.

In summary, this work describes a α-transaminase able to aminate pyruvate using serine as amine donor with high substrate specificity; and two novel ω-transaminases that are more active than the previously described ω-transaminase from *C. violaceum* CV2025 under our reaction conditions and substrates are described; therefore they should be explored further for their implementation in alternative bioconversion processes. We have characterised a novel cascade for the synthesis of chiral amino alcohols that involves a two-step recycle system of the co-product. The implementation of recycling steps within several cascades has been described [36–40], but to the best of our knowledge none of them involve the use of serine as amine donor; as a result our work demonstrates the feasibility of this approach.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.enzmictec.2015.07.003

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