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A Multidisciplinary Approach Toward the Rapid and Preparative-Scale Biocatalytic Synthesis of Chiral Amino Alcohols: A Concise Transketolase-/ω-Transaminase-Mediated Synthesis of (2*S*,3*S*)-2-Aminopentane-1,3-diol

Mark E. B. Smith,[†] Bing H. Chen,[‡,¶] Edward G. Hibbert,[‡,□] Ursula Kaulmann,[§,■] Kirsty Smithies,[†] James L. Galman,[†] Frank Baganz,[‡] Paul A. Dalby,[‡] Helen C. Hailes,[†] Gary J. Lye,[‡] John M. Ward,[§] John M. Woodley,[⊥] and Martina Micheletti*[,‡]

Department of Chemistry, University College London, 20 Gordon Street, London WC1H 0AJ, U.K., Department of Biochemical Engineering, University College London, Torrington Place, London WC1E 7JE, U.K., Institute of Structural and Molecular Biology, University College London, Gower Street, London WC1E 6BT, U.K., and Department of Chemical and Biochemical Engineering, Technical University of Denmark, 2800 Lyngby, Denmark

Abstract:

Chiral amino alcohols represent an important class of value-added biochemicals and pharmaceutical intermediates. Chemical routes to such compounds are generally step intensive, requiring environmentally unfriendly catalysts and solvents. This work describes a multidisciplinary approach to the rapid establishment of biocatalytic routes to chiral aminodiols taking the original synthesis of (2*S*,3*S*)-2-aminopentane-1,3-diol as a specific example. An engineered variant of *Escherichi coli* transketolase (D469T) was used for the initial asymmetric synthesis of (3*S*)-1,3-dihydroxypentan-2-one from the achiral substrates propanal and hydroxypyruvate. A bioinformatics led strategy was then used to identify and clone an ω-transaminase from *Chromobacterium violaceum* (DSM30191) capable of converting the product of the transketolase-catalysed step to the required (2*S*,3*S*)-2-aminopentane-1,3-diol using isopropylamine as an inexpensive amine donor. Experiments to characterize, optimize and model the kinetics of each reaction step were performed at the 1 mL scale using previously established automated microwell processing techniques. The microwell results provided excellent predictions of the reaction kinetics when the bioconversions were subsequently scaled up to preparative scales in batch stirred-tank reactors. The microwell methods thus provide process chemists and engineers with a valuable tool for the rapid and early evaluation of potential synthetic strategies. Overall, this work describes a concise and efficient biocatalytic route to chiral amino alcohols and illustrates an integrated multidisciplinary approach to bioconversion process design and scale-up.

1. Introduction

The pharmaceutical industry today faces significant challenges in bringing new drugs to the market. A key area of interest is in harnessing new technologies to progress from initial drug discovery to the final manufacturing process as rapidly and cost-effectively as possible. Rising costs related to the development of increasingly complex pharmaceuticals (with multiple chiral centers and functional groups) are compounded by increasingly stringent environmental legislation and the drive toward sustainable processes. Working together with industry these are the technologies and challenges that the multidisciplinary UCL Bioconversion - Chemistry - Engineering interface (BiCE) programme aims to address.

While there are extensive synthetic transformations for which chemical (catalytic) conversions are most appropriate, biocatalytic (enzyme and microbial) strategies have been increasingly adopted where mild conditions and high regio- or stereoselectivity are desired.[1,2] The majority of biocatalytic processes reported to date have involved the use of a single isolated enzyme. However, recent developments in metabolic engineering and synthetic biology have highlighted the possibility of using multienzyme synthetic pathways to perform more complex syntheses.[3,4] Furthermore, many of the enzymes that could be used in series do not necessarily exist naturally together in known metabolic pathways. This raises the possibility of creating *de novo* pathways in engineered microorganisms using existing powerful tools such as rDNA technology. As a demonstration of such an approach we have previously devised a synthetic scheme using a transketolase (TK) and a transaminase (TAm) to create an optically enriched 2-amino-1,3-diol using an engineered *Escherichia coli* strain.[5]

Chiral 2-amino-1,3-diols are an important class of pharmaceutically relevant compounds, and their motif is present in antibiotics,[6−10] antiviral glycosidase inhibitors,[11,12] and sphingo-

* Corresponding author. E-mail: m.micheletti@ucl.ac.uk.
† Department of Chemistry, University College London.
‡ Department of Biochemical Engineering, University College London.
§ Institute of Structural and Molecular Biology, University College London.
⊥ Department of Chemical and Biochemical Engineering, Technical University of Denmark.
¶ Current address: Department of Chemical and Biochemical Engineering, College of Chemistry and Chemical Engineering, Xiamen University, Xiamen 361005, China.
□ Current address: Illumina Cambridge Ltd., Chesterford Research Park, Cambridge CB10 1XL, UK.
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lipids.[13,14] A number of chemical methods exist to produce this class of amino alcohols including those starting from the chiral pool materials serine[8,15,16] and glucose,[17] Sharpless asymmetric dihydroxylation with subsequent regioselective azide substitution[18,19] and nucleophilic attack of α,β-epoxy carboxylic esters with azide followed by azide and ester reduction.[20] However, these procedures are either step-intensive and/or make use of toxic catalysts. We have recently developed the first nonenzymatic one-pot synthesis of α,α′-dihydroxyketones via a mimetic of the transketolase reaction.[21] However, no asymmetric variant of this synthesis has been developed to date, and thus reductive amination of the racemic products obtained from this reaction can only be manipulated into mixtures of diastereoisomers. Alternatively, using TK in combination with a TAm potentially offers a highly concise, stereospecific, and benign biocatalytic route to this key class of synthons.

In vivo TK catalyses the transfer of a two-carbon ketol unit from D-xylulose-5-phosphate, to either D-ribose-5-phosphate or D-erythrose-4-phosphate.[22] The enantioselective carbon−carbon bond-forming ability of TK, together with the ability to yield an irreversible reaction when using β-hydroxypyruvate (HPA) as the ketol donor, makes it very attractive as a biocatalyst in industrial synthesis.[23,24] Although nonhydroxylated aliphatic aldehydes can be accepted by TK, the activity is typically very low. Using active-site targeted saturation mutagenesis we have recently identified several mutants with improved activities, notably a novel TK mutant, D469T, with a nearly 5-fold increase in specific activity when screened towards the nonhydroxylated aldehyde acceptor substrate, propionaldehyde (PA), for the production of 1,3-dihydroxypentan-2-one (DHP).[25] Other mutants with improved, and even reversed, enantioselectivity have also been described.[26] ω-Transaminases, such as that isolated from *Vibrio fluvialis*, have been shown to aminate a wide range of aldehyde and ketone substrates. This is not true for α-transaminases which typically have a strong preference for either α-ketoacids or α-amino acids as substrates.[27] However, several transaminases which efficiently aminate an aromatic α,α′-dihydroxyketone have been described.[28] Recently we used the published sequence of the ω-TAm isolated from *V. fluvialis* to conduct a bioinformatics-based search of genome homologues, thereby facilitating the recruitment and characterization of novel ω-TAms.[28] The production of chiral aliphatic 2-amino-1,3-diols from chiral ketodiols using the ω-TAm recruited from *Chromobacterium violaceum* DSM30191 (CV2025) would demonstrate the significant commercial potential of this enzyme and class on bioconversion.

From an engineering perspective, there are several factors contributing to the successful implementation of a multienzymatic process such as a linked TK−ω-TAm bioconversion. In order to achieve a competitive final product yield the reaction rates and initial substrate loading(s) need to be maximised, whilst at the same time overcoming issues of substrate or product inhibition of each enzyme. The identification of the "best" biocatalyst at an early stage is crucial since recent advances in protein engineering have enabled the subsequent modification of enzymes to achieve greater activity, enhanced stability and enantioselectivity, and wider substrate range.[29] Ultimately, experiments performed early during development need to provide insight into the most suitable strategies for process optimization and scale-up in order to maximize the yield of product on substrate and catalyst. In this regard we have recently established a range of automated and microscale (100−1000 μL) experimental techniques to successfully mimic key bioprocess unit operations. An understanding of the engineering fundamentals governing experimentation at this scale underpins the ability to obtain quantitative results capable of predicting larger-scale process performance.[30−32]

The aim of this work is to demonstrate a challenging and novel example of a two-step stereoselective biocatalytic synthesis of 2-amino-1,3-diols using a multidisciplinary approach that integrates (1) enzymes obtained either by directed evolution or bioinformatics-based cloning strategies, (2) microscale experimentation and robotics for rapid process optimisation and scale-up, (3) advanced high-throughput analytical techniques for small-molecule detection and analysis, and (4) establishment of an enzymatic kinetic model from microscale data for the rapid design of the larger-scale bioconversion process. Specifically, as shown in Scheme 1, we have focused on the TK D469T-catalysed conversion of propanal (**1**) and hydroxypyruvate (HPA) (**2**) to (3*S*)-1,3-dihydroxypentan-2-one (DHP) (**3**) and the subsequent CV2025 ω-TAm-catalysed conversion of DHP to (2*S*,3*S*)-2-aminopentane-1,3-diol (APD) (**4**).

2. Results and Discussion

2.1. Multidisciplinary Approach and Strategy. Taking into account the lack of kinetic information available on the TK D469T and CV2025 ω-TAm-catalyzed reactions, a detailed

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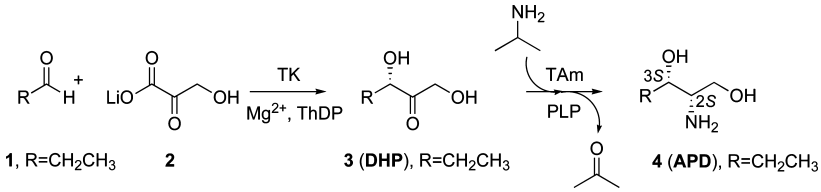
Scheme 1. **Two-step biocatalytic synthesis of 2-amino-1,3-diols using transketolase (TK) and transaminase (TAm)**



initial characterization was considered necessary for both enzymes in order to determine the optimal reaction conditions in terms of substrate concentration ratios, enzyme loading, and in the case of the ω-TAm-catalysed step, potential use of a cosolvent. This lack of initial kinetic information is a common feature at the early stages of bioconversion process evaluation and so is typically the starting point for experimental studies. Automated microscale experimentation was initially used for this purpose, and experiments were planned with a view to subsequent integration of the two steps and ultimately process scale-up. Development of a successful and scalable enzymatic synthesis of APD (**4**) involved specifically: (i) evaluation of the TK D469T-catalysed step (section 2.2), (ii) evaluation of the CV2025 ω-TAm-catalysed step (section 2.3), and (iii) integration of the two enzymatic steps such that a concise synthesis of APD (**4**) could be successfully modeled and then verified experimentally at preparative scale (section 2.4). Underpinning this approach was the establishment of high-throughput assays to characterize the synthetic potential of the enzymes and to quantitatively determine the influence of process conditions on biocatalyst performance.

2.2. TK Evaluation. Mutants of *Escherichia coli* transketolase with improved substrate specificity towards the non-natural aldehyde substrate propanal have been obtained by directed evolution in a previous study.[25] In particular mutant D469T was identified from an active site library of TK mutants as being a likely catalyst, showing a 4.9-fold increase in specific activity with this substrate, compared to wild-type TK. The D469 residue was previously identified as important for substrate binding by Schörken who carried out a kinetic analysis of several *E. coli* transketolase mutants obtained by using site-directed mutagenesis.[33,34] This mutant enzyme was therefore selected for use in the proposed biocatalytic process demonstration (Scheme 1). The availability of an analytical method for the study of the TK-catalyzed reaction was crucial in order to quantify the reaction kinetics. Several TK assays have been reported for product quantification;[23,35−37] however, an HPLC assay was established[25] in order to detect both HPA (**2**) starting material and the product of the propanal reaction, DHP (**3**). The assay time and sample loading needed were minimized to increase the throughput in accord with the microscale experimentation strategy. In-house chemical synthesis[21] of HPA (**2**) and racemic DHP (**3**) provided samples for calibration purposes to ensure complete quantitative analysis of this reaction. As propanal is a rather volatile substrate, control experiments were carried out to confirm that no loss was occurring during sampling and that all mass balances were closed.

The TK D469T-catalyzed reaction required optimization in order to achieve the highest possible product yield and reaction rate. For simplicity of operation, a batch reaction approach to performing the bioconversion was adopted. The final concentration of both propanal (**1**) and HPA (**2**) were dictated to a degree by the necessary requirement to preincubate the enzyme with its cofactors, ThDP and Mg^{2+}, prior to the introduction of the substrates. It has been previously documented that aldehyde substrates react with primary amines forming a Schiff base and may therefore react with enzymes exhibiting these functional groups in accessible positions, thus affecting enzyme activity.[35] A significant effect of glycolaldehyde on holo- and apo-transketolase activity has been shown at a range of aldehyde concentrations.[35] For this reason a strategy of working at an excess of propanal was immediately ruled out. Taking into account the economic implications of working at high HPA concentrations, it was decided on the basis of these observations and requirements to optimize the TK-catalysed step using equimolar and stoichiometric concentrations of propanal and HPA.

All TK D469T evaluation experiments were carried out at 1 mL scale and at room temperature using previously established automated microwell methods.[31] At this scale the reaction pH was controlled at pH = 7 using appropriate buffers and concentrations. Initial experiments were carried out at 50, 100, 200, and 300 mM concentrations of reagents in stoichiometric amounts with 30% v/v cell-free lysate (Figure 1a). At 200 mM substrate concentration the reaction went to completion in 8−10 h, with reaction rates increasing from 22.2 mmol L^{-1} h^{-1} of DHP (**3**) to 57 mmol L^{-1} h^{-1} at 50 and 200 mM initial substrate concentrations, respectively. When the initial substrate concentration was set at 300 mM, an initial rate of 45.3 mmol L^{-1} h^{-1} was obtained, indicating the probable effects of aldehyde inhibition and/or toxicity on the enzyme. Increasing the amount of enzyme used increased the final product yield (Figure 1b). Increasing the lysate concentration from 30% to 50% v/v, whilst maintaining the substrate concentration at 300 mM, allowed complete conversion to DHP (**3**) to be achieved in 5−10 h and with an initial rate of 92.5 mmol L^{-1} h^{-1}. This set of microwell reaction conditions was therefore selected as the optimum for the TK D469T-catalysed synthesis of the intermediate product (3*S*)-1,3-dihydroxypentan-2-one (DHP) (**3**) and was subsequently adopted for the larger-scale reaction (section 2.4).

2.3. TAm Evaluation. The value of the well-known *V. fluvialis* ω-TAm JS17 for synthetic applications has already

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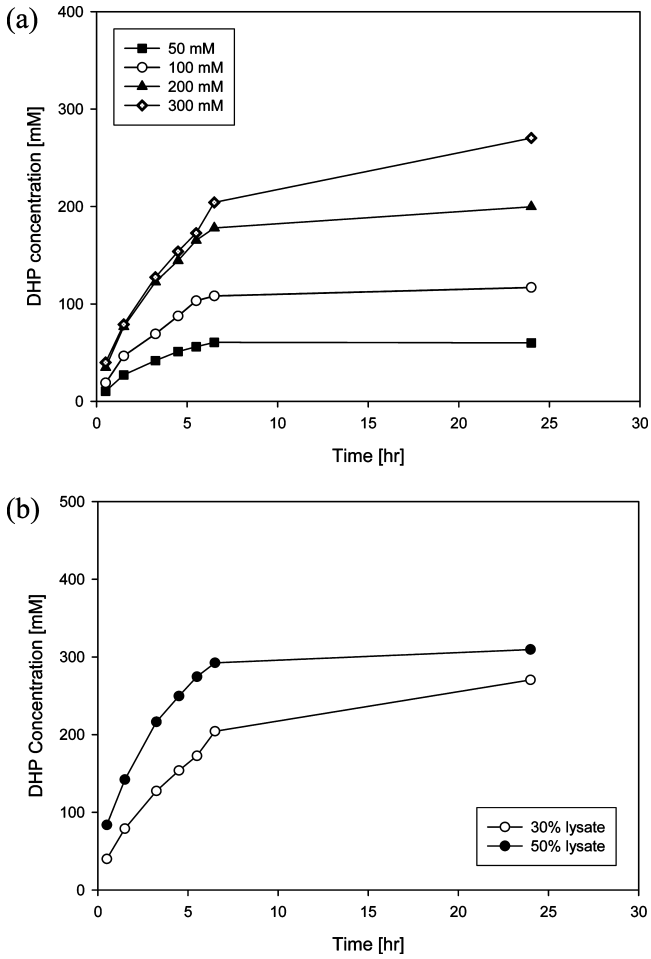

Figure 1. **Characterization of the TK D469T reaction step at 1 mL scale. (a) Effect of substrate, propanal and HPA, concentration on product formation kinetics at stoichiometric conditions with 30% v/v lysate. (b) Effect of enzyme concentration on product formation kinetics at stoichiometric conditions and 300 mM substrate concentration.**

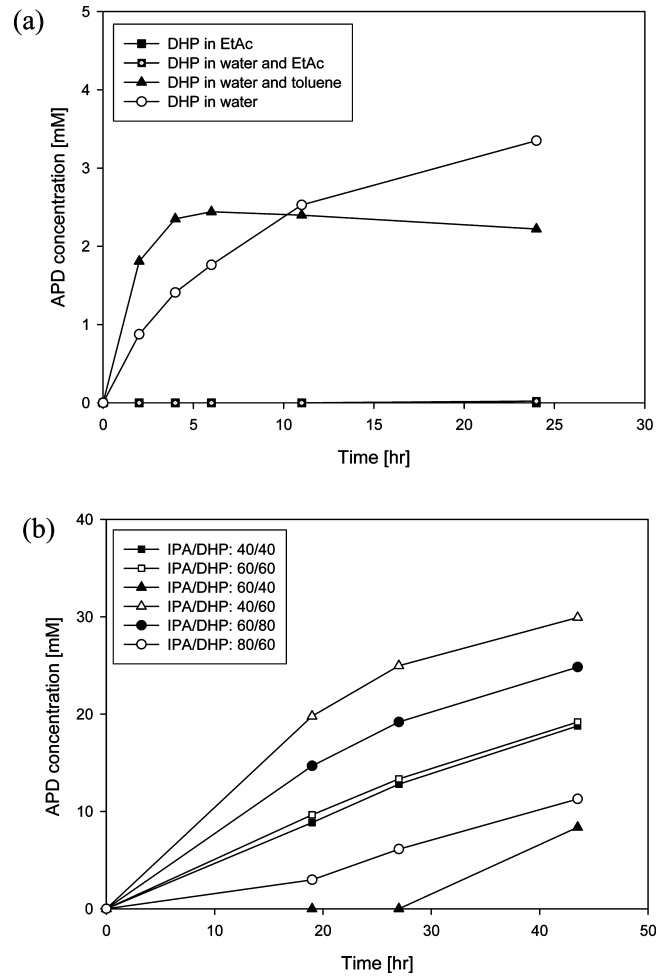

Figure 2. **Characterization of the TAm reaction step at 1 mL scale. (a) Effect of cosolvent and type on product formation kinetics using the CV2025 ω-TAm. (b) Effect of substrate, IPA, and DHP ratio on product formation kinetics.**

been widely recognized. Both the ketol 3-hydroxy-2-butanone (acetoin) and the aromatic amino alcohol 2-phenylglycinol are reported to be accepted by the enzyme as substrates.[38] *V. fluvialis* and related ω-TAms therefore have the potential to be used as biocatalysts in the preparation of chiral 2-amino-1,3-diols. Since the *V. fluvialis* ω-TAm is not publicly available, a detailed search for homologues of this enzyme was carried out in the genome databases of fully sequenced bacteria using the published protein sequence of the enzyme. This led to the identification, cloning into *E. coli*, and partial characterization of 12e ω-TAms from a variety of bacteria. The most efficient ω-Tam[28] for aminodiol synthesis among all those recruited was that from *C. violaceum* DSM30191 (CV2025). Initial experiments using the CV2025 enzyme with DHP (**3**) indicated it was converted to APD (**4**), and so further experiments were performed using this enzyme.

Preliminary experiments to investigate the potential of running a one-pot reaction found that the CV2025 ω-TAm could act upon the TK substrates in the presence of an amine donor (data not shown). Consequently, it was decided to run the synthesis as two sequential steps. Since solvent extraction was envisaged as the likely method for isolation of the intermediate product DHP (**3**) from the initial TK D469T-catalysed reaction, an appraisal of solvent tolerance was carried out for the *C. violaceum* ω-TAm. All evaluation experiments were carried out at the 1 mL microwell scale using buffers for pH control as in the TK studies. Ethyl acetate (EtOAc) and toluene were initially evaluated as solvents with differing polarities and physicochemical properties. The influence of these solvents on the kinetics of the CV2025 ω-TAm-catalysed conversion of DHP (**3**) to APD (**4**) using isopropylamine (IPA) as the amine donor is illustrated in Figure 2a. When the transamination of DHP (**3**) in EtOAc was carried out, no aminodiol product was formed. Similarly, no product was detected when the reaction was carried out in a mixture of EtOAc and water. Using toluene as a cosolvent proved to be more positive with an excellent initial rate of reaction observed. However, after a relatively short period of time the reaction ceased, suggesting that the solvent may be having an adverse effect on the CV2025 ω-TAm over time. It was therefore concluded that use of a cosolvent was not appropriate in the TAm-catalysed step. Consequently, it would be necessary to isolate the intermediate DHP product from the TK D469T-catalysed reaction by solvent extraction prior to the second CV2025 ω-TAm step.

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Table 1. **Characterization of the CV2025 ω-TAm reaction at 1 mL scale: impact of the amount of cell lysate on the extent of APD (4) formation over 24 h based on initial substrate concentrations of 40 mM of IPA and 60 mM of DHP**

lysate (enzyme) concentration (% v/v)	[APD] after 6 h (mM)	[APD] after 24 h (mM)
30	8.7	22.7
40	11.8	22.5
50	12.8	19.6
60	13.5	21.5
70	14.3	20.0
80	18.1	22.0

Given that the product yield from the ω-TAm step will depend upon the equilibrium position of the reaction, another crucial variable with regard to process optimization is the ratio of the amine donor to DHP (**3**). Initial experiments to investigate this were conducted with a number of amine donors (data not shown), systematically varying each of the substrate concentrations while maintaining the other constant. The aim of these experiments was to identify donors with minimal inhibition effects on the enzyme. Preliminary studies conducted with (*S*)-(α)-methylbenzylamine, which has been used in other transaminase systems,[39] showed the detrimental effect of using an excess of this amine donor, probably due to its toxicity towards the enzyme. Attempts were made to reduce this effect by carrying out the reaction in the presence of a nonpolar organic solvent, the aim being to diminish the effective concentration of the amine in the ω-TAm-containing aqueous phase whilst ensuring that the DHP (**3**) remains in the reactive phase. The use of hexadecane allowed for higher concentrations of amine to be present in the reactor; however, the maximum working concentration of (*S*)-(α)-methylbenzylamine was still limited to 40 mM. Alternative amine donors were therefore examined, and similar conversions were achieved with a cheaper, more volatile, and more water-soluble substrate, isopropylamine (IPA). At comparable reaction concentrations the quantity of product **4** formed was similar when using (*S*)-(α)-methylbenzylamine and IPA without the need for additional organic solvent. In addition, any excess IPA (and its product, acetone) could be more easily removed downstream compared to the aromatic amine.

Figure 2a shows the results obtained at different substrate ratios of the selected amine donor, IPA, and DHP (**3**) using 30% v/v lysate. The highest concentration of APD (**4**) achieved was 30 mM, obtained with 40 mM of IPA and 60 mM DHP. In order to further increase the reaction rate, increased quantities of cell lysate were used that allowed for the same amount of product to be formed in shorter periods of time. The results of these experiments are shown in Table 1. Approximately the same amount of product formed using 30% v/v cell lysate in 48 h could be obtained in 24 h using 80% v/v cell lysate. The enzyme activity values, based on initial rates, were 10.8 and 15.6 U g^{-1}_{TAm} for 30% and 80% v/v cell-free lysate concentrations, respectively. A compromise is therefore needed between the cost of preparing an increased amount of cell lysate and the overall reaction time when operating conditions for scale-up are selected. It is noteworthy that the reaction could be potentially driven to completion by feeding of one of the two substrates or by enzymatic[44] or in situ product removal.[45]

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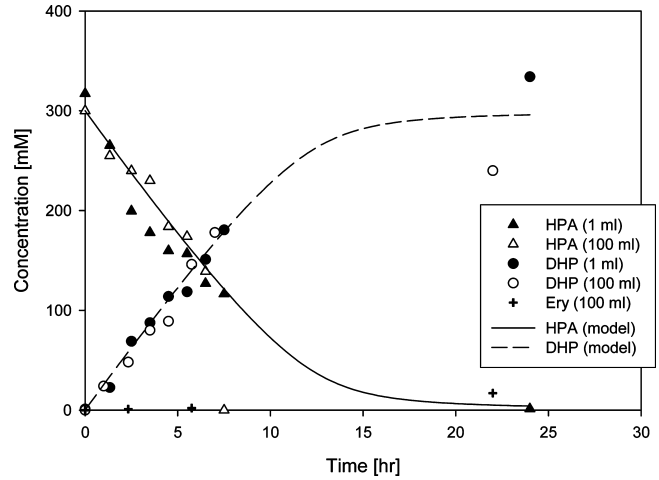

Figure 3. **Comparison of TK D469T reaction kinetics at microwell (open symbols) and 100 mL (solid symbols) scales and with the kinetic model predictions (lines). TK reaction performed at 300 mM stoichiometric substrate concentration and 40% v/v lysate. Model predictions are based on eq 1 using the kinetic parameters shown in Table 2 determined from microscale experiments.**

2.4. TK-TAm Reaction Scale-up, Modeling, and Integration. Once the optimal conditions for each biocatalytic step had been assessed, the reactions optimized at microwell scale were scaled to 100 mL scale for the TK D469T reaction and 333 mL for the ω-TAm reaction. Both reactions were performed in laboratory-scale batch stirred-tank reactors. At the 100 mL scale, the pH of the TK-catalysed reaction was maintained at pH 7.0 by the use of automated pH stat addition of 1 M HCl (aq) to minimize the salt concentration in the reactor outlet and waste stream. The kinetic profiles for the TK D469T reactions conducted at both 100 and 1 mL scale, using 300 mM of both HPA and PA and 40% v/v cell lysate, are shown in Figure 3. The results of the microwell optimized conditions have scaled excellently, showing very similar kinetic profiles after a 100-fold scale-up. Calculated values of the initial reaction rates varied by just 15%, confirming the utility of the automated microwell experiments for rapid process optimization and scale-up. The product DHP (**3**) was extracted from the reaction via multiple solvent extractions into EtOAc. Subsequent removal of the solvent under vacuum gave the product as an oil in 68% yield. The ee of the product was determined by diacetylation and chiral GC to be comparable to that previously reported of 64%.[26] No reduction in ee was thus observed during the transition to preparative-scale operation. This crude DHP material was taken forward without further purification as substrate for the following ω-TAm step of the synthesis.

At this stage in process development a reliable enzyme kinetic model for the TK D469T mutant would be desirable in order to predict reaction rate and yield over a range of bioreactor operating conditions. The general TK reaction mechanism is well-known and leads to the following kinetic expression based on competitive substrate inhibition:

$$\frac{d[Q]}{dt} = \frac{k_{cat}E_i[A][B]}{K_b[A]\left(1 + \frac{[A]}{K_{ia}}\right) + K_a[B]\left(1 + \frac{[B]}{K_{ib}}\right) + [A][B] + \frac{K_a}{K_{iq}}[B][Q] + \frac{K_aK_{ib}}{K_{iq}}[Q]} \quad (1)$$

Solutions to such models are, however, difficult to establish due to the large number of experiments required to determine the specific kinetic constants for a particular enzyme mutant and set of reaction substrates. We have previously reported a model-driven approach to kinetic parameter identification that combines automated microscale experimentation with numerical methods for kinetic parameter estimation from a minimum number of experiments.[40,41] This approach was used here to rapidly determine the kinetic parameters for the new TK D469T mutant for DHP (**3**) synthesis from propanal (**1**) and HPA (**2**).

The specific reaction kinetic parameters obtained are presented in Table 2. Comparing the model parameters it can be seen that there is a major difference in reaction rate between use of propanal (**1**) as substrate compared to the kinetic parameters previously reported for glycolaldehyde.[42] Another key difference is that the Michaelis constant for propanal is 6 times that of glycolaldehyde. The consequence of this for process design is that significantly more TK D469T will be required for the propanal/HPA reaction in order to achieve the same bioconversion rate. Using eq 1 and the kinetic parameters listed in Table 2 from microscale experimentation, Figure 3 also shows the predicted preparative-scale bioconversion kinetics (solid and dashed lines). The good agreement between the experimentally determined preparative-scale reaction kinetics and the model predictions confirms the utility of the modeling approach. Such modeling tools can build confidence for process chemists and engineers in process scale-up, reactor sizing, and first approximations of the process costs.

Following the TK D469T-catalysed synthesis of DHP (**4**), the subsequent CV2025 ω-TAm reaction was performed using 40 mM IPA and 60 mM DHP together with cofactor PLP and 30% v/v clarified lysate in HEPES buffer. After 24 h, an Amberlite IRA-410 resin was added with stirring to the final product mixture to remove any HEPES from solution. The filtrate was then passed through an Isolute SCX-2 (Biotage) ion-exchange column which retained the desired APD product (**4**) whilst allowing all other impurities to be washed away. The APD was then released from the column by elution with 4 M ammonia in methanol, giving the product as a solid in 26% isolated yield. Reports using (*S*)-(α)-methylbenzylamine as the amine donor with ω-TAms and our studies using the *C. violaceum* ω-TAm with aromatic α,α′-dihydroxyketones have indicated selectivity for formation of the *S*-amine.[19,39] The ee of the product **4** at C-2 was determined to be >98% and postulated from previous work to be the 2*S*-isomer. The de of **4** was 61% by chiral HPLC, the impurity being exclusively (2*S*,3*R*)-2-aminopentane-1,3-diol. This indicated that the *C. violaceum* ω-TAm was able to accept both (*R*)- and (*S*)-isomers of **3**, highlighting its potential versatility, and demonstrated excellent stereoselectivity in catalyzing the conversion of the ketone DHP (**3**) to the amine moiety in APD (**4**).

Table 2. **Enzyme kinetic parameters for the TK D469T-catalysed conversion of propanal, PROP (1), and HPA (2) into (3*S*)-1,3-dihydroxypentan-2-one (3)[a]**

kinetic parameters	value
rate constant: K_{cat} (min^{-1})	501
Michaelis constant for HPA: K_a (mM)	12
Michaelis constant for PROP: K_b (mM)	98
inhibition constant for HPA: K_{ia} (mM)	43
inhibition constant for PROP: K_{ib} (mM)	625
inhibition constant for DHP: K_{iq} (mM)	681

[a] Kinetic parameters determined using the method described in section 2.4 from 34 microscale (1 mL) experiments at a range of substrate and enzyme concentrations.

Table 3 provides a summary of the reaction and recovery yields of each step of the TK D469T and CV2025 ω-TAm-catalysed route to APD (**4**). For the TK reaction there is particularly good agreement between the reaction rates (as previously discussed) and yields achieved at the two scales. The isolated yield of the final product at 26% is acceptable at this stage since little attention has been directed toward optimization of the recovery process. This two-step biocatalytic synthesis represents an extremely concise and atom-efficient route to such aminodiols. Indeed, a recent study focused on compound screening for fungicidal activity reported the nine-step synthesis of the *anti*-2*S*,3*R*-isomer of APD.[42] The biocatalytic route reported here thus represents a significant improvement. On the basis of the process design information obtained at the microwell scale (sections 2.2 and 2.3) and subsequently verified here at the preparative scale, Figure 4 shows a feasible process flowsheet of how the complete two-step biocatalytic synthesis might proceed at a manufacturing scale. Standard geometry stirred tank bioreactor configurations would be best suited for the two bioconversion steps since for the TK reaction at least mass transfer limitations have been reported when performed without mixing or shaking at volumes higher than 10 mL.[43] The product of the first biocatalytic reaction, an aqueous mixture of DHP and other lysate components, can be followed by a suitable enzyme recovery step, such as ultrafiltration, from where the TK enzyme could be recycled to the bioreactor. A solvent extraction step using EtOAc in a simple mixer/settler unit is necessary to separate the DHP (**3**) from the other components. After removal of the EtOAc under vacuum, the DHP would then be redissolved in water to the optimal concentration for the following CV2025 ω-TAm reaction. Following enzyme separation the product mixture from this reaction containing ADP (**4**) and unreacted substrates would

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Table 3. **Comparison of microscale and preparative-scale yield data for the TK D469T (batch reactor 1) and CV2025 ω-TAm (batch reactor 2) conversions showing reaction conditions, product concentrations, and isolated product yields (N/D = not determined)**

	batch reactor R1 [D469T, 300 mM HPA/PA, TK 40% v/v]		batch reactor R2 [CV2025, 40 mM IPA/60 mM DHP, TAm 30% v/v]	
reaction scale (mL)	product [DHP] (mM)	isolated yield (%)	product [APD] (mM)	isolated yield (%)
1	300	N/D	22.7	N/D
100	300	68	37.8	26

undergo a simple resin-based purification step, as described above, prior to a final evaporation step to obtain the dry solid product.

3. Conclusion

The two-step biocatalytic preparative synthesis of (2*S*,3*S*)-2-aminopentane-1,3-diol from the achiral starting substrates, propanal and hydroxypyruvate, and the inexpensive amine donor, isopropylamine, represents a concise and efficient route to this class of compound. The synthesis was facilitated by the ability to engineer the TK enzyme to accept the non-natural substrate propanal and the use of bioinformatics-led strategies to identify a suitable ω-TAm able to accept the intermediate TK product and convert it into the final desired synthon. The use of automated microscale processing techniques facilitated rapid optimisation and initial scale-up of the two biocatalytic steps. Such microscale approaches are particularly useful at the early stages of process development where quantities of key intermediates and information of their reaction kinetics are scarce.

While the TK D469T mutant displays desirable reaction rates and yields, alternative recently identified mutants could be used,[25] or the D469T mutant could be further engineered to specifically improve the ee of the product of this reaction step. Similarly, now that a suitable ω-TAm has been cloned and overexpressed, strategies to increase the rate and yield of this step could be explored including directed evolution of the enzyme or the application of techniques to shift the reaction equilibrium to product formation such as enzymatic byproduct removal[44] or *in situ* product removal.[45]

4. Experimental Section

Materials. Unless otherwise noted, solvents and reagents were reagent grade from commercial suppliers (Sigma-Aldrich) and used without further purification. ^{1}H NMR and ^{13}C NMR spectra were recorded at the field indicated using a Bruker AMX300 MHz and AMX500 MHz machines. Coupling constants are measured in hertz (Hz) unless otherwise specified, NMR spectra were recorded at 298 K. Mass spectra were recorded on a Thermo Finnegan MAT 900XP spectrometer. LC-MS was performed on a Finnigan LTQ mass spectrometer and an Agilent 1100 HPLC (G1322A degasser, G1311A quaternary pump, and a G1367A autosampler). The data were processed using Xcalibur Quan browser. Lithium hydroxypyruvate was synthesised as previously described.[46] The TK D469T mutant was engineered, overexpressed in *E. coli*, and obtained as a clarified lysate as reported elsewhere.[25] Similarly the CV2025 ω-TAm from *C. violaceum* was cloned, overexpressed in *E. coli*, and obtained as a clarified lysate as reported previously.[28]

Automated Microscale Bioconversion Methods. TK D469T and CV2025 ω-TAm bioconversions were carried out in 96-deep square well (96-DSW) and 96-standard round well (96-SRW) microplate formats over a range of experimental conditions. Automated microscale process sequences involving bioconversion setup and operation, sample acquisition, and processing were established for both reaction steps shown in Scheme 1. Automation was achieved using a Genesis Workstation (Tecan, Berkshire, UK) equipped with a liquid handling arm and a RoMa arm for plate manipulation. Disposable tips were used for reagent addition. The accuracy and precision of pipetting were determined for the low viscosity liquids and dispense volumes used in this work, and the error was less than 5% in all cases.[32] All the experiments were carried out in a Class II robotic containment cabinet.

Analytical Methods. Progress of the TK D469T-catalysed synthesis of (*S*)-1,3-dihydroxypentan-2-one was monitored by HPLC: 100% of 0.1% TFA in water, 0.6 mL/

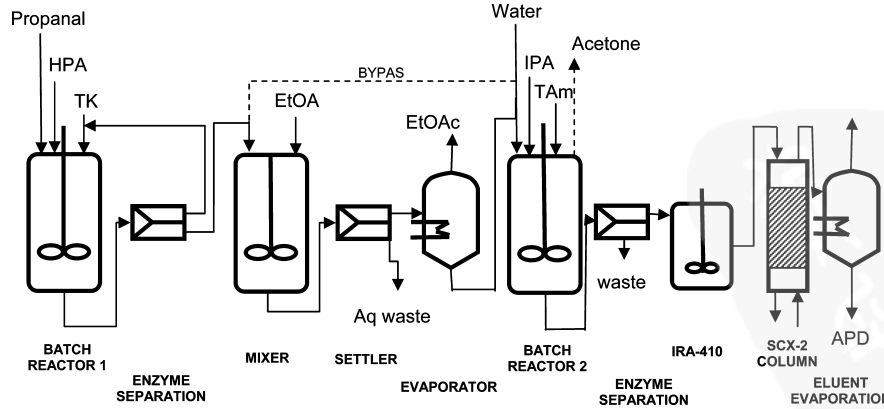

Figure 4. **Proposed manufacturing process flow sheet for the large-scale, two-step TK D469T (batch reactor 1) and CV2025 ω-TAm (batch reactor 2) biocatalytic synthesis and purification of (2*S*,3*S*)-2-aminopentane-1,3-diol.**

min, a 15 cm C18 column, 0.1% TFA (aq), 0.6 mL/min, UV detection at 210 nm, HPA **2** 8.95 min, DHP (**3**) 15.55 min, propanal 22.53 min.[25]

The ee of the (*S*)-1,3-dihydroxypentan-2-one was determined by first derivatising the ketodiol to the diacetate as previously described.[26] The assay was performed against a racemic sample of the diacetate using chiral GC: β-Dex column (Supelco, 30 m $\times$ 0.25 mm); injection volume, 1 μL; carrier gas, He; carrier gas pressure, 15 psi; injector temperature, 250 °C; oven temperature, 60 °C and then increased at 10 °C/min to 160 °C and held; detector temperature, 300 °C; detection, flame-ionised detector (FID). Retention times: (*R*)-isomer, 13.7 min; (*S*)-isomer, 13.9 min.

Progress of the ω-TAm-catalysed synthesis of (2*S*,3*S*)-2-aminopentane-1,3-diol was monitored by LC-MS. Ten microliters of the aminodiol biotransformation reaction mixture was dissolved in 990 μL acetonitrile. The solutions were then homogenised by vortex for 10 s in a 2 mL centrifuge tube. This was followed by centrifugation at 13000 rpm for 5 min. The supernatant was transferred to a glass autosampler vial and 1 μL injected into the chromatographic system. For screening the reaction, separation between DHP and APD was achieved using isocratic conditions of 40% 10 mmol NH_4OAc and 60% acetonitrile at a flow rate of 0.2 mL min^{-1}, and the analytical column was a ZIC-HILIC column (50 mm $\times$ 2.1 mm, i.d., 5 μm particle size) maintained at 20 °C. Operating conditions of the ESI interface in positive ion mode: capillary temperature 245 °C, capillary voltage 2 kV, spray voltage 4 kV, sheath gas 30, auxiliary gas 0, sweep gas 10 arbitrary units. For LC-MS calibration purposes stock standard solutions (10 mmol) of APD were prepared in water. Six calibration standards were prepared (0.6−12 ng/mL) covering the possible concentration range of the reaction (0−10 mmol conversion) and six point calibration curves were generated. Over the concentration ranges linear regression of observed peak areas against concentration gave correlation coefficients from 0.9935 to 0.9996. Limit of quantitation (LOQ) for APD from the low-level spiked matrix with 1 μL injected on a 2.1 mm i.d. column was determined (S/N = 3) to be 0.6 ng/mL.

The ee and de of the (2*S*,3*S*)-2-aminopentane-1,3-diol (**4**) was determined by first derivitising the aminodiol to the tribenzoate. The assay was performed against a four diastereoisomer sample of the tribenzoate (synthesised as described in the Supporting Information) using chiral HPLC: Chiracel-OD column (Daicel); mobile phase, isopropanol/hexane (5:95); flow rate, 0.8 mL/min, detection, UV 214 nm. Retention times; 26.7 min, 31.5 min, 40.6 min, 47.8 min for the (2*R*,3*S*)-, (2*S*,3*S*)-, (2*S*,3*R*)-, and (2*R*,3*R*)-isomers, respectively.

Preparation of (*S*)-1,3-Dihydroxypentan-2-one. Thiamine diphosphate (111 mg, 240 μmol) and $MgCl_2 \cdot 6H_2O$ (183 mg, 900 μmol) were added to a reaction vessel containing H_2O (6.7 mL), and the pH was adjusted to 7.0 using 0.1 M NaOH (aq). To the stirred solution was added clarified TK D469T lysate (40 mL, XL10:pQR412(D469T), containing 1.37 mg/mL of TK by densitometry), and the mixture stirred for 20 min. In another flask, lithium hydroxypyruvate (3.30 g, 300 mmol) and propanal (2.16 mL, 30 mmol) were dissolved in H_2O (53.3 mL), and the pH was adjusted to 7.0 using 1 M NaOH (aq). Following the 20 min enzyme incubation, the solution of hydroxypyruvate and propanal was added to the enzyme solution with stirring, and the reaction commenced. The pH of the reaction was maintained at 7.0 using a pH stat (Metrohm Stat Titrino) *via* addition of 1 M HCl (aq) and the progress of the reaction followed by HPLC. After 22 h, the crude reaction mixture was extracted with EtOAc (10 $\times$ 100 mL), and the combined organics were dried over $MgSO_4$. Filtration and concentration of the organics yielded the desired compound (2.40 g, 68%). The ee of the product was determined by chiral GC to be 61% (*S*-isomer). Found (+HRCI) MH^+, 119.07043; $C_5H_{11}O_3$ requires 119.07082. ^{1}H NMR (300 MHz; D_2O): δ 0.82 (3H, t, *J* 7.5, CH_3), 1.49−1.76 (2H, m, CH_2CH_3), 4.22 (1H, dd, *J* 7.7 and 4.4, C*H*OH), 4.37 (1H, d, *J* 19.4, CH_2OH), 4.46 (1H, d, *J* 19.4, CH_2OH); ^{13}C NMR (75 MHz; H_2O): δ 8.7, 26.5, 65.3, 76.1, 214.5.

Preparation of (2*S*,3*S*)-2-Aminopentane-1,3-diol. (*S*)-1,3-Dihydroxypentan-2-one (2.36 g; 0.5 M in water, 40 mL) was added to a solution of isopropylamine (1.25 mL; 6.7 M in water, 4.54 mL, pH 7.5) in 1 M HEPES (23.3.mL, pH 7.5) containing and pyridoxal 5′-phosphate hydrate (17.1 mg, 69 μmol) To this mixture was further added water (165.5 mL) and clarified CV2025 ω-TAm lysate (100 mL). Lysate was obtained in 100 mM HEPES, pH 7.5, containing 0.2 mM PLP, 0.5 mM phenylmethyl sulfonyl fluoride (PMSF), and 8 U of benzonase (Novagen)/mL of cell extract. The reaction was shaken in a 500 mL Erlenmeyer flask at 100 rpm and 37 °C for 48 h and monitored exclusively by LC-MS. The enzyme was spun down (4500 rpm, at 4 °C for 10 min), and the mixture was additionally filtered through a 0.2 μm sterile filter. Amberlite IRA-410 resin (50 g) was added to the reaction mixture. After 1 h, the resin was removed by filtration and washed with H_2O (300 mL). The aqueous mixture was then passed through an Isolute SCX-2 (Biotage) ion-exchange column (40 g of resin) and the column washed with MeOH (2 $\times$ 200 mL). The column was then eluted with 4 M NH_3 in MeOH (3 $\times$ 200 mL) and the eluent concentrated to yield the desired product as a solid (410 mg, 26%). The ee of the (2*S*,3*S*)-product was determined to be >98% (*S*-isomer at C-2) and the de determined as 61% by chiral HPLC, the impurity being exclusively (2*S*,3*R*)-2-aminopentane-1,3-diol. Found (+HRCI) MH^+, 120.10250; $C_5H_{14}NO_2$ requires 120.10245; ^{1}H NMR (500 MHz; D_2O): δ 0.93 (2.4H, t, *J* 7.5, CH_3), 0.94 (0.6H, t, *J* 6.5, CH_3), 1.40−1.60 (2H, m, CH_2CH_3), 2.76 (0.8H, ddd, *J* 6.3, 5.1, and 4.6, C*H*NH_2), 2.81 (0.4H, ddd, *J* 7.5, 5.9, and 4.1, C*H*NH_2), 3.47−3.76 (3H, m, CH_2OH and C*H*OH);^{13}C NMR (75 MHz; H_2O): δ (2*S*,3*S*) 10.1, 26.3, 55.8, 64.1,73.7; (2*S*,3*R*) 9.9, 25.6, 56.2, 63.7, 74.7.

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NOMENCLATURE

APD	(2*S*,3*S*)-2-aminopentane-1,3-diol
DHP	1,3-dihydroxypentan-2-one
EtOAc	ethylacetate
HPA	β-hydroxypyruvate
IPA	isopropylamine
PA	propionaldehyde
PLP	pyridoxal 5′-phosphate
TAm	transaminase
TFA	trifluoroacetic acid
ThDP	thiamine diphosphate
TK	transketolase

Supporting Information Available

Further detail of experimental methods including synthesis and analysis of the four diastereomer mixture of 2-aminopentane-1,3-diol hydrochloride and the chiral HPLC assay for the determination of ee and de of transaminase-derived 2-aminopentane-1,3-diol and synthesis of related standards. This material is available free of charge via the Internet at http://pubs.acs.org.

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