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Enhancing and Reversing the Stereoselectivity of *Escherichia coli* Transketolase *via* Single-Point Mutations

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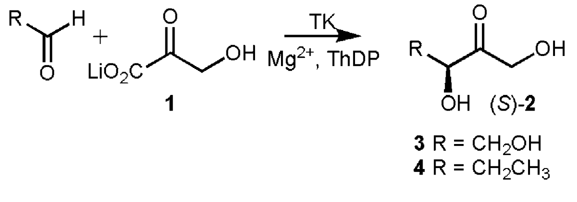
Abstract: Chiral auxiliary methodology and chiral assays have been developed to establish the enantiomeric purities of erythrulose and 1,3-dihydroxypentan-2-one generated using wild-type (WT) *Escherichia coli* transketolase (TK). L-Erythrulose was formed in 95% *ee* and (3*S*)-1,3-dihydroxypentan-2-one in 58% *ee*. Since the latter compound was formed in moderate *ee*, TK libraries were screened to identify higher performing mutants. A colorimetric screen and chiral assay were successfully applied to a 96-well format, and new active TK mutants were identified, which gave 1,3-dihydroxypentan-2-one in high stereoselectivities. Remarkably, active-site single-point mutants were identified that were able to both enhance and reverse the stereoselectivity of TK.

Keywords: biotransformations; α,α′-dihydroxy ketones; enzymes; molecular evolution; transketolase

Introduction

Transketolase (TK) (EC 2.2.1.1) is an important enzyme that has been used in stereospecific carbon-carbon bond formation and requires magnesium(II) ions as well as thiamine diphosphate (ThDP) as cofactors.[1–6] *In vivo* TK catalyses the transfer of a two-carbon ketol unit from D-xylulose 5-phosphate to D-ribose 5-phosphate.[1,2] Although this transfer is reversible, the use of the donor β-hydroxypyruvate (HPA **1**) renders the reaction irreversible.[3] Interestingly, *E.coli* TK more readily accepts **1** than spinach or yeast TK giving up to a 30-fold higher specific activity.[7] The ability of TK to achieve enantioselective carbon-carbon bond formation, and tolerance for a wide range of aldehyde acceptors to give (*S*)-α,α′-dihydroxy ketones **2** (Scheme 1), has made it an attractive enzyme for industrial applications.[2,4] Notably, TK has been used for the continuous production of L-erythrulose in a membrane reactor and process screening for the synthesis of D-xylulose 5-phosphate by TK has been described.[4] TK has been used with a range of α-hydroxyaldehyde acceptors and HPA (free acid or Li salt **1**) where it is stereospecific for the α-(*R*)-hydroxyaldehyde.[5,6] Furthermore, TK can tolerate a range of non-α-hydroxylated aldehydes, although lower relative rates of reaction (typically 5–35% of hydroxylated substrates) are normally observed.[6] For large-scale processes TK variants are required that have high specific activities and stereoselectivities towards substrates. We are particularly interested in using TK in synthetic applications[8] with non-α-hydroxylated aldehydes to expand the use of this enzyme for the preparation of chiral synthons.



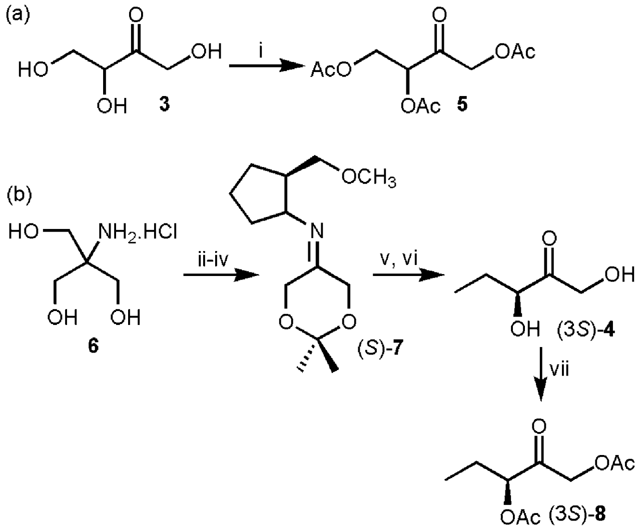
Scheme 1. TK-catalysed reaction to generate α,α′-dihydroxy ketones.

Directed evolution strategies have proven to be extremely successful in generating enzymes with improved properties, and the use of sequence and crystal structure information can be extremely valuable.[9] In previous work we have used saturation mutagenesis targeted to the TK active site to identify enhanced TK activity towards glycolaldehyde ($R = CH_2OH$) and the non-α-hydroxylated aldehyde propanal.[10,11] Although mutants with improved activities were described, stereoselectivities were not established due to a lack of chiral assays. The enantioselectivity of the TK product with chiral α-hydroxy aldehyde acceptors has previously been monitored by comparison to known standards and high enantioselectivities generally observed.[5] With glycolaldehyde and non-α-hydroxylated substrates, although optical rotations have generally been reported, enantiomeric purities are not.[6] For example, when glycolaldehyde was reacted with **1** and spinach TK to give **3**, the authors stated that the 'stereospecificity seems very high' (optical rotation data $[\alpha]_D^{25}$: +7; lit. $[\alpha]_D^{25}$: +11.3[12]), although the enantiomeric purity of the products was not fully assessed.[5a] Other reports describing the formation of **4** using TK are communications with brief experimental data including optical rotation data, however optical purities were not determined.[6b] One exception is the use of spinach TK and **1** with ethanal, methoxyethanal and methylthioaldehydes: *ee*s were reported in the range of 60–76% for the (*S*)-isomer.[6a,13] Our approach was therefore to initially establish suitable assays to determine the enantiomeric purities of products **3** and **4**, when using glycolaldehyde and propanal with WT *E.coli* TK, and then to screen the TK libraries to identify more stereoselective TK variants where suboptimal optical purities were observed.

Results and Discussion

Chiral Assays

Despite the many reports describing the synthesis or enzymatic formation of L-erythrulose, and its commercial applications, the predominant method for assessing enantiomeric purity is optical rotation.[5a,14,15] One problem is that when compounds have low optical rotation values there is a greater risk of introducing errors due to the limits involved in accurately measuring the data. For this reason, and for applications in automated screening protocols or when generating small quantities of **3**, a method using chiral HPLC or GC was sought. For chiral HPLC and GC **3** needed to be derivatised and conversion to the triacetate was investigated. Derivatisation using either acetic anhydride/pyridine/4-dimethylaminopyridine (DMAP) or acetyl chloride/triethylamine led to the formation of polymerised products. The HCl-catalysed esterification of racemic **3**, synthesised using a novel mimetic of the TK reaction recently reported from this laboratory,[16] with acetic anhydride gave racemic triacetate **5** and this material was used to successfully develop a chiral HPLC assay using a Chiralcel AD column. However, using this method to triacetylate a commercially available sample of L-erythrulose (**3**) led to complete racemisation at the α-position. The use of Lewis acid catalysts, including lanthanide triflates, for the acylation of hydroxy groups under mild reaction conditions has been described. In particular erbium(III) triflate has been highlighted as an extremely active acylation catalyst.[17] When racemic **3** was reacted with acetic anhydride in the presence of Er(OTf)$_3$ (0.5 mol%), the triacetylated product **5** was cleanly formed after 17 h in 30% yield (Scheme 2a). Notably, when the reaction was repeated with a commercially available sample of L-erythrulose (**3**), the product **5** had an *ee* of ≥95% by chiral HPLC. In addition, subjecting the sample of L-erythrulose to an extended acylation protocol (48 h) gave no deterioration in the observed *ee* due to racemisation at the α-position, highlighting that Er(OTf)$_3$-catalysed acylation offers an excellent method for the derivatisation of acid-sensitive chiral alcohols such as erythrulose (**3**).



Scheme 2. Synthesis of **5**, (3*S*)-1,3-dihydroxypentan-2-one (**4**) and (3*S*)-**8**. *Reagents and conditions:* (i) 0.5 mol% Er(OTf)$_3$, Ac$_2$O, CH$_3$CN, room temperature; (ii) PSTA, DMF, 2,2-DMP; (iii) NaIO$_4$, KHPO$_4$; (iv) SAMP, PhH, reflux; (v) *t*-BuLi, EtI; (vi) 6N HCl; (vii) Ac$_2$O, pyr, DMAP.

The synthesis of chemical standards and a chiral assay were then established to determine the enantiomeric purity and absolute stereochemistry of **4** when using propanal with WT *E.coli* TK. Using Ender's chiral auxiliary methodology to generate α,α′-dihydroxy ketones in high enantioselectivities, aminotriol **6** was converted to the SAMP hydrazone (*S*)-**7**.[18] Reaction with ethyl iodide and removal of the chiral auxiliary and acetal protecting group gave (3*S*)-**4** (Scheme 2b).[18] Racemic **4**[17] and (3*S*)-**4** were readily converted to the diacetate **8** and a chiral GC method established for screening the enantiomeric purities.

Stereoselectivities of WT *E.coli* TK

Compounds **3** and **4** were prepared using WT *E.coli* TK and converted into **5** and **8** as described above

(Scheme 2). The optical rotation data for **3** was very similar to that reported by Bolte et al for spinach TK ($[\alpha]_D^{25}$: +6.7 measured for **3**; Bolte et al. $[\alpha]_D^{25}$: +7;[5a] lit. $[\alpha]_D^{25}$: +11.3[12]). HPLC analysis of **5**, and comparison to triacetylated L-erythrulose (L-erythrulose available commercially) indicated that **3** had been formed in 95% *ee* (*S* major isomer). Similarly, conversion of **4** to **8** then analysis of **8** by chiral GC, and comparison to (3*S*)-**8** synthesised as described above using SAMP chiral auxiliary methodologies, indicated **4** was formed in 58% *ee* (*S* major isomer). This stereoselectivity was comparable to previous studies using spinach TK and similar acceptors.[6a,13] WT-TK showed good stereoselectivity towards glycolaldehyde but was significantly lower for the non-α-hydroxylated aldehyde propanal. To use TK for the preparation of chiral synthons for applications in synthesis high stereoselectivities are required, and therefore the use of engineered TKs were investigated with the acceptor propanal.

Stereoselectivities with Active-Site Single-Point TK Mutants

We have previously demonstrated that saturation mutagenesis targeted to the enzyme active site can enhance TK activity towards glycolaldehyde and propanal.[10,11] Saturation mutagenesis was targeted to active-site residues selected on either structural grounds (residues within 4 Å of postulated binding of substrate) or phylogenetic variability within bacterial and yeast TKs (also within 10 Å of ThDP cofactor). It has been reported that D477 in yeast TK, the analogous residue to D469 in *E. coli* TK, hydrogen bonds to the C-2 hydroxy group of α-hydroxylated aldehyde substrates and is involved in determining the stereoselectivity of TK (Figure 1, erythrose 4-phosphate adjunct).[19] Furthermore, residues D469, H26 and H100 (*E. coli* TK) form a pocket and interact with the C-2 hydroxy group of natural α-hydroxylated aldehyde substrates.[11] The C-2 hydroxy group of an α-hydroxylated aldehyde substrate has been replaced by the methyl group in propanal, and therefore active mutants identified from the H26, D469, and H100 structural libraries may exert interesting stereochemical effects on the TK reaction and were selected for investigation.

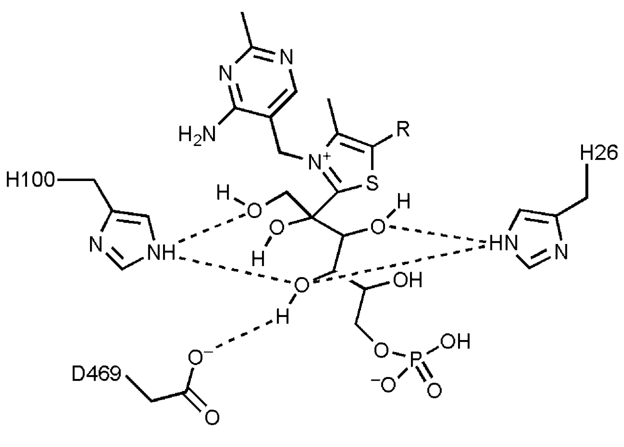


Figure 1. Model of donor (**1**)-substrate (erythrose 4-phosphate)-ThDP adjunct in the active site of TK.[11]

Reaction plates of the *E. coli* TK libraries obtained by saturation mutagenesis of H26, D469, and H100 were incubated with propanal and **1** prior to screening for product **4** with a tetrazolium-based colorimetric assay[20] and for product *ee* using the GC assay. Both assays were successfully applied to a 96-well format allowing for screening in a high-throughput manner (representative data for colorimetric assay in Figure 2 for D469).

The colorimetric assay and GC-based *ee* analysis of the 96-well plates highlighted several interesting features (Table 1). The D469 library displayed good activities with no inactive mutants generated, and single-point mutants that enhanced and reversed the stereoselectivities were identified. It should be noted that the GC assay was more sensitive (>1–2% bioconversions detected) than the colorimetric assay (>8% bioconversions detected[20]), so mutants with low activities not readily detected due to a faint coloration the colorimetric assay, were identified in the GC assay. Mutants showing good *ee*s and conversions to **4**, from the intense coloration in the colorimetric screen, were investigated further and sequenced as D469E [90% *ee* (3*S*)-**4**], D469T [64% *ee* (3*S*)-**4**] and D469Y [53% *ee* (3*R*)-**4**] (Table 2). Several other mutants showed good activities[11] but poor *ee*s were observed against propanal, including D469S [12% *ee* (3*S*)-**4**], and D469A [33% *ee* (3*S*)-**4**].

Analysis of the H26 library showed that, in contrast to the D469 library, most mutants lead to the formation of (3*R*)-**4**. Two mutants gave **4** in an *ee* of >75% in favour of the (3*R*)-product but none gave the (3*S*)-product in high *ee*. The most stereoselective mutant also gave good conversions to **4**, and was sequenced as H26Y, which remarkably gave (3*R*)-**4** in 88% *ee*. Interestingly, activity of the H100 library was reduced compared to WT. This may be because H100 (H103 in yeast) has a key binding interaction to HPA **1** which could be lost or modified unfavourably in the library mutants.[21] Although the (3*S*)-selectivity was largely maintained across the library and 4 mutants demonstrated (3*R*)-selectivity (10–20% *ee*), this set of mutants was not explored further because lower activities and moderate *ee*s were observed.

Reactions using the high performing mutants were performed at 50 mM concentration and preparative quantities of **4** isolated to confirm the *ee* data. The specific activities of D469T and D469Y have been re-

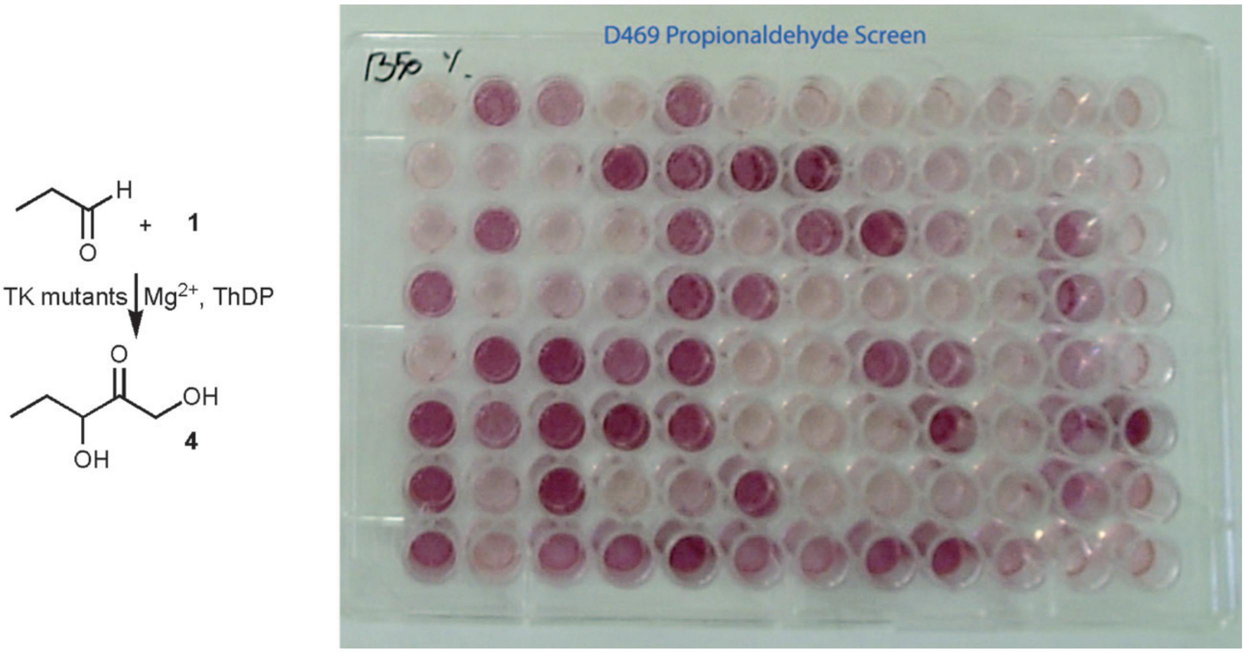


Figure 2. Colorimetric assay for D469 library to give **4**.

Table 1. Stereoselectivity profile for D469 and H26 libraries.

Library	(3*S*)-**4** selective	(3*R*)-**4** selective	*ee* knockouts[a]	Inactive Mutants
D469	69	14	13	0
H26	29	42	12	13
H100	88	4	4	0

[a] *ee* knockouts are for *ee* of $<10\%$.

Table 2. Data for high performing D469 and H26 mutants.[a]

Mutant	Rate [mmol/h]	TK [mg]	Spec. activity [mmol/min mg^{-1}]	Relative Activity	*ee* of **4**
WT	1.49	0.49	0.05	1	58% (3*S*)
D469T	3.74	0.26	0.24	5[b]	64% (3*S*)
D469Y	6.43	0.48	0.22	4	53% (3*R*)
D469E	5.16	0.22	0.39	8	90% (3*S*)
H26Y	2.69	0.25	0.18	4	88% (3*R*)

[a] Reactions carried out using clarified lysate of determined TK concentration with 50 mM HPA, 50 mM propanal, 2.4 mM ThDP and 9 mM $MgCl_2$ in 50 mM Tris at pH 7.0 on 300 μL scale.

[b] From ref.[11]

ported[11] and those for the remaining mutants were determined using clarified lysates of determined TK concentrations (Table 2). All gave better specific activities for the conversion of propanal to **4** than WT: D469E gave an 8-fold improvement and H26Y, 4-fold (Table 2).

The ability of active-site single-point mutations to have such a remarkable effect on the stereoselectivity with a small non-α-hydroxylated acceptor was surprising. Although combination mutants for other enzymes have successfully resulted in reversals in stereoselectivities, there are few examples reported with single-point mutations. These include directed evolution studies with hydantoinases,[22] aldolases,[23] epoxygenases,[24] arylmalonate decarboxylase,[25] cyclohexanone monooxygenase,[26] and hydroxynitrile lyase to form diastereoisomeric products.[27] However, in most cases the single-point mutants were not highly stereoselective and were used in further studies to generate combination mutants. The enhanced *ee* with variant Asp469Glu is probably due to the presence of the extra methylene unit resulting in tighter binding of propanal or reaction intermediates. However, the inverse selectivity observed with Asp469Tyr is harder to rationalise. Similarly, His26Tyr where a positively charged residue is substituted for Tyr, leads to a high reversing variant. It is difficult to predict whether the unexpectedly high *ee* is due to steric or electrostatic effects, or longer range conformational changes.

Conclusions

In summary, assays have been developed to readily screen erythrulose (**3**) and 1,3-dihydroxypentan-2-one

(**4**) for the enantiomeric purities achieved in TK bioconversions. Using WT-TK, L-erythrulose was formed in 95% *ee* and the assay has wide applicability for determining the enantiomeric purity of **3** in biocatalytic or chemical conversions. The synthesis of (3*S*)-1,3-dihydroxypentan-2-one using chiral auxiliary methodology confirmed that this is the major isomer when using WT *E.coli* TK, although only a moderate *ee* was observed (58%). TK libraries were screened to identify higher performing mutants. A colorimetric screen and chiral assay were successfully applied to a 96-well format, and new active TK mutants were identified, which gave **4** in high stereoselectivities. Remarkably, active-site single-point mutants were identified that were able to both enhance and reverse the stereoselectivity of TK towards propanal. Modelling and crystallographic studies are now underway to understand the reasons for these observations.

Experimental Section

General Experimental Methods

Unless otherwise noted, solvents and reagents were reagent grade from commercial suppliers and used without further purification. Dry THF was obtained using anhydrous alumina columns.[28] All moisture-sensitive reactions were performed under a nitrogen or argon atmosphere using oven-dried glassware. Reactions were monitored by TLC on Kieselgel 60 F_{254} plates with detection by UV, potassium permanganate, and phosphomolybdic acid stains. Flash column chromatography was carried out using silica gel (particle size 40–63 μm). ^{1}H NMR and ^{13}C NMR spectra were recorded in $CDCl_3$ at the field indicated using a Bruker AMX300 MHz and AMX400 MHz machine. Coupling constants are measured in Hertz (Hz) and unless otherwise specified, NMR spectra were recorded at 298 K. Mass spectra were recorded on a Thermo Finnegan MAT 900XP spectrometer. Infrared spectra were recorded on a Shamadazu FTIR-8700 infrared spectrophotometer. Optical rotations were recorded on an Optical Activity Limited PolAAR2000 polarimeter at 589 nm, quoted in deg $cm^2 g^{-1}$ and concentration (*c*) in g/100 mL.

5-Amino-5-hydroxymethyl-2,2-dimethyl-1,3-dioxane and 2,2-dimethyl-5-oxo-1,3-dioxane were prepared as previously described.[29] (*S*)-(+)-1-(2,2-Dimethyl-1,3-dioxan-5-ylidenamino)-2-methoxymethylpyrrolidine [(*S*)-**7**] was synthesised as reported by Enders et al.[18] β-Hydroxypyruvate was prepared as the lithium salt as previously described.[6c] Libraries were constructed and reaction plates obtained as previously reported.[10,11]

L-Erythrulose using *E.coli* WT-TK

ThDP (88 mg, 192 μmol) and $MgCl_2.6H_2O$ (146 mg, 718 μmol) were dissolved in H_2O (42 mL) and the pH adjusted to 7 with 0.1 M NaOH. To this stirred solution, at 25 °C, was added WT-TK clarified lysate (8 mL, 39 mg of TK),[10] and the mixture stirred for 20 min. In a separate flask, **1** (440 mg, 4 mmol) and glycolaldehyde (240 mg, 4 mmol) were dissolved in H_2O (30 mL) and the pH adjusted to 7 with 0.1 M NaOH. Following a 20 min enzyme/cofactor pre-incubation, the **1**/glycolaldehyde mixture was added to the enzyme solution and the mixture was stirred at 25 °C for 24 h. During this time, the pH was maintained at 7.0 by addition of 1 M HCl using a pH stat (Stat Titrino, Metrohm). Silica was added and the reaction mixture concentrated to dryness under vacuum then dry loaded onto a flash silica column. Following silica column purification (EtOAc:MeOH, 9:1), L-erythrulose was isolated as an colourless oil; yield: 288 mg (60%). Analytical data were identical to those previously described.[5a] $[\alpha]_D^{25}$: +6.7 (*c* 1.48, H_2O), lit. $[\alpha]_D^{25}$: +11.3 (*c* 4.0, H_2O)[12]

Erythrulose Triacetate (5)

Erythrulose (180 mg, 1.5 mmol) was added to a stirred solution of Ac_2O (425 μL, 4.5 mmol) and $Er(OTf)_3$ (4.6 mg, 0.5 mol% *vs.* erythrulose) in acetonitrile (5 mL). After stirring for 1 h at 25 °C, all erythrulose had dissolved. The mixture was stirred at 25 °C for a further 16 h, and the mixture then partitioned between EtOAc (50 mL) and $NaHCO_3$ (saturated) (50 mL). The organic phase was dried, concentrated, and purified using flash silica chromatography (EtOAc:hexanes, 1:1) to afford **5** as a colourless oil; yield: 109 mg (30%). ^{1}H NMR (300 MHz; $CDCl_3$): δ = 2.06 (3 H, s, $COCH_3$), 2.14 (3 H, s, $COCH_3$), 2.16 (3 H, s, $COCH_3$), 4.36 (1 H, dd, *J* = 12.2 and 5.0, $CHCH_2OAc$), 4.47 (1 H, dd, *J* = 12.2 and 3.4, $CHCH_2OAc$), 4.80 (1 H, d, *J* = 17.6, $COCH_2OAc$), 4.87 (1 H, d, *J* = 17.6, $COCH_2OAc$), 5.38 (1 H, dd, *J* = 5.0 and 3.4, $CHCH_2OAc$); ^{13}C NMR (75 MHz; $CDCl_3$): δ = 20.3, 20.5, 20.6 ($COCH_3$), 62.3 ($CHCH_2OAc$), 66.6 ($COCH_2OAc$), 74.7 ($CHCH_2OAc$), 169.8, 170.0, 170.3 (C=O ester), 198.4 (C=O ketone); MS (+HR-FAB): *m/z* = 269.06301 ($M+Na^+$), $C_{10}H_{14}O_7$ requires 269.06372. Erythrulose triacetate (**5**) obtained *via E.coli* WT-TK $[\alpha]_D^{25}$: −25 (*c* 0.5, $CHCl_3$) and was determined to have an *ee* of (*S*) 95% by chiral HPLC (see below for method).

HPLC Assay for 5

HPLC analysis was performed on a Varian Prostar instrument equipped with a Chiracel AD chiral column (Daicel, 25 cm × 0.46 cm). HPLC conditions: injection volume, 10 μL; mobile phase, *i*-PrOH:hexanes, 5:95; flow rate, 0.8 mL/min; detection, 210 nm; retention times: (*R*)-erythrulose triacetate, 22.0 min, (*S*)-erythrulose triacetate, 25.4 min. Racemic erythrulose standards were synthesised as previously described[16] and acetylated as described above.

(*S*)-(−)-(2,2-Dimethyl-4-ethyl-1,3-dioxan-5-ylidenamino)-2-methoxymethylpyrrolidine[30]

The reaction was carried out under anhydrous conditions. To (*S*)-**7** (0.0446 g, 0.180 mmol) and THF (10 mL) in a three-necked round-bottomed flask was added *t*-BuLi (0.14 mL, 0.20 mmol; 15% in pentane) dropwise by syringe at −78 °C. After stirring for 2 h at this temperature, the mixture was cooled to −100 °C and bromoethane (0.020 g, 0.18 mmol) added slowly. After further stirring for 2 h at −100 °C, the mixture was warmed to room temperature over several hours. The mixture was quenched with pH 7 buffer solution (2 mL) and diluted with Et_2O (80 mL). The organic layer was washed with pH 7 buffer solution (10 mL) and saturated NaCl solution (2×10 mL), dried ($MgSO_4$) and concentrated under vacuum. The monoalkylated SAMP-hydrazone was purified by flash silica chromatography (pentane:Et_2O, 5:1) to afford the title compound as a colourless oil; yield: 18 mg (35%). ^{1}H NMR (400 MHz; $CDCl_3$): δ = 0.97 (3H, t, J = 7.2, CH_2CH_3), 1.39 [6H, s, $C(CH_3)_2$], 1.50–2.08 (6H, m, $NCHCH_2CH_2$, CH_2), 2.40 (1H, m, NC*H*H), 3.34 (3H, s, OCH_3), 2.98–3.45 (4H, m, CH_3OCH_2, NC*H*, NCH*H*), 4.14 (1H, dd, J = 15.6 and 2.0, OC*H*HC=N), 4.25 (1H, m, OC*H*C=N), 4.50 (1H, d, J = 15.6, OCH*H*C=N); ^{13}C NMR (100 MHz; $CDCl_3$): δ = 9.5 (CH_2CH_3), 22.8, 23.9 and 24.4 [$C(CH_3)_2$], 25.2, 26.8, 55.6, 59.2, 59.8, 66.7, 72.0, 75.7, 100.1 [$C(CH_3)_2$], 160.4 (C=N); MS (HR-EI): m/z = 271.20097 (M^+), $C_{14}H_{27}N_2O_3$ requires 271.20162.

(3*S*)-1,3-Dihydroxypentan-2-one (3*S*)-4[31]

(*S*)-(−)-(2,2-Dimethyl-4-ethyl-1,3-dioxan-5-ylidenamino)-2-methoxymethylpyrrolidine (0.100 g, 0.37 mmol) was dissolved in pentane (2 mL) and treated with 6N HCl (0.4 mL). The mixture was vigorously stirred and the reaction followed by TLC analysis. After 20 min the mixture was extracted with Et_2O (3×20 mL), washed with saturated NaCl solution (20 mL) and dried ($MgSO_4$). The crude product was purified by silica gel column chromatography (Et_2O:pentane, 1:2) to give (3*S*)-1,3-dihydroxypentan-2-one[6b] as a colourless oil; yield: 18 mg (40%) . The ^{1}H, and ^{13}C NMR data were identical to those previously reported[6b] and $[\alpha]_D^{25}$: −44.5 (c 3.1, H_2O).

1,3-Diacetoxypentan-2-one (8): General Procedure for Racemic Sample and Preparative Scale Biotransformations

1,3-Dihydroxypentan-2-one (100 mg, 0.85 mmol) was dissolved in pyridine (3 mL) and cooled to 0 °C. DMAP (cat.) was added followed by Ac_2O (1 mL) and the mixture warmed to room temperature. After 3 h, the mixture was evaporated to dryness and the residue re-dissolved in EtOAc (50 mL). The organic phase was washed with 0.3M $KHSO_4$ (50 mL), saturated $NaHCO_3$ solution (50 mL), dried ($MgSO_4$) and the solvent removed under vacuum. The product was purified by silica column chromatography (EtOAc:hexane, 2:3) to yield the titled compound as an oil in quantitative yield. IR (film): ν_{max} = 2977, 2940, 1733 cm^{-1}; ^{1}H NMR (300 MHz; $CDCl_3$): δ = 0.93 (3H, t, J = 7.4, CH_2CH_3), 1.91–1.64 (2H, m, CH_2CH_3), 2.09 (3H, s, $COCH_3$), 2.10 (3H, s, $COCH_3$), 4.72 (1H, d, J = 17.2, C*H*HOAc), 4.77 (1H, d, J = 17.2, CH*H*OAc), 5.01 (1H, dd, J = 7.5 and 5.0, C*H*OAc); ^{13}C NMR (75 MHz; $CDCl_3$): δ = 9.2 (CH_2CH_3), 20.3, 20.5, 24.0, 66.1 (CH_2OAc), 77.5 (CHOAc), 170.0 (C=O ester), 170.4 (C=O ester), 200.7 (C=O ketone); MS (HR-FAB): m/z = 225.07483 ($M+Na^+$), $C_9H_{14}NaO_5$ requires 225.07389.

(3*S*)-1,3-Diacetoxypentan-2-one, (3*S*)-8, *via* SAMP

(3*S*)-1,3-Dihydroxypentan-2-one (18 mg, 0.15 mmol) was dissolved in pyridine (0.1 mL) and Ac_2O (0.015 g) and DMAP (0.020 g) were added. The mixture was stirred for 18 h at room temperature then the solution was concentrated under vacuum and partitioned between EtOAc and saturated $NaHCO_3$ solution. The product was purified by column chromatography (EtOAc:hexane, 2:3) to give the title compound in a quantitative yield. The characterisation data were identical to those above and $[\alpha]_D^{25}$: −23.0 (c 1.2, H_2O).

Screening of Libraries for Activity and Product *ee*

Preparation of cell samples – plate format: The 96-well reaction plates were thawed from −80 °C resulting in the freeze-thaw lysis of the 100 μL cell cultures. A 12× cofactor stock solution (28.8 mM ThDP, 108 mM $MgCl_2$, 50 mM Tris, pH 7.0; 25 μL) was added to each well and the plate was then incubated at 25 °C for 20 min. A 4× HPA stock solution (200 mM in 50 mM Tris, pH 7.0; 75 μL) and a 3× propanal stock solution (150 mM in 50 mM Tris, pH 7.0; 100 μL) were then added to each well and the plate incubated at 25 °C for a further 2 d.

***Assaying reaction plates for production of 4*:** 50 μL of reaction mixture were transferred from each well to a fresh 96-well plate containing 50 μL of 50 mM Tris, pH 7.0 and 20 mg of MP-Carbonate Scavenger Resin (Biotage), which was added to each well using a resin loader (Radleys). After 3 h, Tris (100 μL, 50 mM, pH 7.0) was added to each well, with mixing, and 50 μL from each well transferred to a fresh 96-well plate. A plate reader (Fluostar, BMG-labtech) fitted with an autoinjector was then used to add 2,3,5-triphenyltetrazolium chloride (20 μL, 0.2% solution in methanol) then 3M NaOH (10 μL). The plate was shaken for 10 s, left for 1 min and an absorbance reading taken of each well at 485 nm.

Assaying reaction plates for ee determination of 4 – conversion of 4 into 8: 100 μL of reaction mixture were transferred from each reaction well to a corresponding set of vials each containing EtOAc (300 μL). The vials were shaken and allowed to partition prior to transferring a portion of the organic phase (100 μL) into fresh vials. Pyridine [containing DMAP (10 mg/mL), 20 μL] was added to each vial and *ee* analysis performed. A racemic sample of **4** synthesised as previously described[16] and converted into **8** as described above.

GC assay for ee determination of 8: GC analysis was performed on a Perkin–Elmer Autosystem XL Gas Chromatograph equipped with a β-Dex 225 chiral column (Supelco, 30 m×0.25 mm). GC conditions: injection volume, 1 μL; carrier gas, He; carrier gas pressure, 15 psi; injector temperature, 250 °C; oven temperature, 60 °C then increased at 3 °C/min; detector temperature, 300 °C; detection, flame ionised detector (FID). Retention times: (3*R*)-**8**, 29.9 min; (3*S*)-**8**, 30.3 min.

Obtaining Preparatively Useful Quantities of Selected Mutant Biocatalysts

Streak plates onto Luria-Bertani (LB) agar containing 150 μg/mL ampicillin were made for potentially interesting mutants from the relevant library master plate glycerol stock. Individual colonies were selected and grown in LB broth (10 mL) containing 150 μg/mL ampicillin for 16 h at 37 °C, 200 rpm. The 10 mL innoculum was then transferred into further LB broth (200 mL) containing 150 μg/mL ampicillin and incubated for 16 h at 37 °C, 200 rpm. An innoculum optical density (600 nm) of 5 is typical after this time.

The innoculum was centrifuged at 4000 rpm for 20 min at 10 °C. The supernatant was discarded and the cell pellet resuspended in sodium phosphate (10 mL of buffer per 1 g wet cell paste, 5 mM, pH 7.0). The suspension was sonicated (Sanyo, Soniprep 150) on ice and the crude lysate centrifuged for 20 min at 4000 rpm at 10 °C. The clarified lysate was pipetted into Eppendorf flasks and stored at −20 °C.

Kinetic Characterisation of Selected Mutants

Lysate TK protein concentration: The total protein concentration in lysate samples was measured using a standard Bradford assay using bovine serum albumin solutions of known concentrations as standards. The percentage of total protein present comprising TK was determined by densitometry measurements following the electrophoresis of lysate samples on a 7.5% SDS-PAGE gel using commercially available TK (Sigma) as a band standard. The Coomassie-stained gel was imaged using Imagesoft softmare to calculate the transketolase band density relative to the total band density.

Initial Rate Data: A 12 × cofactor stock solution (28.8 mM ThDP, 108 mM $MgCl_2$, 50 mM Tris, pH 7.0; 25 μL) was added to lysate (100 μL) and the mixture incubated at 25 °C for 20 mins. A 4 × stock solution of **1** (200 mM in 50 mM Tris, pH 7.0; 75 μL) and a 3 × propanal stock solution (150 mM in 50 mM Tris, pH 7.0; 100 μL) were then added and the reaction sampled and quenched after 1 h and 2 h by addition of 50 μL of reaction mixture to 50 μL of 0.1% TFA in water. The concentration of **4** present in the samples was determined by HPLC.

HPLC Assay for Determining Concentration of 4

HPLC analysis was performed on a Dionex equipped with an Aminex HPX-87H ion-exclusion column (250 mm × 4.6 mm, Bio-Rad Laboratories) maintained at 60 °C, using 0.1% (v/v) trifluoroacetic acid in H_2O as mobile phase at a flow rate of 0.6 mL/min. Retention time: **4**, 15.6 min.

TK-Mediated Synthesis of 4

ThDP (88 mg, 192 μmol) and $MgCl_2.6H_2O$ (146 mg, 718 μmol) were dissolved in H_2O (42 mL) and the pH adjusted to 7 with 0.1 M NaOH. To this stirred solution, at 25 °C, was added TK clarified lysate (8 mL, 0.7–2.3 U) and the mixture stirred for 20 min. In another flask, **1** (440 mg, 4 mmol) and propanal (288 μL, 4 mmol) were dissolved in H_2O (30 mL) and the pH adjusted to 7 with 0.1 M NaOH. Following the 20 min enzyme/cofactor pre-incubation, the mixture of **1** and propanal was added to the enzyme solution and the mixture stirred at 25 °C for 24 h. During this time, the pH was maintained at 7.0 by addition of 1 M HCl using a pH stat (Stat Titrino, Metrohm). Silica was added and the reaction mixture concentrated to dryness before being dry loaded onto a flash silica gel column. Following column purification (EtOAc/hexane, 1:1), **4** was isolated as an oil which became a white solid on standing; yield: 354 mg (70% for D469E).

Wild-type TK afforded (3*S*)-**4** in 58% *ee* (36% yield). Mutant D469E afforded (3*S*)-**4** in 90% *ee* Mutant H26Y afforded (3*R*)-**4** in 88% *ee* (63% yield).

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