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α,α'-Dihydroxyketone formation using aromatic and heteroaromatic aldehydes with evolved transketolase enzymes

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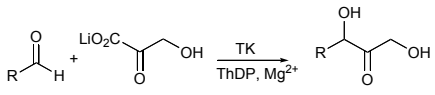
Transketolase mutants have been identified that accept aromatic acceptors with good stereoselectivities, in particular benzaldehyde for which the wild type enzyme showed no activity.

The advantages of using biocatalysts as a sustainable resource in synthesis are well established and includes their potential to achieve high regio- and stereoselectivities.[1,2] Transketolase (TK) (E.C.2.2.1.1) is a thiamine diphosphate (ThDP) dependent carbon-carbon bond forming enzyme.[3] *In vivo* it reversibly transfers a two carbon ketol unit to D-erythrose-4-phosphate and D-ribose-5-phosphate.[3,4] To render the reaction irreversible *in vitro*, β-hydroxypyruvic acid (HPA **1**) has been used extensively as the ketol donor and *E. coli* TK shows higher specific activity towards **1** than yeast and spinach TKs.[5,6] TKs have been used with a range of non-phosphorylated α-hydroxyaldehydes, where good conversion rates and stereospecificities for the (2*R*)-hydroxyaldehyde acceptor were observed, to give (*S*)-α,α'-dihydroxyketones **3** (Scheme 1).[4,7] Wild-type (WT) TKs have been noted to tolerate some non-α-hydroxylated aliphatic aldehydes, but compared to α-hydroxyaldehydes lower substrate activities were reported.[8] *E. coli* TK has also been overexpressed,[9] and there is interest in industrial applications.[10]

To generate TKs with improved properties towards hydrophobic substrates for synthetic applications, saturation mutagenesis libraries were created, each targeted to one TK active-site residue.[11] Single point active-site mutants were identified with improved activity towards propanal (R=CH_2CH_3) that were selective for (3*S*)-**3** and (3*R*)-**3**.[12] Several variants were from the D469X library: D469E with propanal gave 3*S*-**3** in 90% *ee* and D469Y 3*R*-**3** in 53% *ee*.[12] The mutant D469E-TK has also been shown to decrease the acceptance of formaldehyde and glycolaldehyde compared to WT-TK,[13] while a yeast TK crystal structure showed that the equivalent residue hydrogen bonds to the C-2 hydroxy group of erythrose-4-phosphate in the active site.[14] For these reasons the D469X library was investigated with a series of linear aldehydes (C_4 to C_8) and C_3, C_5 and C_6 cyclic carboxaldehydes.[15] Excellent *ees* (86-99%) were observed with the D469E mutant and variable yields (10-58%) which were generally lower with the cyclic aldehydes.[15]

The α,α'-dihydroxyketones **3** produced by TK are valuable building blocks for conversion to other chiral synthons such as 2-amino-1,3-diols.[16] For example, (*rac*)-1,3-dihydroxy-1-phenylpropane-2-one synthesised non-enzymatically, can be converted using a transaminase enzyme to (1*rac*,2*S*)-2-amino-1-phenyl-1,3-propanediol, a motif present in several antibiotics such as thiamphenicol and fluoramphenicol.[17]

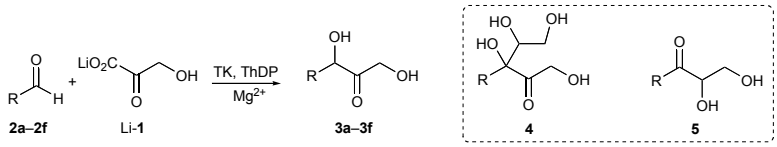


2 HPA Li-**1** **3**

Sch. 1 TK catalysed reaction to generate α,α'-dihydroxy ketones **3**

In the current work the focus was to identify TK variants able to accept aromatic and heteroaromatic aldehydes. Such substrates in terms of their reactivity and structure are far removed from the natural aldosugars used *in vivo*. Previous work has highlighted the possible acceptance of aromatic and heteroaromatic substrates. 2-Furaldehyde has been used with *E. coli* TK but very low V_{rel}s were observed,[8b] as was the case for benzaldehyde and 2-furaldehyde with yeast TK, although 2-thiophene-carboxaldehyde was more readily accepted.[8a] *E.coli* variants D469E and D469A have been used with pyridine carboxaldehydes where conversions were observed, but no reaction occurred with benzaldehyde or 2-furaldehyde.[18] However, as previously highlighted, no data corresponding to the TK products has ever been reported, and reactions were typically monitored by determining HPA **1** consumption, which can undergo slow decomposition.[6,19] The use of aldehydes with aromatic moieties have been reported using WT-TK including phenylacetaldehyde and 2-hydroxy-phenylacetaldehyde, to give products in 26% (isomeric mixture) and 54% yield ((3*S*,4*R*)-isomer), respectively.[8c,20]

Initially, benzaldehyde **2a** and Li-**1** was used with WT-TK, but no reaction was observed (Scheme 2, Table 1). Benzaldehyde was then screened against the D469X library utilising the tetrazolium red colorimetric assay for use with non-α-hydroxylated aldehydes.[19] From this several promising mutants were selected: D469E, D469K, D469S, and D469T. Interestingly, D469E, D469K and D469T had also been identified as suitable variants for use with the cyclic aldehydes where D469E and D469T gave (3*S*)-products in >97% *ee*.[15] An engineered TK mutant F434A was also selected for investigation as this highly conserved first-shell residue is adjacent to the D469 site and the mutation to alanine was therefore predicted to improve substrate acceptance for larger aromatic and heteroaromatic aldehydes by removing hydrophobic and steric interactions within the active site. From the colorimetric assay variant D469S gave significant amounts of product and was explored further. However, product analysis revealed only a trace of **3a**, and instead a double addition product **4** (R = Ph) was isolated,



Sch. 2 Formation of dihydroxyketones **3** using TK and side products **4** and **5**

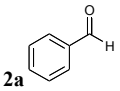
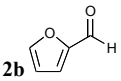
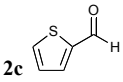
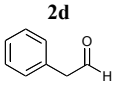
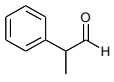
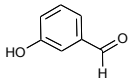
Aldehyde	Product	**WT-TK** *ee* or isomer (yield)	**D469E** *ee* or isomer (yield)	**D469T** *ee* or isomer (yield)	**D469K** *ee* or isomer (yield)	**F434A** *ee* or isomer (yield)
2a 	**3a** (R = Ph)	no reaction	0% (2%) [**4** (5%)]	70% (3*R*) (2%) [**4** (5%)]	82% (3*R*) (2%) [**4** (5%)]	82% (3*R*) (10%) no **4**
2b 	**3b** (R = furyl)	no reaction	- (5%) [**5** (<2%)]	- (3%) [**5** (<2%)]	- (3%) [**5** (<2%)]	- (1%) [**5** (<2%)]
2c 	**3c** (R = thienyl)	no reaction	- (2%) [**5** (<2%)]	- (3%) [**5** (<2%)]	- (2%) [**5** (<2%)]	- (1%) [**5** (<2%)]
2d 	**3d** (R = CH_2Ph)	93% (3*S*) (5%)	90% (3*S*) (50%)	96% (3*S*) (50%)	95% (3*S*) (50%)	97% (3*S*) (48%)
2e 	**3e** (R = $CH(CH_3)Ph$)	88% (3*S*,4*R*) 12% (3*S*,4*S*) (35%)	95% (3*S*,4*R*) 5% (3*S*,4*S*) (30%)	96% (3*S*,4*R*) 4% (3*S*,4*S*) (40%)	95% (3*S*,4*R*) 5% (3*S*,4*S*) (38%)	85% (3*S*,4*R*) 15% (3*S*,4*S*) (35%)
2f 	**3f** (R = *m*-$(OH)C_6H_4$)	no reaction	no reaction	0% (4%)	-	53% (3*R*) (6%)

Table 1 Stereoselectivities for WT-TK and TK mutant reactions with aromatic aldehydes

resulting from the aldol addition of **3a** to glycolaldehyde, presumably generated from decarboxylation of the donor HPA *in situ*. An additional experiment was performed using **3a** (prepared using the biomimetic reaction[21]) with Li-**1** and D469S-TK and no **4** was formed, and was repeated with D469T-TK with the same result. This suggested that the addition to glycolaldehyde might occur in the active site while the intermediate leading to **3a** is still attached to ThDP, or with **3a** when it has been formed. However the formation of **3a**, even as an intermediate product was promising. Benzaldehyde was then used with the other selected mutants and **3a** isolated yields of 2-10% (Table 1), with variant F434A giving the highest yield. Care was taken when isolating **3a** due to the ease of rearrangement to **5** (R = Ph). For the first time these experiments established that as with several other ThDP dependant enzymes, such as pyruvate decarboxylase (PDC), TK-mutants can accept benzaldehyde even though the yields are low. For D469E, D469K and D469T 5% of **4** (R = Ph) was also generated. By comparison, the mutant F434A gave no double addition product **4**, but **3a** in 10% yield, perhaps reflecting that with increased accessibility to the active site, with the substitution of Phe to Ala, the product **3a** can more readily exit the active site region avoiding glycolaldehyde addition. For HPLC analysis of the optical purities of **3a**, racemic **3a** was dibenzoylated and determination of the absolute stereochemistry was achieved by formation of the Mosher's ester at the primary hydroxyl with (*S*)- and (*R*)-MTPACl.[22] Mutant D469E gave **3a** as a racemate, in contrast to the high stereoselectivities observed with this mutant for the aliphatic linear and cyclic aldehydes.[15] Variants D469K, D469T and F434A gave **3a** in 82% *ee*, 70% *ee* and 82% *ee*, respectively, and in all cases the 3*R*-isomer was formed predominantly, comparable to the (*R*)-hydroxyketones formed using ThDP dependant PDC and benzaldehyde lyase. By analysis of an alignment of 382 TPP-dependent enzyme sequences described previously,[23] the position equivalent to F434 is always Phe or Tyr, except in phospho- or sulfo-pyruvate decarboxylases (Asn), and most interestingly is Ala only in benzoyl formate decarboxylase and benzaldehyde lyase enzymes, indicating the potential role of the F434A mutation for acceptance of the benzene ring. Position D469 is typically Asp or Asn and occurs naturally as Lys of Thr only in a few PDC and PDC-related enzymes.

With several TK mutants able to accept benzaldehyde, the use of aromatic and heteroaromatic aldehydes **2b**–**2f** was also investigated. Products **3b**–**3f** were prepared for chiral assay development from aldehydes **2b**–**2f** using the biomimetic reaction.[21] For the determination of *ees* by chiral HPLC **3d** and **3e** were monobenzoylated at the primary alcohol and **3f** dibenzoylated, and Moshers' ester derivatisation of **3d**, **3e**, and **3f** performed.[22] For **2b** and **2c** no conversions to **3b** and **3c** were observed with WT-TK. When using the D469 mutants and F434A products **3b** and **3c** were formed in low yields <5%, with <2% of the rearranged dihydroxyketone **5** and no **4**. Attempts to derivatise **3b** and **3c** as esters for absolute stereochemistry and *ee* determination were not successful: rearrangement to **5** occured. Phenylacetaldehyde **2d** and (*rac*)-2-phenylpropionaldehyde **2e** were initially used with WT-TK and **3d** and **3e** formed in 5% and 35% yield, where the more bulky α-methylated aldehyde was accepted more readily. Phenylacetaldehyde has been used with *E.coli* WT-TK to give **3d** and **5** (R = CH_2Ph) as a mixture of isomers, although here

only **3d** was formed, possibly due to maintenance of the pH during the reaction. The D469 mutants and F434A led to the formation of **3d** in approximately 50% yield, and **3e** in 30-40% yield. For **3d** *ee*s in the range 90-97% (3*S*-isomer) were determined with the highest stereoselectivity observed with F434A. ^{1}H NMR analysis of **3e** formed by D469T indicated the presence of two diastereoisomers, one major and one minor. Monobenzoylation and chiral HPLC analysis revealed two products in a ratio of 96:4. Use of (2*R*)-**2e** in the biomimetic reaction and chiral HPLC peak correlation, together with the Mosher's method indicated that the major product formed using (*rac*)-**2e** with the D469 mutants and F434A was (3*S*,4*R*)-**3e**, and the minor isomer (3*S*,4*S*)-**3e**. The D469 mutants were therefore enantioselective for (2*R*)-**2e**, and stereoselectively formed the (3*S*)-isomer. When (2*R*)-**2e** was used with D469T only (3*S*,4*R*)-**3e** was formed. 2-Hydroxyphenylacetaldehyde has been used with *E. coli* WT-TK and generates the (3*S*,4*R*)-isomer.[20] Despite removal of the key hydrogen-bonding interaction between the aldehyde C-2 hydroxyl and D469, H100, H26, indicated from yeast TK studies, and replacement with a methyl group this aldehyde enantioselectivity was maintained.[11,14] To probe whether a hydroxylated benzaldehyde might influence active site hydrogen bonding interactions, the aldehyde **2f** was used. No reaction was observed with WT-TK or D469E, but with D469T racemic **3f** and F434A (3*R*)-**3f** formed (53% *ee)*.

These results are extremely interesting, for the first time it has been shown that selected TK mutants can accept benzaldehyde, however the stereoselectivity observed is the opposite to that reported for the aliphatic series. In addition, phenylacetaldehyde and 2-methyl phenylacetaldehyde, gave products in good yields and high *ee*s when using the single point mutants. Higher reactivities with phenylacetaldehydes compared to benzaldehyde may reflect increased conformational flexibilities, less steric interactions, and higher reactivities. The yields for the formation of **3a**–**3c** and **3f** probably reflects low rates due to poor access to the active site, since the use of F434A clearly enhanced product formation. However, for more productive reactions, the yield may also be influenced by product inhibition or enzyme deactivation: aldehyde solubility is not limiting with these substrates. This work also highlights the importance of product isolation: TK assays based on HPA consumption or colorimetric detection of hydroxyketones can not distinguish between products **3** and **4**. With identification of the first TK mutants to definatively accept benzaldehyde, further studies are now underway to produce improved combination mutants.

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Notes and references

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