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Rational substrate and enzyme engineering of transketolase for aromatics

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The uses of 3-formylbenzoic acid and 4-formylbenzoic acid as molecular probes along with previous and new transketolase mutants revealed the factors governing the rate of reaction between transketolase and aromatic aldehydes. The novel α,α-dihydroxyketones were produced at 15 to 30-fold higher yields and up to 250-fold higher specific activities with D469T TK when compared to those obtained for benzaldehyde.

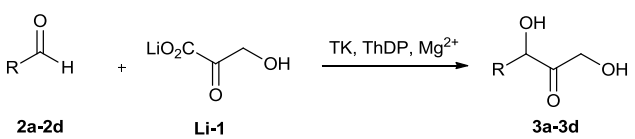
Introduction

Stereospecifically controlled carbon-carbon bond formation is a key goal of organic synthesis. While many carbon-carbon bond forming chemical reactions can be achieved in a single step, several challenges still exist,[1] and enzymes such as transketolase (TK) offer an attractive alternative due to their high stereo-specificity and selectivity.[2-8]

Transketolase (E.C. 2.2.1.1) is a thiamine diphosphate (ThDP) dependent enzyme which plays a crucial role in the non-oxidative phase of the pentose phosphate pathway and Calvin cycle,[9] where it catalyses a reversible transfer of a two-carbon ketol group from a ketose sugar to aldose.[10, 11] The use of hydroxypyruvate as the ketol donor leads to the liberation of carbon dioxide and hence an effectively irreversible reaction, which is synthetically attractive due to the higher yields achieved. The dihydroxyketone functionality generated in these reactions is present in a wide range of natural products,[4, 12, 13] and TK has been used in multi-enzyme cascades to synthesise several chiral sugars and their analogues,[4, 11, 14] (+)-*exo*-brevicomin,[15] and chiral amino-diols.[16, 17]

Several residues around the TK active-site are highly conserved across species and also throughout the ThDP-dependent enzyme family.[18] Many are directly involved in substrate recognition and binding, which dictate the substrate preference. Transketolases from different species can accept a wide range of hydroxylated and non-hydroxylated aliphatics, as well as cyclic and aromatic aldehydes,[10, 11, 19, 20] and nitroso aromatics.[21] However, the hydroxylated substrates are far more readily accepted due to highly conserved histidine and aspartate residues, which directly interact with the hydroxyl groups of acceptor substrates. D469 (*E. coli* TK) also governs the stereospecificity and stereoselectivity of transketolase.[22, 23] Directed evolution of TK, especially with mutations at D469, has been shown to enhance and reverse the stereoselectivity of TK and improve the reaction towards non-hydroxylated substrates.[23-25] This has increased the spectrum of aliphatic substrates accepted by transketolase and its potential in organic synthesis applications.

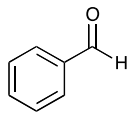
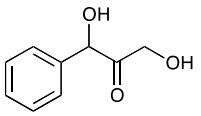
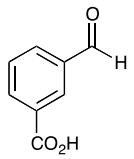
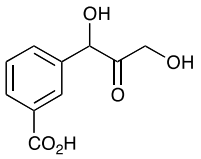
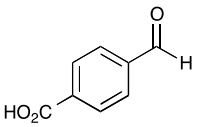
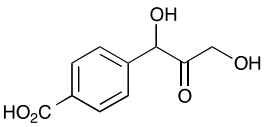
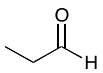
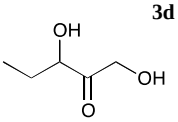
Improving the acceptance of aromatic aldehydes by TK would pave the way for the biocatalytic synthesis of novel aromatic dihydroxyketones, and via subsequent transamination, access aromatic aminodiols as the basis of novel routes to antibiotics such as chloramphenicol analogues. Wild-type (WT) *E. coli* TK has been reported to accept only a few aromatic aldehydes such as phenylacetaldehydes, and with low product yields from overnight reactions, often containing by-products.[20] More challenging are benzaldehyde and the heteroaromatic aldehydes such as 2-furaldehyde and 2-thiophene-carboxaldehyde which are not accepted by wild-type *E. coli* TK before the hydroxypyruvate substrate degrades,[20, 26] although some limited activity has been reported for yeast TK.[10] Recent mutants of *E. coli* TK: D469E; D469T; D469K and F434A, each resulted in the acceptance of benzaldehyde, 2-furaldehyde and 2-thiophene-carboxaldehyde, but again with very limited activity and product yields of just 1-10%.[26]



2a-2d **Li-1** **3a-3d**

Scheme 1. The reaction between hydroxypyruvate and aldehyde catalysed by TK to generate dihydroxyketones.

Table 1. Aldehyde substrates used and products formed.

Aldehyde TK substrate	Dihydroxyketone product
Benzaldehyde **2a**	**3a**
	
3-Formylbenzoic acid (3-FBA) **2b**	**3b**
	
4-Formylbenzoic acid (4-FBA) **2c**	**3c**
	
Propanal **2d**	**3d**
	

The current work aimed to identify the factors governing the bioconversion of aromatic aldehydes by transketolase, beginning with the known D469 mutants of *E. coli* TK. As the poorly accepted benzaldehyde **2a** has few features that would allow the enzyme to form specificity enhancing interactions, carboxylated analogues 3-formylbenzoic acid (3-FBA) **2b** and 4-formylbenzoic acid (4-FBA) **2c** were chosen to re-establish electrostatic and hydrogen bonding interactions similar to those found with the natural phosphorylated substrates. To confirm that these substrates were interacting with the phosphate binding residues in TK, a series of rationally designed mutants were created to probe the importance of each residue for the observed activity with aromatic substrates. Computational docking of 3-FBA **2b** and 4-FBA **2c** into energy-minimised mutant structure models provided further insights into the impact of each mutation upon activity and substrate binding.

Results and Discussion

Screening for novel activities

TK crystal structures and earlier mutagenesis studies revealed several highly conserved residues that contribute to substrate specificity, including interactions with the phosphate moiety in natural substrates.[27-30] Furthermore, ribose-5-phosphate (R5P) can bind the TK active site in its cyclic six-membered ring form (cR5P),[30] as shown in Figure 1a. This suggested that some previously generated *E. coli* TK mutants might accept the aromatic ring of benzaldehyde in a similar manner and that presentation of negatively charged 3- or 4- substituents towards the phosphate binding residues could improve substrate affinity. This hypothesis was supported by the docking of 3-FBA **2b** into D469T containing the ThDP-enamine intermediate (Figure 1b), and comparing this to the crystal structure for *E. coli* TK bound to cR5P (Figure 1a).

In the crystal structure residues R358, S385, H461, and R520 interact with the phosphate group of cR5P,[30] via a mixture of hydrogen bonds and charge-charge interactions, similar to those found in the yeast TK structure complexed with erythose-4-phosphate.[28] Similar interactions were found between the negatively-charged carboxylate group and residues R358, S385, H461, and R520 when 3-FBA **2b** was docked into D469T. The orientation of the six-membered ring in cR5P was also found to be similar to the aromatic ring of 3-FBA **2b** although the latter was pulled further away from the ThDP cofactor. The new substrates 3-FBA **2b** and 4-FBA **2c**, therefore provide negatively charged moieties for potential electrostatic interactions with the phosphate binding residues of TK, with carboxylate pK_as of 3.8 and 3.7, for 3-FBA **2b** and 4-FBA **2c** respectively.[31]

Table 2. Performance of TK mutants towards benzaldehyde derivatives.

Mutant	Reported isolated yield[a]		Conversion yield[b]	
	2a	3-HBA	3-FBA **2b**	4-FBA **2c**
WT	0%	0%	0%	0%
D469T	2%	4%	65%	30%
D469Y	4%	n.d.	0%	0%
D469K	2%	n.d.	n.d.	n.d.
D469E	2%	0%	n.d.	n.d.
F434A	10%	6%	0%	0%

[a]Reported,[26] except D469Y where the reaction was carried out for 48 h. Isolated yields were obtained after purification and therefore incur minor losses relative to reaction conversion yields. (n.d. is not determined).

[b]Conversion yields were determined from the consumption of the aldehydes after 24 h at 50 mM 3-FBA **2b** and 50 mM HPA, or 30 mM 4-FBA **2c** and 30 mM HPA, in 50 mM Tris buffer, pH 7.0, 2.4 mM ThDP and 9 mM $MgCl_2$ [32] using 10% v/v clarified lysate in a total reaction volume of 300 μL.

To test the hypothesis experimentally, the activities of WT-TK and the previously identified mutants D469T, D469Y and F434A towards 3-FBA **2b**, 4-FBA **2c** were investigated, and compared to isolated yields for benzaldehyde **2a** and 3-hydroxybenzaldehyde (3-HBA) using the TK variants (Table 2). A 24 hour time-course reaction was performed using 3-FBA **2b** and 4-FBA **2c** with WT-TK, D469T, D469Y and F434A and monitored by HPLC. Only D469T gave any significant conversion of 3-FBA **2b** or 4-FBA **2c**, producing **3b** and **3c**, respectively. While 3-FBA **2b** gave **3b** in 65% conversion yield, 4-FBA **2c** gave rise to a 30% conversion yield (Table 2). These yields were both considerably higher than those obtained previously with any benzaldehyde substrate and mutant combination (0-10%). The reaction of 3-FBA **2b** with TK variant D469T reached a final yield of 65% yield within 2 hours, compared to the 2% yield reached after 17 hours with **2a**.[26] This potentially represents an increase in specific activity of up to 250-fold with 3-FBA **2b** relative to **2a**, although enzyme inactivation by exposure to unreacted aldehyde could also be a factor for the slower reaction with **2a**. Since D469T TK appears to accept aromatic aldehydes, it was tested against aromatic aldehydes bearing non-acid groups at the 3- and 4-positions, namely 3- and 4-methylbenzaldehyde, 3- and 4-methoxybenzaldehyde and 3- and 4-isopropylbenzaldehyde (see

Table S1) in order to confirm the importance of the negatively charged species at 3- and 4- positions. In all cases no conversions were noted which confirms the importance of the acidic functionality and hydrophobic interaction alone was insufficient to obtain high turnover for benzaldehyde derivatives. Although, the bioconversions of two hydrophobic heteroaromatic aldehydes; 2-phenylpropanal and phenylacetaldehyde, by D469T mutant were reported to achieve 50% conversion,[26] the reactivity and factors influencing their reactions with D469T cannot be compared with benzaldehyde derivatives. These aldehydes are significantly more reactive than the benzaldehydes due to electronic and steric effects.

The reaction using D469T and 3-FBA **2b** was scaled-up and carried out over 18 hours, and after purification **3b** was isolated in 88% yield. Notably the D469T reaction with **2a** also produced a double addition by-product (5% isolated yield), whereas with 3-FBA **2b** only a single product **3b** was observed by HPLC and isolated. The solubility of 3-FBA **2b** is much greater than **2a,** which may have enhanced the reaction rate. However, no biphasic behaviour was observed with either **2a** or 3-FBA **2b** at 50 mM concentrations.

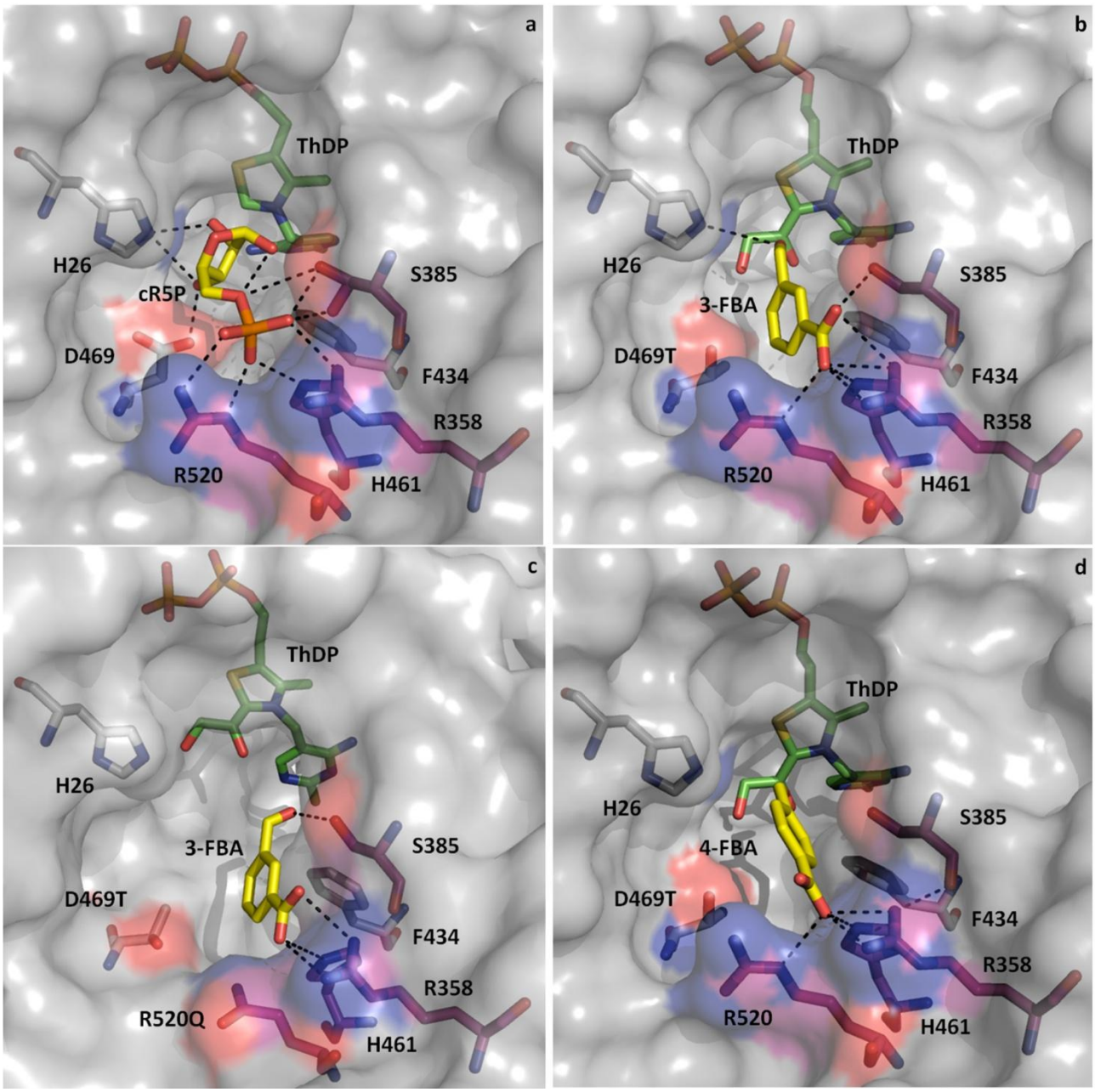


Figure 1. Computational docking of 3-FBA **2b** and 4-FBA **2c** into the active sites of D469T-based TK , and comparison with the binding of cyclic ribose-5-phosphate in the wild type active site. (a) Crystal structure of ribose-5-phosphate bound to *E. coli* WT-TK (PDB: 2R5N). Computational docking of 3-FBA **2c** in models of D469T (b), and D469T/R520Q (c), each derived from the wild-type structure (PDB: 1QGD). (d) Computational docking of 4-FBA **2c** in D469T. Hydrogen bonds to substrates are represented by black dotted lines. ThDP-enamine bound TK structure was obtained from computational docking of energy-minimised ThDP-enamine into modelled mutant structures

The activity with D469T reflects the generally higher yields (2-6%) previously obtained for this mutant relative to the other D469 mutants on benzaldehyde **2a**, 3-hydroxybenzaldehyde and hetero-aromatic aldehydes.[26] Crystal structures of yeast and *E. coli* TK reveal that D469 interacts with the α-hydroxyl group of substrates,[22] and replacing this negatively charged residue with the more hydrophobic and uncharged threonine was reported to enhance the activity toward non-hydroxylated substrates, due to the more favourable interactions with non-polar groups.[23-25] For similar reasons, D469T also previously gave a low activity towards benzaldehyde that was not observed in the WT enzyme. A decrease in steric hindrance for benzaldehyde to access the active site may also play a role in its reactivity with D469T. Docking of 3-FBA **2b** into D469T (Figure 1b) and alignment of the WT-TK structure (not shown) reveals that the carboxyl moiety of D469 would be just 2.9 Å from the aromatic ring of 3-FBA **2b**, compared to a distance of 4.1 Å from the D469T side chain hydroxyl group. This supports steric hindrance as a factor, but more important is that the D469T mutation avoids the burial of a charged group when 3-FBA **2b** is bound.

Our observations with D469Y are consistent with steric hindrance being a key factor. While D469Y accepts the non-hydroxylated aliphatic substrate propanal **2d** to give **3d** in high yield, and even reverses the product *ee*,[23] it only gave a 4% isolated yield after 48 hours with benzaldehyde **2a**, and did not accept the larger 3-FBA **2b** and 4-FBA **2c** derivatives (Table 2). The aromatic tyrosine side chain has much greater steric bulk than threonine, which may restrict the access of benzaldehydes **2a-c** to the C-2 carbon of the thiazolium ring resulting in poor or no activity. Interestingly, F434A did not accept 3-FBA **2b** or 4-FBA **2c** (Table 2), even though activity has been previously reported for **2a**.[26] This suggested that while F434A produces a hydrophobic pocket large enough to accept benzaldehyde (10% yield), it may not accept the aromatic ring in an orientation required to accommodate the added steric bulk or charge from the 3-FBA **2b** or 4-FBA **2c**. This view is consistent with the lower isolated yield observed previously with 3-HBA (6%) which is uncharged and slightly smaller than either 3-FBA **2b** or 4-FBA **2c**.

Kinetics of mutants with 3-FBA 2b and 4-FBA 2c

To determine the rate enhancing influence of interactions between the phosphate binding residues R358, H461, or R520, and the carboxyl groups of 3-FBA **2b** and 4-FBA **2c**, a series of mutations of these residues were made in D469T based on previously identified functional mutations. Mutations at these residues in yeast TK were previously shown to lower the affinity towards phosphorylated substrates,[28] and improve the activity towards non-phosphorylated substrate.[27] Due to their influences in the activities towards phosphorylated and non-phosphorylated substrates, they were chosen as targets for studying the enzyme-substrate affinity where the phosphate group was mimicked by a carboxylate. The yields and kinetic parameters for D469T and six further mutants of D469T are shown in Table 3 (see Figure S1-S2 for Michaelis-Menten plots). For 3-FBA **2b**, all mutants gave similar conversion yield after 24 hours, except for R358L/D469T which gave <10% yield but was also less active and R358P/D469T/R520Q with 14% yield which also has high affinity towards 3-FBA and was subject to stronger substrate and product inhibitions. The yield was independent of kinetic differences between mutants, while up to 20% Li-HPA (**1**) also remained, indicating possible product inhibition by the same extent for all mutants. The rates and product yields for the mutants with 4-FBA **2c** were found to be much lower than for 3-FBA **2b**, and the activities for H461S/D469T and H461S/D469T/R520Q were too low to obtain rate data. Lower yields at 24 hours were due to lower activities, although enzyme inactivation and HPA degradation over the longer timescales may also have had a significant influence. For example, after 24 hours 30% of 4-FBA **2c** was converted by D469T, yet only 50% HPA remained, suggesting 20% HPA degradation. The causes of HPA degradation were further investigated. HPA was incubated for 18 hours, in the presence and absence of ThDP, for lysates of overexpressed wild type TK, *E. coli* XL-10 Gold cells with no overexpressed TK, and for a Tris buffer control. Background degradation of HPA was between 2% and 14%, with or without ThDP, even for the Tris buffer control. 5% of the background appears to be catalysed by overexpressed WT TK. This demonstrates that other host enzymes in the lysate, including background WT-ThDP, do not significantly contribute to this degradation. However, 96% HPA degradation did occur with lysates containing D469T or D469T/R520Q TK and additional ThDP (see Table S2). This suggests that HPA degradation is catalysed by D469T and D469T/R520Q containing mutants over long timescales, which is itself an interesting result.

A surprising result is that R358P/D469T increased the relative specific activity with 50 mM 3-FBA **2b** by 20% compared to D469T. k_{cat}/K_M values for R358P/D469T and D469T/R520Q also increased to 550 ± 80 $s^{-1} M^{-1}$ and 470 ± 170 $s^{-1} M^{-1}$, respectively, from 240 ± 50 $s^{-1} M^{-1}$ for D469T while the highest k_{cat}/K_M value was found to be 630 ± 115 $s^{-1} M^{-1}$ in the triple mutant R358P/D469T/R520Q. Remarkably, these mutations therefore bring the k_{cat}/K_M of 3-FBA **2b** to higher values than those observed with WT *E. coli* TK and glycolaldehyde (283 $s^{-1} M^{-1}$) and with D469T and propanal (91 $s^{-1} M^{-1}$),[25, 27] yet are still much lower than those with yeast TK and the natural phosphorylated substrates erythrose-4-phosphate ($1.7x10^6$ $s^{-1} M^{-1}$) and ribose-5-phosphate ($3.2x10^5$ $s^{-1} M^{-1}$).[22, 28] The activity of D469T/R520Q was similar to D469T at low 3-FBA **2b** concentrations but slightly lower than D469T at above 30 mM 3-FBA. By contrast, R358L/D469T had a severely impaired activity, and H461S/D469T had the lowest activity at all concentrations but was slightly recovered by introduction of R520Q in the triple mutant H461S/D469T/R520Q. The activity of R358L/D469T and yield of product obtained was too low to obtain kinetic parameters. Substrate inhibition was also observed at above 40 mM for all mutants and so inhibiting concentrations were excluded from the kinetic analysis.

Each single charge-neutralising arginine mutation, R520Q or R358P in D469T, surprisingly increased k_{cat}/K_M for 3-FBA **2b.** While k_{cat} decreased slightly from 13 s^{-1} in D469T to 6 s^{-1}, 11 s^{-1}, and 3.4 s^{-1} for D469T/R520Q, R358P/D469T, and R358P/D469T/R520Q respectively, their K_M values decreased more significantly from 56 mM in D469T to 13 mM for D469T/R520Q, and 20 mM for R358P/D469T.

Table 3. Kinetic parameters of TK mutants towards 3-FBA **2b**, 4-FBA **2c** (see Figure S1-S2 for Michaelis-Menten plots).

Mutant	Substrate	Specific activity (relative to D469T)	Conversion yield (%)	K_M (mM)	k_{cat} (s^{-1})	k_{cat}/K_M (s^{-1} M^{-1})
WT	3-FBA **2b**	0	0	n.d[b]	n.d.	n.d.
D469T	3-FBA **2b**	1.0[a]	67 (1.0)	56 (10)	13 (1.5)	236 (50)
H461S/D469T	3-FBA **2b**	0.08	65 (0.6)	29 (15)	0.8 (0.2)	28 (16)
D469T/R520Q	3-FBA **2b**	0.75	66.7 (0.1)	13 (4)	6 (0.75)	468 (170)
H461S/D469T/R520Q	3-FBA **2b**	0.21	62.7 (0.7)	11 (3)	1.5 (0.2)	138 (45)
R358P/D469T	3-FBA **2b**	1.2	65 (2.5)	20 (2.5)	11.0 (0.7)	553 (80)
R358L/D469T	3-FBA **2b**	<0.1	<10	n.d.	n.d.	n.d.
R358P/D469T/R520Q	3-FBA **2b**	0.42	14 (1.3)	5.5 (1)	3.4 (0.2)	625 (115)
WT	4-FBA **2c**	0.0	0.0	n.d.	n.d.	n.d.
D469T	4-FBA **2c**	1.0[a]	30(1.7)	251 (240)	5.0 (4.4)	20 (26)
D469T/R520Q	4-FBA **2c**	0.45	13(0.4)	25 (7.6)	0.20 (0.03)	8 (2.8)
R358P/D469T	4-FBA **2c**	<0.005	1(0.1)	n.d.	n.d.	n.d.
R358L/D469T	4-FBA **2c**	0.30	5(0.1)	n.d.	n.d.	n.d.
R358P/D469T/R520Q	4-FBA **2c**	<0.001	<1	n.d.	n.d.	n.d.

Standard errors are in parentheses. Specific activities and percentage yields were determined with equimolar substrates at 50 mM 3-FBA **2b** and HPA, or 30 mM 4-FBA **2c** and HPA, in 50 mM Tris buffer, pH 7.0, 2.4 mM ThDP and 9 mM $MgCl_2$ using 10% v/v clarified lysate in a total volume of 300 μL. The conversion yields were determined from the consumption of the aldehydes and the productions of **3b** and **3c**.

[a]The specific activity of D469T towards 3-FBA **2b** and 4-FBA **2c** are 4.6 μmol mg^{-1} min^{-1} and 0.45 μmol mg^{-1} min^{-1} respectively.

[b] not determined (n.d.) stated where the activities were too low to obtain kinetic constants

The H461S mutation in H461S/D469T had a similar though weaker effect by decreasing the K_M to 29 mM, though this mutant also led to a significant drop in k_{cat} to just 0.8 s^{-1}. Unexpectedly, the highest affinity was found to be 5.5 mM when both R358 and R520 residues were mutated to neutral amino acids in R358P/D469T/R520Q. This significant improvement in the K_M is the major factor that improves the k_{cat}/K_M of R358P/D469T/R520Q mutant. However, the specific activity of R358P/D469T/R520Q at 50 mM of 3-FBA **2b** is only 42% of D469T due to the very low k_{cat} compared to D469T. Since H461 seems to provide a weak interaction, the strong affinity and severely impaired k_{cat} in R358P/D469T/R520Q may arise from an exposure of a certain positively charged arginine such as R91 which provides a new 3-FBA **2b** binding site upon the mutations. Yet, binding to this new binding site is rather unproductive. These results indicate an increased affinity for 3-FBA **2b** when a positive charge is removed from the phosphate binding region. This may be in part explained by the role of these residues in binding a phosphate which has a charge of -2, compared to 3-FBA **2b** which has a charge of -1. Therefore, the close proximity of two positively charged arginine residues (R358 and R520), and the positively charged H461, appears to be destabilising when interacting with a substrate that has only a single negative charge. Removal of a positive charge via R520Q, R358P or H461S removes the destabilisation caused by burying the additional charge, and hence improved the affinity of D469T TK for 3-FBA **2b** even when both arginine residues were removed. These results differ to the decreased affinities observed for natural phosphorylated substrates using mutations at equivalent residues in yeast TK,[28] as in that case mutations led to the destabilising burial of an excess single negative charge on the substrate.

The effect of H461S on the K_M for 3-FBA **2b** in either H461S/D469T or the triple mutant is relatively low compared to the effects of R520Q or R358P, which suggests that H461 may not contribute a major electrostatic interaction with the carboxylate group in 3-FBA **2b**, but rather a weaker hydrogen bond. Therefore, the substitution to serine could still provide the necessary hydrogen bond donor, although the shorter side chain in serine may have brought it out of range of the substrate. However, simultaneous mutation at H461 and R520 in the triple mutant left only one positive charge at R358, which led to the greatest affinity between TK and 3-FBA **2b** at 11 mM.

All of the mutations caused a decrease in k_{cat} with 3-FBA **2b** which implied that removing one of the charged residues results in a movement of the substrate which in turn repositions the aldehyde relative to the active centre of the ThDP-enamine intermediate. The greatest loss of k_{cat} was due to the mutation H461S in H461S/D469T, though this was slightly restored in the triple mutant H461S/D469T/R520Q. It is also possible that the more significant loss of k_{cat} in H461S/D469T is related to a decrease in enzyme stability or rearrangement of active site residues. Indeed, H461S was recently found to dramatically decrease the activity of TK towards glycolaldehyde and propanal **2d** when combined with other active-site mutations, while the mutation R520Q stabilised certain D469 mutations that otherwise disrupted a coevolved network of residues within the active site.[33] Furthermore, the instability of H461S was also indicated by the lower soluble fraction of H461S which was found to be 10% compared with 16% in wild type.[33] The activity of each mutant towards **2d** was therefore tested as a control. Propanal is uncharged and also too small to interact with the phosphate binding residues when bound in a productive conformation. H461S was found to decrease the specific activity for **2d** from 1.39 μmol mg^{-1} min^{-1} with D469T, to just 0.08 μmol mg^{-1} min^{-1}

with H461S/D469T, consistent with a minor active-site rearrangement or partial destabilisation of the enzyme. The specific activity increased slightly to 0.22 μmol mg^{-1} min^{-1} in H461S/D469T/R520Q, consistent with stabilisation by R520Q.

For D469T, the specific activity towards 4-FBA **2c** was 0.45 μmol mg^{-1} min^{-1}, approximately 10-fold lower than for 3-FBA **2b**, consistent with a 12-fold lower k_{cat}/K_M. The K_M values for D469T TK indicate that 4-FBA **2c** binds with 5-fold lower affinity than 3-FBA **2b** even though both substrates carry the same charge, while k_{cat} was 2.5-fold lower for 4-FBA **2c**. This suggests that the relative orientations of the aldehyde and carboxylate moieties are important for both productive binding and catalysis. While the *para*-carboxylate in 4-FBA **2c** is expected to electronically favour nucleophilic attack at the aldehyde relative to the meta-carboxylate in 3-FBA **2b**, such an electronic effect is clearly not a major influence on the observed kinetics for 3-FBA **2b** and 4-FBA **2c**. The reaction using D469T and 4-FBA **2c** was scaled-up and carried out over 18 hours to give **3c** in a 40% conversion yield. The product **3c** was prone to rearrangement to the propan-1-one isomer on purification and was isolated in 8% yield.

Overall, the bioconversion of 4-FBA **2c** was significantly impaired when any one of R358, H461 or R520 were mutated to a neutral amino acid, when considering either specific activity or yield. However, at least in the case of R520Q, this was not due to the loss of affinity towards 4-FBA **2c**, where the K_M decreased 10-fold. For all mutants where K_M was measured, the removal of at least one positive charge in R358, H461 or R520 led to an improved affinity for both 3-FBA **2b** and 4-FBA **2c**, consistent with an increase in stability achieved by bringing the active-site net charge closer to neutral when bound to these substrates.

While the R520Q mutation led to a 10-fold improved affinity for 4-FBA **2c** in D469T/R520Q, k_{cat} decreased almost 25-fold. This contrasts to 3-FBA **2b** where the affinity improved 4-fold in D469T/R520Q but k_{cat} only decreased to 50% that of D469T. Moreover, the K_M of D469T/R520Q towards 4-FBA **2c** is only 2-fold higher than for 3-FBA **2b**, whereas the k_{cat} for 4-FBA **2c** is 30-fold lower than for 3-FBA **2b**. Again this suggests that the affinity gained by creating a specific interaction between the substrate carboxylate-moieties and the mutated phosphate binding pocket in TK serves to differentiate the two substrates by placing the aldehyde group of 3-FBA **2b** in a more productive orientation for catalysis than with 4-FBA **2c**. The conversion yield of **2c** with R358P/D469T was 60-fold lower than that for D469T at 30 mM of HPA and 4-FBA **2c**, consistent with the much lower specific activity. This contrasts with the activity found for R358P/D469T with 3-FBA **2b**, which was higher even than the activities of D469T and D469T/R520Q (Table 3). Therefore the R358P mutation improves the affinity to 3-FBA **2b** but severely impairs the activity towards 4-FBA **2c**. The R358L mutation results in a partial loss of activity towards 4-FBA **2c** and a slightly greater loss for 3-FBA **2b**.

The removal of these positively charged amino acids has illustrated the change in charge distribution within the active site and it could provide a better microenvironment for the binding of benzaldehyde. However, the bioconversion of benzaldehyde by D469T/R520Q was less than 3% which is not significantly improved from D469T. This still implied that hydrophobic alone is insufficient for high bioconversion of aromatic aldehydes. The triple mutants have low performance, so they were not further investigated.

Computational Docking of 3-FBA 2b and 4-FBA 2c

The computational docking of 3-FBA **2b** into D469T showed that the carboxylate group of 3-FBA **2b** is in close proximity to R358, S385, H461, and R520 in D469T (Figure 1b). 3-FBA **2b** was found to have two major binding clusters in D469T and the free binding energy of both clusters were similar. In one cluster the aldehyde group of 3-FBA **2b** forms a hydrogen bond with H26 to bring the aldehyde into close proximity to the enamine (4.89 Å). However, in the second cluster the aldehyde was at a non-productive distance from the enamine (7.21 Å) due to a hydrogen bond formed between the aldehyde O-atom and H473, and could potentially even inhibit enzyme function. It should be noted that benzaldehyde did not dock into the D469T active site with any distinct cluster of structural poses.

The interactions between the carboxylate group of 3-FBA **2b** and D469T/R520Q were slightly different to those in D469T, whereby a small shift towards R358 removed a hydrogen bond with S385 in D469T/R520Q (Figure 1c). This also moved the 3-FBA **2b** molecule to bring the aldehyde group much further away (6.59 Å) from the enamine than in D469T, consistent with the 2-fold lower k_{cat} observed experimentally for D469T/R520Q.

Only one binding cluster was predicted for 4-FBA **2c** in the D469T active site (Figure 1d). While 4-FBA **2c** formed hydrogen bonds between one of the oxygen atoms of the carboxylate group in 4-FBA **2c** and residues R358, H461, and R520, it lacked several others found with 3-FBA **2b**, consistent with the 5-fold higher K_M for 4-FBA **2c**. In particular, 4-FBA **2c** lacked the hydrogen bond to S385 and also all interactions with the second carboxylate O-atom. The aldehyde was placed 5.05 Å away from the enamine, which is only 0.16 Å further than for 3-FBA **2b** in D469T which may play some part in the 2.5-fold lower k_{cat} for 4-FBA **2c** relative to 3-FBA **2b**. However, this was much closer than the distance between the aldehyde of 3-FBA **2b** and the enamine in D469T/R520Q, which gave a similar k_{cat} to 4-FBA **2c** in D469T, indicating that factors additional to proximity, such as orientation of the aldehyde carbonyl relative to the enamine intermediate, are also a factor. Finally, D469T/R520Q was found to have four highly populated binding clusters with similar binding energies when docking with 4-FBA **2c**. These gave aldehyde to enamine distances of 4.6 Å, 6.5 Å, 8.3 Å and 8.9 Å, with 35%, 10%, 16% and 26% occupancies respectively, consistent with the poor k_{cat} observed.

Although the prediction of binding sites and the positions of 3-FBA **2b** and 4-FBA **2c** by AutoDock agreed with the kinetic studies, the free energies of binding (Table 4) were consistent with experimental data only for wild-type and D469T TK, whereas D469T/R520Q calculations deviated considerably from the best line of fit to the plot of experimental versus calculated -log(K_d) values (supplementary information Figure S3 and Table S3). The deviations for D469T/R520Q calculations were likely to be due to small active-site structure changes during the energy minimisation of the D469T/R520Q double mutant using CharmM. Small structure shifts may have resulted in a systematic shift in the calculations of binding free energy in Autodock relative to those in wild-type of D469T.

Table 4. The free energy of binding, predicted K_d and K_M values of 3-FBA **2b** and 4-FBA **2c** for D469T and D469T/R520Q.

Mutant	ΔG predicted (kcal/mol)		K_d predicted (mM)		K_M experiment (mM)		Distance of aldehyde to enamine (Å)	
	3-FBA **2b**	4-FBA **2c**	3-FBA **2b**	4-FBA **2c**	3-FBA**2b**	4-FBA **2c**	3-FBA **2b**	4-FBA **2b**
D469T	-3.35	-3.15	3.30	4.64	56	251	4.89	5.05
D469T/R520Q	-1.96	-1.91[a]	35.3	38.7	13	25	6.59	4.6-8.9[b]

The K_d values were calculated from $\Delta G = -RT.\ln(K_d)$ where ΔG is free energy of binding.

[a]Population weighted average from four docking clusters (ranged -1.87 to -1.96 kcal/mol).

[b]Range observed for four docking clusters.

Conclusion

We have illustrated that the naturally existing phosphate binding pocket in the *E. coli* transketolase active site can be used to improve the bioconversion of benzaldehyde derivatives by appropriate positioning of a carboxylate moiety on the aromatic ring. Reliance on only hydrophobic interactions between the enzyme and benzaldehyde leads to inefficient catalysis, but aromatic substituents can provide the basis for hydrogen bonding and electrostatic interactions for high catalytic conversion. In particular, the *meta*-carboxylate group significantly improves the activity observed in 3-FBA **2b** relative to benzaldehyde. Decreasing the total net positive charge in the phosphate binding site by mutation of arginine residues led to an improved binding affinity to carboxylated benzaldehyde derivatives.

Materials and Methods

Chemicals and reagents

All chemical reagents were obtained from Sigma-Aldrich, unless otherwise stated. Lithium hydroxypyruvate was prepared as described previously.[20] Propanal **2d** was purchased from AcroSeal (Acros Organics, Fisher Scientific, UK). 1,3-Dihydroxypentan-2-one **3d** was synthesised as described previously,[5] as a product standard for HPLC calibration.

Transketolase library

All transketolases were expressed under the control of *tktA* gene promoter in the plasmid pQR791 in XL-10 Gold (Stratagene, La Jolla, California, USA).[34] WT-TK, D469T, D469T/R520Q, D469Y, and F434A were available from our previous published works. Additional mutants were constructed using the Quikchange™ site directed mutagenesis kit (Stratagene) transformed into XL-10 gold competent cells (Stratagene), and confirmed by DNA sequencing. Primers used for additional mutant construction were as follows, where the target codons are in bold, and the changed bases underlined:

R358P: GAAAATCGCCAGC**C**<u>**CA**</u>AAAGCGTCTCAGAATG
R358L: GAAAATCGCCAGC**C**<u>**TT**</u>AAAGCGTCTCAGAATG
H461S:
GGTGATGGTTTACACC<u>**AG**</u>**C**GACTCCATCGGTCTGG
D469Y: TCGGTCTGGGCGAA<u>**T**</u>**AC**GGGCCGACTCACCAG
R520Q: GATCCTCTCCCAG**C**<u>**AG**</u>AACCTGGCGCAG
F434A: CGTACACCTCCACC<u>**GC**</u>**C**CTGATGTTCGTGG
D469T: TCGGTCTGGGCGAA<u>**AC**</u>**C**GGGCCGACTCACCAG

Enzyme preparation and quantification

Glycerol stocks of all mutants were streaked on LB plates containing 150 μg/mL ampicillin and cultured overnight at 37 °C. Single colonies were picked and cultured in 250 mL flask containing 20 mL of LB with 150 μg/mL of ampicillin for 16 hours at 37 °C, shaking at 250 rpm. Cells were harvested by centrifugation, the pellet resuspended in 50 mM Tris buffer, pH 7.0, then sonicated (MSE Soniprep 150 probe, Sanyo) on ice with 10 s on, 15 s off for 10 cycles. The lysate was clarified by centrifugation at 17,700 g for 10 min at 4 °C. The supernatant was aliquoted and stored at -80 °C. Total protein concentrations were determined by Bradford assay using BSA as a standard. The concentration of TK was determined by SDS-PAGE and densitometry as previously described.[27]

Screening assay when using 3-FBA 2b and 4-FBA 2c

WT TK, mutants D469Y and D469T/R520Q previously reported to enhance the activity toward non-hydroxylated aldehydes,[23-25, 33] and F434A which was previously found to have some activity with benzaldehyde and other aromatic substrates,[26] were initially screened for activity with 3-FBA **2b** and 4-FBA **2c**. Clarified lysate (30 μL) was incubated for 20 minutes with 140 μL of 50 mM Tris buffer, pH 7.0, and 30 μL of 10x cofactor solution (24 mM ThDP and 90 mM $MgCl_2$).[32] The reaction was started by addition of 100 μL of 3x substrate solution (150 mM HPA and 150 mM 3-FBA **2b** or 90 mM 4-FBA **2c** and 90 mM HPA, in 50 mM Tris buffer, pH 7.0, prepared in glass vials). After 24h at 25 °C, 20 μL samples were taken and added to 180 μL of 0.1% TFA, centrifuged at 13,000 rpm for 3 minutes, and the supernatants analysed by HPLC with UV detection (210 nm). The Aminex HPx-87H, 300x7.8mm HPLC column (Bio-Rad), was maintained at 60 °C, and analysis performed by isocratic flow of 0.1% TFA in water at 0.6 mL/min. The consumptions of the starting materials were monitored and the retention times for both 3-FBA **2b** and 4-FBA **2c** were 64 minutes.

Enzyme kinetics with 3-FBA 2b and 4-FBA 2c

Due to previously observed instabilities with purified TK mutants,[33] clarified lysate was used throughout this study. Enzyme reactions were prepared as for the screening method, except 3-FBA **2b** concentrations were varied from 10 mM to 40 mM, with HPA kept constant at 50 mM. Reactions were carried out in triplicate in glass vials at 22 °C. Samples of 20 μL were taken every 4 minutes for at least 20 minutes and added to 380

μL of 0.1% TFA, then analysed by HPLC on an ACE5 C18 reverse phase column (150 x 4.6 mm), using 0.1% TFA at 1.0 mL/min with a gradient of acetonitrile from 15% to 72% over 9 minutes. The gradient was followed by a 2 min equilibration. The retention times of 3-FBA **2b** and the product **3b** were 5.47 and 2.97 minutes, respectively. The K_M and k_{cat} of each mutant were determined by non-linear regression fitting of the Michaelis-Menten plot. Repeat kinetics were obtained with enzyme concentrations at between 0.04 – 0.07 mg/mL with negligible effect on the final kinetic parameters. Kinetics for 4-FBA **2c** were obtained in the same way but limited to 6-30 mM 4-FBA **2c** due to precipitation upon quenching in TFA at above 30 mM. The retention times of the 4-FBA **2c** and the product **3c** were 5.41 and 2.45 minutes, respectively. For 4-FBA **2c**, the TK concentration in the reaction was 0.1 mg/mL for D469T and 0.17 mg/mL for D469T/R520Q. Substrate and product standards of 3-FBA **2b** and 4-FBA **2c** were used to calibrate the conversion yield.

Propanal activity

Clarified lysate was incubated with 10x cofactor as above for 3-FBA. The reaction was started by addition of 100 μL of 3x substrate solution (150 mM HPA, 150 mM propanal **2d** in 50 mM Tris buffer, pH 7.0). Samples of 20 μL were taken every 20 minutes for 2 hours, quenched in 180 μL of 0.1% TFA, centrifuged at 13,300 rpm for 3 minutes, then analysed by HPLC as for 3-FBA **2b** screening. A standard of chemically synthesised **3d** was used to calibrate the product concentrations obtained.

General methods

Flash column chromatography was carried out using silica gel (particle size 40-63 μm). ^{1}H NMR and ^{13}C NMR spectra were recorded at the field indicated using Bruker AMX300 MHz and Avance-600 MHz machine. Coupling constants are measured in Hertz (Hz) and NMR spectra were recorded at 298 K. Mass spectra were recorded on a Thermo Scientific LTQ Orbitrap XL spectrometer. Infrared spectra were recorded on a Perkin Elmer Spectrum 100 FTIR spectrometer.

3-(1,3-Dihydroxy-2-oxopropyl)benzoic acid 3b

$MgCl_2$ (390 μL of 100 mg/mL solution, 0.41 mmol) was added to ThDP (22 mg, 48 mmol) in water (10 mL) at pH 7. Variant D469T TK cell free lysate (2 mL; approx. 1 mg/mL) was added and the mixture stirred for 20 minutes. In a separate flask, Li-HPA (110 mg, 1.00 mmol) was dissolved in water (10 mL) with 3-formylbenzoic acid (0.150 g, 1.00 mmol) and the pH adjusted to 7 using sodium hydroxide solution (1 M). This was then added to the enzyme suspension and the reaction was stirred for 18 h. The pH was maintained at 7.0 by the addition of 1 M HCl using a pH stat (Stat Titrino, Metrohm). Silica was added to the reaction mixture and the solvent was removed *in vacuo*. The product was purified by flash silica chromatography (MeOH:CH_2Cl_2, 1:4) to afford 3-(1,3-dihydroxy-2-oxopropyl)benzoic acid as an amorphous solid (186 mg, 88%). M.p. decomp.; R_f 0.57 (MeOH:CH_2Cl_2, 1:4); $[\alpha]^{25}_D$ = +37.8 (*c* 0.8, MeOH); ν_{max}(neat)/cm^{-1} 3252, 2946, 2843, 1693, 1606; ^{1}H NMR (300 MHz; CD_3OD) δ 8.08 (1H, br s, 2-H), 7.96 (1H, dt, *J* 7.7 and 1.4 Hz, 4-H), 7.61 (1H, d, *J* 7.7 Hz, 6-H), 7.45 (1H, t, *J* 7.7 Hz, 5-H), 5.36 (1H, s, C*H*OH), 4.46 (1H, d, *J* 19.2 Hz, C*H*HOH), 4.39 (1H, d, *J* 19.2 Hz, CH*H*OH); ^{13}C NMR (150 MHz; D_2O) δ 211.6 (*C*=O), 175.0 (*C*O_2H), 139.8, 139.5, 130.4, 130.1, 129.2, 128.9, 78.9, 66.0; *m/z* (NSI-) 209 ([M-H]$^-$, 100%), 179 (27), 149 (27); *m/z* (NSI-) found [M-H]$^-$ 209.0453. $C_{10}H_9O_5$ requires 209.0455.

4-(1,3-Dihydroxy-2-oxopropyl)benzoic acid 3c

$MgCl_2$ (390 μL of 100 mg/mL solution, 0.41 mmol) was added to ThDP (22 mg, 48 mmol) in water (10 mL) at pH 7. D469T TK cell free lysate (2 mL; approx. 1 mg/mL) was added and the mixture stirred for 20 minutes. In a separate flask, Li-HPA (110 mg, 1.00 mmol) was dissolved in water (10 mL) with 4-formylbenzoic acid (0.150 g, 1.0 mmol) and the pH adjusted to 7 using 1 M NaOH. This was then added to the enzyme suspension and the reaction was stirred for 18 hours. The pH was maintained at 7.0 by the addition of 1 M HCl using a pH stat (Stat Titrino, Metrohm). Silica was added to the reaction mixture and the solvent was removed *in vacuo*. The product was purified by flash silica chromatography (MeOH:CH_2Cl_2, 1:4) to afford 4-(1,3-dihydroxy-2-oxopropyl)benzoic acid as an amorphous solid (16 mg, 8%). M.p. decomp.; R_f 0.57 (MeOH:CH_2Cl_2, 1:4); $[\alpha]^{25}_D$ = +25.0 (*c* 0.8, MeOH); ν_{max}(neat)/cm^{-1} 3356, 2946, 1690, 1634, 1412; ^{1}H NMR (600 MHz; CD_3OD) δ 8.02 (2H, d, *J* 8.3 Hz, 3-H), 7.53 (2H, d, *J* 8.3 Hz, 2-H), 5.35 (1H, s, C*H*OH), 4.42 (2H, s, CH_2OH); ^{13}C NMR (150 MHz; CD_3OD) δ 210.7 (*C*=O), 170.4 (*C*O_2H), 144.9, 132.5, 131.0, 127.8, 78.6, 65.9; *m/z* (NSI-) 209 ([M-H]$^-$, 100%), 179 (15), 149 (86), 75 (47); *m/z* (NSI-)found [M-H]$^-$ 209.0453. $C_{10}H_9O_5$ requires 209.0455.

Computational docking of 3-FBA 2b and 4-FBA 2c into TK mutant structures

A ThDP-enamine initial structure, 3-FBA **2b**, and 4-FBA **2c** structures were drawn in chem3D ultra v.10 and energy minimized by MM2 calculation. The energy-minimised ThDP-enamine was docked into the active site and compared with the yeast-ThDP-enamine crystal structure (1GPU).[35] The structures of D469T, H461S/D469T, and D469T/R520Q were obtained from WT *E. coli* TK crystal structure (PDB: 1QGD) by creating the mutations and then energy minimising the structures around the ThDP-enamine, using the CharmM forcefield, Adopted Basic NR, Implicit Generalised Born solvent model, True SHAKE constant, and 1000 steps in Discovery Studio 2.0 (Accelrys, Inc. San Diego, California, USA). Docking of 3-FBA **2b** and 4-FBA **2c** was carried out using Autodock 4.2 and AutoDock tools 1.5.4,[36] with the grid centred at 11.777, 27.078, 37.195 and a grid size of 30 Å x 30 Å x 30 Å.

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Notes and references

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Table of contents entry

The presence of carboxylate substituents on the benzaldehyde ring improves transketolase activity towards aromatic substrates by reconstituting interactions similar to those with cyclic ribose 5-phosphate.