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FULL PAPER

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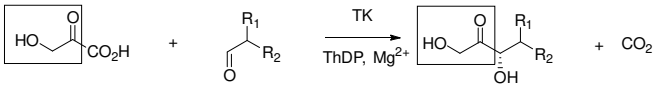
Abstract: Thermostable TK_{gst} was successfully engineered for the synthesis of aliphatic acyloins with various carbon backbone lengths (C_5-C_{10}). Based on structure guided studies, efficient TK_{gst} variants with enhanced activities toward aliphatic aldehydes as acceptors together with aliphatic pyruvate homologues as donors were identified. The TK_{gst} single variant L382F was able to catalyze efficiently the transfer of the ketol group from HPA on all targeted aliphatic aldehydes (C_3-C_8) to give the corresponding 1,3-dihydroxy ketones with good yields and excellent enantioselectivity. The combination of H102L/H474S previously designed for the improvement toward aliphatic pyruvate homologues, with F435I gave a new variant H102L/H474S/F435I able to transfer the acyl goup of 2-oxobutyrate and 2-oxovalerate to aliphatic aldehydes, giving mono hydroxylated ketones never obtained before with this enzyme.

Introduction

From a chemical viewpoint, acyloins (α-hydroxy ketones) are of particular value as fine chemicals because of their utility as building blocks for the production of larger molecules particularly used in pharmaceuticals.[1] In this family of compounds, aliphatic acyloins display other properties such as flavors[2] or nonionic surfactants.[3] Several purely chemical synthetic approaches have been proposed to afford acyloins,[4] together with some organocatalytic strategies (thiazolium-based carboligations,[5] aldolization[3]). However, many of these chemical approaches are not straightforward, lack selectivity, or are economically unattractive because of the large number of chemical steps required. Different enzymatic ways have been proposed by dynamic kinetic resolutions (DKRs) catalyzed by lipases,[6] whole-cell redox processes,[7] and thiamine diphosphate-dependent (ThDP) lyases.[8] This last family of enzymes are all capable of acyloin-type condensation reactions with a nucleophilic donor and an electrophilic acceptor (aldehyde) substrates in water under mild conditions, leading to chiral acyloins. ThDP enzymes react with the donor to form an active enamine carbanion which subsequently attacks the acceptor to form the α-hydroxy ketone product. As a relevant example, benzaldehyde lyase (BAL) catalyzes the carboligation of both aromatic and aliphatic aldehydes, thus forming many enantiopure (*R*)-α-hydroxy ketones.[8] The use of pyruvate decarboxylases (PDC) at industrial scale has been demonstrated with the synthesis of *L*-phenylacetylcarbinol (PAC) precursor of ephedrine.[8c] PDC has also been described for the synthesis of volatile acyloins with flavor properties obtained by condensing either aldehydes with pyruvate or 2-keto acids with acetaldehyde.[8c] Transketolase (TK) is another ThDP enzyme yielding α-hydroxy ketones by stereospecifically transfering a ketol unit from a "natural" donor polyhydroxylated ketose phosphates or hydroxypyruvate (HPA) to an aldehyde acceptor (scheme 1).[9] While the other ThDP enzymes show very high substrate specificity for their nucleophilic donor substrates, we recently engineered the thermostable transketolase from *Geobacillus stearothermophilus* (TK_{gst}) by directed evolution and we succeeded in the discovery of TK_{gst} variants able to utilize pyruvate and higher aliphatic homologues as nucleophiles for irreversible acyl transfer instead of ketol transfer from the natural polyhydroxylated ketose phosphates or HPA.[10] Another interest of TK catalyzed reaction compared to the other ThDP enzymes is the large range of aldehydes used as acceptors particularly (2*R*)-polyhydroxylated (phosphorylated or not) and non hydroxylated aldehydes with a short carbon chain. To improve and broaden the acceptor aldehyde spectra using HPA as donor, we discovered TK_{gst} variants that efficiently improved TK_{gst} activity toward (2*R*) and (2*S*)-configured polyhydroxylated aldehydes [11,12] and also arylated and aliphatic aldehydes with high stereocontrol.[13] Similar studies were also reported for the TK from *E. coli*.[14]



Donor HPA | Acceptor Aldehydes | Product (3*S*)-hydroxyketone

Scheme 1. Irreversible reaction catalyzed by TKs in the presence of hydroxypyruvate (HPA) as donor substrate with aldehydes as acceptor substrates.

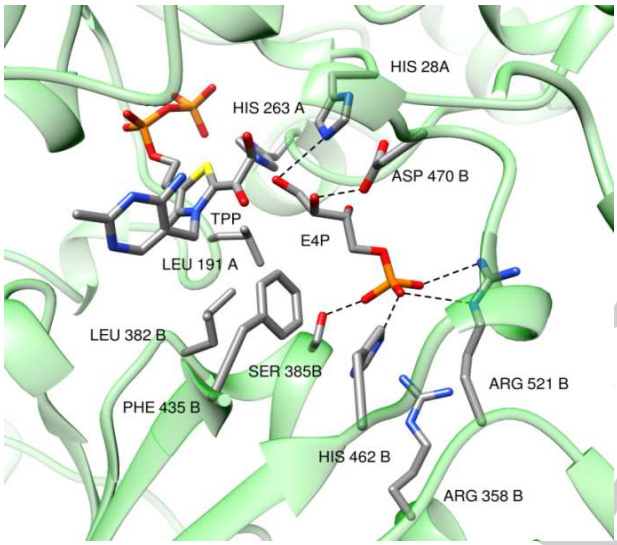
Our previous results obtained with TK_{gst} showing evolvability,[10-13] robustness against temperature and cosolvent,[15,16] these advantages appeared particularly suitable for the synthesis of aliphatic acyloins. It should be noted that these compounds display a particular interest in a program focused on the valorization of vegetable oil derivatives (*i.e.* fatty α-hydroxyketones)[17] in which they will be used as model structures. In this work, efficient TK_{gst} variants has been designed in using a structure-guided engineering by combining the hot spots found individually to improve TK_{gst} activity toward HPA or aliphatic pyruvate homologues as donors (2-oxobutyrate and 2-oxovalerate) as well as aliphatic aldehyde acceptors (C_3-C_8). The best TK_{gst} variants have been selected, purified and characterized through analytical studies and then used with the appropriated substrates for obtaining the corresponding acyloins.

Results and Discussion

Construction of TK_{gst} variant libraries.

To improve TK_{gst} activity toward aliphatic pyruvate homologues as donors as well as aliphatic aldehyde acceptors we combined hot spots identified in our previous studies by semi-rational engineering approach focusing on all residues that are in direct contact to the CH_2OH moiety of the natural donor (HPA) and to hydroxyl groups of natural acceptors (polyhydroxylated ketoses).[10-13] Since the three-dimensional structure of TK_{gst} is unknown, homology models of the TK_{gst} active-site pocket containing its natural phosphorylated acceptor aldose, D-erythrose-4-phosphate (E4P) or HPA as donors were built based on the X-ray crystal structure of TK_{ban} as a template, which, belonging to the same microbial species, has 74% identity (Figure 1).[14]

a)



b)

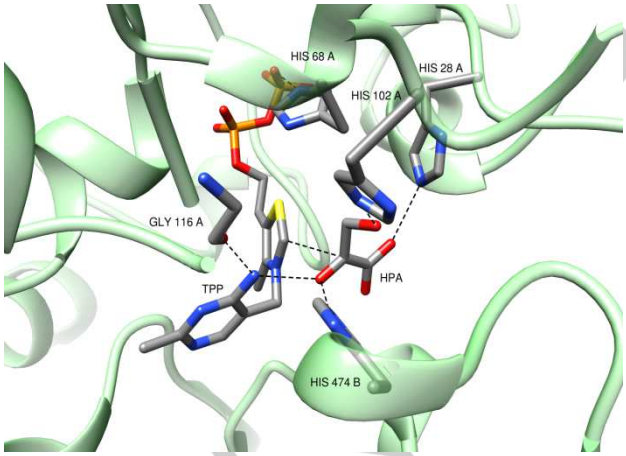


Figure 1. Model of wild-type TK_{gst} based on the X-ray crystal structure of TK_{ban} (PDB entry 3M49) with a) natural acceptor substrate D-erythrose-4-phosphate (E4P) and b) hydroxypyruvate (HPA) as donor. The model was built using Modeler 9.14 and Chimera.

For the improvement of TK_{gst} activity toward aliphatic aldehydes with long carbon chain as acceptors, we targeted the residues Leu191, Leu382, Phe435, and Asp470 arranged in space surrounding the C2 atom of the acceptor. Site Saturation Mutagenesis (SSM) applied on these positions revealed D470I, L382F, F435I, L191I as the best candidates for aliphatic acceptor substrates providing an hydrophobic contribution to substrate binding.[13]

For the improvement of TK_{gst} activity toward aliphatic pyruvate homologues as non-natural nucleophilic donors, we based our strategy on the well-known similarity among the ThDP-dependent enzyme family and the highly conserved sequence motif for the cofactor-binding site. We previously used a structure-guided analogy study with TK from *E.coli* (TK_{eco}) and another ThDP-dependent enzyme 1-deoxy-D-xylulose-5-phosphate synthase (DXS, EC 2.2.1.7), which can utilize pyruvate as a natural donor for decarboxylative carboligation and transfer of the acetyl group from the donor to an aldehyde. [10] We targeted some key positions (Gly116, His68, His102 and His474) closed to the CH_2OH moiety of the ketol. The screening of variant libraries on these combined positions revealed the double variant H102L/H474S as the best candidate for significantly enhanced substrate affinity toward aliphatic pyruvate homologues. Apparently, an exchange of the two histidine residues by smaller side chains was required to make room for the larger nucleophilic substrates.

Finally, the hot spots found individually to improve TK_{gst} activity toward each targeted donor and acceptor substrates were combined in expecting a cooperative effect. In this study, we produced four single TK_{gst} variants (L382F, D470I, F435I, L191I), one double TK_{gst} variant (H102L/H474S) and four new triple TK_{gst}variants(H102L/H474S/L382F, H102L/H474S/D470I, H102L/H474S/F435 and H102L/H474S/L191I).

Screening of TK_{gst} variants toward donor and acceptor substrates.

TK_{gst} variants were expressed in *E. coli* BL21(DE3)pLysS strain and after cell lysis, purified by Ni^{2+} chelating affinity column chromatography. Their specific activities with aliphatic acceptors **2a-f** coupled with HPA or aliphatic pyruvate homologues **1b** and **1c** as donors were compared to wild-type TK_{gst} (Chart 1, 2 and 3). To determine specific activities, we used an efficient, direct, quantitative high-throughput colorimetric assay developed earlier and based on pH changes caused upon TK catalyzed oxoacids consumption in the presence of phenol red as pH indicator.[18] The decarboxylation of oxoacids used as TK_{gst} donor substrates, causes release of bicarbonate and the pH to rise, rendering measurements independent of the structure of donor and acceptor substrates. Absorbance variation at 560 nm was measured at 50°C at initial time and then overnight.

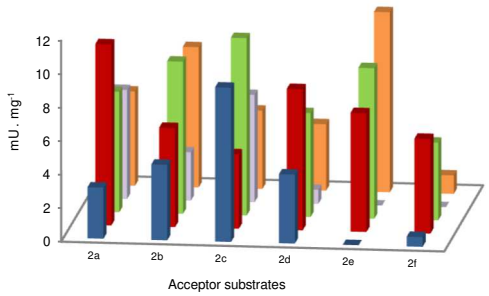


Chart 1. Specific activities of ■ wild type, ■ L382F, ■ D470I, ■ F435I, ■ L191I TK_{gst} toward aliphatic aldehydes **2a-f** as acceptors and HPA as donor. Enzyme reactions were carried out with **2a-f** (100 mM), HPA (50 mM), purified enzyme (0.4 mg . mL^{-1}), at pH 7.2 and 50 °C and incubated overnight.

For the further synthesis of 1,3-dihydroxyketones, aliphatic aldehydes (C_3-C_8) **2a-f** were investigated as acceptor substrates of single variant TK_{gst} (D470I, L382F, F435I, L191I) previously identified with HPA as donor (Chart 1). The best variant activities toward propanal **2a**, hexanal **2d** and octanal **2f** compared to wild type TK_{gst} were obtained with L382F while D470I allowed higher improvement toward butanal **2b** and pentanal **2c**. L191I appeared more efficient to increase TK_{gst} activity toward heptanal. F435I afforded no improvement compared to wild type except with propanal but with lower activities than L382F and D470I.

For the further synthesis of mono α-hydroxylated ketones, D470I, L382F, F435I, L191I were individually combined with H102L/H474S identified in our previous studies as the best candidate for aliphatic pyruvate homologues in place of HPA as donor.[10] The resulting variants were screened with increase carbon chain length aliphatic aldehydes **2a-f** in the presence aliphatic pyruvate homologues as donors, 2-oxobutyrate **1b** and oxavalerate **1c**.

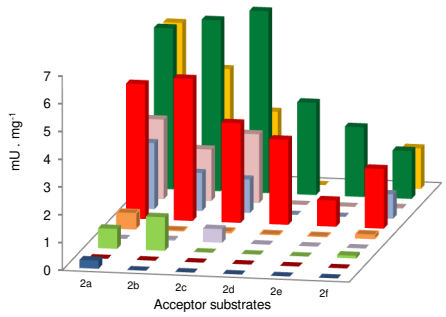


Chart 2. Specific activities of ■ wild type, ■ L382F, ■ D470I, ■ F435I, ■ L191I , ■ H102L/H474S, ■ H102L/H474S/L382F, ■ H102L/H474S/D470I, ■ H102L/H474S/F435I, ■ H102L/H474S/L191I toward aliphatic aldehydes **2a-f** as acceptors and 2-oxobutyrate **1b** as donor. Enzyme reactions were carried out with **2a-f** (100 mM), **1b** (50 mM), at, purified enzyme (0.4 mg . mL^{-1}), at pH 7.2 and 50 °C and incubated overnight.

With 2-oxobutyrate **1b** as donor (Chart 2), we observed that the single variants (L382F, D470I, F435I, L191I), designed for the improvement toward aliphatic aldehyde showing previously high activities with HPA as donor, became inefficient (Chart 2.). Among the four triple variants obtained by combination of the hot spot H102L/H474S (designed for the improvement toward aliphatic pyruvate homologues) and D470I, L382F, F435I, L191I respectively, H102L/H474S/F435I gave the best enhancement of TK_{gst} activity compared to the activities obtained with the double variant H102L/H474S. The TK_{gst} improvement was particularly significant with propanal **2a**, butanal **2b** and pentanal **2c** reaching a level almost equivalent to that obtained previously with HPA. We noted that the single variant F435L was previously discarded for its less efficiency toward aliphatic aldehydes in the presence of HPA. H102L/H474S/L191I was the only triple variant giving almost the same activity as H102L/H474S/F435I but only in the presence of propanal **2a** and octanal **2f**.

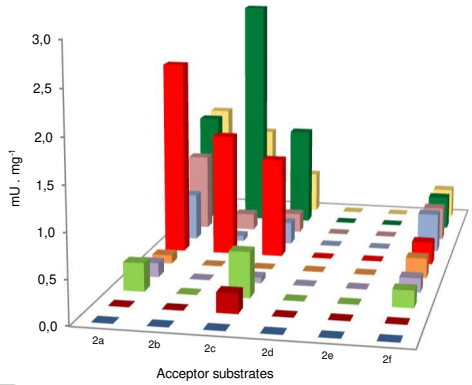


Chart 3. Specific activities of ■ wild type, ■ L382F, ■ D470I, ■ F435I, ■ L191I , ■ H102L/H474S, ■ H102L/H474S/L382F, ■ H102L/H474S/D470I, ■ H102L/H474S/F435I, ■ H102L/H474S/L191I toward aliphatic aldehydes **2a-f** as acceptors and 2-oxovalerate **1c** as donor. Enzyme reactions were carried out with **2a-f** (100 mM), **1c** (50 mM), at, purified enzyme (0.4 mg . mL^{-1}), at pH 7.2 and at 50 °C and incubated overnight.

With 2-oxovalerate **1c** as donor (Chart 3), as mentioned earlier with 2-oxobutyrate**1b**, the single variants led to very low or no activity in the presence of aliphatic aldehydes while the double variant H102L/H474S restored a significant TK_{gst} activity but only in the presence of propanal **2a**, butanal **2b** and pentanal **2c**. The expected cooperative effect by combination of L382F, D470I, F435I, or L191I with H102L/H474S observed earlier with 2-oxobutyrate, was only visible in the case of H102L/H474S/F435I in the presence butanal **2b**. H102L/H474S/L191I also led to improve TK_{gst} activity but at a level almost equivalent to that obtained with double variant H102L/H474S showing no real benefic effect of L191I. No variant was able to restore TK_{gst} activity in the presence of hexanal **2d** and heptanal **2e** while the single variant D470I, F435L, L191I and the four triple variants showed a slight increase of TK_{gst} activity with octanal **2f**.

In conclusion of this analytical study, the best variant activities toward HPA with propanal **2a,** hexanal **2d** or octanal **2f** were obtained with L382F while D470I allowed the highest improvement toward butanal **2b** with pentanal **2c**. H102L/H474S/F435I was the best candidate toward 2-oxobutyrate **1b** as donor with all aliphatic aldehydes and toward 2-oxovalerate **1c** as donor with butanal **2b** while H102L/H474S allowed the highest enhancement toward 2-oxovalerate **1c** and propanal.

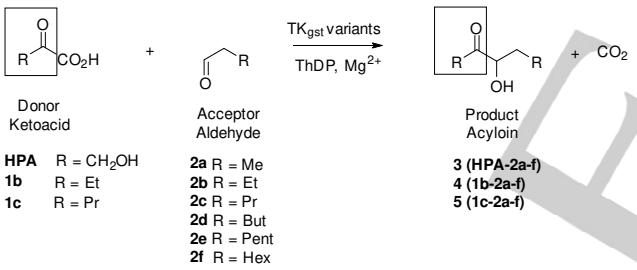
Table 1. Results of TK_{gst} variant-catalyzed reaction for the synthesis of 1,3-dihydroxyketones **3a-f** with HPA and aliphatic aldehydes **2a-2f** and 4-hydroxyketones **4a-f** and **5b** with oxobutyrate**1b** and 3-metyl-2-oxobutyrate **1c** respectively.

Substrates Donor-acceptor[a]	Product	TK_{gst} variant	TK_{gst} variant specific activity U . mg^{-1}	TK_{gst} improvement relative to WT	Reaction time (h)	Product Yield (%)[c]	*e.e.* (%)[d]
1a-2a	**3a**	L382F	11	4	24	56[13a]	94
1a-2b	**3b**	L382F	6	6	24	43[13a]	99
1a-2c	**3c**	L382F	4	0.5	24	44	99
1a-2d	**3d**	L382F	8	2	24	41	98
1a-2e	**3e**	L382F	7	∞	24	21	87
1a-2f	**3f**	L382F	6	6	24	6	ND
1b-2a	**4a**	H102L/H474S/F435I	5.8	20	24	33	12
1b-2b	**4b**	H102L/H474S/F435I	6.2	∞	24	27	6
1b-2c	**4c**	H102L/H474S/F435I	6.5	∞	24	28	31
1b-2d	**4d**	H102L/H474S/F435I	3.3	∞	24	25	33
1b-2e	**4e**	H102L/H474S/F435I	2.5	∞	24	<5	ND
1c-2b	**5b**	H102L/H474S/F435I		∞	24	<5	ND

[a] Reactions were carried out with 6-30 mg of TK_{gst} variants, 0.1 mM of ThDP, 1 mM of $MgCl_2$, 50 mM of aldose, 50-550 mM of donor at pH 7 and at 60°C. [b] Donor conversion determined by *in situ* ^{1}H NMR analysis. [c] Isolated yields obtained after purification except for **4e** and **5b** (yields determined by *in situ* NMR) [d] determined by chiral GC analysis after derivatization.

Synthesis of acyloins with TK_{gst} variants.

The targeted aliphatic acyloins **3**, **4** and **5** were synthetized in the presence of the most appropriate TK_{gst} variant identified previously with donor (HPA, **1b, 1c**) and acceptor **2a-f** substrates (Scheme 2, Table 1).



Scheme 2. Aliphatic acyloin synthesis catalyzed by TK_{gst} variants in the presence of aliphatic pyruvate homologues as donors with aliphatic aldehydes as acceptors.

All reactions were carried out at 50°C. A study of the thermostability of TK_{gst} variants showed a total recovery of enzymatic activity after 5 days at 50°C. At this temperature, TK_{gst} activity was 7 times higher than at 20°C, thus allowing significantly shorter reaction times.[14] In addition, **1b** and **1c** were completely stable at 50°C, while HPA showed a slight degradation (80% of remaining HPA after 24h at 60°C). Aldehydes **2a**-**f** were added continuously during the first 18 h by a syringe pump. The pH was kept constant at 7 by pH-stat control. The consumption of donors HPA, **1b** and **1c** was quantified by ^{1}H NMR analysis of aliquots taken from the reaction mixture at certain time intervals, allowing the measurement of the final conversion levels of oxoacids (Table 1).

For the synthesis of 1,3-dihydroxyketones **3a-f** with HPA and aliphatic aldehydes **2a**-**2f** the efficient single TK_{gst} variant L382F was used taking into account its higher enantioselectivity described previously compared to D470I.[ref] As expected according the previous analytical studies, the donor substrate HPA with all aliphatic aldehydes **2a-f** in the presence of L382F gave higher conversion rates and product isolated yields compared to that obtained with oxobutyrate **1b** with the same aldehydes. With both donors HPA and **1b**, the values of these parameters decreased with the increase carbon chain length of the aldehydes. All 1,3-dihydroxyketones **3a-f** were obtained with good yields and excellent enantioselectivity.

The synthesis of 4-hydroxyketones, was catalyzed by the triple variant H102L/H474S/F435I with **1b** and **1c** as donors and **2a-f** and **2b** as aliphatic aldehydes respectively. This new triple variant H102L/H474S/F435I, never investigated before, gave with oxobutyrate **1b** and all aliphatic targeted acceptor substrates except octanal **2f**, the corresponding products **4a-e**. We note that the *ee* values were lower compared to that obtained previously with HPA as donor and L382F. However, considering the further applications of these aliphatic mono hydroxylated ketones (potential surfactants or mimic structures of vegetable oil derivatives) high enantiomeric purity is not required but could be improved with additional mutagenesis cycles. With the donor substrate **1c,** the acceptor butanal **2b** only showing the best activity with H102L/H474S/F435I allowed a partial conversion of substrates **(1c** and **2b)** into **5b**.

Conclusion

In summary, thermostable TK_{gst} was successfully engineered for the synthesis of aliphatic acyloins with various carbon backbone lengths (C_5-C_{10}). The single TK_{gst} variant L382F was able to catalyze efficiently the transfer of the ketol group from HPA on all aliphatic aldehydes (C_3-C_8) for obtaining the corresponding 1,3-dihydroxyketones **3a-f** with good yields and excellent enantiomeric purity. The combination of H102L/H474S previously designed for the improvement toward pyruvate homologues (**1b** and **1c)** with the mutation F435I, gave a new triple variant H102L/H474S/F435I able to significantly improve TK_{gst} activity toward both aliphatic donors **1b** and **1c** and aliphatic acceptor substrates **2a-e** and **2b** respectively. This triple variant allowed the coupling of aliphatic oxoacids donors with aliphatic acceptors

as TK_{gst} substrates giving the corresponding 4-hydroxyketones **4a-e** and **5b** never reported before with this enzyme**.**
This procedure is an attractive alternative to other enzymatic and chemical strategies and paves the path to enhance TK_{gst} activities toward longer carbon chain aliphatic oxoacids and aldehydes for obtaining corresponding high-carbon acyloins. To this end, the evolvability, the robustness against temperature and cosolvent make TK_{gst} an appropriated enzyme for the synthesis of such compounds.

Experimental Section

General. All chemicals were purchased from Sigma-Aldrich, Alfa-Aesar, TCI Chemicals and CarboSynth. Bradford reagent was obtained from Bio-Rad and Ni-NTA resin from QIAGEN. Oligonucleotides were synthesized by Eurofins Genomics (Ebersberg, Germany). QuikChange Lightning Site-Directed Mutagenesis Kit (Agilent, USA) was used to build the TK_{gst} mutants libraries. Gene sequencing was performed by Eurofins Genomics (Ebersberg, Germany). Plasmidic DNA was extracted with the GenElute™ Plasmid Miniprep kit or with the GenElute™ Plasmid Midiprep kit (Sigma-Aldrich). Li-HPA was synthesized according to the procedure described in the literature.[ref] Aliphatic aldehydes (propanal, butanal, pentanal, hexanal, heptanal and octanal) were freshly distilled before use. Proteins and enzymes were acquired from Sigma-Aldrich. Lyophilisation was carried out with Triad LABCONCO dryer. UV-visible absorbance was measured using a Spark control 10 microplate reader from TECAN. MARCHEREY-NAGEL GmbH & Co KG 60/40-63 mesh silica gel for Liquid Flash Chromatography and MACHEREY-NAGEL GmbH & Co KG 60 F254 silica gel TLC plates with *p*-anisaldehyde or phosphomolybdic acid stain for detection were used. The pH of the enzymatic reactions was automatically adjusted with a TitroLine®7000 autotitrator. The optical rotation was determined with a polarimeter P-2000 JASCO PTC-262 at the given temperature and wavelength (Na-D-line 589 nm) in a cell 10 cm long cell; [α] values are given in 10^{-1} deg cm^2 g^{-1} (concentration given as g.100 mL^{-1}). NMR spectra were recorded in D_2O, $CDCl_3$ or CD_3OD on a 400MHz Bruker Avance III HD spectrometer. Chemical shifts are referenced to the residual solvent peak. The following multiplicity abbreviations are used: (s) singlet, (d) doublet, (t) triplet, (m) multiplet, (br) broad signal, (dd) doublet of doublets, (dt) doublet of triplets. GC analyses were performed on ThermoScientific™ Trace GC Ultra instrument coupled with mass spectrometry detector Trace DSQ (scanning between 30 and 400 m/z). Restek™ chiral column Rt™-bDEXsm with the following characteristics was used: length 30 m, inner diameter 0.25 mm, film thickness 0.25 µm.
Site-directed mutagenesis. Site-directed mutagenesis was performed using the QuikChange XL II Site-Directed Mutagenesis Kit (Agilent Technologies) according to the manufacturer's instructions. For the PCR, the modified synthetic TK_{gst} gene[15] or the double variant H102L/H474S[10] were used as template for single and triple (pr quadruple) variants, respectively, using appropriate oligonucleotides (SI). The PCR products were transformed by heat shock into XL-10 Gold competent cells. The resultant clones were cultured on Luria-Bertani (LB) medium agar plate containing 30 µg. mL^{-1} of kanamycin, overnight. Plasmids were extracted for sequencing to evaluate the quality of mutagenesis and to check the correct amino acid substitutions. The TK_{gst} variant plasmids were transformed into BL21(DE3)pLysS chemicals competent cells for protein expression.
Expression of TK_{gst}. Expression was carried out in *E. coli* BL21(DE3)pLysS strain. This strain was transformed by heat shock with the following TK_{gst} variants: artificial wild type TK_{gst},[15] TK_{gst}L382F,[13a] TK_{gst}D470I,[13a] TK_{gst}F435I,[13b] TK_{gst}L191I,[13b] TK_{gst}H102L/H474S,[10] TK_{gst}H102L/H474S/L382F, TK_{gst}H102L/H474S/D470I, TK_{gst}H102L/H474S/F435I, TK_{gst}H102L/H474S/L191I. These strains were stored at – 80 °C in glycerol 60 % (10 % final). A 100 µl culture aliquot of each of the clones was transferred into 50 ml liquid LB meduium containing kanamycin (30 µg. mL^{-1}) and grown at 27°C, 200 rpm for 12h. The pre-culture (20 ml) was used to inoculate 1 liter of culture medium containing kanamycin (30 µg. mL^{-1}) and grown at 37°C, 200 rpm. Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.5 mM was added when the OD_{600nm} range reached 0.6 - 0.8 A.U. Then, cells were grown at 30°C, 200 rpm, overnight. After that, cells were recovered by centrifugation (8,000 rpm, 4°C for 15 min), washed twice with phosphate buffer $NaH_2PO_4.2H_2O$ (50 mM), NaCl (300 mM) at pH 8.0 and finally harvested (4,000 rpm, 4°C for 15 min). The culture medium was removed and the bacterial pellets were stored at – 25 °C (≈ 5 g of wet bacterial pellet. L^{-1} of biomass for TKs).
Purification of TK_{gst}. The pellets were re-suspended in 35 mL of phosphate buffer (50 mM) containing NaCl (300 mM) at pH 8.0. The cells were disrupted by sonication on ice for 30 min and insoluble pellets were discarded after centrifugation at 14,000 rpm for 15 min at 4°C. Crude extracts were applied to a Ni-NTA column equilibrated with phosphate buffer. After washing with phosphate buffer, the His6-tagged TKs were finally eluted with phosphate buffer (50 mM) containing NaCl (300 mM) and imidazole (300 mM) at pH 8.0. The fractions containing the eluted proteins were collected and dialyzed against triethanolamine buffer (2 mM, pH 7.5) and then against water (pH 7.5) through dialysis tubing (cut-off 14,000 g. mol^{-1}) at 4°C. Finally, these protein solutions were lyophilized. Protein concentration was determined by the Bradford method and bovine serum albumin (BSA) was used as the standard. The specific activities of lyophilized wild-type and variant TK_{gst} measured at 25°C were between 0.01 U. mg^{-1} and 1.477 U. mg^{-1} of total protein (SI). The purity and molecular mass of these samples were analyzed by SDS-PAGE using Precision PlusProtein™ All Blue Standards#161-0373 (10–250 kDa, BioRad) as standard. The proteins were revealed with Coomassie Blue G-250 (SI).
Determination of TK_{gst}. One unit of TK_{gst} activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of ketose product per minute at 25°C in glycylglycine buffer (100 mM, pH 7.5). TK_{gst} enzymatic assay was performed in the presence of L-erythrulose and D-ribose-5-phosphate (DR5P) leading to D-sedoheptulose-7-phosphate (DS7P) and glycolaldehyde. The glycolaldehyde formed was reduced by yeast alcohol dehydrogenase (ADH) to ethylene glycol in the presence of nicotine adenine dinucleotide reduced form (NADH). L-erythrulose (100 mM), D-R5P (9.1 mM), ThDP (0.1 mM), $MgCl_2$ (0.5 mM), NADH (0.2 mg · mL^{-1}), 5µL of ADH (25 U · mL^{-1}) and the TK_{gst} suspension (10 µL) were added to disposable plastic cuvettes and completed to 1 mL with glycylglycine buffer. The disappearance of NADH was followed by spectrophotometry at 340 nm (value of ε_{NADH} at 340 nm is 6220 $M^{-1}.cm^{-1}$). All measurements were performed in triplicate.
Screening of TK_{gst}. Selected variants were screened by a colorimetric pH-based assay using phenol red as pH indicator[17]. In a 96-well plate, 50 µL of HPA or **1b** or **1c** (100 mM) were mixed with 160 µL of aliphatic aldehyde (50 mM), 10 µL of TK (4 mg. mL^{-1}), 10 µL of $MgCl_2$ (25 mM) and 10µL of ThDP (2.5 mM). Plates were covered by a plastic foil and incubated at 50 °C and at pH 7.2, 500 rpm, overnight. After incubation, 10 µL of phenol red (2 mM) were added and the OD_{560nm} were measured.
Monitoring of the donor conversion rate. The donor substrate concentration in the reaction mixture of wild-type or variant TK_{gst}-catalyzed reaction was determined by an enzymatic assay using L-lactate dehydrogenase (L-LDH) from rabbit muscle to determine the conversion rate after 8 and 24 hours. 10 µL of reaction mixture (diluted if necessary) was introduced in a well containing 10 µL of $NADH,H^+$ (10 mg. mL^{-1}), 2 µL of L-LDH (550 U. mg^{-1}) and 228 µL of HEPES buffer (pH 8.0, 50 mM). The disappearance of NADH was followed by spectrophotometry at 340 nm and the difference between initial and final absorbance was used to calculate the donor concentration using the Beer–Lambert law. All measurements were performed in triplicate.
GC assay method for enantiomeric excess determination.
Derivatization conditions: Samples of products **3** were trifluoroacetylated before injection using trifluoroacetic anhydride (TFAA). In a GC vials, 5 mg of samples were dissolved in 200 µL of chloroform and 50 µL of TFAA. Samples were stirred during 60 min at room temperature. Then 200 µL of 5 % aqueous ammonia solution were added to remove the formed trifluoroacetic acid. The organic layer was dried with $MgSO_4$ and filtered before injection. Samples of products **4** were trimethylsilylated before injection using N,O-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1 % of trimethylchlorosilane (TMCS). In a GC vial, 5 mg of sample were dissolved in 700 µL of acetonitrile and 300 µL of the mixture BSTFA + 1 % TMCS. Samples were agitated during 30 s and incubated at 60 °C

during 15 min. After cooling samples were directly injected. Commercial racemic 4-hydroxyhexan-3-one and synthetized racemic dihydroxyketones obtained with low stereoselectivity TK_{gst} variant (D470I)[13a] were used as standards.

GC conditions: Helium was used as carrier gas (1 mL.min^{-1}). The injector were set at 200 °C, 2 µL of samples were injected with a split (ratio 1:30). Initial column temperature was set at 80 °C, which was held for 10 min. Then, the temperature was raised to 90 °C at the rate of 1 °C. min^{-1} and finally to 230 °C at the rate of 5 °C. min^{-1} and held for 5 min.

General procedure of TK_{gst} catalyzed reaction. In a 100 mL, three-neck, round bottom flask, ThDP (1 mg, 0.1 mM) and $MgCl_2{\cdot}6H_2O$ (4 mg, 1 mM) were dissolved in H_2O (20 mL, total volume) and the pH adjusted to 7.5 using 0.1 M NaOH. To this solution, the lyophilized TK_{gst} (L382F, H102L/H474S or H102L/H474S/F435I) enzyme (18 mg for HPA or 36 mg for sodium 2-oxobutyrate) was added and the mixture stirred at 150 rpm for 30 min at 50 °C. After 30 min donor (HPA 110 mg, 50mM; sodium 2-oxobutyrate, 124 mg, 50 mM) and aldehyde acceptor (20 mM, 0.4 eq.) were added under stirring. The pH of the reaction mixture was automatically maintained at 7 throughout time by addition of 0.1 M HCl using a pH autotitrator. Aldehyde acceptor (1.8 eq.) was added continuously during the first 18 h by a syringe pump. After 24 h, reactions were stopped and enzymes were removed by precipitation and centrifugation. Then, reaction mixtures were extracted by continuous extraction using diethyl ether as solvent. Products were isolated after concentration under vacuum.

1,3-dihydroxyheptan-2-one 3c. **3c** was isolated as a white powder (66 mg, 44 % yield with TK_{gst} variant L382F). NMR data for **1** were consistent with those previously described[14]. ^{1}H NMR (400 MHz, Deuterium Oxide) δ 4.58 (d, J = 19.3 Hz, 1H, $H_{1\alpha}$), 4.50 (d, J = 19.3 Hz, 1H, $H_{1\beta}$), 4.39 (dd, J = 8.3, 4.1 Hz, 1H, H_3), 1.85 – 1.72 (m, 1H, $H_{4\alpha}$), 1.71 – 1.57 (m, 1H, $H_{4\beta}$), 1.46 – 1.24 (m, 4H, H_5 and H_6), 0.89 (t, J = 7.1 Hz, 3H, H_7). ^{13}C NMR (101 MHz, Deuterium Oxide) δ 214.41 (C_2), 74.64 (C_3), 64.90 (C_1), 32.50 (C_4), 26.30 (C_5), 21.65 (C_6), 13.06 (C_7). m/z HRMS: calculated for $C_{14}H_{28}NaO_6$ $[2M+Na]^+$ 315.1776; found 315.1778.

1,3-dihydroxyoctan-2-one 3d. **3d** was isolated as a white powder (68 mg, 41 % yield with TK_{gst} variant L382F).^{1}H NMR (400 MHz, Deuterium Oxide) δ 4.49 (d, J = 19.3 Hz, 1H, $H_{1\alpha}$), 4.40 (d, J = 19.3 Hz, 1H, $H_{1\beta}$), 4.30 (dd, J = 8.2, 4.1 Hz, 1H, H_3), 1.74 – 1.63 (m, 1H, $H_{4\alpha}$), 1.60 – 1.48 (m, 1H, $H_{4\beta}$), 1.39 – 1.13 (m, 6H, H_5, H_6 and H_7), 0.79 (t, J = 7.3 Hz, 3H, H_8). ^{13}C NMR (101 MHz, Deuterium Oxide) δ 214.42 (C_2), 74.65 (C_3), 64.90 (C_1), 32.75 (C_4), 30.62 (C_6), 23.74 (C_5), 21.71 (C_7), 13.19 (C_8).m/z HRMS: calculated for $C_{16}H_{32}NaO_6$ $[2M+Na]^+$ 343.2091; found 343.2088.

1,3-dihydroxynonan-2-one 3e. 3e was isolated as a white powder (15 mg, 8 % yield with TK_{gst} variant L382F).^{1}H NMR (400 MHz, Methanol-d_4) δ 4.46 (d, J = 19.4 Hz, 1H, $H_{1\alpha}$), 4.43 (d, J = 19.0 Hz, 1H, $H_{1\beta}$), 4.19 (dd, J = 8.2, 4.2 Hz, 1H, H_3), 1.81 – 1.67 (m, 1H, $H_{4\alpha}$), 1.66 – 1.50 (m, 1H, $H_{4\beta}$), 1.50 – 1.25 (m, 8H, H_5-H_8), 0.93 (t, J = 6.6 Hz, 3H, H_9). ^{13}C NMR (101 MHz, Methanol-d_4) δ 214.32 (C_2), 76.42 (C_3), 66.52 (C_1), 34.93 (C_4), 32.87 (C_7), 30.17 (C_6), 26.06 (C_5), 23.64 (C_8), 14.38 (C_9). m/z HRMS: calculated for $C_{18}H_{36}NaO_6$[2M+Na]$^+$ 371.2404; found 371.2403.

1,3-dihydroxydecan-2-one 3f. **3f**…….?

^{1}H NMR (400 MHz, Methanol-d_4) δ 4.48 (d, J = 19.5 Hz, 1H, $H_{1\alpha}$), 4.42 (d, J = 19.1 Hz, 1H, $H_{1\beta}$), 4.18 (dd, J = 8.2, 4.2 Hz, 1H, H_3), 1.81 – 1.67 (m, 1H, $H_{4\alpha}$), 1.65 – 1.52 (m, 1H, $H_{4\beta}$), 1.49 – 1.27 (m, 10H, H_5-H_9), 0.92 (t, J = 6.8 Hz, 3H, H_{10}). ^{13}C NMR (101 MHz, Methanol-d_4) δ 214.32 (C_2), 76.42 (C_3), 66.53 (C_1), 34.93 (C_4), 32.96 (C_8), 30.46 (C_6 or C_7), 30.30 (C_6 or C_7)), 26.10 (C_5), 23.69 (C_9), 14.40 (C_{10}).

4-hydroxyhexan-3-one 4a. **4a** was obtained as a pale yellow liquid (41 mg, 33 % yield with TK_{gst} variant H102L/H474S). ^{1}H NMR (400 MHz, Chloroform-d) δ 4.17 (dt, J = 6.9, 3.3 Hz, 1H, H_4), 3.49 (br, 1H, OH), 2.61 – 2.35 (m, 2H, H_2), 1.98 – 1.80 (m, 1H, $H_{5\alpha}$), 1.70 – 1.49 (m, 1H, $H_{5\beta}$), 1.12 (t, J = 7.3 Hz, 3H, H_1), 0.93 (t, J = 7.4 Hz, 3H, H_6).^{13}C NMR (101 MHz, Chloroform-d) δ 212.94 (C_3), 77.13 (C_4), 31.22 (C_2), 26.98 (C_5), 8.96 (C_6), 7.72 (C_1). m/z HRMS: calculated for $C_6H_{13}O_2$ $[M+H]^+$ 117.0910; found 117.0912.

4-hydroxyheptan-3-one 4b. 4b was obtained as a pale yellow liquid (34 mg, 27 % yield with TK_{gst} variant H102L/H474S). ^{1}H NMR (400 MHz, Chloroform-d) δ 4.19 (dt, J = 7.8, 4.1 Hz, 1H, H_4), 3.49 (d, J = 4.9 Hz, 1H, OH), 2.66 – 2.36 (m, 2H, H_2), 1.87 – 1.72 (m, 1H, $H_{5\alpha}$), 1.59 – 1.23 (m, 3H, $H_{5\beta}$-H_6), 1.12 (t, J = 7.3 Hz, 3H, H_1), 1.02 – 0.86 (m, 3H, H_7).^{13}C NMR (101 MHz, Chloroform-d) δ 213.08 (C_3), 76.19 (C_4), 36.09 (C_5), 31.20 (C_2), 18.28 (C_6), 14.04 (C_7), 7.77 (C_1). m/z HRMS: calculated for $C_7H_{15}O_2$ $[M+H]^+$ 131.1067; found 131.1069.

4-hydroxyoctan-3-one 4c. 4c was obtained as a pale yellow liquid (41 mg, 28 % yield with TK_{gst} variant H102L/H474S).^{1}H NMR data for **7** were consistent with those previously described.[14] ^{1}H NMR (400 MHz, Chloroform-d) δ 4.18 (dd, J = 7.4, 3.8 Hz, 1H, H_4), 3.47 (br, 1H, OH), 2.64 – 2.33 (m, 2H, H_2), 1.99 – 1.75 (m, 1H, $H_{5\alpha}$), 1.70 – 1.23 (m, 5H, $H_{5\beta}$-H_6-H_7), 1.12 (t, J = 7.3 Hz, 3H, H_1), 0.91 (t, J = 7.1 Hz, 3H, H_8). ^{13}C NMR (101 MHz, Chloroform-d) δ 213.04 (C_3), 76.34 (C_4), 33.70 (C_5), 31.21 (C_2), 27.05 (C_6), 22.67 (C_7), 14.04 (C_8), 7.76 (C_1). m/z HRMS: calculated for $C_8H_{17}O_2$ $[M+H]^+$ 145.1223; found 145.1224.

4-hydroxynonan-3-one 4d. 4d was obtained as a pale yellow liquid (16 mg, 10 % yield with TK_{gst} variant H102L/H474S/F435I). ^{1}H NMR data for **8** were consistent with those previously described.[14]^{1}H NMR (400 MHz, Chloroform-d) δ 4.19 (dd, J = 7.3, 3.8 Hz, 1H, H_4), 2.65 – 2.37 (m, 2H, H_2), 1.87 – 1.75 (m, 1H, $H_{5\alpha}$), 1.69 – 1.20 (m, 7H, $H_{5\beta}$-H_6-H_7-H_8), 1.12 (t, J = 7.3 Hz, 3H, H_1), 0.94 – 0.84 (m, 3H, H_9).^{13}C NMR (101 MHz, Chloroform-d) δ 213.06 (C_3), 76.84 (C_4), 33.98 (C_5), 31.79 (C_6), 31.22 (C_2), 24.62 (C_7), 22.64 (C_8), 14.13 (C_9), 7.78 (C_1). m/z HRMS: calculated for $C_9H_{19}O_2$ $[M+H]^+$ 159.1380; found159.1379.

Acknowledgements

This work was funded by the region Auvergne Rhône-Alpes through the "Pack Ambition Recherche" programme (project VALCOUPENZ, 2017).

Keywords: biocatalysis •acyloins• transketolase• C-C bond formation • mutagenesis • molecular modelling

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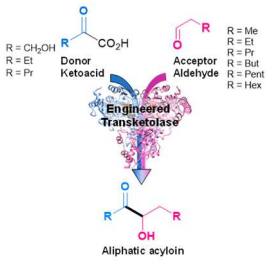

Efficient variants of Transketolase from *Geobacillus steorothermophilus* gave 1,3-dihydroxy and 4-hydroxyketones from hydroxypyruvate and oxobutyrate respectively with aliphatic aldehydes (C_3-C_8). The combination of H102L/H474S previously designed for the improvement toward pyruvate homologues with the mutation F435I, gave a new triple variant H102L/H474S/F435I able to significantly improve TK_{gst} activity toward both aliphatic donor and aliphatic acceptor substrates.