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KEYWORDS. Biocatalysis, Transketolase, C-C bond cleavage, Mutagenesis, α-hydroxy ketones

ABSTRACT. The reaction catalyzed by ubiquitous thiamine pyrophosphate-dependent transketolase engaged in cells in the pentose phosphate pathway can be applied in vitro to the cleavage of aliphatic α-hydroxy ketones with thermostable transketolase variants from *Geobacillus stearothermophilus* obtained through rational design. The simple variant F435I gave the best activity toward (3*S*)-1,3-dihydroxyhexan-2-one **3** leading to the corresponding product with 92% yield after only 2 h reaction time. Three new triple variants H102L/H474 (S, G or A) / F118I were found to cleave (±)-4-hydroxyhexan-3-one **6** giving the corresponding product with 90, 82 and 79 % yield respectively after 24 h, whereas wild-type transketolase was almost

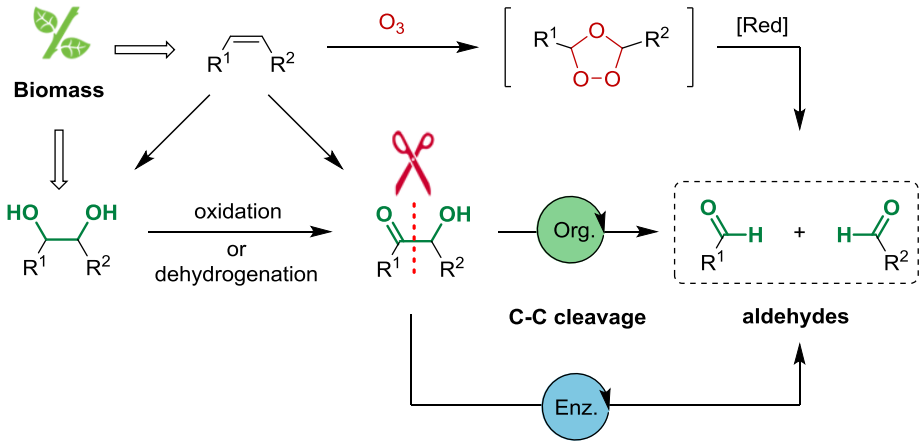
ineffective. With respect to the cleavage of α hydroxy ketones, this one-step biocatalytic strategy offers an attractive alternative to other enzyme or chemical strategies developed so far.

INTRODUCTION

In their respective fields, chemists and biochemists have developed a range of synthetic strategies for the formation of C-C bonds in order to synthesize complex compounds from simple and abundant raw materials Recently, the use of biomass as a renewable feedstock has been recognized as one of the ways to face the depletion of fossil resources and to limit CO_2 emissions. The valorization of biomass implies, in that case, to split existing (natural) molecules to make compounds with higher added value (scheme 1). In this context, new synthetic methodologies for C-C bond cleavage, including enzymatic ones, should be developed.

Particularly, aliphatic α-hydroxy ketones can be cleaved to yield free aldehydes of great interest for various applications such as aromas and intermediates for the synthesis of surfactants or for the preparation of polyesters and polyamides.[1] The α-hydroxyketone function can be found as such in several natural products such as dihydroxyacetone (DHA), acetoin, erythrulose or in the masked form in xylulose, sorbose and fructose. Moreover, α-hydroxyketones can be formed by Benzoin condensation, enolate oxidation, C-H oxidation and several other means.[2] More importantly, they can be readily prepared in one-step by enzymatic-,[3] metallic-[4] or electrochemical[5] selective oxidation or by dehydrogenation[6] of the corresponding 1,2-diols. These methods allow the preparation of α-hydroxyketones derived from renewable resources as the 1,2-diol moiety is widespread in Nature (*e.g.* glycerol, carbohydrates, etc.).

The retro-acyloin method allows the cleavage of aliphatic α-hydroxy ketones to yield free aldehydes and could offer an alternative to reductive ozonolysis, which is a prominent reaction for the clean and efficient production of fatty aldehydes from a double bond (scheme 1).[7]

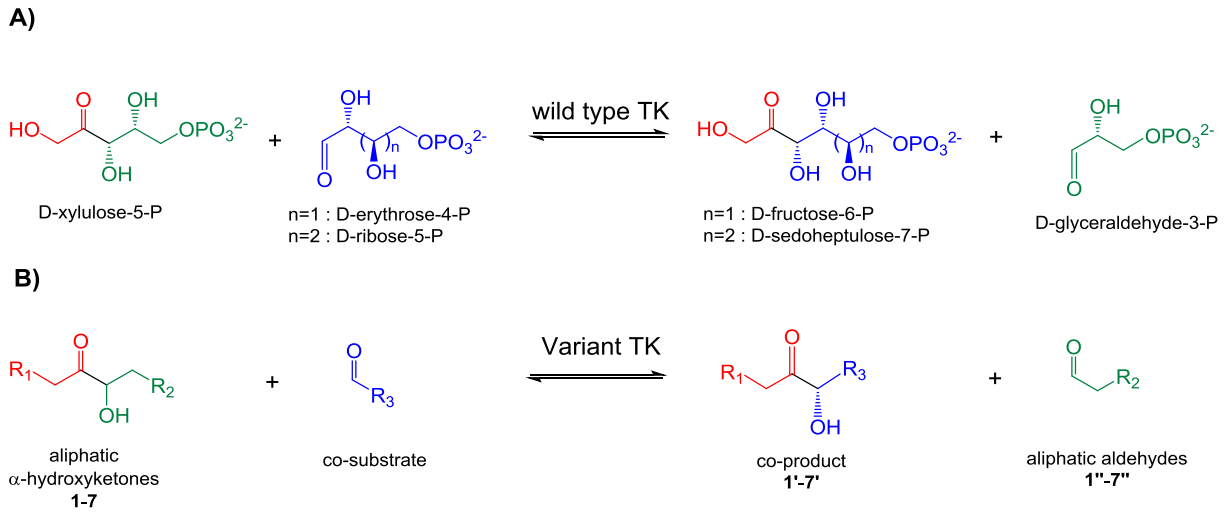


Scheme 1. Synthetic strategies for the preparation of aldehydes through C-C bond cleavage.

Despite the economy atoms achieved during the implementation of the ozonolysis reaction, the use of ozone (O_3), which is both highly toxic and explosive presents the major drawback of this synthetic method. If the (asymmetric) acyloin condensation has been studied extensively for the synthesis of acyloins (α-hydroxy ketones) by chemical and enzymatic ways,[8,9] its reversibility has been demonstrated in only a few cases[10,11] and has been underexploited.[12,13]

Recently, an efficient chemical retro-acyloin method was reported for obtaining aliphatic free aldehydes by cleavage of fatty α-hydroxy ketones catalyzed by thiazolylidene.[14] The continuous distillation of the targeted aldehydes allowed the reaction equilibrium shift under anaerobic conditions. The main problems of this retro-acyloin strategy are the harsh temperature (180 °C) and the need to perform the reaction under reduced pressure (1 to 100 mbar). To overcome these limitations, enzymatic alternatives requiring mild conditions warrant investigation. A multienzymatic cascade was designed and evaluated for the cleavage of renewable fatty acids of

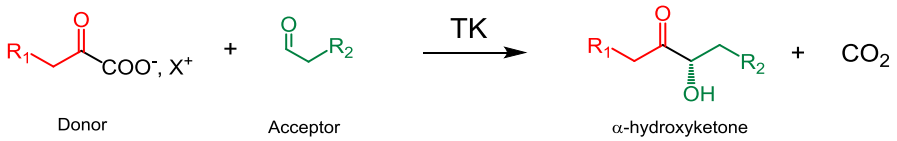
plant origin (e.g., oleic acid, ricinoleic acid) successively catalyzed by alcohol dehydrogenase, Baeyer-Villiger monooxygenase and lipase, leading to the production of long-chain free α,ω-dicarboxylic acids and ω-hydroxycarboxylic acids (C_9–C_{13}).[15] The enzymatic cleavage of aliphatic α-hydroxy ketones to obtain the corresponding aldehydes in one step by a mimetic chemical retro-acyloin process remains a challenge (scheme 2B). The transketolase enzyme (TK) is involved in carbohydrate pathway metabolism. In biochemistry, its is able to catalyse this type of reaction since it uses thiamine diphosphate, the active form of vitamin B1 (natural carbene), as cofactor (Scheme 2A).[16]



Scheme 2. TK-catalyzed reaction with wild type in the pentose phosphate pathway A) and with variants for *in vitro* α-hydroxyketone cleavage B)

Incells, TK is engaged in the pentose phosphate pathway where this enzyme reversibly transfers a two-carbon unit, an α-hydroxy carbonyl (ketol) group from a ketose phosphate to an aldose phosphate.. Previous *in vitro* studies for biocatalytic applications showed that non-phosphorylated ketose and aldose compounds could also be used as TK substrates. In particular,

the ketoacid hydroxypyruvate (HPA) as donor confers a major advantage on the TK-catalyzed reaction by rendering the reaction irreversible through the release of carbon dioxide. Thus, TK appears as a powerful enzyme for the asymmetric synthesis of α-hydroxy ketones and related acyloins(Scheme 3).[17]



Scheme 3. Reaction catalyzed by TK for the synthesis of α-hydroxy ketones from α-ketoacid as donor and aldehydeas acceptor substrates.

When HPA was used as donor, the TK enzyme exhibited the best activities for non-phosphorylated short carbon chain α-hydroxy aldehydes (C_2–C_3). Regarding the TK stereoselectivity, , the newly-formed asymmetric carbon of the product has an (*S*) configuration. In addition, TK preferentially accepts (2*R*)-hydroxy aldehydes, yielding (3*S*, 4*R*)α-hydroxy ketones . For syntheticpurposes , mesophilic TKs from *Saccharomyces cerevisiae* (TK_{sce}) and *Escherichia coli* (TK_{eco}) have been widely used[12] and optimized by mutagenesis.[18,19] We discovered and charaterized the first thermostable TK from *Geobacillus stearothermophilus* (TK_{gst}), displaying high stability at elevated temperature and robustness toward non-usual reaction conditions.[20-22] The TK_{gst} substrate specificity and enantiopreference were improved and broadened using rational mutagenesis approach toward hydroxylated or non-hydroxylated aldehydes as acceptors[23,24] and aliphatic α-ketoacids as donors.[25,26] We showed in particular that engineered TK_{gst},[26] like TK_{eco},[18d] facilitated the synthesis of aliphatic α-hydroxy ketones (C_5–C_{10}) from combinations of aliphatic acceptor and and donor substrates.

Based on these last results, we studied the reversibility of the TK reaction (mimetic of retro-acyloin condensation) for the cleavage of aliphatic α-hydroxy ketones with appropriate TK_{gst} variants, never hitherto explored to our knowledge (scheme 1B). This work is part of a research program on the valorization of vegetable oil derivatives in which these compounds will be used as model structures of fatty α-hydroxy ketones employed in the chemical retro-acyloin condensation reported earlier.[14] Here, we describeappropriate TK_{gst} variants obtained by mutagenesis applied at targeted positions of the active site, the identification of best TK_{gst} variants for the cleavage of aliphatic mono-,di- and tri-α-hydroxy ketones of various carbon chain length, and the quantification and characterization of the products.

RESULTS AND DISCUSSION

Construction of TK_{gst} variant libraries. In the pentose phosphate pathway, –the phosphorylated aldoses (C_3–C_5) and ketoses (C_5–C_7) are the TK acceptor and donor substrates respectively. To improve TK_{gst} activity toward non-phosphorylated α-hydroxy ketones **1**–**7** (Table 1), we modified the TK_{gst} active site at targeted positions by semi-rational mutagenesis (site saturation mutagenesis, SSM). The 3D structures of microbial TKs such as TK_{sce},[27] TK_{ec}[28] and TK from *Bacillus anthracis* (TK_{ban})[29] have a strong protein sequence homology.[20,21] The key residues stabilizing the TK substrates are identical and have similar orientation to ThDP. The 3D structure of TK_{gst} being unknown, a model of the TK_{gst} active-site pocket containing its phosphorylated acceptor aldose, D-erythrose-4-phosphate (E4P), was constructed using the X-ray crystal structure of TK_{ban} as a template, which belongs to the same microbial species and has a high percentage of identity (74%) (Figure 1).[20,21]

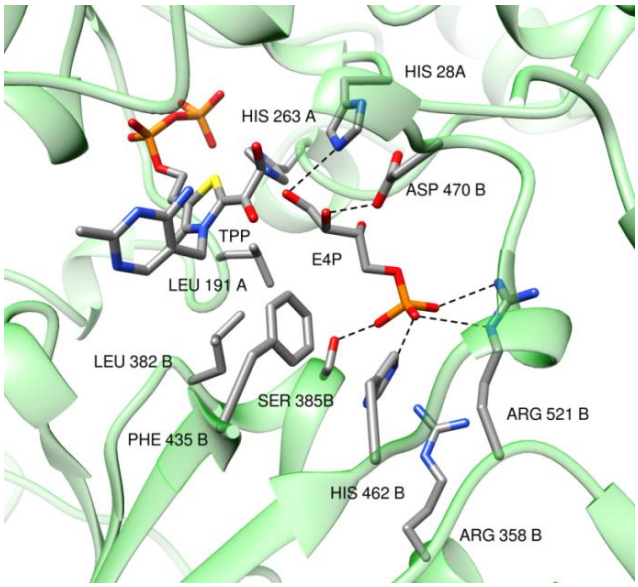
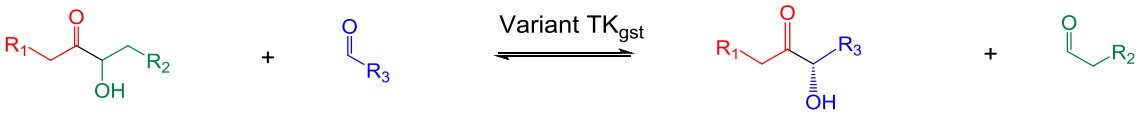


Figure 1. Model of wild-type TK_{gst} active site based on the X-ray crystal structure of TK_{ban} (PDB entry 3M49) with D-erythrose-4-phosphate (E4P) as acceptor substrate. Modeler 9.14 and Chimera were used to construct the model.

To improve TK_{gst} activity toward the cleavage of (3*S*)-1,3-dihydroxy ketones **1**-**5**, the residues Leu191, Leu382, Phe435, and Asp470 were individually targeted. SSM applied earlier on these positions showed that D470I, L382F, F435I and L191I were the best variants offering a hydrophobic environment to substrate binding. These variants were used for the formation of these 1,3-dihydroxy ketones **1**–**5** obtained with hydroxypyruvate as donor and corresponding aliphatic acceptor substrates (Table 1).[24-26]

Table 1. Selected positions in the TK_{gst} active site for the improvement of TK_{gst} activity toward α-hydroxyketone **1**–**7** cleavage.



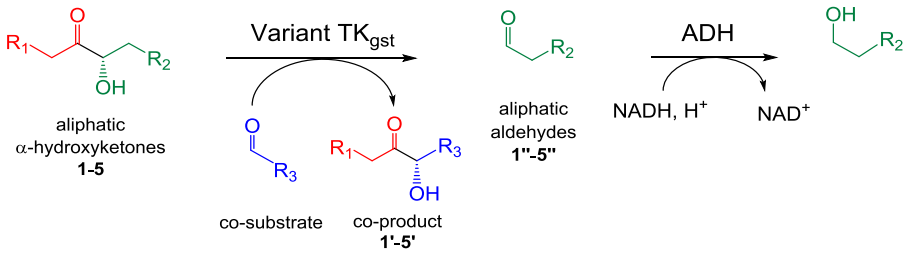
α-hydroxyketones **1-7** + Co-substrate ⇌ co-product **1'-7'** + aldehydes **1''-7''**

α-hydroxy ketones	R_1	R_2	Targeted positions in TK_{gst} active site
1	-OH	-OH	D470I L382F F435I L191I
2	-OH	$-CH_3$	
3	-OH	$-CH_2-CH_3$	
4	-OH	$-(CH_2)_2-CH_3$	
5	-OH	$-(CH_2)_3-CH_3$	
6	$-CH_3$	$-CH_3$	H102L/H474S (or X)/D470X H102L/H474S (or X)/L382X H102L/H474S (or X)/F435X H102L/H474S (or X)/L191X H102L/H474S (or X)/L118X
7	$-CH_2-CH_3$	CH_2-CH_3	

To identify plausible positions for improving TK_{gst} activity toward the cleavage of aliphatic 4- or 5-hydroxy ketones **6** or **7,** we based our strategy on positions previously found to increase TK_{gst} activity for the formation of the same aliphatic α-hydroxy ketones obtained with pyruvate analogs as donors and aliphatic aldehydes as acceptors. Previous results showed that the double variant H102L/H474S was the best variant to improve the substrate affinity toward pyruvate analogs including oxobutyrate.[25] The presence of smaller and less polar side chains in place of the two histidine allowed enlargement of active site for biger hydrophobic donor substrates and kept the catalytic mechanism intact. The two positions were combined successively with mutations on Asp470, Leu382, Phe435 or Leu191 as the best positions reported earlier for aliphatic acceptor substrates.[24, 26] In addition, we selected position L118, which is involved in the stabilization of the thiazolium cycle of ThDP and which could also stabilize the aliphatic substrates by interacting with an appropriate longer carbon chain residue than Leu. For the design of focused TK_{gst} variant libraries, SSM was applied on each of the five positions using

NDT codon degeneracy to reduce the screening efforts.screening efforts. Indeed,[27] this strategy involves 12 codons (in place of 20 with NNK systems) corresponding to 12 amino acids (Phe, Leu, Ile, Val, Tyr, His, Asn, Asp, Cys, Arg, Ser, Gly) which are representatives of polar and nonpolar, aliphatic and aromatic, and negatively and positively charged side chains.Five libraries were thereby created, H102L/H474X/D470X (or L382X, or F435X or L191X or L118X) (Table 1).

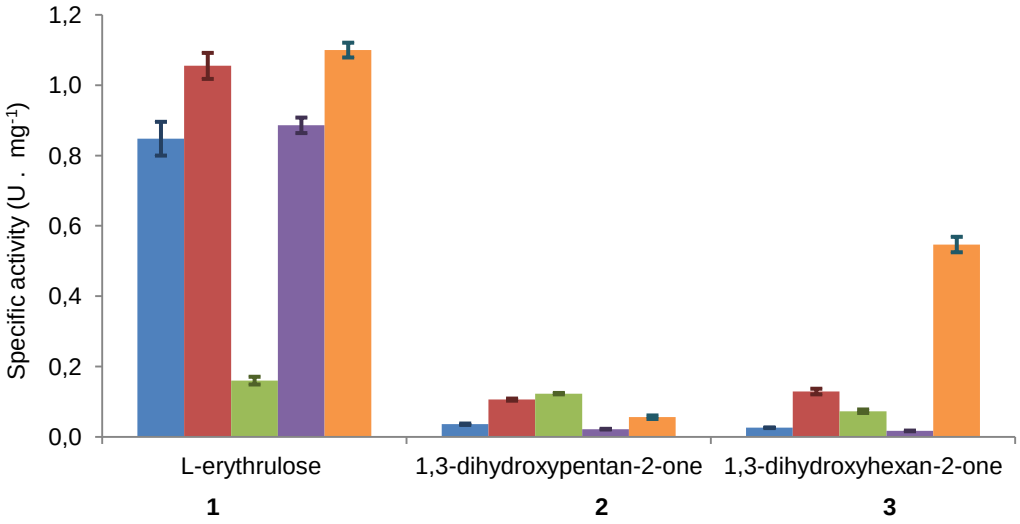
Screening of TK_{gst} variant libraries with aliphatic α-hydroxy ketones 1–5. To determine the specific activities of TK_{gst} variants (enzymatic lysates or purified enzymes) toward cleavage of aliphatic α-hydroxy ketones **1**–**5**, the release of aldehydes **1′′–5′′** was detected by our spectrophotometric assay at 340 nm *via* consumption of the reduced form of nicotinamide adenine dinucleotide (NADH) and using yeast alcohol dehydrogenase (ADH) as an auxiliary enzyme.[28] This method offers a continuous measurement of TK initial velocity by shifting the TK_{gst} equilibrium. TK being a transferase, the overall process requires an aldehyde as acceptor substrate. The absence of acceptor drastically decreases TK_{gst} activity (data not shown). The phosphorylated aldose, D-ribose-5-phosphate (D-R5P), the TK acceptor in the pentose phosphate pathway or D-erythrose, one of the best non-phosphorylated acceptors for TK, were chosen as co-substrates because they are not substrates of ADH (Scheme 4).



Scheme 4. Screening assay to determine TK_{gst} activity toward cleavage of α-hydroxy ketones **1**–**5** by coupling with yeast alcohol dehydrogenase (ADH).

To select the best variants against cleavage of (3*S*)-di- or trihydroxy ketones **1**–**5**, the single TK_{gst} variants D470I, L382F, F435I and L191I were expressed in *E. coli* BL21(DE3)pLysS strain and purified by Ni^{2+} chelating affinity column chromatography. TK_{gst} variant activities with (3*S*)-hydroxy ketones **1**–**3** were compared to those obtaianed with wild-type TK_{gst} (Chart 1).

Chart 1. Determination of TK_{gst} variant activities with (3*S*)-hydroxy ketones **1**–**3** and wild-type , L382F, D470I, L191I, F435I



Enzyme reactions were carried out in triplicate with L-erythrulose **1,** 1,3-dihydroxypentan-2-one **2,** 1,3-dihydroxyhexan-2-one **3** (50 mM), D-ribose-5-phosphate (2.5 mM), purified enzyme (1–5 mg·mL^{-1}), wt TK_{gst} L382F, D470I, L191I, F435I at pH 8 and 25 °C.

(3*S*)-1,3,4-trihydroxybutan-2-one **1** (L-erythrulose), one of the best non-phosphorylated substrates for wild-type TKs was chosen as a reference.[28] As expected, with wild type and variants, **1** gave the best activities, except with D470I, the only polar residue replaced by an aliphatic chain. We note the strong influence of the nonpolar residue Ile in place of Asp, leading to a marked decrease in activity due probably to the lack of interaction with the hydroxyl group of **1** C4. The mutations of other nonpolar residues by the aliphatic chain had no influence on activity. The (3*S*)-1,3-dihydroxy ketones **2** and **3** with increasing non-hydroxylated carbon chain

length showed lower activities than **1** with wild type and variants except with F435I and **3**. In this last case, (3*S*)-1,3-dihydroxyhexan-2-one **3** gave the best TK_{gst} improvement compared to wild type (18 fold) owing to the presence of Ile in place of Phe, allowing an interaction with the C6 of **3** (Figure 2).

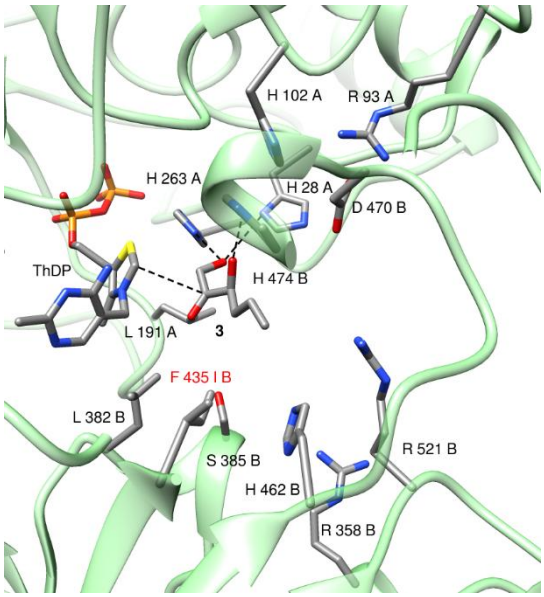


Figure 2. Model of F435I TK_{gst} with (3*S*)-1,3-dihydroxyhexan-2-one **3** based on the X-ray crystal structure of TK_{ban} (PDB entry 3M49) showing the main residues of the active site. Modeler 9.14 and Chimera were used to construct the model (SI).

The mutation of the other residues too far from the active center of ThDP gave no enhanced activity. No or very low activities were obtained with longer carbon chain (3*S*)-1,3-hydroxy ketones **4** and **5**, which can be explained by a poor stabilization by the targeted residues and a lower solubility compared to compounds **1–3.**

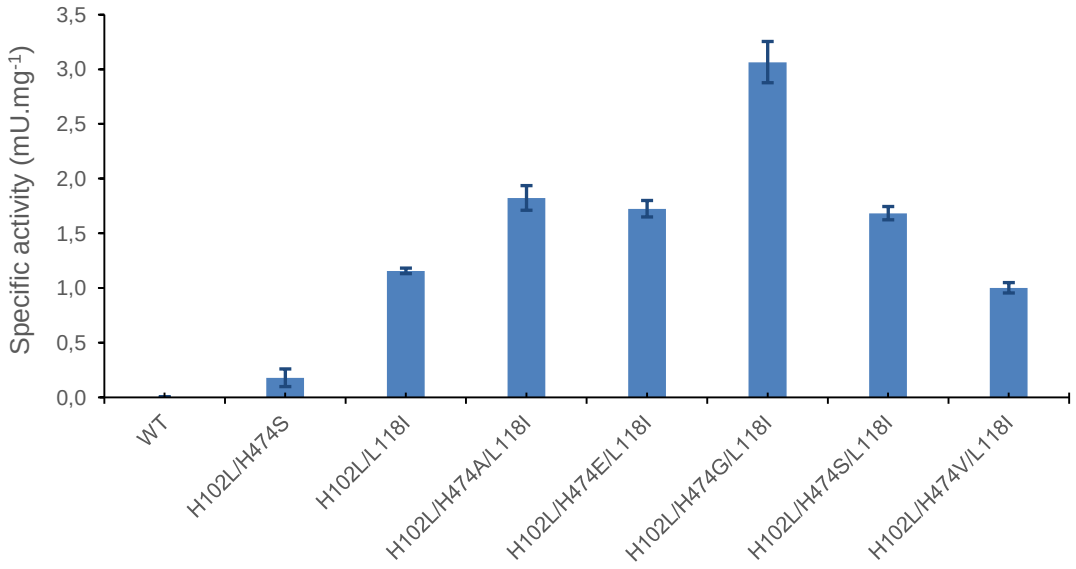
The single variants tested previously with α-hydroxy ketones **1–3** did not cleave the C-C bond of (±) 4- or 5-hydroxy ketones **6** and **7** (data not shown). We turned to triple TK_{gst} variant libraries (H102L/H474X/D470X or L382X, or F435X, or L191X or L118X) combining the best positions identified earlier toward the reverse reaction (formation of α-hydroxy ketones using

aliphatic ketoacids as donors and fatty aldehydes as acceptors). The libraries were expressed in the *E. coli* BL21(DE3)pLysS strain and after lysis by sonication and centrifugation, crude extracts were first screened against α-hydroxy ketones **6** and **7** and compared to wild-type TK_{gst}. Each assay (125 µL) contains a TK_{gst} variant lysate with (±)-4- or 5-hydroxy ketones **6** or **7** and D-erythrose as acceptor, one of the best non-phosphorylated acceptor substrate for TK used in place of D-R5P, considering the high cost incurred by the large numbers of TK_{gst} variants to be screened and the weakening recognition of this polyhydroxylated and phosphorylated acceptor by TK_{gst} variants.

The percentages of hits in each library were first estimated on sight by thin layer chromatography (TLC) based on the spot intensity of products **6′** or **7′**. We did not observe the formation of **7′** with any TK_{gst} variant libraries, whereas product **6′** was observed on TLC with several TK_{gst} variant lysates (SI). We observed that the library H102L/H474S/L118X gave the greatest number of active TK_{gst} variants. Considering these results, the 22 best hits were sequenced, of which 17 revealed the presence of an Ile in place of Leu118. Ile induces a new position of ThDP favoring the interaction of the unipolar head of **6** with H102L (Figure 2). For further improvement, we set out to create another library keeping L118I and also H102L and creating diversity by SSM on the H474 position, expecting higher activity with a nonpolar residue in place of Ser.

All the best candidates were finally purified by affinity chromatography and their specific activities toward **6** were determined using the spectrophotometric assay described earlier (Chart 2). For comparison purpose, wild-type TK_{gst}, and H102L/H474S TK_{gst} variant were used as controls since they were already described[25,26] to improve TK_{gst} activity for the formation of C-C with pyruvate homologues as donors and aliphatic acceptors.

Chart 2. Determination of the TK_{gst} variant activities with (±)-4-hydroxyhexan-3-one **6** compared to wild type.



Enzyme reactions were carried out in triplicate with **6** (200 mM), D-erythrose (50 mM), purified enzyme (5 mg . mL^{-1}), at pH 8 and 25 °C.

While wild-type TK_{gst} displayed no activity toward **6,** all variants containing Ile in place of Leu at 118 position gave higher activities compared to H102L/H474S showing the cooperative effect afforded by this mutation for the C3-C4 bond cleavage of **6**. As expected, the results (Chart 2) showed that replacing His 474 by a nonpolar residue such as Gly (H102L/H474G/L118I) notably improved activity (7, 5, 3, and 1,7 times higher than that observed with H102L/H474S, H102L/L118I, H102L/H474A/L118I and H102L/H474S/L118I respectively).

The hydrophobic environment given by Gly and the two other aliphatic residues favors the stabilization of the nonpolar head (ethyl group) of **6** (Figure 3).

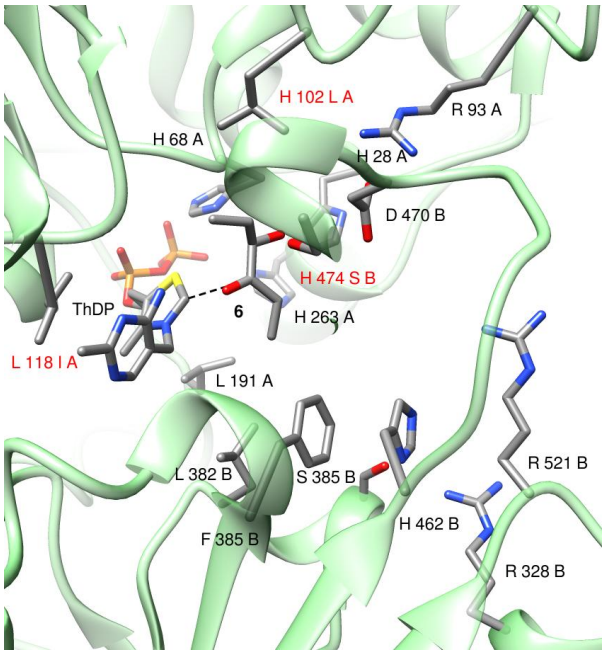


Figure 3. Model of H102L/H474S/L118I TK_{gst} with (±)-4-hydroxyketone **6** based on the X-ray crystal structure of TK_{ban} (PDB entry 3M49) showing the main residues of the active site. The model was built using Modeler 9.14 and Chimera (SI).

The nonpolar Ala or Val residues in place of His 474 (H102L/H474A/L118I and H102L/H474V/L118I) gave lower activities compared to Gly owing to their higher steric hindrances, which impede the interaction of the nonpolar head of **6** with H102L. The polar and/or charged Ser and Glu in place of H474 (H102L/H474E/L118I) also led to lower activities compared to Gly, explained by the polar side chain of Ser and by a negative charge of Glu in conflict with D470. This environment destabilizes the position of the nonpolar head of **6** toward H102L.

Preparative scale study of aliphatic α-hydroxyketone cleavage with the best TK_{gst} variants

The cleavage reactions of (3*S*)-1,3-dihydroxyhexan-2-one **3** and (±)-4-hydroxyhexan-3-one **6** was investigated at preparative scale in the presence of glycolaldehyde. This cheap, non-volatile, and highly reactive co-substrate is the most plausible candidate for further aliphatic aldehyde **3** " or **6** " separation by *in situ* distillation of reaction mixture.

The reactions were carried out at 50 °C and at pH 7.5 with purified WT and variants identified earlier (Table 2). All substrates, reagents and variants were fully stable at 50 °C. The reactions were regularly monitored by ^{1}H NMR analysis with TSP-*d4* as reference. We followed the disappearance of **3** and **6** concomitantly with the appearance of products **3′** and **6′** to prove the transfer of the hydroxyacetyl group of **3** or acyl group of **6** on the glycolaldehyde (Table 2). The *in situ* quantification of aldehyde products **3″** and **6″** would be less reliable because of their volatility. To shift the equilibrium toward the products, four equivalents (200 mM) of α-hydroxy ketones **3** or **6** were required against one equivalent (50 mM) of glycolaldehyde.

The TK_{gst} variant F435I identified earlier at analytical scale as the best candidate showed a total recovery of activity after 5 days at 50 °C and 7 fold higher activity than at 25 °C, thus allowing appreciably shorter reaction times. The *in situ* ^{1}H NMR study of the reaction with **3**, the best substrate for this variant (Chart 1) showed the conversion of 92% (46 mM) of glycolaldehyde after 2 h and the formation of product **3′** with an *in situ* yield of 92% (46 mM), demonstrating the cleavage of the C2-C3 bond of (3*S*)-1,3-hydroxyhexan-2-one **3** by F453I (Table 2).

The triple variants H102L/H474 (S, G, or A) /L118I were selected to cleave (±)-4-hydroxyhexan-3-one **6**. As expected, considering the lower specific activities obtained previously (Chart 2 compared to Chart 1), a total or almost total conversion of glycolaldehyde was obtained after 24 h.

Table 2. Results of TK_{gst} wild type (WT) and variant-catalyzed cleavage of α-hydroxy ketones **3** and **6** in the presence of glycolaldehyde (GoA) .

α–Hydroxy ketones	TK_{gst}[a]	Time (h)	α-hydroxy ketones **3** or **6**			GoA		Products **3′** or **6′**		
			Initial[b] mM	Final[b] mM	*ee* %[c]	Initial[b] mM	Final[b] mM	Product[b] mM	Yield %	*ee*[c] %[c]
3	WT	2	200	164	-	50	34	34	68	-
	F435I	2	200	148	-	50	4	46	92	-
6	WT	24	200	200	0	50	4	0	0	0
	H102L/H474S/L118I	24	200	155	0	50	0	45	90	98
	H102L/H474A/L118I	24	200	158	1	50	0	41	82	98
	H102L/H474G/L118I	24	200	160	1	50	2	39	78	95

[a]Enzyme reactions were carried out with purified enzyme (1 $mg \cdot mL^{-1}$ with 3 and 5 $mg \cdot mL^{-1}$ with 6), and incubated from 2 h to 24 h at pH 7.5 and 50 °C. [b]Concentrations determined by in situ NMR (SI). [c]Enantiomeric excess (*ee*) determined by chiral GC analysis after derivatization (SI).[20a]

H102L/H474S/L118I gave the best results with 90% yield of product **6′** (45 mM) against 82% (41 mM) with H102L/H474A/L118I and 78% (39 mM) with H102L/H474G/L118I, whereas the conversion of glycolaldehyde was total. This can be explained by the self-condensation of glycolaldehyde giving L-erythrulose already reported and observed on ^1H NMR spectra (SI). This by-product had not previously been identified with F435I and **3,** the reaction time being shorter. It can be noted that we did not observe the compound corresponding to the condensation of the ketol group of L-erythrulose which could act as TK_{gst} donor substrate on propanal **6’’**. In addition, the results revealed that H102L/H474S/L118I was slightly more efficient that H102L/H474 (G or A) /L118I, in line with the thermostability study of these variants giving a higher thermostability over time (at 50 °C) compared to H102L/H474 (G or A) / L118. The molecular modeling of the H102L/H474S/L118I active site showed that the presence of Ser in position 474 favors the interaction with Asp 470, which seems important to stabilize the bridge

between Asp 470 from chain A or B and Arg 93 from chain B or A, explaining the greater thermostability than H102L/H474 (G or A) /L118. The importance of a nonpolar residue at the 474 position (particularly Gly or Val) in place of Ser showed a weaker influence on product **6′** formation when reactions were conducted at 50 °C for a long reaction time compared to the effect of these mutations on specific activities measured earlier at analytical scale at 25 °C. Finally, (±)-4-hydroxyhexan-3-one **6** being racemic, the stereoselectivity of the three triple variants were determined. The enantiomeric excess of product **6′** (ee > 95 %) revealed the presence of one predominant enantiomer, suggesting a high stereoselective control of the C3 formation by TK_{gst} variants. Given the stereoselectivity of H102L/H474S reported earlier in the reverse reaction (formation of aliphatic α-hydroxy ketones),[26,27] a 3*S* configuration is expected.

We also investigated the removal of aldehyde product by continuous distillation of the reaction mixture heated at 50°C under reduced pressure. This experiment was performed with H102L/H474S/L118I from (±)-4-hydroxyhexan-3-one **6** at 4 mmole scale, this compound being cheap and commercially available compared to (3*S*)-1,3-dihydroxyhexan-2-one **3** which was obtained in lower quantities by enzymatic synthesis.[26] In addition, considering that this work is taking part of a research program on the valorization of vegetable oil derivatives, aliphatic α-hydroxyketone **6** was chosen as it is more structurally related to fatty α-hydroxyketones than **3.** After 48h of reaction time, only a very small quantity of aldehyde **6"** was recovered, the boiling points of **6** and **6"** being close (64°C and 49°C respectively). Nevertheless, the other product **6'** was purified by extraction with diethyl ether and obtained with 8 % yield, without any optimization. The compound **6'** was characterized by NMR and GC analysis given an enantiomeric excess of 95% (SI).

CONCLUSION

This study shows for the first time that rationally designed TK_{gst} variants can cleave carbon-carbon bond of aliphatic α-hydroxy ketones **1**, **2**, **3**, **6** with a carbon chain from 4 to 8 atoms to release the corresponding products. The simple variant F435I displayed high activity with (3*S*)-1,3-dihydroxyhexan-2-one **3** leading to the formation of product **3′** with 92% yield after only 2 h reaction time. The combination of L118I with the double-site mutation H102L/H474S previously identified for improving activity toward pyruvate homologues for synthetic purposes[25] gave three new triple variants TK_{gst} H102L/H474 (S, G and A) /L118I that were found to cleave (±)-4-hydroxyhexan-3-one **6** to give product **6′** with 90, 82 and 79% yield respectively, while wild-type and H102L/H474S were respectively ineffective and only weakly effective. The improvement of TK_{gst} activity through rational design enables us to propose an enzymatic one-step reaction which offers an attractive alternative to other multi-enzyme or chemical strategies developed for the cleavage of aliphatic α-hydroxy ketones with longer carbon backbone lengths so far. by.

Experimental Section

General. All chemicals were purchased from Sigma-Aldrich, Alfa-Aesar, TCI Chemicals, and CarboSynth, Bio-Rad and QIAGEN. A QuikChange Lightning site-directed mutagenesis kit (Agilent, USA) was used to construct the TK_{gst} variant libraries. Oligonucleotides synthesis and gene sequencing were performed by Eurofins Genomics (Ebersberg, Germany). The GenElute™ Plasmid Miniprep kit or with the GenElute™ Plasmid Midiprep kit (Sigma-Aldrich) was used for Plasmid DNA extraction. Chiral (3*S*)-1,3-hydroxy ketones **1** was purchased from Sigma **2**, **3**, **4** and **5** were synthesized using the procedure described in the literature. [26] (±)-hydroxy ketones **6**

or **7** were purchased from TCI Europe. NMR spectra were recorded in D_2O or $CDCl_3$ on a 400 MHz Bruker Avance III HD spectrometer. GC analyses were performed on a ThermoScientificTM Trace GC Ultra instrument coupled with a Trace DSQ mass spectrometry detector (scanning between *m/z* 30 and 400). Enantiomeric excess was determined using a RestekTM chiral column RtTM-bDEXsm (length 30 m, inside diameter 0.25 mm, film thickness 0.25 μ).

Site-directed mutagenesis. The QuikChange XL II site-directed mutagenesis kit (Agilent Technologies)was used . Single variants (D470I, L382F, F435I and L191I) and triple variants (H102L/H474S/D470I or H102L/H474S/L382F or H102L/H474S/F435I or H102L/H474S/L191I) were previously described.[26] For the construction of triple variant NDT libraries, the double variant TK_{gst} H102L/H474S[25] was used as template using appropriate oligonucleotides (SI). The PCR products were transformed by heat shock into XL-10 Gold competent cells which were cultured in Luria-Bertani (LB) broth medium containing 30 μg . mL^{-1} of kanamycin overnight. Plasmids were extracted and sequenced to evaluate the quality of mutagenesis and to check the correct substitutions. *E. coli* BL21(DE3)pLysS competent cells were used for TK_{gst} protein expression.

Expression of TK_{gst}. *E. coli* BL21(DE3)pLysS strain was used for TK_{gst} expression. This strain was transformed by heat shock with the TK_{gst} plasmids expressing synthetic wild-type TK_{gst},[15] TK_{gst} L382F, TK_{gst} D470I, TK_{gst} F435I, TK_{gst} L191I, TK_{gst} H102L/H474S, TK_{gst} H102L/H474S/L382F, TK_{gst} H102L/H474S/D470I, TK_{gst} H102L/H474S/F435I or TK_{gst} H102L/H474S/L191I. These strains were stored at –80 °C in glycerol 60% (10% final). Apre-culture (20 ml), containing kanamycin (30 $\mu g.mL^{-1}$) grown at 27 °C, 200 rpm for 12 h, was used to inoculate 1 liter of culture medium containing kanamycin (30 $\mu g.mL^{-1}$) grown at 37 °C,

200 rpm. Isopropyl β-D-1-thiogalactopyranoside (IPTG) at 0.5 mM was added when the OD 600 nm range reached 0.6–0.8 A.U. Cells were then grown at 30 °C, 200 rpm overnight. After centrifugation (8000 rpm, 4 °C for 15 min), the recovered cells were 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 recovered bacterial pellets were stored at −25 °C (≈ 5 g of wet bacterial pellet . L^{-1} of biomass).

Expression of TK_{gst} libraries. Each clone was transferred with a replicator from glycerol stock to individual wells in 96-well plates containing 200 µl per well of LB growth media and 30 µg . mL^{-1} kanamycin. The plates were sealed with breathable sealing film to prevent evaporation, and then incubated at 37 °C, 900 rpm overnight. Aliquots of 50 µl were added from each well to 96-deepwell plates containing 800 µl per well of LB-kanamycin growth medium. The plates were sealed with breathable sealing film and then incubated at 37 °C, 900 rpm for 3 h. 20 µL of IPTG solution (4 mM) was added to each well to start the induction phase.. The plates were sealed with breathable sealing film and then incubated at 30 °C, 900 rpm for 21 h. After centrifugation at 3000 rpm for 20 min, the recovered cell pellets were stored at –80 °C for at least 30 min. For cell lysis, the cell pellets were added in 50 µl of buffer (1/10 BugBuster solution (Novagen), 0.5 mg . mL^{-1} lysozyme (Roth)) and 4 U.mL^{-1} benzonase endonuclease (Novagen), and incubated in a shaker for 1.5 h at 37 °C. The cell lysate was then centrifuged at 3000 rpm for 1 h and stored at 4 °C before screening.

Purification of TK_{gst} (wild type and variants). The pellets were intoduced in 35 mL of phosphate buffer (50 mM) containing NaCl (300 mM) at pH 8.0 and then disrupted by sonication on ice for 30 min. After centrifuging (14,000 rpm at 4 °C, 15 min), Insoluble pellets were discarded. Crude extracts containing His6-tagged TKs were purified using Ni-NTA column

equilibrated with phosphate buffer. After washing with phosphate buffer, the elution of His6-tagged TKs was performed 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 (cutoff 14,000 $g \cdot mol^{-1}$) at 4 °C. Finally, the protein solutions were lyophilized. The Bradford method using bovine serum albumin (BSA) as the standard was used for the determination of protein concentration. The specific activity of lyophilized wild-type and variant TK_{gst} were measured at 25 °C and gave 0.01 $U.mg^{-1}$ to 1.4 $U.mg^{-1}$ of total protein (SI). SDS-PAGE with Precision PlusProteinTM All Blue Standards#161-0373 (10–250 kDa, BioRad) as standard was used to determine the purity and molecular mass of the samples revealed with Coomassie Blue G-250 (SI).

Determination of WT TK_{gst} specific activity. One unit of TK_{gst} activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of α-hydroxy ketone product per minute at 25 °C in HEPES buffer (50 mM, pH 8). TK_{gst} enzymatic assay was carried out with L-erythrulose and D-ribose-5-phosphate (DR5P) leading to D-sedoheptulose-7-phosphate (DS7P) and glycolaldehyde. The yeast alcohol dehydrogenase (ADH) was used to reduce the released glycolaldehyde to ethylene glycol with nicotine adenine dinucleotide reduced form (NADH). 50 µL L-erythrulose (250 mM), 50 µL D-R5P (12.5 mM), 10 µL ThDP (2.5 mM), 10 µL $MgCl_2$ (25 mM), 10 µL NADH (10 mg. mL^{-1}), 10 µL of ADH (25 U . mL^{-1}) and 10 µL TK_{gst} solution (1 mg . mL^{-1}) were added in a well of a 96-well multiplate and adjusted to 250 µL with with HEPES buffer (pH 8, 50 mM). 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} variants with TLC. Screening reactions were performed in 96-well microplates at room temperature and 400 rpm overnight. First, a premix containing 10 mL of (4*S*,4*R*)-hydroxyhexan-3-one **6** (500 mM), 2 mL of glycolaldehyde (625 mM), 250 µL of $MgCl_2$ (100 mM), 250 µL of ThDP (10 mM) and 2.5 mL of distilled water was prepared and pH was adjusted to 7.5 before use. In a well, the reaction was started by adding 68 µL of premix with 45 µL of cellular lysate. After one night of incubation, product **6′** was visualized by TLC using *p*-anisaldehyde stain (solvent system: ethyl acetate/cyclohexane 9/1, R_f **6′** = 0.44).

Determination of the best TK_{gst} variant activities.

*Protocol with dihydroxy ketones (**2**, **3**, **4** and **5**).* One unit of TK_{gst} activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of α-hydroxy ketone product per minute at 25 °C in HEPES buffer (50 mM, pH 8). TK_{gst} enzymatic assay was carried out with dihydroxy ketone (**2**, **3**, **4** or **5**) and DR5Pleading DS7Pand the corresponding aldehyde (propanal, butanal, pentanal or hexanal respectively). ADH was used to reduce the released aldehyde to the corresponding alcohol with NADH. 50 µL dihydroxy ketone (250 mM), 50 µL D-R5P (12.5 mM), 10 µL ThDP (2.5 mM), 10 µL $MgCl_2$ (25 mM), 10 µL NADH (10 mg . mL^{-1}), 10 µL of ADH (25 $U.mL^{-1}$) and 10 µL TK_{gst} solution (1–5 $mg.mL^{-1}$) were added in a well of a 96-well microplate and adjusted to 250 µL with HEPES buffer (pH 8, 50 mM). 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.

Protocol with (±)*-4-hydroxyhexan-3-one **6**.* One unit of TK_{gst} activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of α-hydroxy ketoneproduct per minute at 25 °C in HEPES buffer (50 mM, pH 8). TK_{gst} enzymatic assay was carried out with

(4*S*,4*R*)-hydroxyhexan-3-one **6** and D-erythrose as acceptor leading to the corresponding ketose and propanal. ADH was used to reduce the released propanal to propanol with NADH. 110 µL (4*S*,4*R*)-hydroxyhexan-3-one **6** (250 mM), 50 µL D-erythrose (250 mM), 10 µL ThDP (2.5 mM), 10 µL $MgCl_2$ (25 mM), 10 µL NADH (10 mg . mL^{-1}), 10 µL of ADH (25 U . mL^{-1}) and 50 µL TK_{gst} solution (5 mg . mL^{-1}) were added in a well of a 96-well multiplate and adjusted to 250 µL with HEPES buffer (pH 8, 50 mM). 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.

General procedure of TK_{gst} (wild type and variants)-catalyzed cleavage of α-hydroxy ketones. In a 2 mL Eppendorf tube were mixed 800 µL of (di)-hydroxy ketones **2**, **3** and **6** (500 mM) with 80 µL of glycolaldehyde (1.25 M), 20 µL $MgCl_2$ (100 mM), 20 µL ThDP (10 mM) and 680 µL of distilled water. pH was adjusted to 7.5 and 400 µL of TK_{gst} WT or variants (25 mg . mL^{-1}) were added to start the reaction. Reactions were monitored by TLC (solvent system: ethyl acetate/cyclohexane 9/1, stain : *p*-anisaldehyde) and ^1H NMR. The concentration of substrates and products were determined by *in situ* ^1H NMR.

GC assay method for enantiomeric excess determination. Samples of compounds **6** and **6′** were trimethylsilylated before injection using *N,O*-bis(trimethylsilyl)trifluoroacetamide (BSTFA) with 1% of trimethylchlorosilane (TMCS). First, 250 µL of reaction mixture was filtered using Centricon (10 min, 14,500 rpm, cutoff 30,000 $g.mol^{-1}$) to remove the enzyme, and the filtrate was then mixed with 250 µL of chloroform to extract **6** and **6′** from the aqueous phase. Finally, the organic layer was mixed with 75 µL of the BSTFA mixture and with 1% of TMCS. Samples were stirred for 30 s and incubated at 60 °C for 15 min. After cooling, samples were directly injected. The carrier gas was helium (1 mL . min^{-1}). The injector temperature was

set at 200 °C. 2 μL aliquots were injected with a split ratio of 1:30. Initial column temperature was 80 °C, held for 10 min. The temperature was then ramped to 90 °C at 1 °C. min^{-1}, to 230 °C at 5 °C. min^{-1}, and then held for 5 min.

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ASSOCIATED CONTENT

Supporting Information.

Creation of TK_{gst} variant libraries.
Production and purification of best hits.
Screening of TK_{gst} libraries by TLC.
Monitoring of TK_{gst}-catalyzed reaction by *in situ* ^{1}H NMR.
In situ ^{1}H NMR spectra of reaction mixtures.
Cleavage of compound 6 at preparative scale.
Molecular modeling of TK_{gst} active site.

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All the authors jointly wrote the article. All the authors have given their approval to the final version of the manuscript.

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BRIEFS

Rationally designed TK_{gst} variants cleaved aliphatic α-hydroxy ketones with a carbon chain of 4–8 atoms to release the corresponding aldehydes.

SYNOPSIS