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Review

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Enzymatic carbon–carbon bond formation catalyzed by transketolase: from nature to selective and sustainable synthesis of α-hydroxyketones

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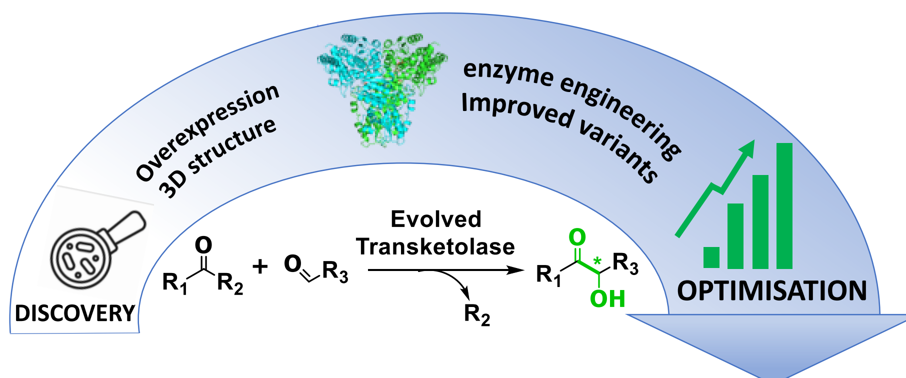
Abstract

Transketolase (TK), a thiamine diphosphate (ThDP)-dependent enzyme, plays a pivotal role in the pentose phosphate pathway, catalyzing the reversible transfer of a two-carbon ketol unit from a donor ketose to an aldose acceptor. Beyond its metabolic function, TK has emerged as a versatile biocatalyst for stereoselective carbon–carbon bond formation, enabling the efficient synthesis of a wide range of chiral α-hydroxyketones under mild, aqueous conditions. This review traces the historical development, mechanistic insights, and protein engineering approaches to broaden substrate scope and improve enzyme stability, providing a comprehensive framework for researchers seeking to exploit this enzyme in green and stereoselective organic synthesis.



Keywords: Biocatalysis, carbon-carbon formation, transketolase, α-hydroxyketones, enzyme engineering

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1. Introduction

The efficient and stereoselective formation of carbon–carbon (C–C) bonds constitutes the foundation for constructing complex molecules in organic chemistry, enabling synthesis of natural products, pharmaceuticals, agrochemicals, and fine chemicals.[1] Classical methods, such as the aldol reaction, Mannich reaction, organometallic couplings, and organocatalysis, enable high degrees of selectivity. However, these approaches often require harsh reaction conditions, transition metal catalysts, protecting groups, and multiple purification steps. Consequently, there is an ongoing drive to develop mild, selective, and sustainable alternatives, which has brought biocatalysis to the forefront of synthetic strategies. Enzymes are natural catalysts designed to accelerate specific reactions, offering chemo-, regio-, and stereoselectivity, operating under aqueous, mild conditions compatible with environmentally friendly synthesis.[2,3] These features, combined with the potential for protein engineering, make biocatalysis an increasingly attractive alternative to conventional chemical catalysts, particularly to produce enantiopure compounds. The optimization of enzymes to address technical challenges, such as a broad substrate range, high selectivity, and stability, is made possible by the

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implementation of directed evolution.[4] A growing number of methodologies and computational tools have been developed to identify suitable enzyme activities, and numerous applications have proved the efficiency of these integrated strategies in biocatalytic processes.[5] The purpose of this review is to highlight such methodologies through a thiamine diphosphate enzyme, transketolase (TK), for obtaining chiral α-hydroxyketones by C-C bond formation in green and stereoselective manner. The α-Hydroxyketones display numerous properties and serve as key components in pharmaceuticals, flavoring agents, and fragrances, and are also valuable precursors for the synthesis of high-value compounds such as vicinal diols and amino alcohols.[6]

2. From Transketolase Discovery to First Application in Organic Synthesis

2.1 Thiamine diphosphate-dependent enzymes

Among enzymes capable of catalyzing C–C bond formation, thiamine diphosphate (ThDP)-dependent enzymes occupy a prominent position.[7] ThDP (Figure 1A) is the active form of vitamin B1 and an essential cofactor for enzymes in key metabolic pathways. ThDP-dependent enzymes display the formation of a C–C bond between two carbonylated compounds (aldehyde or ketone) according to a common mechanism (Figure 1B) involving the activated ThDP **I** to produce a highly reactive intermediate (**III**) via polarity reversal (Umpolung reaction) characterized by two mesomeric forms (carbanion-enamine or Breslow intermediates). The carbanion subsequently attacks the carbonyl group of an electrophile (acceptor) substrate, leading to the formation of the C-C bond (**IV**) and to the release of the product. The stereo control of the new asymmetric carbon depends on the type of ThDP enzyme and the structure of the substrates.

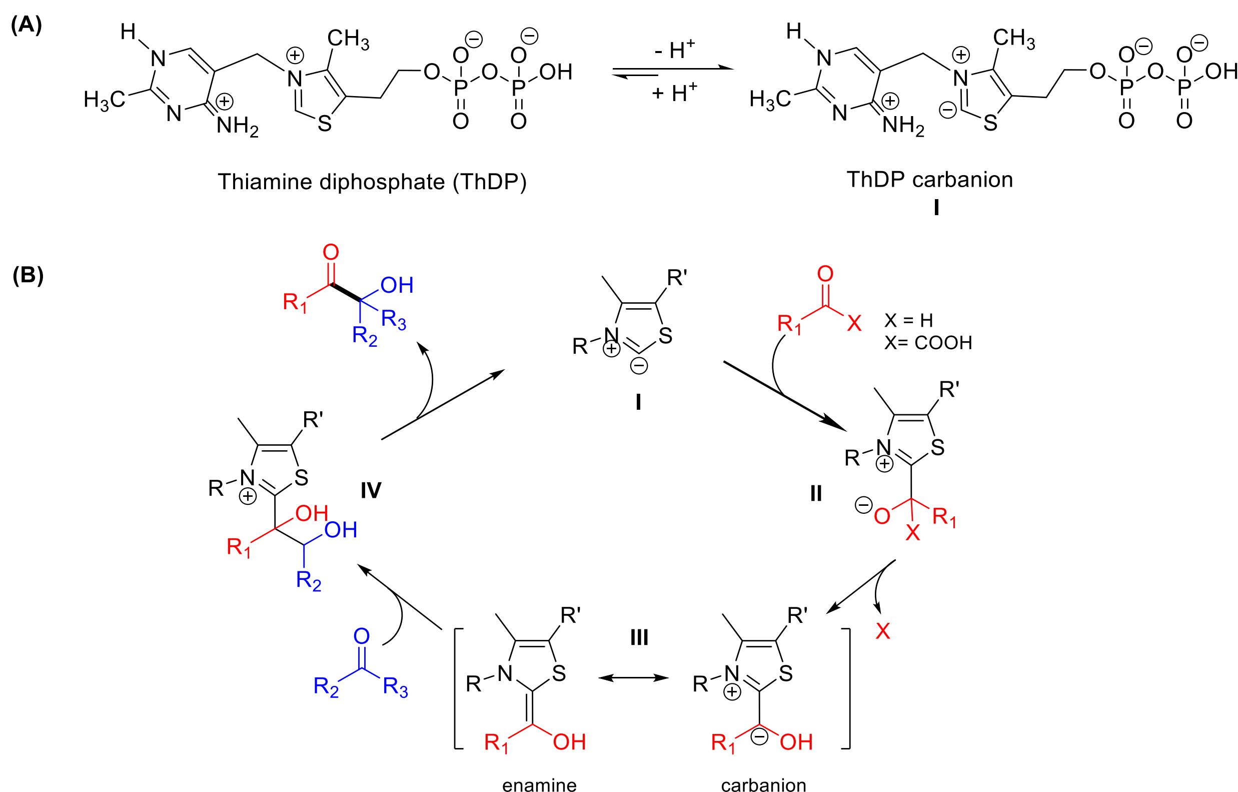

Figure 1. Activation of thiamine diphosphate A); catalytic cycle of ThDP-dependent enzymes B).

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2.2 Transketolase properties

Historically, transketolase (TK, EC 2.2.1.1) was first characterized in the early 1950s during studies of carbohydrate metabolism.[8] Then, its biochemical significance has been recognized ever since in animal, plant, and bacterial systems.[9] The discovery of its dependence on ThDP marked an important milestone in understanding cofactor-mediated enzymatic reactions, highlighting the complex interplay between vitamins and primary metabolism. TK occupies a central role in cellular metabolism, particularly within the pentose phosphate pathway. TK catalyzes the reversible transfer of a two-carbon ketol unit from a ketose phosphate donor to an aldose phosphate acceptor, creating a new chiral center with the (*S*)-configuration, thereby facilitating the interconversion of sugars of varying chain lengths (Figure 2).

D-xylulose-5-phosphate + n = 1 : D-erythrose-4-P; n = 2 : D-ribose-5-P ⇌ (TK; ThDP, Mg^{2+}) D-glyceraldehyde-3-P + n = 1 : D-fructose-6-P; n = 1 : D-sedoheptulose-6-P

Figure 2. Transketolase-catalyzed reaction in the pentose phosphate pathway.

The enzymatic activity of TK is not only crucial for the generation of NADPH and D-ribose-5-phosphate, which are essential for anabolic reactions and nucleotide biosynthesis, but it also plays a key role in maintaining the balance of glycolytic and pentose phosphate intermediates.

2.3 Microbial transketolase sources

For TK applications as a biocatalyst in organic synthesis, microbial sources were more suitable for producing the enzyme in large quantities. TK from *S. cerevisiae* (TK_{sce}) was first overexpressed [10] and then TK from *E. coli* (TK_{eco}),[11,12] offering high expression and activity levels. Both TKs typically exhibit optimal activity range between 20–40 °C and lose activity rapidly at higher temperatures due to irreversible aggregation and unfolding. However, we will see later that protein engineering strategies enabled the enhancement of its thermostability.[13]

Thermophilic sources provide TKs with superior thermal and solvent stability, making them highly attractive for industrial biocatalysis. TK from *Geobacillus stearothermophilus* (TK_{gst}) discovered in 2013, exhibits optimal activity between 60 °C and 70 °C, 11-fold activity enhancement at 60 °C compared with that obtained at 20 °C, and maintains its activity during five days at 60 °C.[14] This robustness offers easy purification from crude extract in a single heat treatment step, **s**tability in non-usual media (organic solvents, ionic liquids), and renders this enzyme suitable for optimization by mutagenesis. In addition to the development of large-scale synthesis, TK_{gst} was immobilized on clays, giving recycling at least six times without loss of activity.[15] For even more extreme conditions, TKs from hyperthermophilic organisms such as *Thermotoga maritima* (TK_{tmar}) are particularly valuable. This TK retains activity at temperatures above 70 °C and demonstrates exceptional resistance to thermal denaturation, while its native substrate scope is somewhat limited.[16]

Over the past twenty years, the TKs most commonly used for synthetic applications were TK_{eco}, TK_{sce}, and TK_{gst} (Table 1). The selection of one over the others depends on the desired combination of substrate scope, stereoselectivity, stability, and operational conditions.

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Table 1. Comparative TKs from different sources

Property	TK_{eco}	TK_{sce}	TK_{gst}
Molecular weight	70 kDa (homodimer)	65–68 kDa(homodimer)	72 kDa(homodimer)
Cofactors	ThDP + Mg^{2+}/Mn^{2+}	ThDP + Mg^{2+}/Ca^{2+}	ThDP + Mg^{2+}
Temperature optimum	37–40 °C	30–35 °C	55–60 °C
pH optimum	7.0–7.5	5.5–6.5	7.0–8.0
Stability	Moderate	Moderate	Very high (thermostable)
Solvent tolerance	Low	Moderate	High (organic cosolvent

2.4 Transketolase three-dimensional structures

The crystallographic structures of microbial TKs used in biocatalysis TK_{sce}[10] TK_{eco}, [12] and more recently TK_{gst},[17] were determined notably with both cofactors ThDP and divalent cation. These TKs are homodimers and exhibit a high degree of homology (>70%). For TK_{tmar},[16] despite a low sequence homology (< 36%) compared to other TKs, the key active-site residues remain highly conserved, comprising approximately 35 amino acids, found in other ThDP-dependent enzymes.[18] The superimposition of the active sites of these TKs shows the same residues in a similar position to ThDP and divalent cation Mg^{2+}.

The direct interactions of physiological substrates with the residues of the active sites of three different TK_{sce}, TK_{eco}, and TK_{gst}, commonly used in biocatalysis, have been mainly studied. TK_{sce} has been co-crystallized with one of its natural acceptor substrates, D-Erythrose-4-phosphate (D-E4P).[19] Four conserved amino acids R528, S386, H469, and R359, are responsible for interacting with the phosphate group of the acceptor substrate, anchoring it within the deep cavity of the enzyme's active site. The remarkable ability of TK_{sce} to accommodate aldehydes of varying sizes (ranging from three to seven carbon atoms) is attributed to the flexibility provided by these four residues (Figure 4).[20]

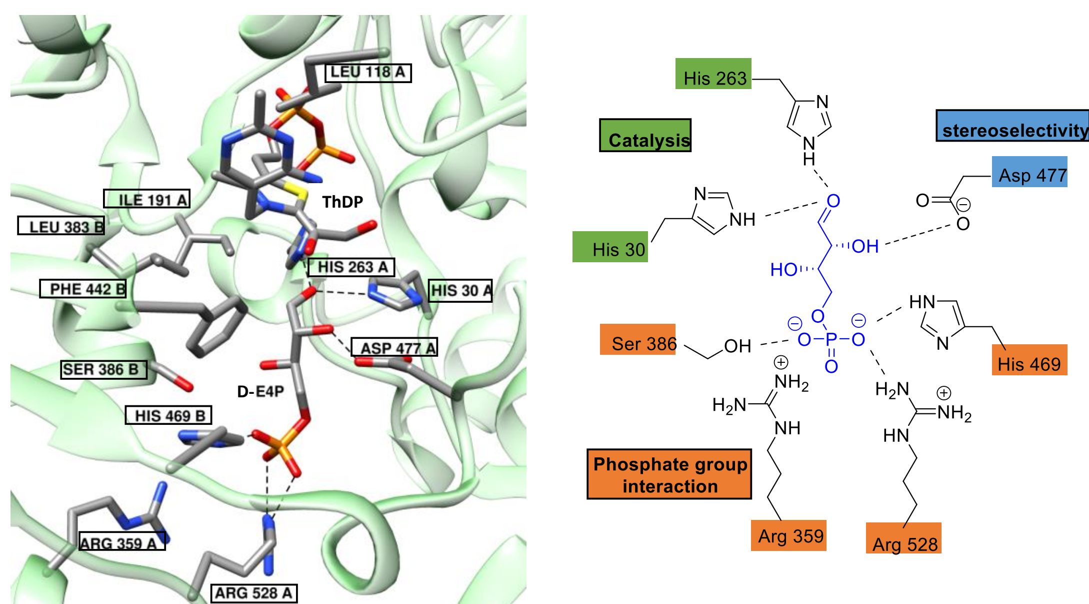

Figure 3. A) TK_{sce} active site co-crystallized with its natural acceptor substrates, D-Erythrose-4-phosphate (DE4P) and ThDP. B) representation of direct interactions of D-E4P with active site residues.

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The carbonyl group of D-E4P is stabilized by both histidines, H30 and H263, through hydrogen bonding explaining the stereoselectivity in the nucleophilic attack on this carbonyl group. Two histidines H263 and H481 are both directly involved in the proton transfer required for the cleavage and the formation of the C–C bond in the catalytic mechanism. Additionally, the C2 hydroxyl group of the aldehyde function interacts with the carboxylate group of the residue D477, explaining the enantioselectivity towards (2*R*)-hydroxyaldehyde.[21] The role and corresponding numbering of the active site residues of each TK sources mainly used in biocatalysis are given in Table 2.

Table 2. Key amino acids in TK_{sce}, TK_{eco}, TK_{gst} active sites

Residue properties	TK_{sce}	TK_{eco}	TK_{gst}
Catalysis	H30	H26	H28
	H263	H261	H263
	H481	H473	H474
	H103	H100	H102
Enantioselectivity	D477	D469	D470
	H469	H461	H462
Phosphate group Stabilization	R359	R358	R358
	S386	S385	S385
	R528	R520	R521

2.5 Transketolase reaction mechanism

The reaction mechanism catalyzed by TK_{sce} was the first elucidated using a ketose phosphate as donor and an aldose phosphate as acceptor substrates (Figure 4). Studies conducted on TK_{eco} and TK_{gst} 3D structures have highlighted the same residues involved in the catalytic process as those in TK_{sce}, with only a different numbering of residues. The catalytic reaction begins with the deprotonation of the C2 atom of the thiazolium ring of ThDP, giving the "activated" form of ThDP **I** (also known as the ylide form).[22, 23] Then, the TK reaction proceeds according a "Bi-Bi, ping-pong" mechanism with the successive binding of the donor substrate and then the acceptor to the active (Figure 4A).[24]

In the first step (**a**)**,** the carbanion of the activated form of ThDP **I** attacks the carbonyl group of the donor substrate **D**, the oxygen of the hydroxyl group being protonated by the iminium group of the pyrimidine ring or by residue H481. [25] The latter hypothesis is assumed in the mechanism depicted in Figure 4, giving the intermediate **II**. Subsequently, residues H103 and H30 maintain **D** in an optimal conformation.[26] In step **b**, residue H263 is then ideally positioned to abstract the proton from the hydroxyl group at C3 of **D** bound to ThDP, leading to the cleavage of the C2-C3 bond of **D**[27] and the formation of **III** intermediate, also known as the "activated ketol donor," stabilized by mesomeric effect. The product **P1** is then released. In step **c**, the carbanion of DHE-ThDP **III** complex attacks the *re*-face of the aldehyde group of acceptor substrate **A**, giving **IV** intermediate, the oxygen being protonated by H263. The newly formed asymmetric carbon has an (*S*)-configuration. In the final step **d**, the C–C bond cleavage of **IV**, via deprotonation of the hydroxyl group by H481, leads to the release of the final product **P2**, which has a (3*S*,4*R*)-configuration and the regeneration of activated ThDP **I** (Figure 4B).

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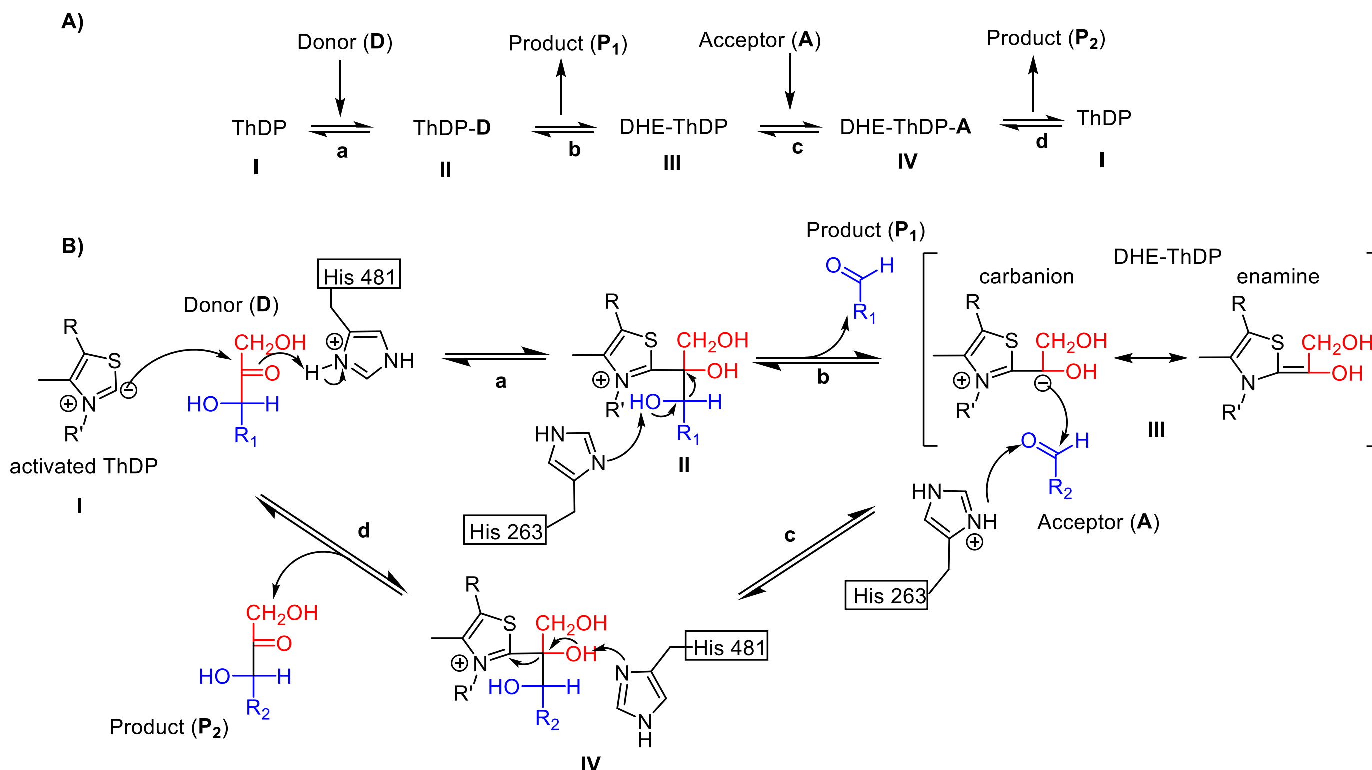

Figure 4. Mechanism of the TK_{sce}-catalyzed reaction. A) Bi-Bi type mechanism. B) ketol group transfer mechanism.

2.6 Substrate scope of wild-type Transketolases

Over the past several decades, the interest in TK has extended far beyond its biological function, encompassing its potential applications in synthetic organic chemistry. A significant breakthrough came in the late 1980s and early 1990s, with the demonstration that TK isolated from spinach leaves and TK_{sce} catalyzes the asymmetric transfer of a ketol group from an α-ketoacid, hydroxypyruvate (HPA), to non-phosphorylated aldehydes, resulting in an irreversible reaction due to carbon dioxide release (Figure 5).[28, 29]

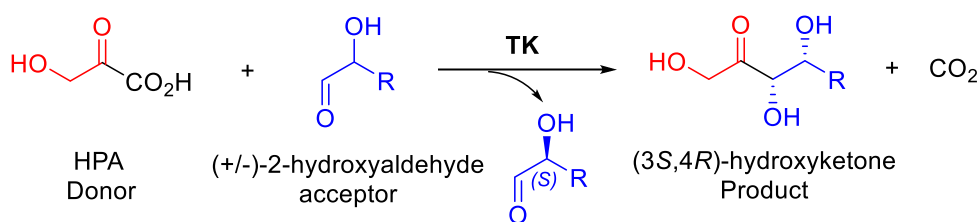

Figure 5. Irreversible reaction catalyzed by TKs in the presence of HPA.

Then, using HPA as donor, TK_{sce}, TK_{eco}, and TK_{gst} acceptor-substrate scopes were intensively studied, showing very similar profiles consistent with the high homology of the TK active sites. TKs accept non-phosphorylated aldehydes with a preference towards polyhydroxylated aldehydes with short carbon chain (C2-C4) in (2*R*)-configuration, meaning that the enzyme can be employed not only for the synthesis of a single product (3*S*,4*R*)-hydroxyketone from a racemic 2-hydroxyaldehydes but also in the kinetic resolution of aldehyde substrate.[30] The only difference observed with TK_{gst} compared to the other microbial sources is a

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significant activity towards (2*S*)-hydroxylated aldehydes with three and four carbon atoms. TK_{gst} being thermostable, studies conducted at 60 °C showed an improvement in activities by a factor of four to five towards C3 (L-glyceraldehyde, L-lactaldehyde) and C4 aldoses (L-erythrose, and D-threose) compared to results obtained at 25 °C.[31] With all TKs, a significant decrease in activities was observed with aldehydes displaying a long carbon chain length (C5-C6) or without a hydroxyl group at the α or β positions or hydrophobic and aromatic aldehydes.

From the early 2000s, various groups interested in this enzyme have sought to increase the efficiency of TKs and to broaden the substrate spectrum by engineering to access a diverse range of α-hydroxyketones from new donor and acceptor substrates.

3. Strategies for New TK Discovery and Engineering

3.1 Molecular biology coupled with molecular modeling

Over the last 20 years, TKs have provided an excellent model for testing new strategies for enzyme engineering in order to improve stability and extend substrate scope.

Thermostability improvement offers additional resources for industrial applications, where enzyme stability is paramount. Metagenome mining and gene splitting have recently been investigated, allowing the construction of chimeric enzymes by combining functional elements from different homologs, and thereby exploring new regions of sequence space. Hence, novel TK has been reconstituted from two separate polypeptide chains encoded by a ‘split-gene’ identified in the genome of the hyperthermophilic bacterium, *Carboxydothermus hydrogenoformans*.[32] Another strategy was applied based on mining of putative split TKs from metagenomes. Two enzymes expressed by hyperthermophilic organisms (*T. maritima* and *M. jannaschii*) exhibited enhanced high-temperature tolerance compared to other TK examples from the literature, such as partial activity after heating at 90 °C or 100 °C for 1 hour, respectively.[33] Furthermore, recent work conducted with TK_{eco} indicates that four mutations (H192P/A282P/I365L/G506A, known as 4M) increases the rigidity of the enzyme and, consequently, its stability. This improvement is highlighted by a thermal transition midpoint temperature (Tm), defined as the midpoint at which the protein transitions from its native to its denatured state, of 68.9 ± 0.04, higher than that of the wild-type protein (65.7 ± 0.4).[34]

To extend the substrate scope of TK_{eco}, TK_{sce}, and TK_{gst} to obtain new α-hydroxyketones, protein engineering by rational or semi-rational mutagenesis techniques was intensively used over the last 20 years (Figure 6).

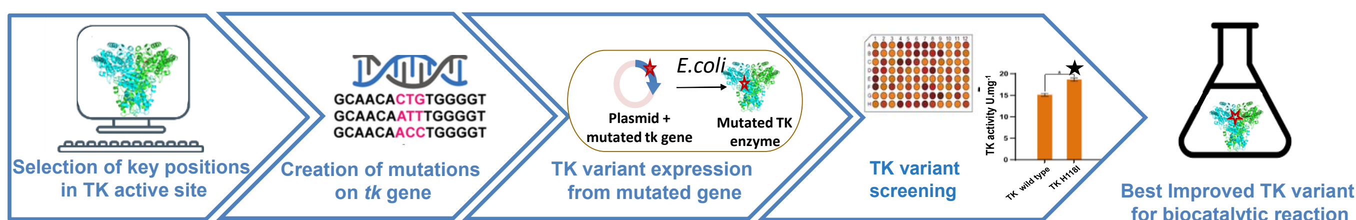

Figure 6. Workflow of the transketolase (TK) engineering process.

Through rational design, amino acid residues involved in substrate binding, catalysis, or stability are substituted (mutated) by other amino acids based on structural and mechanistic insights. While the

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replacement by non-natural amino acids has been investigated to explore how novel amino-acid side chains might further enable substrate specificity to be improved,[35] the substitution by natural L-amino acids remains the technique classically reported in the literature to broaden the substrate scope of TK variants.[36,37]

The substitutions are performed upstream at one or multiple positions simultaneously in the nucleotide sequence corresponding to the identified position in the protein sequence. In 1978, Michael Smith introduced the site-directed mutagenesis (SDM) technique, allowing the substitution of one targeted amino acid in the wild-type protein with one of the other nineteen canonical amino acids.[36] An extension of SDM is site saturation mutagenesis (SSM), which consists of a semi-rational approach where each targeted amino acid of the wild-type sequence is replaced by the nineteen other amino acids. This approach, known as the Combinatorial Active-Site Saturation Test (CAST), was introduced in 2005.[37] To reduce the number of variants and screening efforts, particularly when multiple positions are targeted, different techniques provide functional diversity without redundancy by avoiding stop codons while encoding several representative amino acids in each category: anionic, cationic, aliphatic hydrophobic, aromatic hydrophobic, hydrophilic.

The choice of positions to be mutated is a critical aspect guided by *in silico* analysis of the TK active sites within 4 Å or less from ThDP. It is noteworthy that the residues located within this sphere are highly conserved and largely play a crucial role in substrate binding and the catalytic mechanism of TK. Another crucial point is detecting the most active variants from the TK variant libraries with a suitable assay.

3.2 Enzymatic assays for Transketolase activity detection

Screening and selection assays are central to protein engineering, as they enable the identification of improved enzyme variants from large mutant libraries.[38] Selection assays directly link enzyme performance to cell survival, growth, or a selectable phenotype, allowing the interrogation of very large libraries with minimal experimental handling. Our group developed the proof-of-principle of a cell-based selection assay enabling the identification of active TK variants by directly linking catalytic activity to the production of an essential metabolite, D-methionine, in an auxotrophic host cell. But the limiting step of this strategy was the prior chemical and/or enzymatic synthesis of the TK probes required as substrates, which are then transformed by auxotrophic cells into D-methionine.[39] In addition, this type of assay is not generic and limited to the detection of one targeted improvement or modification depending on the TK probe structure.

Over the last 20 years, studies have focused more specifically on the development of fluorometric or colorimetric screening assays, either directly in cells for qualitative visual detection and/or with isolated TK variants for quantitative activity measurements (Figure 7).[40] These two types of assays are complementary. Indeed, cell-based tests are intended for pre-screening, particularly for large libraries and quantitative assays, which enable the determination of the activities of the best-selected candidates.

An ideal screening assay must be sensitive, robust, reproducible, and compatible with high-throughput formats, while reliably reporting the desired catalytic property. Depending on the target reaction, screening strategies can be based on activity, selectivity, and stability**.** The main goal in the case of TK-catalyzed reaction is to extend the substrate scope of donor and/or acceptor substrates. For this, a generic assay relevant to various α-hydroxyketone products obtained from a wide range of α-ketoacids and aldehydes as substrates is required.

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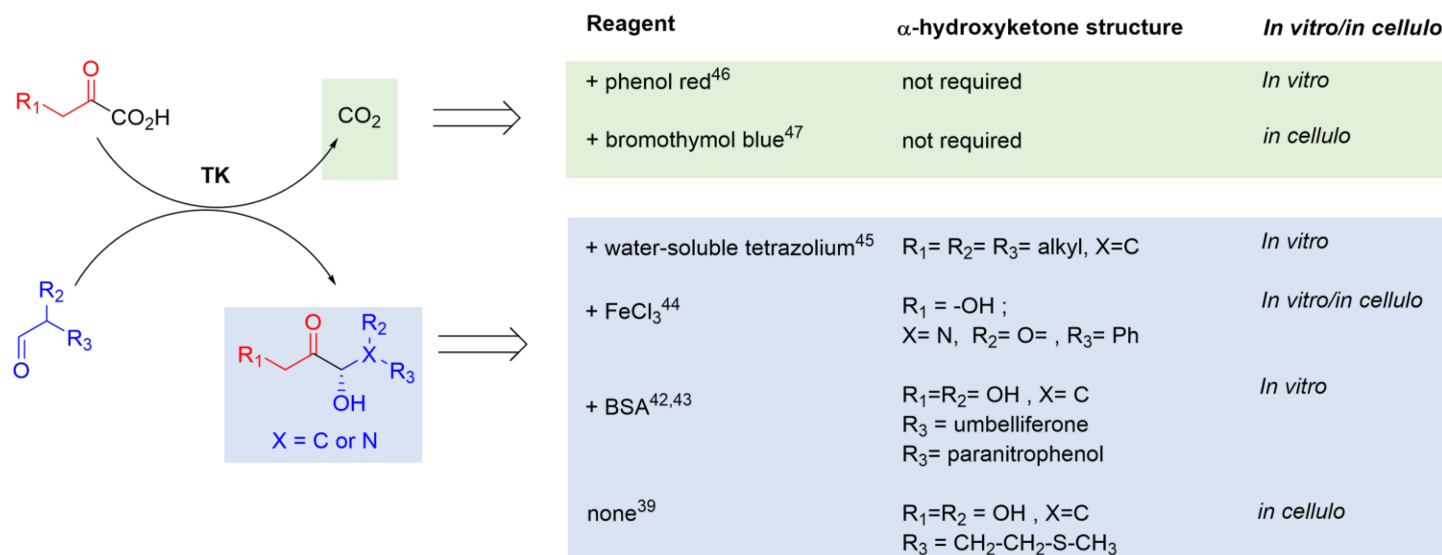

Figure 7. Screening assays developed for TK variant activity detection.

Different strategies have been developed, such as droplet-based microfluidics,[41] cleavable chromophore[42] and fluorophore[43], TK-based reaction to produce *N*-aryl hydroxamic acids,[44] colorimetric assay using WST-1 (water-soluble tetrazolium),[45] but suffered from different limitations such as enabling ultra-high-throughput and/or costly and/or not applicable on a wide range of α-hydroxyketones.

The detection of carbon dioxide offers a generic assay independent of the α-hydroxyketone and substrate structures. For this purpose, a strategy based on pH variation generated by decarboxylation of the α-ketoacids was proposed.[46] Indeed, during the reaction catalyzed by TK in the presence of the α-ketoacid as donor, a proton is consumed in each cycle, which releases an equivalent of bicarbonate ions (HCO_3^-), leading to a pH increase of the reaction medium (Figure 8A).

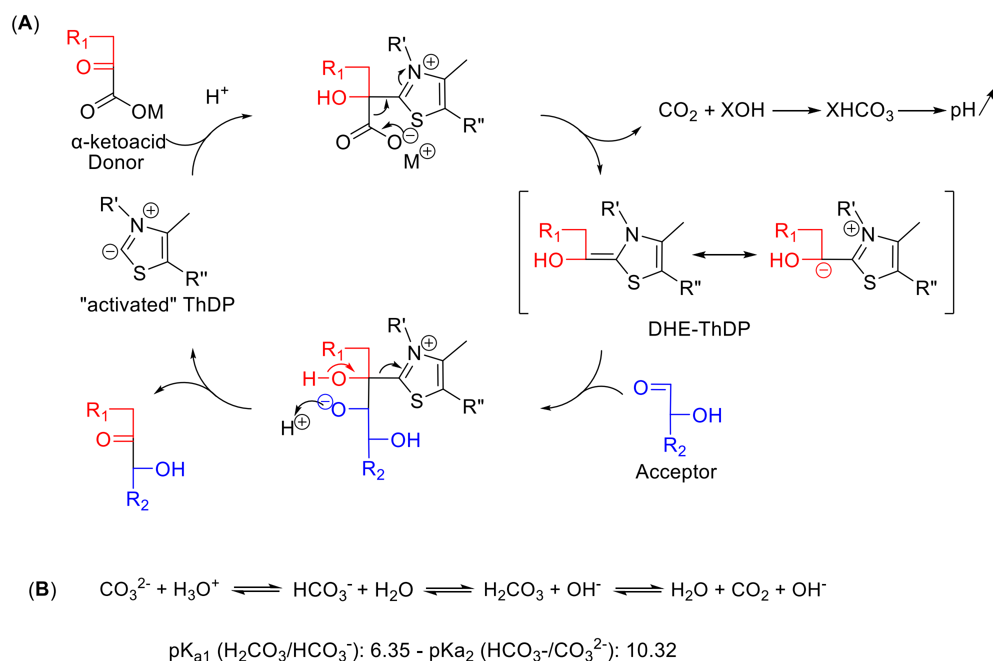

Figure 8. Catalytic cycle of TK in the presence of α-ketoacid, adapted from the literature[46] (A), dissociation equilibrium of the bicarbonate ion in solution in water (B).

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The bicarbonate ion is the dissociated form of carbonic acid (H_2CO_3) and is involved in a dynamic equilibrium of dissociation in water, which leads to a basification of the solution, due to the partial formation of hydroxide ion at the pH of the medium, which is approximately 7.5 (Figure 8B).

The pH increase in the reaction mixture can be determined photometrically by the color change of the pH indicator using a low buffer concentration (triethanolamine 2 mM, pH 7.5). Phenol red was selected for its pKa of 7.4 and its turning zone (6.8 < pH < 8.2) compatible with the optimal pH of the reaction catalyzed by TK, which is approximately 7.5. Phenol red changes from bright yellow at acidic pH to bright pink at basic pH (Figure 10 A) a detection by naked eye and a quantification by spectrophotometry with high sensitivity (ε = 56000 $M^{-1}.cm^{-1}$ at λ max = 557 nm). Thus, it offers a wide spectrum of colors, allowing easy visualization of the reaction progress. Finally, phenol red, in its deprotonated form, has a high molar absorption coefficient (ε = 56000 $M^{-1}.cm^{-1}$ at λ max = 557 nm), allowing its detection by spectrophotometry with high sensitivity. This rapid, generic and cheap assay allows continuous monitoring, quantitative determination of the kinetic parameters, and can be applied in microplates for high throughput screening (Figure 10 A).

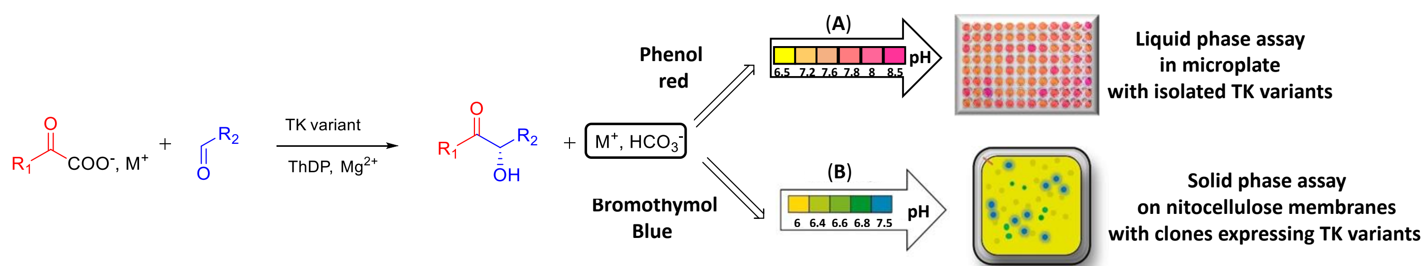

Figure 10. Colorimetric pH-based assays using phenol red required for quantitative activity determination in liquid phase (A) or using bromothymol blue required for visual solid phase assay (B).

The principle of this assay was also applied to clones expressing TK variants growing on a solid phase and then transferred to a nitrocellulose membrane. This membrane was placed on a semi-solid medium containing the substrates, cofactors, and the pH indicator bromothymol blue (giving more contrast compared to phenol red used in the liquid phase). The colonies became blue if TK variants are active towards the substrates (Figure 10 B).[47]

In conclusion, the pH-based assays can be used whatever the structures of α-ketoacids or aldehydes and are applicable on the solid phase for visual pre-screening of TK variants directly in clones or in the liquid phase for quantitative activity determination of extracted TK variants.

4. TK Variants for the Synthesis of New α-Hydroxyketones

As previously mentioned, mesophilic TKs (TK_{eco}, TK_{sce}) and, more recently, thermophilic TKs such as the most-studied TK_{gst} have been used in organic synthesis. From the early 2000s, TK variants were designed by protein engineering to broaden the substrate spectrum to obtain a wide range of α-hydroxyketones (polyhydroxylated, alkyled, arylated) from new α-ketoacid donor and aldehyde acceptor substrates. In contrast, wild-type TKs in cellular metabolism are limited to phosphorylated ketoses (Figure 11).

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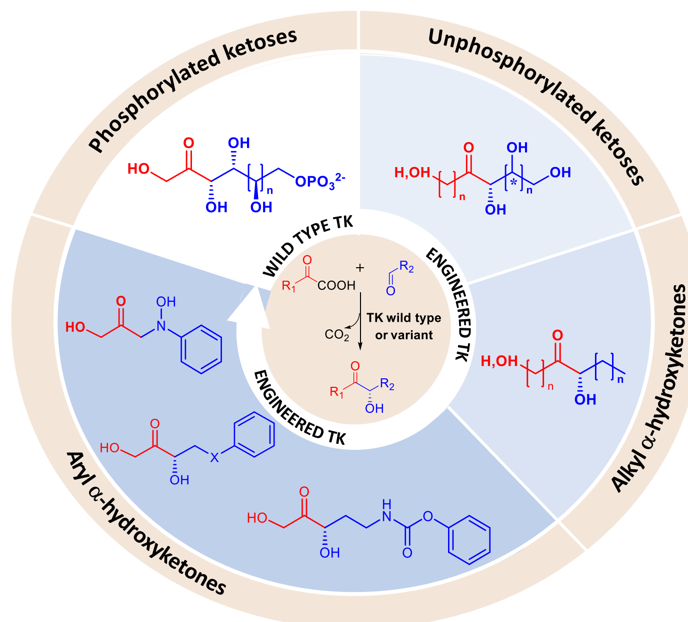

Figure 11. From natural phosphorylated ketoses to novel α-hydroxyketones obtained using engineered TKs.

4.1 Synthesis of unphosphorylated α-hydroxyketones

4.1.1. synthesis of ketoses. Unphosphorylated and polyhydroxylated α-hydroxyketones, (3*S*)-ketoses have important biological functions in the pharmaceutical, agricultural, and food sectors,[48] as well as in synthetic chemistry, where they are considered valuable enantiopure building blocks for the preparation of glycomimetics.[49] Because most of the ketoses are not readily available, this fundamental class of carbohydrates still requires the development of enantioselective synthesis. Typical chemical synthetic routes to higher-carbon sugars involve homologation of lower-carbon sugars, requiring the introduction of new stereogenic centers in a controlled manner with tedious protection/deprotection manipulations.[50] Isomerization of aldoses to ketoses has been extensively explored by chemocatalytic routes in the presence of Lewis acid or alkali catalysts[51] and by using isomerases and epimerases, even though the reaction equilibrium is most often unfavorable for ketose formation.[52,53] These processes can suffer from low yields, a complicated isomer separation step, and are limited by the substrate scope of isomerases.

As attractive alternatives to chemical chain elongation, enzymes that catalyze stereospecific C–C bond formation, such as aldolases and TKs are most popular. However, aldolase-catalyzed reactions most often require phosphorylated substrates and additional enzymes to liberate free long-carbon-chain rare ketoses differently configured on C3 and C4.[54]

To improve and extend the substrate scope towards non-phosphorylated long-chain (2*R*)-aldoses as acceptors, TK_{eco}[55] and TK_{gst}[56] were optimized by mutagenesis for obtaining the corresponding Cn+2 ketoses from HPA as nucleophile (Figure 12).

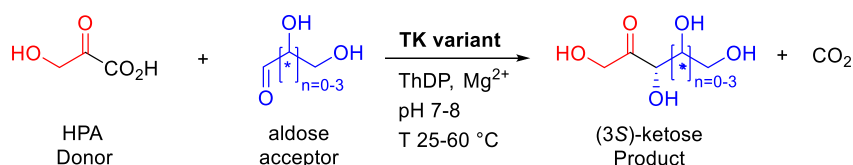

Figure 12. TK variant-catalyzed reaction for non-phosphorylated ketose synthesis.

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To enhance activities towards long-chain (2*R*)-aldoses, suitable positions in TK active sites were selected by molecular modeling (Figure 13).

TK_{gst} variants showed improved activities towards C4 to C6 non-phosphorylated aldoses, such as L382F/F435Y towards tetroses (D-threose and its (3*S*)-epimer L-erythrose), R521Y/S385/H462N towards pentoses (D-ribose and its (3*S*)-epimer D-xylose), and R521V/S385D/H462S towards hexoses (D-allose and its (3*S*)-epimer D-glucose). These aldoses led to the corresponding Cn+2 ketoses (C6−C8) with good yields (59-84%) and high diastereoselectivities (>95%) at 60 °C under near-neutral pH.[56] These ketoses have important biological functions, such as D-tagatose (C6), a hypocaloric sweetener,[57] L-psicose (C6) precursor of glucosidase inhibitor,[58] D-altro-heptulose (D-sedoheptulose) (C7), a marker of sugar metabolism disorders,[59] D-ido-heptulose glucosidase, inhibitors useful for the treatment of hyperglycemic symptoms,[60] D-glycero-D-altro-octulose (C8) plant antioxidant.[61]

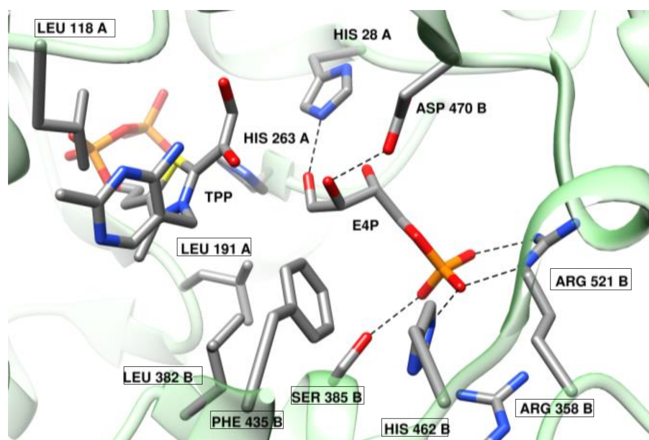

Figure 13. Model of wild-type TK_{gst} with natural acceptor substrate D-erythrose-4-phosphate (E4P). The model was built using Modeler 9.14 and Chimera.

4.1.2 Synthesis of deoxyketoses. The access to 6-, 7-, or 8-deoxyketoses was first investigated using HPA coupled with 4-, 5- or 6- deoxyaldoses (Figure 14 A) such as 6-deoxy-L-mannose (also called L-rhamnose, a biosourced aldose naturally abundant in the pectin fraction of plant cell wall polysaccharides).[62] In the presence of a suitable TK_{gst} variant R521H/S385D/H462I able to enhance activities towards this acceptor, the 8-deoxy-L-*glycero*-L-*galacto*-octulose was obtained with excellent yield (80 %) and diasteroselectivities (>95 %).[62]

The access to 1- and 1,2-deoxyketoses was rendered possible with the identification of TK_{gst} variants capable of accepting pyruvate analogs instead of the commonly used HPA (Figure 14B).[63] The mutation of two positions H102L and H474 (S, G) near the nucleophile was crucial for the transfer of the acyl group (Figure 15A). Analytical assays using pyruvate analogs coupled with glycolaldehyde (as model acceptor) showed that H102L gave 7-fold higher activities than wild-type TK_{gst}, H102L/H474S 14-fold higher activities towards 2-oxobutyrate, and H102T 5-fold higher activities towards 3-methyl-2-oxobutyrate (Figure 15B).

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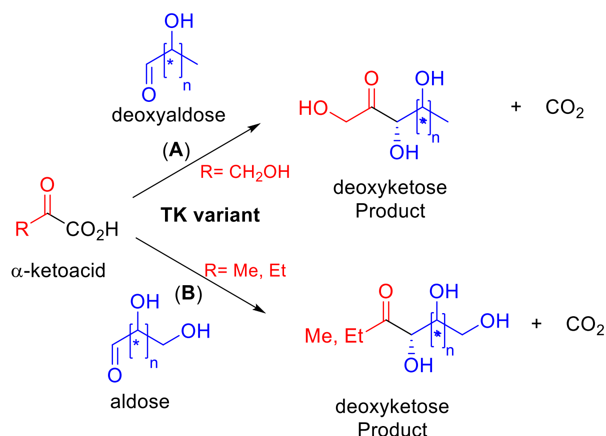

Figure 14. Two pathways for obtaining deoxyketoses: from ketoacid coupled with deoxyaldoses (A) or with pyruvate analogs coupled with aldoses (B).

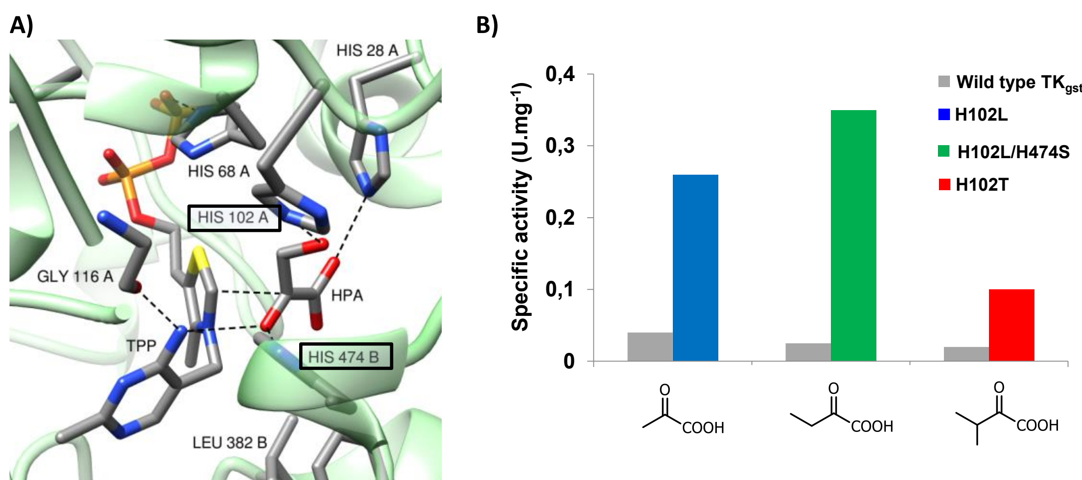

Figure 15. A) model of wild-type TK$_{gst}$ based on the X-ray crystal structure of TK$_{ban}$ (PDB entry 3M49) with donor substrate hydroxypyruvate (HPA). The model was built using Modeler 9.14 and Chimera. B) specific activities of TK$_{gst}$ variants H102L (blue), H102L/H474S (green), H102T (red) compared to wild type TK (grey) determined with glycolaldehyde as acceptor and pyruvate, 2-oxobutyrate, and 3-methyl-2-oxobutyrate.[63]

The combination of these two mutated positions H102L/H474S with L118I, required to enhance activities towards aldoses such as D-erythrose (C4), D-ribose (C5), and D-xylose (C5), gave 1- and 1,2-deoxyketoses from pyruvate and 2-oxobutyrate with good to excellent isolated yields (60 %-86 %) and high diastereoselectivities.[62,64]

4.1.3 Novel transketolase pathway for (deoxy)ketose synthesis. A breakthrough for obtaining (deoxy)ketoses was the discovery of a promiscuous cross acyloin condensation reaction (Figure 16, pathway B) catalyzed by TK variants recently published showing that two different aldehydes can be used as TK substrates one playing the role of nucleophile (analog of the aliphatic α-ketoacid donor used in the common pathway A) and the other the role of electrophile (the same aldose used as acceptor in pathway A, figure 16).[65,66]

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The condensation of hydrophobic aldehydes (ethanal, propanal, *iso*-butanal) in place of corresponding α-ketoacids (pyruvate, 2-oxobutyrate, or 3-methyl-2-oxobutyrate)[65] on aldoses as electrophiles was catalyzed by TK_{gst} variants H102L/L118I/H474G. Glycolaldehyde as nucleophile was also investigated in place of HPA. Its condensation on aldoses used as electrophiles was catalyzed by a TK_{eco} variant including four mutations H192P/A282P/I365L/G506A (4M) giving enzyme stabilization already described,[34] coupled with double mutations I189Q/D469E for better interaction with substrates.[66] The aldehydes used as nucleophiles coupled to aldoses (C_4-C_6) as electrophiles gave, by cross acyloin condensation, the corresponding (deoxy) ketoses, which are the same as those obtained through pathway A with equivalent yields (Figure 16).

In pathway A, the α-ketoacid nucleophile is decarboxylated in the TK active site, while in pathway B the nucleophilic aldehyde is deprotonated, giving finally the same intermediate **II** (acylThDP carbanion) able to react with an aldehyde electrophile. The pathway B avoids the use of costly and /or unstable α-ketoacids and their decarboxylation, thereby allowing atom economy.

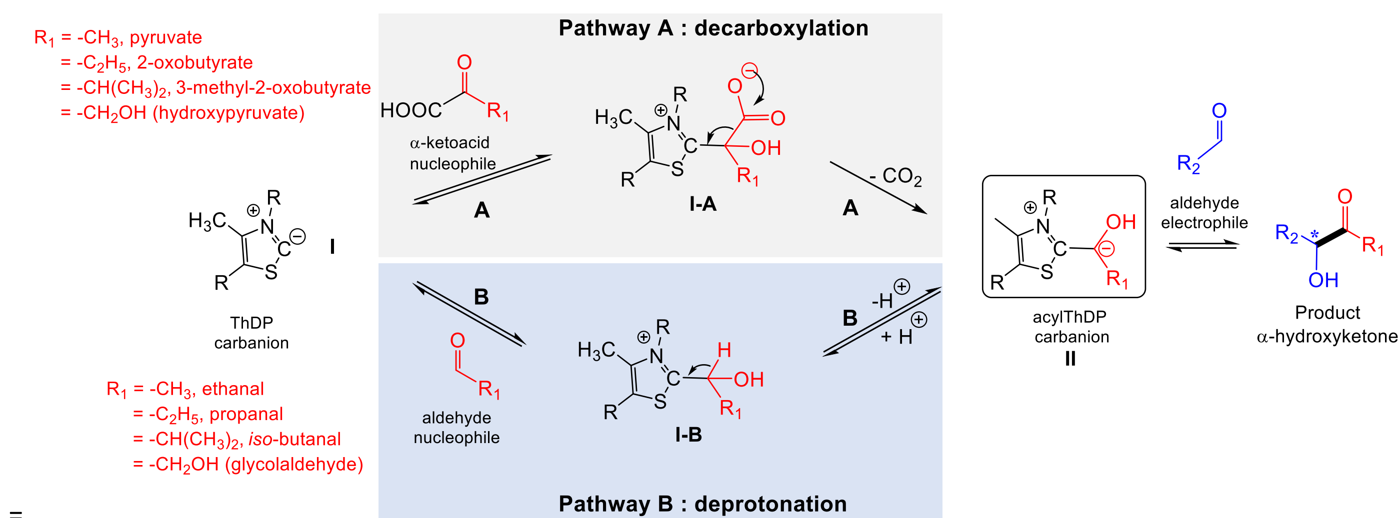

Figure 16. TK-catalyzed reaction according to pathway A from α-ketoacid as nucleophile through decarboxylation or pathway B from aldehyde as nucleophile through deprotonation.

4.2 Alkyl-substituted α-hydroxyketones

Alkyl substituted α-hydroxyketones display numerous properties, such as flavors [67] or nonionic surfactants.[68] Several purely chemical synthetic approaches have been proposed to afford unsymmetrical acyloins, together with some organocatalytic strategies (aldolization,[68] thiazolium-based carboligations,[69]). However, many of these chemical approaches are not straightforward, lack selectivity, or are economically unattractive because of the large number of chemical steps. The use of HPA (Figure 17A) and pyruvate analogs (Figure 17B) as nucleophiles with non-hydroxylated aldehydes having various carbon backbone lengths (C5-C10) was studied with TK_{eco}[70] and TK_{gst} variants.[71]

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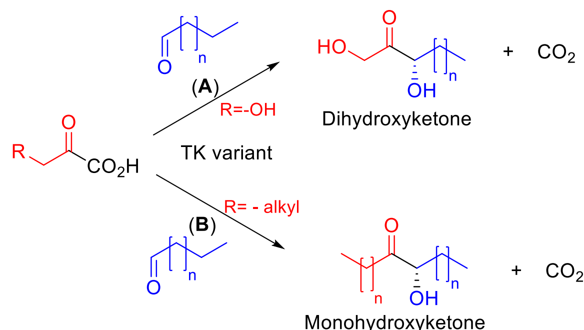

Figure 17. Pathways for obtaining alkyl substituted α-hydroxyketones s from non-hydroxylated aldehyde either with hydroxypyruvate (R = -OH, pathway A) or with pyruvate analogs (R= -alkyl, pathway B) coupled with aldoses.

For example, TK_{gst} single variant L382F was able to catalyze efficiently the transfer of the ketol group from HPA on all targeted aliphatic aldehydes (C3-C8) with 4- to 6-fold higher efficiency compared to wild type to give the corresponding 1,3-dihydroxyketones (C5-C10) with good yields (21-56%) and excellent enantioselectivity (*ee* > 87-99%). The combination of H102L/H474S previously designed for the improvement toward aliphatic pyruvate homologs, with F435I gave a new triple variant H102L/H474S/F435I able to transfer the acyl group from 2-oxobutyrate and less efficiently from 2-oxovalerate to aliphatic aldehydes (C3-C7), giving mono-hydroxylated ketones (C5-C10) never obtained before with this enzyme but the isolated yields (25-47%) were moderate and *ee* values (6-33%) were lower compared to that obtained with HPA as nucleophile.

4.3 Aryl substituted α-hydroxyketones

A breakthrough is required for the conversion of generic aryl-containing compounds as TK substrates to broaden the scope of this enzyme class for novel applications. The TK_{gst} L382X/D470X library was constructed to furnish suitable residue combinations that could accommodate even a bulky phenyl moiety.[72] The natural acceptor substrates having a terminal phosphate group, the residues forming the phosphate-binding site might reasonably be assumed to offer sufficient flexibility to also adapt a phenyl structure having a diameter similar to that of a phosphate ester moiety (Figure 13) although its polarity is quite different.

After screening, the most effective (active and selective) TK_{gst} variant was L382F/D470S showing rate accelerations up to 28-fold for the conversion of various non-functionalized phenyl-alkanals of the generic structure $Ph(CH_2)_n$ CHO (n = 0–2) **a-b**, and up to 12-fold for the conversion of related arylated substrates **c-e**, allowing with HPA as donor the synthesis of the corresponding 1,3-dihydroxyketones in good yields (50–73%) and virtually complete (3*S*)-stereo-selectivity (>99% *ee*) (Figure 18). Interestingly, reactions of both the aryl ether components (**c**, **d**) showed almost identical activity but reactions were consistently somewhat less accelerated than those of pure arylakanals (**a**, **b**), despite the fact that the oxygenated compounds should profit from a higher electrophilic reactivity. Possibly, the oxygen atom is experiencing electrostatic repulsive interactions not found for the aliphatic chains, or the extent of hydrate formation.

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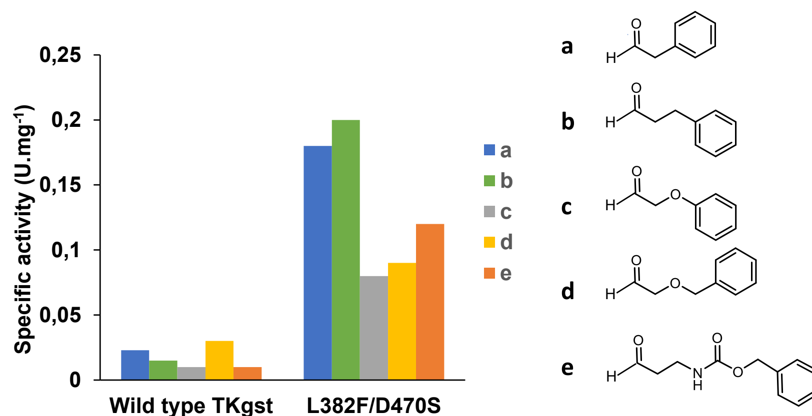

Figure 18. Rate acceleration obtained with TK_{gst} variant L382F/D470S using hydroxypyruvate as donor (HPA) and different arylated aldehydes as acceptors **a** (blue), **b** (green), **c** (grey), **d** (yellow), **e** (orange).[72]

Hydroxamic acids (HA) are a well-studied class of compounds of importance across many areas of life sciences. Their O=C-N-OH functionality can act as a bidentate ligand that forms strong chelate complexes with different metal ions (Figure 19). This chelating capacity confers high pharmacological versatility that includes anti-cancer, anti-inflammatory, anti-bacterial, anti-fungal, anti-malarial, anti-tubercular, and anti-oxidant activities.[73]

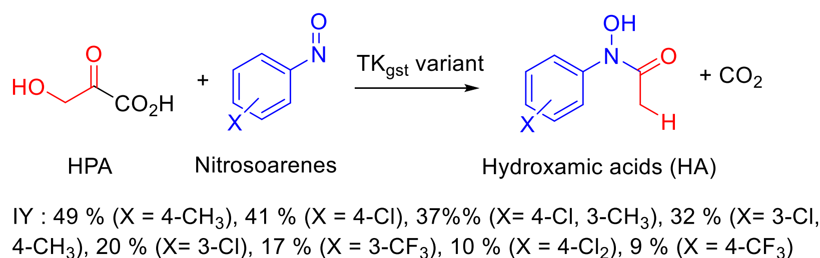

Figure 19. TK_{gst} variant L382N/D470S catalyzed hydroxamic acids (HA) synthesis from hydroxypyruvate (HPA) and nitrosarenes. Reaction conditions: 50 °C, pH 7.5, co-solvent (20% of acetone or 8% of EtOH or DMSO).

HAs are usually synthesized by chemical acylation of hydroxylamine with an activated carboxylic acid derivative,[74] or by direct amidation of aldehydes with nitroso compounds via *N*-heterocyclic carbene catalysis.[75] For obtaining such compounds, the enzymatic nucleophilic ketol transfer from HPA to nitrosoarene electrophiles was catalyzed by TK_{gst} variant L382N/D470S (selected earlier for HPA/benzaldehyde).[76] This reaction is analogous to the acyloin carboligation but rather is to be classified as an *N*-acyl condensation. The TK_{gst} variant L382N/D470S showed about 7-fold higher initial rates with nitrosoarenes as compared to the wild-type enzyme. Among the tested set of various *para*-, *meta*-, and *ortho*-mono- and disubstituted nitrosoarenes, electron-withdrawing substituted groups accelerated the reactivity, whereas the electron-donating *p*-dimethylamino compound was not converted. By this method, using HPA as nucleophile, fifteen different *N*-aryl HA have been prepared and obtained with 9 to 49% yields. From a co-solvent screen, acetone (20 % in the aqueous reaction medium) proved particularly beneficial in view of a significantly simplified work-up and highest overall yields. These new compounds showed chelating capacity with various metal ions, offering a broad scope of pharmacological activities.[76]

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5. Transketolase in Cascade Reaction

Cascade process avoids the purification of sometimes unstable and/or toxic intermediates and thus leads to a reduction in waste and an increase in overall yield.[77] The different enzymatic reactions can be carried out independently or within the same "one-pot" reactor, either sequentially (enzymes are introduced successively) or simultaneously (enzymes are introduced together). Finally, this type of process represents an effective strategy to shift the equilibrium of a reversible enzymatic reaction. In this context, the irreversibility of the reaction catalyzed by TK makes it possible to shift the equilibrium of the enzymatic reactions with which it is coupled. In the following section, we will describe some examples reported with different TKs (wild type or variants) coupled to enzymes allowing the *in-situ* generation of the α-ketoacid donor, and/or the acceptor substrates or that lead to the structural modification of the a-hydroxyketone produced by TKs.

5.1 Cascade for *in situ* generation of transketolase acceptor substrate

The generation of optically pure L-glyceraldehyde-3-phosphate (L-G3P) was investigated by opening of racemic epoxide with an epoxide hydrolase from *Aspergillus niger* (Figure 20). Subsequently, the optically pure aldehyde L-G3P was used as an acceptor substrate for TK_{sce} in the presence of L-erythrulose as donor, to synthesize 4-deoxy-D-fructose-6-phosphate, a potential inhibitor of enzymes involved in the metabolism of monosaccharides such as aldolases.

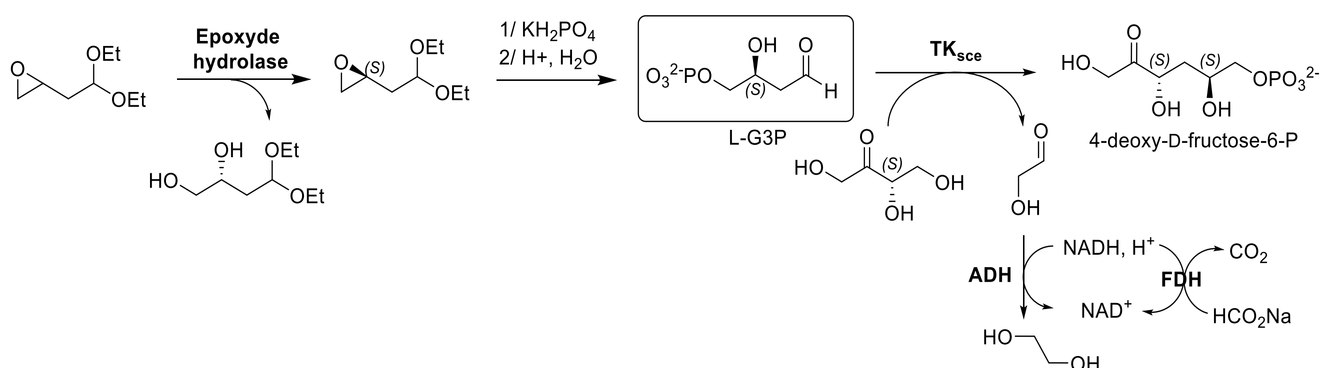

Figure 20. In situ generation of L-G3P catalyzed by an epoxide hydrolase coupled with TK_{sce} and alcohol dehydrogenase (ADH) and formate dehydrogenase (FDH) for the synthesis of 4-deoxy-D-fructose-6-phosphate.

A simultaneous “one-pot” process, in which D-glyceraldehyde-3-phosphate (D-G3P) synthesis was performed from D-fructose-1,6-bisphosphate (D-F-1,6P) with D-fructose-1,6-bisphosphate aldolase (FruA) and triose-phosphate isomerase (TPI). D-G3P was then used as an acceptor substrate for TK_{eco}, in the presence of HPA as a donor substrate, allowing the synthesis of D-xylulose-5-phosphate (D-X5P) with a yield of 82% on a gram scale order. This phosphorylated pentose is an intermediate of interest for the study of metabolic diseases but also an essential substrate for the study of numerous enzymes (Figure 21).[79]

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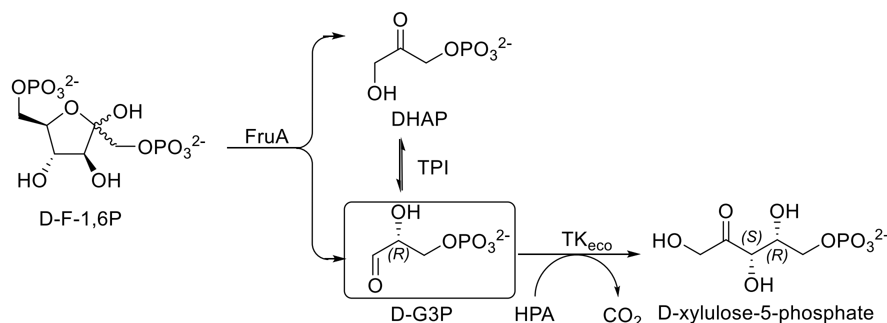

Figure 21. "One-pot" synthesis of D-X5P based on the *in situ* generation of D-glyceraldehyde-3-phosphate (DG3P) from D-fructose-1,6-bisphosphate with D-fructose-1,6-bisphosphate aldolase (FruA) and triose phosphate isomerase (TPI) coupled with transketolase (TK_{eco}).

5.2 Cascade for *in situ* generation of transketolase donor substrate

The use of HPA or its lithium salt as nucleophile, is commonly used for biocatalytic applications, but this compound is commercial and very expensive. The chemical synthesis makes it possible to obtain its lithium salt with modest yields, by hydrolysis of bromopyruvate in the presence of lithium hydroxide, which is a toxic compounds.[80] In addition, the instability of HPA at 25 °C in a buffered medium[14] is even more marked at higher temperatures.[28] Thus, several groups have considered generating this compound *in-situ* starting from the corresponding amino acid, serine, using transaminases (TAs) or amino acid oxidases (AAOs) coupled in one pot with TK, avoiding HPA degradation since it is consumed by TK as soon as it is generated by TAs or DAAOs.

5.2.1 *In situ* generation of HPA with Transaminase

As thermostable TKs have been used to catalyze the synthesis of α-hydroxyketones, several studies have first focused on thermostable TAs or DAAO to perform "one pot" synthesis at high temperatures. Thermostable L-a-TAs from *Thermosinus carboxydivorans* (TA_{tca}),[81] was first discovered and used to generate HPA from L-serine in one pot reaction at 60°C with TK_{gst} (Figure 22).

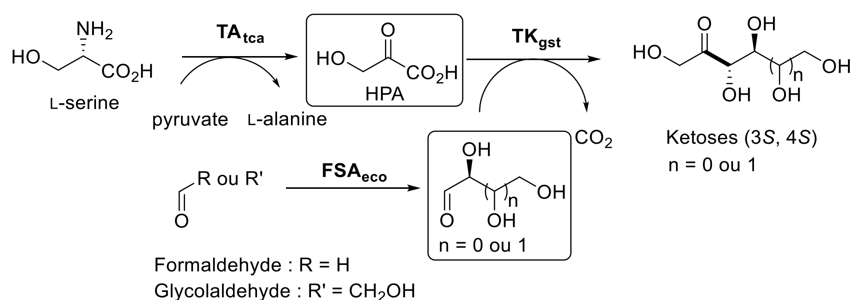

Figure 22. *In situ* generation of HPA from L-serine catalyzed by thermostable Transaminase (TA_{tca}) coupled to transaminase (TK_{gst}) and sequential *in situ* generation of aldehydes as TK acceptors by fructose-6-phosphate-aldolase (FSA_{eco}).

Different (3*S*)-hydroxyketones were synthesized without accumulation of HPA due to a suitable ratio of TA_{tca}/TK_{gst}. In addition, the TK-acceptor substrates such as D-threose and L-erythrose can be obtained from formaldehyde and glycolaldehyde using fructose-6-phosphate-aldolase from *E. coli* (FSA_{eco}) in a sequential step (Figure 22).[82]

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An advance in the HPA generation with TA was obtained with a process starting from a racemic mixture of serine involving both *Thermobifida fusca* (TA_{tfu}) and *Geobacillus stearothermophilus* (TA_{gst}), which showed opposite stereoselectivities retaining approximately 70% of their activity after 24 hours of incubation at 60°C (Figure 23).[83]

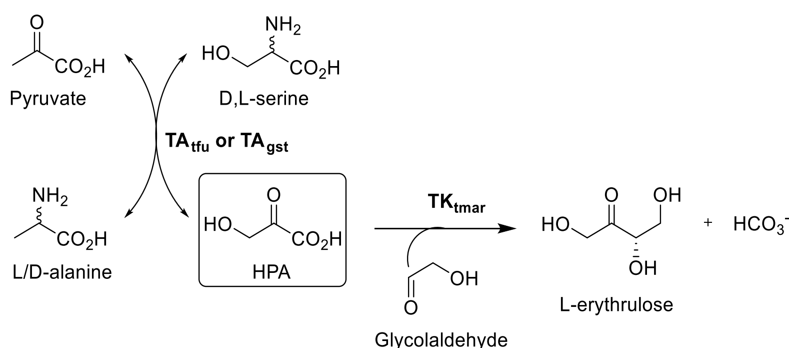

Figure 23. *In situ* generation of HPA from D,L-serine catalyzed by two thermostable TAs coupled to TK_{tmar}, in the presence of glycolaldehyde (GOA), for the synthesis of L-erythrulose.[1]

The main disadvantage of TA strategies is the use of an a-ketoacid as co-substrate, leading to the release of the corresponding amino acid, which is a by-product that is not in favorable to atom economy. To circumvent this drawback, an original strategy has been developed for the synthesis of (2*S*, 3*R*)-2-amino-1,3,4-butanetriol (ABT), an amino alcohol used as a precursor to numerous molecules with therapeutic activities. To obtain this compound, an amine-serine pyruvate w-TA from *Rhodobacter sphaeroides* (amine-S:P-TA) was coupled to a TK-catalyzed reaction.[84] The major interest of TA is its ability to generate HPA from L-serine together with the amination of the TK product L-erythrulose into ABT. However, amine-S:P-TA also uses glycolaldehyde, the TK acceptor substrate and generates ethanolamine as a by-product. (Figure 24).

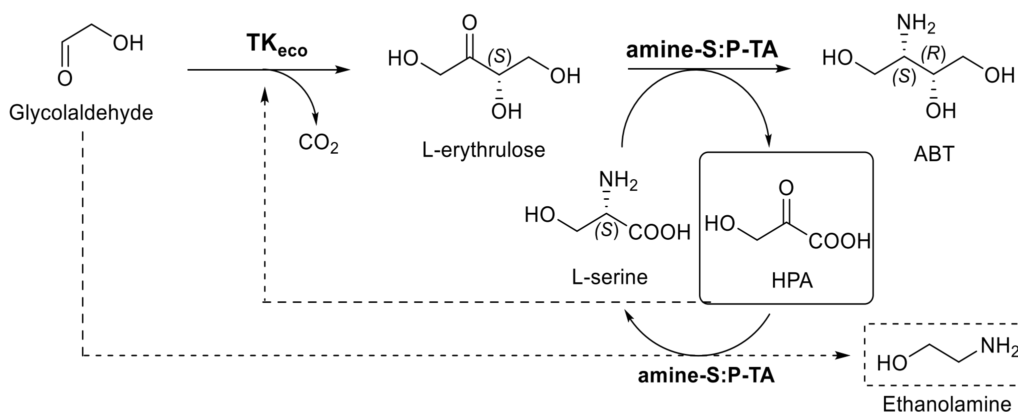

Figure 24. *In situ* generation of HPA from L-serine catalyzed by transaminase (amine-S:P-TA) coupled with transketolase (TK_{eco}) for (2*S*, 3*R*)-2-amino-1,3,4-butanetriol (ABT) synthesis.

Whatever the TA-catalyzed strategy for HPA *in situ* generation, a by-product is released not in favor of atom economy.

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5.2.2 *In situ* generation of α-ketoacid with amino oxidase and threonine dehydratase. Another enzymatic strategy has been considered from D-serine, allowing the oxidation of the amine function by a D-amino acid oxidase (DAAO) in the presence of O_2 and catalase to dismutate the released hydrogen peroxide. DAAO is required because L-AAO does not accept L-serine. DAAO reaction is irreversible and avoids the use of a co-substrate compared to TA.

The first application of rapid *in situ* generation of HPA from D-serine was reported with a microbial DAAO from *Rhodotorula gracilis* ($DAAO_{Rg}$).[85] The $DAAO_{Rg}$ reaction was coupled with TK_{gst} in a one-pot, two-step sequential cascade ketose because $DAAO_{rg}$ is not thermostable (Figure 25). Different aldehydes (hydrophobic or polyhydroxylated with increased carbon chain length) were used as TK_{gst} acceptor substrates and were introduced in the reaction mixture at the same concentration as D-serine giving a complete conversion of all substrates in 8 hours. In addition, D-serine was produced in a sequential step from achiral glycine and formaldehyde by D-threonine aldolase (DTA) with high stereoselectivity (ee > 99 %) within high yield recovery (>70%) in collaboration with Proteus by Seqens (Figure 25).[86]

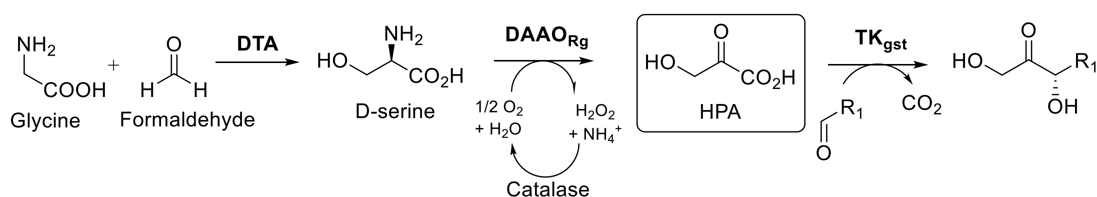

Figure 25. *in situ* generation of HPA from achiral glycine and formaldehyde catalyzed by D-threonine aldolase (DTA), and D-amino acid oxydase ($DAAO_{Rg}$) coupled with Transketolase variants (TK_{gst}).

The DAAO strategy was extended to the generation of other α-ketoacids such as pyruvate and 2-oxobutyrate from the corresponding D-amino acids D-alanine and D-homoalanine, respectively, using a thermostable AAO4536 provided by Prozomix Limited.[64] L-Threonine and D-Isoleucine were also used as precursors of corresponding a-ketoacids with threonine dehydratase from *E. coli* and $DAA0_{Rg}$, respectively, coupled with TK_{gst} variant H102L/L118I/H474S and pentanal or (2*R*)-2-methyl butanal for obtaining a natural hazelnut aroma precursor (filbertone) holding promise for the industrial production (Figure 26).[87]

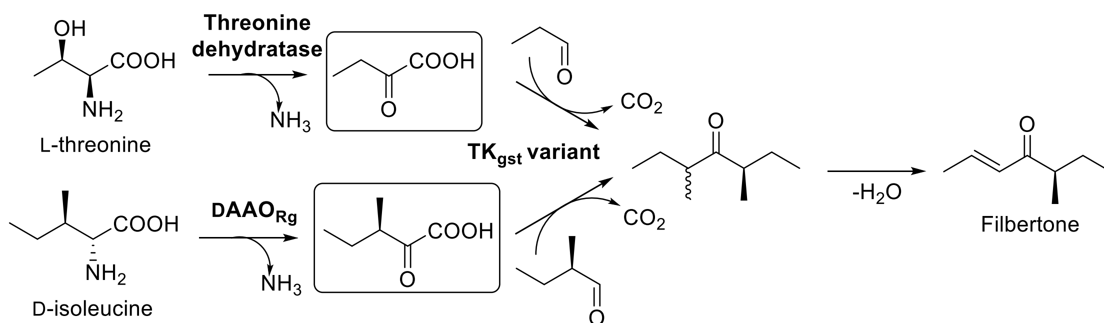

Figure 26. *In situ* generation of 2-oxobutyrate and (3R)-3-methyl-2-oxovalarate from L-Threonine and D-Iioleucine with threonine dehydratase from *E. coli* and $DAA0_{Rg}$ respectively coupled with TK_{gst} variant H102L/L118I/H474S for filbertone) holding.

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5.3 Cascade reaction for Transketolase product modification

The coupling of TK with isomerase has been investigated to get ketose stereoisomers by epimerization[88] or ketose/aldose isomerization. In this latter case, an example is the synthesis of 5-thio-D-xylopyranose (thioaldose) used as an inhibitor of β-D-xylosidases but also as a building block for the preparation of D-xylopyranosides, compounds with antithrombotic activity.[89] TK_{sce}-catalyzed reaction was applied for the synthesis of the corresponding ketose, 5-thio-D-xylulofuranose using TK_{sce} in the presence of (*R*,*S*)-3-thioglyceraldehyde and HPA, previously prepared chemically from acrolein diethyl acetal. The ketose was isomerized into the corresponding aldose, 5-thio-D-xylopyranose with D-glucose isomerase (GlcI, EC 5.3.1.5). 5-thio-D-xylulofuranose was obtained with a yield of 48 % and quantitatively isomerized into 5-thio-D-xylopyranose (Figure 27).

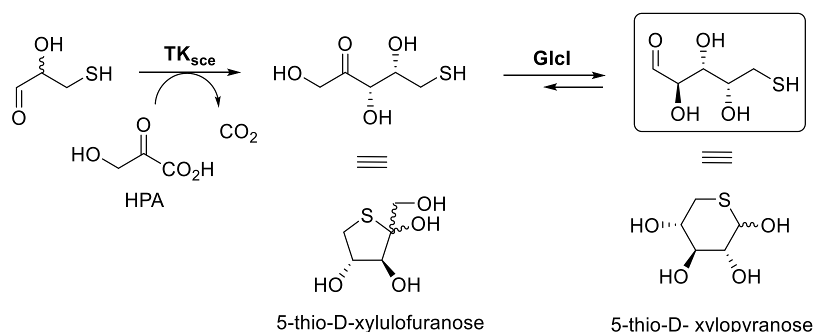

Figure 27. Two-step Transketolase (TK_{sce})-D-glucose isomerase catalyzed the synthesis of 5-thio-D-xylopyranose

TK was also coupled with TA for obtaining chiral amino-alcohols. Expeditious and stereoselective synthetic methodologies of such compounds from simple starting materials are of significant interest in pharmaceuticals.[90]

As described previously, L-erythrulose produced by TK from HPA and glycolaldehyde was converted into (2*S*, 3*R*)-2-amino-1,3,4-butanetriol (ABT) using an amine-S:P-TA but in this case (*S*)-α-methylbenzylamine (MBA) was used as co-substrate releasing acetophenone (AP) (Figure 28).[84] To obtain quickly full conversion a microreactor-based approach was developed with free-coupled TK and TA in batch and continuous-flow systems. Using the compartmentalization of the reactions, the microreactor cascade, avoids inhibitory effects of the TK cofactor ThDP on TA reaction, increases the activity per unit volume, and optimizes individual reaction conditions, giving a full conversion of MBA in two hours and a final yield of 100% ABT.[91]

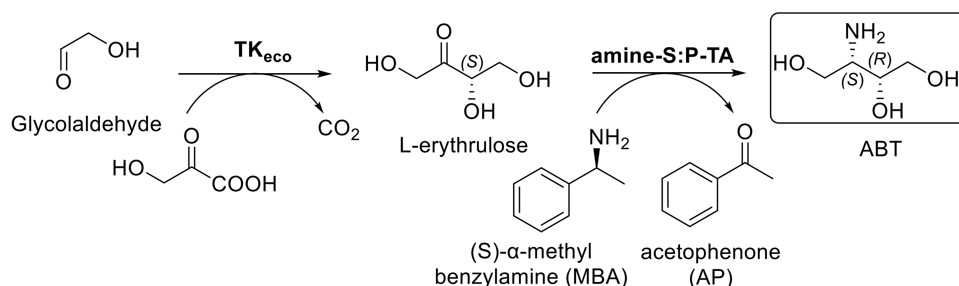

Figure 28. Two-step Transketolase (TK_{eco})- transaminase (amine-S:P-TA) catalyzed (2*S*, 3*R*)-2-amino-1,3,4-butanetriol (ABT) synthesis.

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The synthesis of an aminodiol, (1*R*,2*R*)-*p*-methylsulfonyl phenylserinol, intermediate of florfenicol derivative of thiamphenicol widely used in veterinary medicine for its high antibacterial activity and safety was investigated from industrial raw material 4-(methylsulfonyl) benzaldehyde by coupling TK_{eco} variant H26Y/F434Y/L466F and ω-TA variant F225H/I157H/F122C/V69A (figure 29). Multiple rounds of structure-guided iterative site-saturation mutagenesis were conducted to reverse TK_{eco} enantioselectivity from (*S*) (93% *ee*) to (*R*) (95% *ee*), and also to invert ω-TA ATA117 (a homolog of TA from *Arthrobacter* sp.) enantiopreference from *E*(*S*) = 9 to *E*(*R*) = 12 and ketone/aldehyde TA substrate selectivity which is a rare event for ω-TA. Pyruvate released by the TA reaction was reduced into lactic acid by LDH/NADH coupled with GDH/glucose ensuring NADH regeneration. Finally, (1*R*,2*R*)-*p*-methylsulfonyl phenylserinol was biosynthesized with good yield (76%) and high stereoselectivity (96% de and >99% *ee*) in sequential one-pot cascade reaction which provided the most direct chiral intermediate for the preparation of florfenicol.[92]

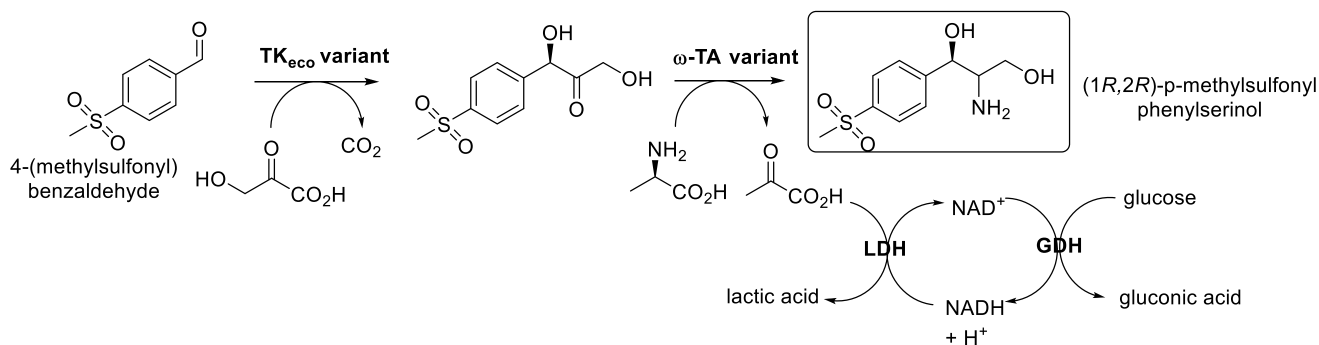

Figure 29. Two-step transketolase (TK)-transaminase (w-TA) catalyzed synthesis of (1*R*,2*R*)-*p*-methylsulfonyl phenylserinol using lactate dehydrogenase (LDH) for removal of pyruvate coupled with glucose dehydrogenase (GDH) for NADH regeneration.

Conclusions

Following extensive research on TK structure and its biochemical properties, the natural substrates, phosphorylated ketoses and aldoses, have been replaced by nucleophiles and electrophiles with increasingly different structures, showing the potential of this enzyme in terms of evolvability and significant untapped potential in biocatalysis for chiral *α*-hydroxyketone synthesis by C-C bond. New sources of TKs from metagenomic libraries or resulting from optimization by mutagenesis could contribute to the development of even more efficient and atom-efficient syntheses, in particular by integrating TK into multi-enzyme cascades allowing the generation of donor and acceptor substrates through judiciously chosen auxiliary enzymes. This review thus opens up promising avenues for future developments, directing research towards more efficient and environmentally friendly chemical processes for chiral *α*-hydroxyketone synthesis and further industrial applications for pharmaceuticals and fine chemicals.

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Laurence Hecquet is professor of Organic Chemistry at the University Clermont Auvergne. After a PhD through a Sanofi fellowship, she began her career in 1992 as Associate Professor and spent in 1999 a research stay at the Institute Karolinska of Stockholm (Sweden). In 2000, after obtaining her "Habilitation à Diriger des Recherches" (HDR), she was appointed full Professor at the University Clermont Auvergne and she develops her research works at the Institute of Chemistry of Clermont-Ferrand (ICCF, UMR 6296 CNRS–UCA) of which she is deputy director since 2017. Her research focuses on biocatalysis and enzyme engineering, particularly the enzymatic synthesis of chiral molecules by carbon-carbon formation. She has published over 100 papers and chapter books and is actively involved in scientific leadership, national evaluation committees, and international research collaborations and programs. She is president of the French biocatalysis network CBSO (Club Biocatalysis in Organic synthesis) and president of the chemistry and biotechnology group of the French Chemical Society. She was named a distinguished member of the French Chemical Society in 2015 and received the UCA Foundation Research Prize in 2018. In 2023 she was conference chair of the 16th International Symposium Biotrans organized in La Rochelle (France).

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