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## One-pot, two-step cascade synthesis of naturally rare L-erythro (3S,4S) ketoses by coupling thermostable transaminase and transketolase

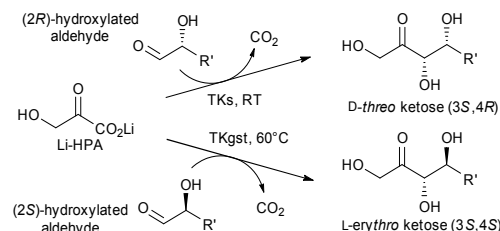
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An efficient simultaneous cascade of two enzymatic steps catalyzed by thermostable transaminase and transketolase was performed at elevated temperature allowing the synthesis of naturally rare L-erythro (3S,4S) ketoses. L-ribulose, 5-deoxy-L-ribulose, D-tagatose and L-psicose, which are highly valuable chiral building blocks and displaying prominent biological properties, were obtained on a preparative scale with excellent stereoselectivities and good yields. Thermostable transketolase from *Geobacillus stearothermophilus* catalyzed at high temperature the stereospecific synthesis of L-erythro (3S,4S)-configured ketoses from (2S)-hydroxylated aldehydes and β-hydroxyppyruvate in which the latter is generated in an unprecedented manner *in situ* from natural L-serine and pyruvate using a novel thermostable L-α-transaminase from the thermophilic bacterium *Thermosinus carboxydvorans*. Overall, this cascade synthesis prevents the thermal decomposition of the labile β-hydroxyppyruvate and offers an efficient and environmentally friendly procedure.

### Introduction

In polyol and carbohydrate chemistry, the inherent problems of regioselectivity, chemoselectivity and stereoselectivity is a challenge for traditional methods of organic synthesis requiring sophisticated protective group strategies. By contrast, regio- and stereoselectivity of enzymes avoid the use of protective groups and enable reaction pathways with fewer steps in mild reaction conditions. To date, enzymes catalyzing the stereospecific C-C bond formation have found some useful applications in the synthesis of complex carbohydrates, sugar analogues and related biologically important natural compounds, widely used as pharmaceutical or nutraceutical products, food additives and cosmetics.<sup>1,2</sup> The enzyme transketolase (TK), a thiamine diphosphate (ThDP)-dependent enzyme, yields D-threo ketoses having a (3S,4R)-configuration by C-C bond formation.<sup>3</sup> TK is highly specific for ketol donor substrates, stereospecific for forming the new asymmetric center in (S) configuration and enantioselective towards 2-hydroxyaldehyde acceptor substrates with an (R) configuration. Interestingly, commercial lithium β-hydroxyppyruvate (Li-HPA) can be used as a

donor substrate, rendering the ketol transfer irreversible owing to its decarboxylation catalyzed by TK.<sup>4</sup> Hence, Li-HPA is extensively used in biocatalysis and makes TK an ideal tool for synthetic purposes.<sup>5</sup> Mesophilic TK enzymes from yeast (TK<sub>sce</sub>) and from *E. coli* (TK<sub>eco</sub>) have been largely used for organic synthesis<sup>6</sup> (Scheme 1). Moreover, efficient TK<sub>eco</sub> variants have been obtained by active-site directed mutagenesis to improve TK properties towards hydroxylated and non-hydroxylated acceptors.<sup>7</sup>



**Scheme 1** Irreversible TK reaction with lithium β-hydroxyppyruvate (Li-HPA) and (2R) α-hydroxyaldehydes at room temperature (RT) using TK<sub>sce</sub>, TK<sub>eco</sub>, and TK<sub>gst</sub> or with (2S) α-hydroxyaldehydes at 60 °C using TK<sub>gst</sub>.

Thermostable enzymes offer many advantages, such as improved solubility of organic substrates at elevated temperatures, increased tolerance toward unconventional media and greater resistance to protein destabilizing factors introduced by mutagenesis.<sup>8</sup> Thermostable enzymes, which have been found mainly in thermophilic organisms, have led to a number of commercial

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†Electronic Supplementary Information (ESI) available: Electrophoresis, TA<sub>cca</sub> gene sequence, kinetic parameters of TK<sub>gst</sub> cofactors, cascade reaction monitoring, products NMR spectra. See DOI: 10.1039/x0xx00000x

applications.<sup>9</sup> Advances in this area have been made possible by the availability of new genome sequences from thermophilic microbial sources, providing DNA from hardly cultivatable organisms. We recently cloned and overexpressed the first thermostable TK (A0A0I9QGZ2) from the thermophilic bacterium *Geobacillus stearothermophilus* (TK<sub>gst</sub>). TK<sub>gst</sub> has an optimum temperature range of 60–70 °C and retained full activity for one week at 50 °C and for 3 days at 65 °C.<sup>10</sup> At high temperature, the stereospecificity of TK<sub>gst</sub> proved identical to that of other TK sources, generating a (3S) configuration. TK<sub>gst</sub>, like other TK sources, preferentially accepts (2R)-hydroxylated aldehydes with a short carbon chain. In addition to these properties, TK<sub>gst</sub> offers new synthetic opportunities. Its robustness in ionic media, used as co-solvent in aqueous solution, has been used to increase the conversion rate of D-ribose into D-altro-heptulose.<sup>11</sup> Remarkably, at high temperature, TK<sub>gst</sub> was also able to accept (2S)-hydroxylated aldehydes yielding (3S,4S) ketoses stereospecifically. None of the mesophilic TKs have been reported to catalyze the conversion of (2S)-configured hydroxylaldehydes (Scheme 1).<sup>12,13</sup> This side activity of TK<sub>gst</sub> with L-glyceraldehyde was 32 times weaker at 25 °C than that obtained with D-glyceraldehyde for instance. To improve TK<sub>gst</sub> properties, TK<sub>gst</sub> variants were designed that displayed enhanced activities towards various aldehydes.<sup>13</sup>

For synthetic purposes, the main problem of TK<sub>gst</sub>-catalyzed reactions at high temperature is the limited stability of the artificial donor substrate Li-HPA. Instead of the costly commercial material, this compound can also be synthesized by hydrolysis of bromopyruvic acid with lithium hydroxide. However, Li-HPA is recovered in moderate yield through a tedious purification step.<sup>14</sup> Generation of HPA *in situ* for coupling to the TK-catalyzed reaction has already been described at room temperature,<sup>15–16</sup> in particular with a microbial transaminase (TA) from natural and affordable L-serine in a sequential cascade.<sup>16</sup> TAs are pyridoxal phosphate (PLP)-dependent enzymes which catalyze the reversible transfer of an  $\alpha$ -amino group between a donor substrate and a carbonyl acceptor. The TA family is highly diverse, including many ubiquitous L- $\alpha$ -transaminases (L- $\alpha$ -TAs) that play a key role in most proteinogenic L- $\alpha$ -amino acid metabolisms. The potential of TAs for chiral amines synthesis has been largely demonstrated, while more promiscuous enzymes are still in high demand.<sup>17</sup>

Here we report on the identification and characterization of a novel thermostable serine-glyoxylic acid L- $\alpha$ -TA (A1HUC4) from the thermophilic bacterium *Thermosinus carboxydivorans* DSM 14886 (TA<sub>tca</sub>) and its use in an efficient two-step simultaneous cascade catalyzed by a TA<sub>tca</sub> / TK<sub>gst</sub> tandem at elevated temperature for the preparation of highly valuable and naturally rare L-erythro (3S,4S)-ketoses. Such a configuration is currently inaccessible with mesophilic TKs and to our knowledge, no such thermostable L- $\alpha$ -TA has been used previously for the biocatalyzed synthesis of HPA at high temperature and on preparative scale. Within the TA<sub>tca</sub> / TK<sub>gst</sub> cascade, the overall equilibrium of the TA<sub>tca</sub>-catalyzed reaction from L-serine and pyruvic acid is shifted towards HPA and L-alanine by coupling to the irreversible TK<sub>gst</sub>-catalyzed reaction. The procedure was applied for the synthesis of L-erythro (3S,4S)-pentuloses, L-ribulose and its analogue 5-deoxy-L-ribulose. L-ribulose is an important chiral lead molecule<sup>19</sup> particularly used for the preparation of L-ribose, a high-value rare sugar needed for the synthesis of antiviral L-nucleosides.<sup>20</sup> We also prepared two L-erythro (3S,4S)-hexuloses such as D-tagatose, important for numerous applications in nutrition and pharmaceuticals<sup>21</sup> and L-psicose, which is a chiral building block used for the synthesis of other rare L-sugars<sup>22</sup> and recently reported as an antiviral

compound.<sup>23</sup> The irreversible C-C bond formation catalyzed by TK and the stereoselectivity of this enzyme are major advantages to improve environmental impact allowing the total conversion of the donor and acceptor substrates and the formation of only one stereoisomer of each product. In comparison, other enzymatic methods can lead to incomplete conversion of the substrate other the formation of additional stereoisomers of the product due to the reversibility of the synthetic reaction and/or low stereoselectivity of enzymes, respectively. For example, the L-arabinose isomerase catalyzed reversible enzymatic isomerization of L-arabinose or D-galactose does not favor the products L-ribulose or D-tagatose respectively,<sup>24</sup> ribitol was only partially dehydrogenated into L-ribulose by *Gluconobacter oxydans*,<sup>25</sup> and also the non stereoselective aldolase-catalyzed addition to L-glyceraldehyde led to a mixture of L-psicose and L-sorbose.<sup>26</sup> Any undesired stereoisomer of the product as well as the remaining substrate, with properties similar to the product, must be removed by tedious purification steps including significant amounts of solvents or salts to obtain optically pure final product.

## Results and discussion

### Lithium $\beta$ -hydroxypyruvate stability and kinetic parameters measured with TK<sub>gst</sub>

The key donor substrate used for making the TK<sub>gst</sub> reaction irreversible is Li-HPA.<sup>14</sup> We first determined kinetic parameters relative to this donor and compared the values with those obtained with TK<sub>eco</sub> and TK<sub>sce</sub>, both commonly used in biocatalysis (Table 1). Li-HPA showed a better affinity for TK<sub>gst</sub> than for the other TKs (7 and 13 times lower  $K_M$  and 2 to 68 times higher  $v_{max}$  compared to TK<sub>eco</sub> and TK<sub>sce</sub> respectively). Therefore, Li-HPA can be considered as a better substrate for TK<sub>gst</sub> than for the other TK sources.

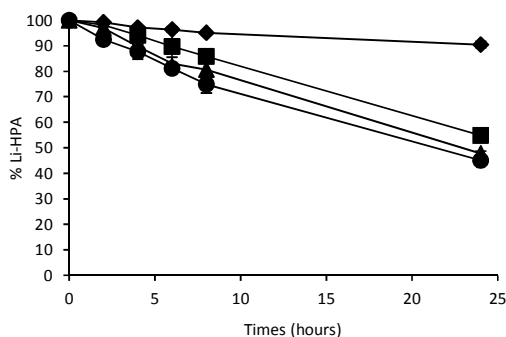
**Table 1** Kinetic parameters relative to Li-HPA for different TK sources at 25 °C.

TK	$K_M$ mM	$v_{max}$ U.mg <sup>-1</sup>	$v_{max}/K_M$ U.mg <sup>-1</sup> .mM
TK <sub>gst</sub>	2.5 ± 0.2	135 ± 7	54 ± 7
TK <sub>eco</sub>	18 <sup>27</sup>	60 <sup>27</sup>	3
TK <sub>sce</sub>	33 <sup>28</sup>	2 <sup>28</sup>	0.06

Reaction conditions: ThDP (2.4 mM), MgCl<sub>2</sub> (9 mM), glycolaldehyde (50 mM), Li-HPA (0.5–50 mM), TK (0.0125–0.25 U.mL<sup>-1</sup>), glycylglycine buffer (50 mM, pH 7.5). Li-HPA consumption was determined by spectrophotometry at 340 nm from aliquots added to an assay solution containing L-lactic dehydrogenase and NADH.

Li-HPA stability had been studied previously in water and buffered solutions and the results clearly showed increased degradation at high temperature and low decomposition in pure water.<sup>10,28</sup> Other studies reported that basic conditions<sup>29</sup> enhanced HPA degradation while acidic conditions increased HPA stability at high temperature.<sup>14</sup> Considering these data and the optimum pH of TK<sub>gst</sub>, Li-HPA stability was studied at different temperatures in water over 24 h and in the presence of both TK cofactors at pH 7. In this study, pH was continuously adjusted at 7 by pH-stat control (Fig 1). We observed that high concentrations of TK cofactors previously used in biocatalysis (2.4 mM ThDP, 9 mM MgCl<sub>2</sub>) promote Li-HPA degradation. For this reason, we decreased these concentrations to 0.1 mM ThDP and 1 mM MgCl<sub>2</sub> in accordance with TK<sub>gst</sub> kinetics

parameters (ESI). After 24 h at 20°C to 65°C, 90 % to 45 % of initial Li-HPA were recovered respectively in the reaction mixture. Even under such optimized conditions, Li-HPA decomposition over time remains problematic above 20 °C, particularly for biocatalytic applications of TK<sub>gst</sub> with poor acceptor substrates that require extended reaction periods. HPA degradation was observed irrespective of the counter-ion used (Li<sup>+</sup>, Na<sup>+</sup> or K<sup>+</sup>). The analysis of the mixture by <sup>1</sup>H NMR after 24 h showed the formation of glycolic acid as major product and traces of erythrulose. Glycolic acid has never been reported in the literature as an ultimate degradation product of Li-HPA. It may be formed by decarboxylation of HPA through oxidative C-C bond cleavage. In the literature, the formation of glycolaldehyde had been described together with the formation of erythrulose resulting of chemical condensation of glycolaldehyde from residual Li-HPA.<sup>14,30</sup>

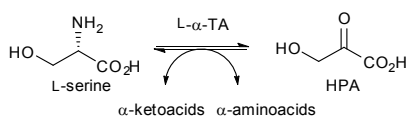


**Fig. 1** Li-HPA stability over time in H<sub>2</sub>O at pH 7 (controlled by pH stat) and at 25°C (◆), 50°C (■), 60°C (▲) and 65°C (●). Li-HPA (50 mM), ThDP (0.1 mM), MgCl<sub>2</sub> (1 mM).

To prevent HPA decomposition, by-products formation at high temperature and consequently to optimize the TK<sub>gst</sub> reaction, we opted for *in situ* generation of HPA, which should be continuously consumed by TK<sub>gst</sub>. For this purpose and also to develop an environmentally friendly HPA synthesis, we turned to an enzymatic transamination of L-serine.

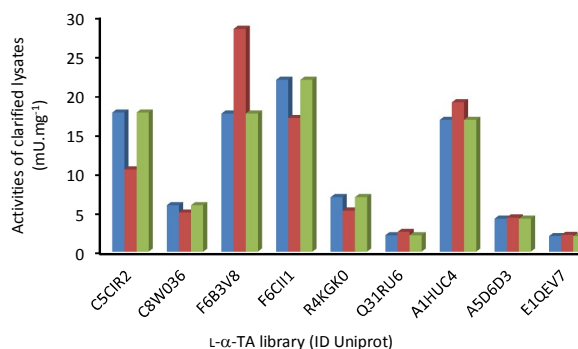
#### Identification and characterization of a novel L-α-transaminase

Our goal was to produce *in situ* the α-keto acid HPA from the naturally occurring α-amino acid L-serine at high temperature, a procedure reported only at room temperature with microbial mesophilic L-α-TAs.<sup>16</sup> To find such a potentially thermostable L-α-transaminase, we screened a 232 member library of α-TAs from Genoscope strain collection.<sup>18</sup> This library containing putative D/L-α-TAs was previously screened using L- or D-cysteine sulfinic acid (CSA) as an irreversible amino donor and with various keto acids, including Li-HPA as acceptors.<sup>18</sup> Among these enzymes, we selected 9 putative L-α-TAs showing the best activities with Li-HPA. These 9 enzymes were screened again using L-serine as donor substrate and either one of three common α-keto acids as acceptor substrate: glyoxylic, pyruvic and α-ketoglutaric acids (Scheme 2).



**Scheme 2** L-α-transaminase (L-α-TA) reaction with L-serine yielding hydroxyppyruvate (HPA).

The assay was based on the selective detection of 2-hydroxy ketone by reaction with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulphophenyl)-tetrazolium (WST-1) as a water-soluble tetrazolium salt. WST-1 was also used for the determination of activity of enzymes<sup>31</sup> including pyruvic acid decarboxylase, TK and TA. TA activity was measured following a discontinuous assay in which aliquots of TA-catalyzed reactions were regularly taken out and were assayed separately with WST-1 under basic conditions at 60°C. The reaction generates a blue formazan color which was followed by measuring its absorbance at  $\lambda = 600 \text{ nm}$  ( $\epsilon = 42000 \text{ M}^{-1}\text{cm}^{-1}$ ).<sup>32</sup> The results obtained with L-serine as amino donor showed that 4 TAs displayed significant activities above  $10 \text{ mU}\cdot\text{mg}^{-1}$  at 20°C whatever the ketoacid assayed (Fig. 2). Among these 4 TAs, only one TA, annotated as serine-glyoxylic acid TA (ID Uniprot A1HUC4) from the thermophilic bacterium *Thermosinus carboxydivorans* DSM 14886 (TA<sub>tca</sub>), showed an increased activity at 60°C and remained active at this temperature for hours. The gene encoding for TA<sub>tca</sub> (ESI) was cloned into an expression vector with a His<sub>6</sub>-tag at the N-terminus of the enzyme for purification purposes. Sequencing validation showed that the protein sequence of TA<sub>tca</sub> is identical to A1HUC4 of *Thermosinus carboxydivorans* Nor1, used as a template for primer design.



**Fig. 2** L-α-TA specific activities in clarified lysates towards L-serine as amino donor and α-ketoacid as acceptor such as ■ pyruvic acid, ■ glyoxylic acid and ■ α-ketoglutaric acid. Reaction conditions: L-serine (5 mM), α-ketoacids (5 mM), clarified lysates (5 μL), PLP (0.2 mM), HEPES buffer (100 mM), 20°C, pH 7.5.

After expression in *Escherichia coli* strain BL21(DE3)pLysE, TA<sub>tca</sub> was extracted by sonication. The expressed His<sub>6</sub>-tagged protein was applied to a Ni<sup>2+</sup> chelating affinity column for purification. By this procedure, 203 mg of purified TA<sub>tca</sub> was obtained from the cells grown in 1 L of culture (Table 2).

**Table 2** Purification of TA<sub>tca</sub> from recombinant *E. coli* cells

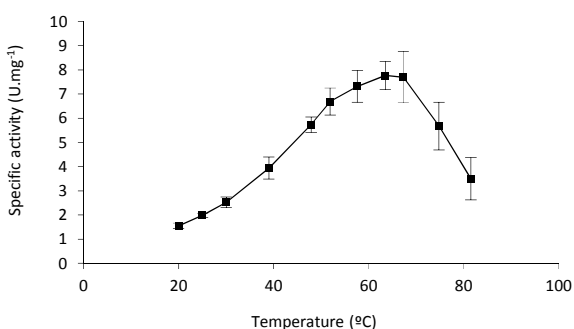
Purification step	Protein <sup>a</sup> mg	Total activity <sup>b</sup> U	Specific activity <sup>b</sup> U·mg <sup>-1</sup>	Yield %	Purification XX-fold
Clarified lysate	572	929	1.1	-	-
Purified TA <sub>tca</sub>	203	717	3.5	77.2	3.2

<sup>a</sup> Crude extract obtained after sonication of *E. coli* cells overexpressing TA<sub>tca</sub> harvested from 1 L of culture. <sup>b</sup> Activity measured in the presence of L-serine and α-keto glutaric acid as donor and acceptor substrates, respectively, in HEPES buffer, at 25°C. HPA released was measured spectrophotometrically at 600 nm via a discontinuous assay involving WST-1 (Experimental part).

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Throughout this study,  $TA_{tca}$  activity was measured with L-serine and  $\alpha$ -ketoglutaric acid, the most frequently used ketoacid acceptor of  $\alpha$ -TA. Purified  $TA_{tca}$  displays a specific activity of  $3.5 \text{ U}\cdot\text{mg}^{-1}$  at  $25^\circ\text{C}$ .  $TA_{tca}$  can be lyophilized or precipitated with ammonium sulfate and stored at  $4^\circ\text{C}$  without loss of activity for several months. The influence of temperature on  $TA_{tca}$  activity was studied in the range between  $20$ – $80^\circ\text{C}$  after 40 minutes of incubation (Fig. 3).  $TA_{tca}$  showed an optimum temperature of  $60^\circ\text{C}$ , giving an up to 5-fold improvement relative to the activity measured at  $20^\circ\text{C}$ . The optimal activity of  $TA_{tca}$  was determined around pH 8.2–8.5 when assayed at  $25^\circ\text{C}$ .  $TA_{tca}$  thermostability at  $60^\circ\text{C}$ , corresponding to the optimum temperature of this enzyme, was studied by measuring the residual enzymatic activity over 10 days with L-serine and  $\alpha$ -ketoglutaric acid.  $TA_{tca}$  maintained 100% of its activity for 4 days and 50% for 10 days. Other serine-pyruvic acid TAs from thermophilic bacteria recently reported in the literature were stable for only 10 minutes at  $70^\circ\text{C}$ .<sup>33</sup>



**Fig. 3** Thermal activity profile of  $TA_{tca}$ . Activity was measured after 30 minutes in the presence of L-serine (100 mM),  $\alpha$ -ketoglutaric acid (3 mM), PLP (0.2 mM), and HEPES (100 mM) at pH 7.5.

Kinetic parameters  $K_M$  and  $v_{max}$  of L-serine and pyruvic acid were determined at  $25^\circ\text{C}$  and were calculated from the Hanes-Woolf plot according to the least-squares method as summarized in table 3. Pyruvic acid was chosen as acceptor substrate because we observed an inhibition of  $TA_{tca}$  when using glyoxylic acid and  $\alpha$ -ketoglutaric acid above 5 mM and 3 mM, respectively. However, when using pyruvic acid, noticeable inhibition only occurred at concentrations higher than 50 mM. The results showed higher affinity towards the acceptor pyruvic acid when compared to the amino donor L-serine, as reported with other  $\alpha$ -TAs used in biocatalysis.<sup>16</sup>

**Table 3** Kinetic constants of  $TA_{tca}$

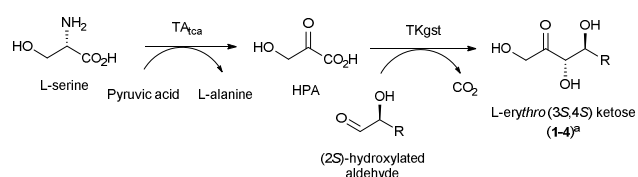
Substrates	$K_M$ app (mM)	$v_{max}$ app ( $\text{U}\cdot\text{mg}^{-1}$ )	$v_{max}/K_M$ ( $\text{U}\cdot\text{mg}^{-1}\cdot\text{mM}^{-1}$ )
Donor L-serine <sup>a</sup>	22.4 $\pm$ 0.3	3.3 $\pm$ 0.1	0.14 $\pm$ 0.01
Acceptor Pyruvic acid	12.8 $\pm$ 0.7	8.1 $\pm$ 0.5	0.63

<sup>a</sup>50 mM of pyruvic acid and L-serine concentrations varying from 1 mM to 250 mM were used. <sup>b</sup>250 mM of L-serine and pyruvic acid concentrations varying from 0.25 mM to 50 mM were used.

#### Preparative scale synthesis of L-erythro (3S,4S) ketoses

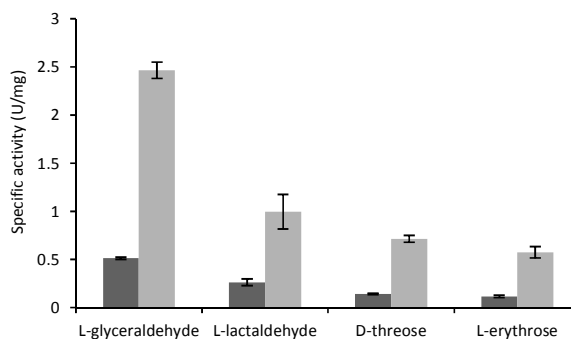
We have demonstrated before that  $TA_{tca}$  and  $TK_{gst}$  are thermostable enzymes exhibiting high efficiency at  $60^\circ\text{C}$ . Moreover, we previously

showed that at high temperature,  $TK_{gst}$  catalyzed the synthesis of L-erythro (3S,4S) ketoses from (2S)-hydroxylated aldehydes and synthetic Li-HPA. We reported the synthesis of L-ribulose and 5-deoxy-L-ribulose from (2S)-glyceraldehyde and (2S)-lactaldehyde that were not hitherto mentioned as possible substrates of mesophilic TKs.<sup>12</sup> The main problem of  $TK_{gst}$ -catalyzed reactions at high temperature was the instability of Li-HPA. To prevent its decomposition, we investigated the coupling of  $TA_{tca}$  and  $TK_{gst}$  in a single reactor at  $60^\circ\text{C}$ , for an integrated synthesis of different L-erythro (3S,4S) ketoses from appropriated (2S)-hydroxylated aldehydes, L-serine, and pyruvic acid (Scheme 3, Table 4).



**Scheme 3** Enzymatic cascade synthesis of L-erythro (3S,4S) ketoses catalyzed by  $TA_{tca}$  and  $TK_{gst}$  at  $60^\circ\text{C}$ . <sup>a</sup>R =  $-\text{CH}_2\text{OH}$  (L-ribulose **1**); R =  $-\text{CH}_3$  (5-deoxy-L-ribulose **2**); R =  $-\text{CHOH(R)}-\text{CH}_2\text{OH}$  (D-tagatose **3**); R =  $-\text{CHOH(S)}-\text{CH}_2\text{OH}$  (L-psicose **4**).

Firstly, we compared initial  $TK_{gst}$  velocities at  $25^\circ\text{C}$  and  $60^\circ\text{C}$ , using chemically synthesized Li-HPA as donor toward several (2S)-hydroxylated aldehydes having 3 to 6 carbon chain length. This included L-glyceraldehyde and L-lactaldehyde (3 carbon atoms), D-threose and L-erythro (4 carbon atoms), D-arabinose and D-lyxose (5 carbon atoms) and D-mannose (6 carbon atoms) (Fig. 4). We note that  $TK_{gst}$  activities decreased with the carbon chain length as reported earlier for (2R)-hydroxylated aldehydes.<sup>34</sup> The best  $TK_{gst}$  activities were obtained with L-glyceraldehyde and L-lactaldehyde while D-threose and L-erythro gave lower  $TK_{gst}$  activities. A temperature increase from  $25^\circ\text{C}$  to  $60^\circ\text{C}$  enhanced  $TK_{gst}$  activities 4.8-fold when using L-glyceraldehyde, 3.8-fold using L-lactaldehyde, and 5-fold with D-threose and L-erythro (Fig. 4). No detectable activities were observed with D-arabinose, D-lyxose, and D-mannose.



**Fig. 4** Effect of temperature  $25^\circ\text{C}$  ■ and  $60^\circ\text{C}$  ■ on  $TK_{gst}$  activity toward different (2S)-hydroxylated aldehydes. The activities were measured using aldehydes (100 mM), Li-HPA (50 mM), ThDP (0.1 mM),  $\text{MgCl}_2$  (1 mM), triethanolamine buffer (2mM), at pH 7.15.

Then, we performed at  $60^\circ\text{C}$  the one-pot, two-step simultaneous cascade catalyzed by  $TA_{tca}$  and  $TK_{gst}$  from L-serine and pyruvic acid as  $TA_{tca}$  substrates using the best four (2S)-hydroxylated aldehydes as  $TK_{gst}$  substrates, L-glyceraldehyde, L-lactaldehyde, D-threose and

**Table 4** Reaction conditions of the one pot bi-enzymatic cascade synthesis yields and d.e. of L-erythro-(3S,4S) ketoses.

Aldehydes	Products	TA <sub>tca</sub> /TK <sub>gst</sub> (mg)	Conversion rate of aldehyde (%) <sup>b</sup>	Reaction time (h)	Isolated product yield (%) <sup>a</sup>	d.e. (%)
L-glyceraldehyde CHO-CHOH(S)-CH <sub>2</sub> OH	L-ribulose <b>1</b>	4.8/6	100	24	56	>95
L-lactaldehyde CHO-CHOH(S)-CH <sub>3</sub>	5-deoxy-L-ribulose <b>2</b>	4.8/6	100	24	98	>95
D-threose CHO-CHOH(S)-CHOH(R)-CH <sub>2</sub> OH	D-tagatose <b>3</b>	4.8/10	>95	72	52	>95
L-erythrose CHO-CHOH(S)-CHOH(S)-CH <sub>2</sub> OH	L-psicose <b>4</b>	4.8/10	>95	96	51	>95

<sup>a</sup>Reactions conditions: L-serine (150mM), aldehydes (50mM), pyruvic acid (50mM for L-glyceraldehyde and L-lactaldehyde and 100 mM for D-threose and L-erythro conversion) were used. <sup>b</sup>Determined using quantitative <sup>1</sup>H NMR relative to 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate (TSPd4) as internal standard.

L-erythrose for the synthesis of the corresponding L-erythro (3S,4S) ketoses, L-ribulose **1**, 5-deoxy-L-ribulose **2**, D-tagatose **3** and L-psicose **4** (Scheme 3). To study the influence of different amounts of both enzymes and substrate concentrations, the reaction was monitored by *in situ* <sup>1</sup>H NMR, using 3-trimethylsilyl-2,2,3,3-tetradeuteriopropionate (TSP-d4) as an internal standard. It was hence possible to quantify the conversion rates of aldehydes and pyruvic acid along with the formation of ketose products. It should be noted that all the different substrates assayed at 60°C were found to be stable except for pyruvic acid, which slowly decomposed with a half-life of 4 days. The best conversion rate of L-glyceraldehyde was achieved using 3 equivalents of L-serine in combination with 1 equivalent of pyruvic acid. The amounts of TA<sub>tca</sub> and TK<sub>gst</sub> were adjusted to prevent the accumulation of HPA that also decomposed gradually at 60 °C. Under these conditions, the time course of the reaction showed the total conversion of pyruvic acid and L-glyceraldehyde after 24 h along with the concomitant quantitative formation of the ketose product (ESI). These conditions were also applied for the conversion of L-lactaldehyde and the conversion results were similar (ESI). For product **2**, being less hydrophilic than **1**, **3** and **4**, purification was facilitated hence this compound **2** was isolated with higher yield (98%) as compared to the other products (51-56%) (Table 4). In the case of D-threose and L-erythrose for which TK<sub>gst</sub> shows low activities (Fig. 4), 2 equivalents of pyruvic acid and a greater quantity of TK<sub>gst</sub> (4.8 mg TA<sub>tca</sub>/10 mg TK<sub>gst</sub>) were used (Table 4). To avoid the inhibition of TA<sub>tca</sub> by pyruvic acid above 50 mM, this compound was added in two portions, the second portion being introduced after 48 h (complete disappearance of the first portion). We observed the total conversion of D-threose and L-erythrose and simultaneously the quantitative formation of the ketose products **3** and **4**, respectively (ESI). Finally, L-ribulose **1**, 5-deoxy-L-ribulose **2**, D-tagatose **3** and L-psicose **4** were obtained with good to excellent isolated yields: 56%, 98%, 52% and 51%, respectively, after reasonable reaction times of 24 to 96 h. It is noteworthy, that the isolated yields of L-ribulose and 5-deoxy-L-ribulose obtained with this cascade process were 5 and 3 times higher (56 % and 98%) than those obtained with synthetic Li-HPA (11% and 33%) as reported earlier.<sup>12</sup> The four L-erythro (3S,4S) ketoses were obtained in optically pure forms with high diastereoselectivity (d.e. >95 %, by <sup>13</sup>C NMR). Furthermore, the absolute configurations of **1-4** were confirmed by comparing the optical rotations with those reported in the literature. The characterization data confirmed that the four TK<sub>gst</sub> catalyzed reactions led to a single ketose with the L-erythro (3S,4S) configuration and was consistent with the stereoselectivity

observed in TK<sub>gst</sub> reactions at high temperature with other aldehyde acceptors.<sup>10,12,13</sup>

Beside the interests of the products and the efficiency of the biocatalytic process, the environmental impact assessment must be compared to other synthetic strategies:

(i) The sub-products formed are natural L-serine and L-alanine, together with CO<sub>2</sub> which in solution forms bicarbonate ions. The removal of these sub-products did not require an additional purification step because they were easily separated from the desired major product by simple chromatography. L-Amino acids were recovered and could be recycled for other reactions. The biocatalytic one-pot, two-steps cascade process avoids the commonly used chemical synthesis of Li-HPA from bromopyruvate,<sup>14</sup> which generates toxic wastes (incomplete yield, hydrobromic acid and bromine fumes) and requires additional purification steps (washing, crystallization and filtration using salts and chemicals).

(ii) The amount of wastes generated by biocatalytic approaches must be considered to evaluate the environmental impact of a process. Some metrics especially the *E* factor ( $m_{\text{wastes}}/m_{\text{product}}$ )<sup>46,47</sup> allow a fast quantification of wastes for comparison to other process despite of some shortcomings<sup>48</sup> (*E* factor neither includes energy use or nature of waste). A value of 0 is optimal with no generation of sub-products and 100% yield. For the synthesis of compounds **1**, **2**, **3** and **4** using the one-pot two-steps sequence generating HPA *in situ*, the values of *E* factors are around 7, 4, 7.5 and 7.5 respectively. In the case of products **1** and **2**, we have compared these *E* factor values to those obtained using the process we have recently published involving two individual steps, in which Li-HPA was firstly synthesized chemically from bromopyruvate and then used as TK<sub>gst</sub> donor substrate.<sup>12</sup> We obtained *E* factor values around 74 for **1** and 53.5 for **2**, higher than those obtained using the cascade synthesis, essentially due to the low yields of both steps (50% yield for Li-HPA synthesis, 11% and 33% for **1** and **2** synthesis respectively). The major advantage of the cascade procedure is the total conversion of the TK<sub>gst</sub> aldehyde acceptor allowed by the gradual use of HPA by TK<sub>gst</sub> leading to higher yields (56 % for **1** and 98% for **2**) as mentioned before and avoiding the decomposition of HPA occurring with the sequential process using chemically prepared Li-HPA. A comparison of these results to *E* factors for other biocatalytic or organocatalytic strategies is not easy. Particularly, when the final product is not fully purified or no isolated yield is given, this renders the corresponding *E* factor undervalued. For example, the equilibrium constants for the reversible isomerization process to convert D-galactose into D-

tagatose or L-arabinose into L-ribulose are not in favor of the products (90/10), so that the reaction mixture contained remaining substrate but the final product was not purified.<sup>24</sup> An interesting aldolase-catalyzed process using an engineered *Corynebacterium glutamicum* strain<sup>26</sup> has been studied for the synthesis of L-psicose from L-glyceraldehyde and dihydroxyacetone phosphate (generated intracellularly via the glycolytic pathway) but the conversion rate was low, and the non-stereoselective process led mostly to the stereoisomeric product L-sorbose (60/40), which was not removed by purification. An aldol reaction catalyzed by (S)-proline has also been used for the synthesis of ketoses such as L-ribulose or 5-deoxy-L-ribulose from a synthetic dioxanone (adding its own *E* factor) in good yield but the major drawbacks were the use of the organic solvent DMF and the need for an additional deprotection step to obtain the final product in unprotected form.<sup>47</sup>

(iii) The procedures of enzyme purification should also be considered in environmental evaluations as mentioned in a recent review.<sup>47</sup> Generally, the purification of tagged enzymes is performed by affinity chromatography using significant amounts of salts, buffers and other chemicals. Thermostable enzymes from thermophilic origins expressed in standard *E. coli* expression systems are gaining relevance because target enzymes are easily purified by a single heat treatment step that removes inactivated host proteins. We have already reported that this type of TK<sub>gst</sub> purification yields enzyme of the same purity as that obtained by affinity chromatography. This procedure can be applied for the purification of both thermostable TA<sub>tca</sub> and TK<sub>gst</sub> to improve the environmental impact of the process.

## Conclusions

The synthesis of L-erythro (3S,4S) ketoses such as L-ribulose, 5-deoxy-L-ribulose, D-tagatose and L-psicose, highly valuable compounds, was performed at 60°C from corresponding (2S)-hydroxylated aldehydes with excellent stereoselectivity and high yields using an efficient one-pot, two-step cascade sequence catalyzed by thermostable TA<sub>tca</sub> and TK<sub>gst</sub>. The L-erythro (3S,4S) configuration is otherwise inaccessible using mesophilic TKs, which exclusively lead to D-threo (3S,4R) configured ketoses from (2R)-hydroxylated aldehydes. The efficiency of this approach was based on a novel thermostable TA<sub>tca</sub>, characterized in this study, allowing the *in situ* generation of HPA, the TK<sub>gst</sub> donor substrate, at 60 °C from natural L-serine and pyruvate. Thereby, sensitive HPA is readily converted by TK<sub>gst</sub>-catalysis, which avoids HPA accumulation and decomposition. This environmentally friendly procedure, never investigated at elevated temperatures, reveals an interesting alternative to the conventional chemical synthesis of HPA. TK<sub>gst</sub> activities towards the (2S)-α-hydroxylated aldehydes, which are generally poor TK<sub>gst</sub> substrates, were greatly enhanced by performing the reactions at higher temperature, leading to excellent conversion rates within a reasonable time (24 h to 96 h). This straightforward approach could be applied with other types of aldehydes using wild-type TK<sub>gst</sub> or variants. Furthermore, this novel TA<sub>tca</sub> could be used in other cascade reactions involving thermostable HPA-dependent enzymes.

## Experimental

### General experimental

*Thermosinus carboxydivorans* DSM 14886 was purchased from DSMZ, *E. coli* strains BL21(DE3)pLysE strain from Invitrogen. Reagents for molecular biology were obtained from Life Technologies. Oligonucleotides and reagents were purchased from Sigma-Aldrich. D-(+)-mannose was provided by Fluka AG. D-lyxose was got from DIFCO. Sodium chloride was procured by ROTH. The WST-1 blue dye was obtained from Interchim (Biotech Grade). L-serine, α-ketoglutaric acid, pyruvic acid, imidazole, ammonium sulfate and potassium chloride were purchased from Alfa Aesar. Pyridoxal 5-phosphate monohydrate (PLP) was procured by Acros Organics. L-glyceraldehyde was acquired from Molekula Society and was beforehand purified. Hydroxypyruvate was prepared as the lithium salt by the method of Dickens and Williamson.<sup>14</sup> L-lactaldehyde was synthesized chemically following the literature<sup>35</sup> from D-threonine in the presence of ninhydrine. Bradford reagent was obtained from Bio-Rad. Ni-NTA resin was acquired from QIAGEN. The 96-wells microplates were shaken and incubated in a Titramax 1000 incubator from Heidolph. Lyophilisation was carried out with Triad LABCONCO dryer. UV-visible absorbances were measured using a SAFIRE II-Basic plate reader from TECAN and a spectrophotometer Perkin Elmer Lambda 25 enabling Peltier-effect temperature control. MARCHEREY-NAGEL GmbH & Co KG 60 F254 silica gel TLC plates and MARCHEREY-NAGEL GmbH & Co KG 60/40-63 mesh silica gel for Liquid Flash Chromatography were used. NMR spectra were recorded in D<sub>2</sub>O and CD<sub>3</sub>OD on a Bruker Avance 400 spectrometer. The optical rotation was determined with a polarimeter P-2000 JASCO PTC-262 at the given temperature and wavelength (Na-D-line: D = 589 nm) in a cell 10 cm long cell. [α]<sub>D</sub> values are given in 10<sup>-1</sup>deg.cm<sup>2</sup>.g<sup>-1</sup> (concentration given as g/100 mL).

### *Thermosinus carboxydivorans* Transaminase preparation

**L-α-transaminase screening.** An α-transaminase library representative as possible of the biodiversity of this family, has been previously constructed.<sup>18</sup> 9 putative L-α-TAs leading to the best activities with Li-HPA were selected for the screening. 5 μL of each 9 clarified lysates were transferred to individual wells in a 96-wells plates containing L-serine (5 mM), an α-keto acid (α-ketoglutaric acid: 5 mM; pyruvic acid: 5 mM; glyoxylic acid: 5 mM) and PLP (0.2 mM), in HEPEs buffer (100 mM, pH 7.5). The total volume in each well was 200 μL. Plates were then incubated at 25°C during 2 hours. HPA formation in each reaction was determined against time using a discontinuous assay involving a WST-1 blue dye, as described by Bommer and Ward.<sup>34</sup> Aliquots of the medium (20 μL) were added over time to 180 μL of a solution containing NaOH (90 mM) and WST-1 (0.1 mg.mL<sup>-1</sup>) in a new 96-well microplate. The total volume in each well was 200 μL. The plate was then incubated during 10 minutes at 60°C and the absorbance increase of medium resulting from formazan formation was measured at 600 nm by plate reader.

**Transaminase cloning and expression.** The TA<sub>tca</sub> gene was amplified by PCR from genomic DNA of *Thermosinus carboxydivorans* DSM 14886 with PCR primers designed on the gene encoding for A1HUC4: TATca-5PRIM-AAAGAAGGAGATAGGATCATGCATCATCACCATCACCATTTACAAAACCGTATTTGA and TATca-3PRIM-GTGTAAATGGATAGTGATCTTATCCCTCCATAAGTATTCTTGC. A hexahistidine tag was introduced after the starting ATG (underlined in the sequence of TATca-5PRIM) in the N-terminal part of the gene.

Specific extensions are added to the primers for cloning into our plasmid pET22b(+) (Novagen) modified for ligation independent cloning.<sup>36</sup> *Escherichia coli* strain BL21(DE3)pLysE was used as the expression host. A 100 mL pre-culture was grown at 37 °C in LB medium with 100 µg.mL<sup>-1</sup> ampicillin overnight. 20 mL of the pre-culture was used to inoculate 1 liter of culture medium containing 100 µg.mL<sup>-1</sup> ampicillin. Isopropyl β-D-thiogalactoside (IPTG) at 1 mM was introduced when the OD range was 0.7-0.8. The cells were then grown overnight at 30°C and harvested (4.45 g.L<sup>-1</sup> of biomass).

**Transaminase purification.** The harvested recombinant cells from 1 L culture were suspended in 31 mL of 50 mM phosphate buffer containing KCl (300 mM) and imidazole (10 mM) at pH 8.0. The cells were disrupted by sonication for 30 min on ice and the insoluble pellet was discarded after centrifugation (4,000 rpm, 30 min, 4°C). The crude extract was mixed with 3.5 mL of PLP (10 mM) and applied to a Ni-NTA (QIAGEN) Ni<sup>2+</sup> chelating affinity column equilibrated with the phosphate buffer mentioned above. After washing the column with the same buffer, the His6-tagged TA<sub>tca</sub> was finally eluted with 50 mM phosphate buffer containing 300 mM KCl and 250 mM imidazole (pH 8.0). Protein concentration of each fraction was determined by the method of Bradford and bovine serum albumin (BSA) was used as the standard. The fractions containing proteins were collected, mixed with 300 µL of PLP (10 mM) and dialyzed against ammonium sulfate solution (3 M, pH 8). After purification from 1 L culture, 203 mg of total proteins was obtained with a total TA<sub>tca</sub> activity of 717 U, corresponding to a specific activity of 3.5 U.mg<sup>-1</sup>, at 25°C. Precipitated proteins were stored in ammonium sulfate solution (3 M, pH 8) at 4°C.

**Transaminase activity.** One unit of TA<sub>tca</sub> activity is defined as the amount of TA that catalyzes the formation of 1 µmol of HPA per minute at 25°C in HEPES buffer (100 mM, pH 7.5). TA<sub>tca</sub> enzymatic assay was performed in the presence of L-serine and α-keto glutaric acid leading to HPA and L-glutamic acid. 30 µL of diluted sample of purified TA<sub>tca</sub> (10 µg.mL<sup>-1</sup>, 2.3 µg per well) were transferred to individual wells in a 96-wells plates containing L-serine (250 mM), α-ketoglutaric acid (3 mM) and PLP (0.2 mM) in HEPES buffer (100 mM, pH 7.5). The total volume in each well was 200 µL. Then, the plate was incubated at 25°C during 20 minutes. The residual activities were measured at different time interval by using the sequential assay described above (Experimental part: L-α-transaminase library screening). All measurements were performed in triplicate.

#### Transaminase optimum temperature and pH

**Transaminase optimum temperature.** The optimum temperature of TA<sub>tca</sub> was examined in the range of 20 to 80 °C in HEPES buffer (100 mM, pH 7.5) with L-serine and α-ketoglutaric acid as substrates. The temperature of the mixture was controlled by a Peltier effect controller. Enzyme solutions (10 µg.mL<sup>-1</sup>) were first incubated during 30 minutes at various temperatures with 3 mM of α-ketoglutaric acid and 0.2 mM of PLP. When the desired temperature was reached, L-serine (100 mM) was added to initiate the reaction. The residual activities were measured at different time interval by using the sequential assay described above (Experimental part: L-α-transaminase library screening). All measurements were performed in triplicate.

**Transaminase optimum pH.** The effect of pH on TA<sub>tca</sub> was determined by measuring TA<sub>tca</sub> in the range of 5 to 9.2 with L-serine and α-ketoglutaric acid in different buffers: sodium citrate buffer (pH 5-6.5), sodium phosphate (pH 6.3-7.6), Tris-HCl buffer (pH 7-8.5) and sodium borate buffer (pH 7.8 - 9.2) at 100 mM final concentration. Enzyme solutions (10 µg.mL<sup>-1</sup>) were incubated at

different pH during 20 minutes at 25°C in the presence of α-ketoglutaric acid (3 mM) and PLP (0.2 mM). Then, L-serine (100 mM) was added to initiate the reaction. The residual activities were measured at different times by using the sequential assay described above (Experimental part: L-α-transaminase library screening). All measurements were performed in triplicate.

#### Transaminase thermostability

The thermostability of TA<sub>tca</sub> was studied at 60 °C with L-serine (170 mM) and α-ketoglutaric acid (3 mM). Enzyme solutions (10 µg.mL<sup>-1</sup>) were incubated with of PLP (0.2 mM) at 60°C up to 10 days in HEPES buffer (100 mM, pH 7.5). The residual activities were measured at different time interval by using the sequential assay described above (Experimental part: L-α-transaminase library screening). All measurements were performed in triplicate.

#### Transaminase kinetic parameters

Apparent kinetic parameters were determined for L-serine and pyruvic acid using the sequential assay described above (Experimental part: L-α-transaminase library screening), at 25°C. Apparent kinetic parameters of pyruvic acid were obtained by the measurement of initial velocities of TA<sub>tca</sub> by varying pyruvic acid concentration from 0.25 to 50 mM while fixing L-serine concentration to 250 mM. Apparent kinetic parameters of L-serine were obtained by the measurement of initial velocities of TA<sub>tca</sub> by varying L-serine concentration from 1 to 250 mM while fixing pyruvic acid concentration to 50 mM. Hanes-Woolf plots were used to determine apparent kinetic parameters values. All measurements were performed in triplicate.

#### *Geobacillus stearothermophilus* Transketolase preparation

**Transketolase expression.**<sup>10</sup> Expression was carried out in *E. coli* BL21 (DE3) Gold strain. This strain was first transformed by electroporation with pGEN717 for the expression of TK<sub>gst</sub> and plated on LB medium with kanamycin (30 µg.mL<sup>-1</sup>). The *E. coli* BL21 (DE3) strain was then transformed by electroporation with pGEN768 for the expression of the TK<sub>gst</sub> from the synthetic *tk* gene. A 100 mL pre-culture was grown at 37 °C in LB medium with 30 µg.mL<sup>-1</sup> kanamycin overnight. 20 mL of the pre-culture was used to inoculate 1 liter of culture medium containing 30 µg.mL<sup>-1</sup> kanamycin. Isopropyl β-D-thiogalactoside (IPTG) at 1 mM was introduced when the OD range was 0.7-0.8. The cells were then grown overnight at 30°C and harvested (3.73 g.L<sup>-1</sup> of biomass).

**Transketolase purification.**<sup>10</sup> Harvested recombinant cells from 1 L culture were suspended in 26 mL of 50 mM phosphate buffer containing 300 mM NaCl (pH 8.0). The cells were disrupted by sonication for 30 min on ice and the insoluble pellet was discarded after centrifugation (4,000 rpm, 30 min, 4 °C). The crude extract was applied to a Ni-NTA (QIAGEN) Ni<sup>2+</sup> chelating affinity column equilibrated with the phosphate buffer mentioned above. After washing the column with the same buffer, the His6-tagged TK<sub>gst</sub> was finally eluted with 50 mM phosphate buffer containing 300 mM NaCl and 500 mM imidazole (pH 8.0). Protein concentration of each fraction was determined by the method of Bradford and bovine serum albumin (BSA) was used as the standard. The fractions containing proteins were collected and dialyzed against triethanolamine buffer (2 mM, pH 7.5) and then against water (pH 7.5) through dialysis tubing. After purification from 1 L culture, 160 mg of total proteins was obtained with a total TK<sub>gst</sub> activity of 324 U, corresponding to a specific activity of 2 U.mg<sup>-1</sup>, at 25°C. Proteins in water solution at pH 7.5 were then lyophilized and stored at 4°C.

**Transketolase activity.** One unit of  $TK_{gst}$  activity is defined as the amount of TK that catalyzes the formation of 1  $\mu$ mol of ketose product per minute at 25°C in glycyglycine buffer (100 mM, pH 7.5).  $TK_{gst}$  enzymatic assay was performed in the presence of L-erythrulose and D-ribose-5-phosphate (D-R5P) leading to D-sedoheptulose-7-phosphate (D-S7P) and glycolaldehyde.<sup>35</sup> The GA formed is reduced by yeast alcohol dehydrogenase (ADH) to ethylene glycol in the presence of nicotine adenine dinucleotide, reduced form (NADH). L-erythrulose (100 mM), D-R5P (9.1 mM), ThDP (0.1 mM),  $MgCl_2$  (0.5 mM), NADH (0.2 mg.mL<sup>-1</sup>), 5  $\mu$ L of ADH (25 U. mL<sup>-1</sup>) and the  $TK_{gst}$  suspension (10  $\mu$ L) were added to disposable plastic cuvettes and completed to 1 mL with glycyglycine buffer (100 mM, pH 7.5). The disappearance of NADH was followed by spectrophotometry at 340 nm (value  $\epsilon_{NADH\ 340\ nm} = 6220\ M^{-1}.cm^{-1}$ ).

#### Transketolase acceptor substrate specificity

The assay reaction was carried out in the presence of phenol red as pH indicator<sup>34</sup> on 1 mL disposable plastic cuvettes. The reaction mixture containing  $TK_{gst}$  (200-450  $\mu$ g.mL<sup>-1</sup>), acceptor substrates: L-glyceraldehyde, L-lactaldehyde, D-threose, or L-erythrulose (100 mM), ThDP (0.1 mM),  $MgCl_2$  (1 mM), phenol red (84  $\mu$ M) in triethanolamine buffer (2 mM, pH 7.15) was first incubated at 25°C and 60°C during 20 minutes. Then, Li-HPA was added at 50 mM final concentration to initiate the reaction and the absorbance increase was measured at 560 nm during 30 minutes. Absorbance was recorded and converted into concentration of  $HCO_3^-$  according to the calibration curve for the activity calculation.<sup>12</sup> All measurements were performed in triplicate.

#### Transketolase kinetic parameters towards lithium $\beta$ -hydroxypyruvate

Kinetic parameters relative to Li-HPA were determined by discontinuous titration of Li-HPA against time, at 25°C. Glycolaldehyde was used as acceptor substrate. The solution contained ThDP (2.4 mM),  $MgCl_2$  (9 mM), glycolaldehyde (50 mM) and Li-HPA (0.5-25 mM) in glycyglycine buffer (100 mM, pH 7.5).  $TK_{gst}$  (0.0125-0.25 U.mL<sup>-1</sup>, 0.37-37  $\mu$ g) was added to initiate the reaction. Li-HPA consumption was determined against time from aliquots of the mixture as described below (Experimental part: determination of lithium  $\beta$ -hydroxypyruvate concentration). Different slopes of Li-HPA consumption were obtained according to Li-HPA concentration tested.  $v_{max}$  and  $K_M$  values were determined using Hanes-Woolf plots.

#### Determination of lithium $\beta$ -hydroxypyruvate concentration

Li-HPA concentration was determined by an enzymatic assay using L-lactic dehydrogenase. Sample of diluted Li-HPA was introduced in a cuvette containing 20  $\mu$ L of NADH (0.2 mg.mL<sup>-1</sup>), 20  $\mu$ L of L-lactic dehydrogenase (4U.mL<sup>-1</sup>) in triethanolamine buffer (100 mM, pH 7.5). The disappearance of NADH was followed by spectrophotometry at 340 nm and difference between initial absorbance and final absorbance gave the Li-HPA concentration using the Beer Lambert law (value  $\epsilon_{NADH\ 340nm} = 6220\ M^{-1}.cm^{-1}$ ).

#### Preparative scale enzymatic cascade synthesis

**General procedure.** ThDP (0.1 mM),  $MgCl_2.6H_2O$  (1 mM), PLP (0.2 mM) and pyruvic acid (50 mM) were dissolved in H<sub>2</sub>O and the pH was adjusted to 7 with 0.1 M NaOH. To this stirred solution was added  $TK_{gst}$  (6 mg in the case of L-glyceraldehyde and L-lactaldehyde ; 10 mg in the case of D-threose and L-erythrulose) and  $TA_{tea}$  (4.8 mg) and the mixture was stirred for 20 min at 60°C. In another flask, (2S)

$\alpha$ -hydroxylated aldehydes (50 mM) and L-serine (150 mM) were mixed and the pH adjusted to 7 with 0.1 M NaOH. After preincubation of enzymes, cofactors and pyruvic acid, L-serine and (2S)  $\alpha$ -hydroxylated aldehydes were added and the mixture was stirred at 60°C. The final volume was 20 mL. The pH was maintained at 7 by adding 0.1M HCl using a pH stat (Radiometer Analytical). The reaction was monitored by measuring pyruvic acid and (2S)  $\alpha$ -hydroxylated aldehydes consumption by *in situ* <sup>1</sup>H NMR. In the case of D-threose and L-erythrulose, the complete disappearance of pyruvic acid was observed after 48 h and a second portion of pyruvic acid (50 mM) was added. After total conversion of pyruvic acid and (2S)- $\alpha$ -hydroxylated aldehydes (24-96 hours), silica was added to the supernatant and the suspension was concentrated to dryness under reduced pressure before dry loading onto a flash silica column. After silica gel chromatography using  $CH_2Cl_2/CH_3OH$  v/v 90:10 as eluent (for L-ribulose and 5-deoxy-L-ribulose) and ethyl acetate/ $CH_3OH$  v/v 100:0-90:10 as eluent (for D-tagatose and L-psicose), L-ribulose, 5-deoxy-L-ribulose, D-tagatose and L-psicose were isolated.

***In situ* <sup>1</sup>H NMR measurements.** Progress of preparative scale enzymatic synthesis were monitored by using quantitative *in situ* <sup>1</sup>H NMR relative to 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP-d4) as internal standard. Aliquots of medium were removed over time (450  $\mu$ L) and mixed with 50  $\mu$ L of TSP-d4 (50 mM in D<sub>2</sub>O).

**(3S,4S)-1,3,4,5-tetrahydroxypentan-2-one (L-ribulose) 1.** 1 was isolated as yellow oil; yield: 84 mg, 56 %. R<sub>f</sub> 0.43 (DCM/ $CH_3OH$ , 8:2).  $[\alpha]_D^{20} = +15.8$  (c 0.1, H<sub>2</sub>O) lit.  $[\alpha]_D^{20} = +16.5 \pm 1.5$  (c 0.1 H<sub>2</sub>O).<sup>38</sup> NMR data were identical to those previously described<sup>39</sup>: (ratio:  $\alpha$ -anomer/ $\beta$ -anomer/keto isomer=63/23/14; lit. ratio:  $\alpha$ -anomer/ $\beta$ -anomer/keto isomer=58/24/18.<sup>39</sup> <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) :  $\delta$  = (keto isomer) 213.9 (C-2), 76.8 (C-3), 74.0 (C-4), 67.9 (C-1), 62.7 (C-5);  $\delta$  = ( $\alpha$ -anomer) 104.0 (C-2), 72.9 (C-5), 71.9 (C-3), 64.2 (C-4), 71.6 (C-1);  $\delta$  = ( $\beta$ -anomer) 107.1 (C-2), 77.2 (C-3), 72.2 (C-5), 72.0 (C-4), 63.9 (C-1). *m/z* HRMS found  $[M+Cl]^-$  185.0212, C<sub>5</sub>H<sub>10</sub>O<sub>5</sub>Cl requires 185.0217.

**(3S,4S)-1,3,4-trihydroxypentan-2-one (5-deoxy-L-ribulose) 2.** 2 was isolated as yellow oil; yield: 131 mg, 98 %. R<sub>f</sub> 0.27 (DCM/ $CH_3OH$ , 9:1).  $[\alpha]_D^{20} = -25.6$  (c 0.25,  $CH_3OH$ ) lit.  $[\alpha]_D^{20} = -26.5$  (c 0.7,  $CH_3OH$ ).<sup>12</sup> NMR data were identical to those previously described<sup>12</sup>: <sup>1</sup>H NMR (400 MHz, CD<sub>3</sub>OD)  $\delta$  = 1.19 (d, *J* = 6.4 Hz, 3H, H<sub>3</sub>); 3.92-3.99 (m, 1H, H<sub>3</sub>); 4.08 (d, *J* = 5.1 Hz, 1H, H<sub>4</sub>); 4.44 (d, *J* = 19.4 Hz, 1H, H<sub>1b</sub>); 4.55 (d, *J* = 19.4 Hz, 1H, H<sub>1a</sub>). <sup>13</sup>C NMR (101 MHz, CD<sub>3</sub>OD)  $\delta$  = 18.6 (C-5), 68.0 (C-1), 70.0 (C-4), 80.3 (C-3), 213.1 (C-2). *m/z* HRMS found  $[M+HCOO]^-$  179.0553, C<sub>5</sub>H<sub>11</sub>O<sub>4</sub> requires 179.0556.

**(3S,4S,5R)-1,3,4,5,6-pentahydroxyhexan-2-one (D-tagatose) 3.** 3 was isolated as a white powder; yield: 93 mg, 52%. R<sub>f</sub> 0.31 (DCM/ $CH_3OH$ , 8:2).  $[\alpha]_D^{25} = -2.9$  (c 2, H<sub>2</sub>O) lit.  $[\alpha]_D^{20} = -2.5$ .<sup>40</sup> NMR data were identical to those previously described<sup>41</sup>: (Ratio:  $\alpha$ -D-tagatato-2.6-pyranose/ $\beta$ -D-tagatato-2.6-pyranose/ $\alpha$ -D-tagatato-2.5-furanose/ $\beta$ -D-tagatato-2.6-furanose: 72/22/2/4.; lit. ratio:  $\alpha$ -D-tagatato-2.6-pyranose/ $\beta$ -D-tagatato-2.6-pyranose/ $\alpha$ -D-tagatato-2.5-furanose/ $\beta$ -D-tagatato-2.6-furanose: 79/14/2/5<sup>42</sup>). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O)  $\delta$  = ( $\alpha$ -D-tagatato-2.6-pyranose): 99.0 (C-2), 71.8 (C-4), 70.6 (C-3), 67.2 (C-5), 64.7 (C-1), 63.1 (C-6);  $\delta$  = ( $\beta$ -D-tagatato-2.6-pyranose): 99.1 (C-2), 70.6 (C-4), 70.1 (C-5), 64.5 (C-3), 64.3 (C-1); 60.9 (C-6) ;  $\delta$  = ( $\alpha$ -D-tagatato-2.5-furanose): 105.8 (C-2), 80.0 (C-5), 77.5 (C-3), 71.9 (C 4), 63.2 (C-1), 62.6 (C-6) ;  $\delta$  = ( $\beta$ -D-tagatato-2.6-furanose): 103.4 (C-2), 80.9 (C-5), 71.8 (C-4), 71.5 (C3), 63.4 (C-1), 61.8 (C-6). *m/z* HRMS found  $[M+Cl]^-$  215.0319, C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>Cl requires 215.0322.

**(3S,4S,5S)-1,3,4,5,6-pentahydroxyhexan-2-one (L-psicose) 4.** 4 was isolated as a white powder; yield: 92 mg, 51%. R<sub>f</sub> 0.31 (DCM/ $CH_3OH$ , 8:2).  $[\alpha]_D^{20} = -2.3$  (c 0,1 H<sub>2</sub>O) lit.  $[\alpha]_D^{20} = -2.4$ .<sup>43</sup> Data for

D-psicose: + 3.02 (c = 1.16 in H<sub>2</sub>O)<sup>44</sup>, +3.1 (c = 1.62 in H<sub>2</sub>O).<sup>45</sup> NMR data were identical to those previously described for the D enantiomer<sup>41,44</sup>: (ratio: α-D-psico-2.6-pyranose/β-D-psico-2.6-pyranose/α-D-psico-2.5-furanose/β-D-psico-2.6-furanose:25/24/37/13; lit. ratio: α-D-psico-2.6-pyranose/β-D-psico-2.6-pyranose/α-D-psico-2.5-furanose/β-D-psico-2.6-furanose: 22/24/39/15<sup>42</sup>): <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O) δ = 3.43 (d, J = 11.7 Hz), 3.87 - 3.51 (m), 4.11 - 3.91 (m), 4.09 (s), 4.33 (dd, J = 7.6, 4.7 Hz). <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O) δ = (α-D-psico-2.6-pyranose) 98.6 (C-2), 72.6 (C-4), 66.8 (C-5), 66.4 (C-3), 64.0 (C-1), 58.9 (C-6); δ = (β-D-psico-2.6-pyranose) 99.3 (C-2), 71.12 (C-3), 69.9 (C-5), 66.0 (C-4), 65.1 (C-6), 64.9 (C-1); δ = (α-D-psico-2.5-furanose) 104.2 (C-2), 83.6 (C-5), 71.2 (C-4), 71.2 (C-3), 64.2 (C-1), 62.2 (C-6); δ = (β-D-psico-2.6-furanose) 106.5 (C-2), 83.6 (C-5), 75.5 (C-3), 71.9 (C-4), 63.7 (C-6), 63.3 (C-1). m/z HRMS found [M+Cl]<sup>+</sup> 215.0318, C<sub>6</sub>H<sub>12</sub>O<sub>6</sub>Cl requires 215.0322.

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## One-pot, two-step cascade synthesis of naturally rare *L-erythro* (3*S*,4*S*) ketoses by coupling thermostable transaminase and transketolase

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Naturally rare *L-erythro* (3*S*,4*S*)-ketoses were prepared at elevated temperature through a simultaneous two-step enzymatic cascade synthesis with excellent stereoselectivity and good yields.

