Convergent *in situ* Generation of Both Transketolase Substrates *via* Transaminase and Aldolase Reactions for Sequential One-Pot, Three-Step Cascade Synthesis of Ketoses

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We describe an efficient three-enzyme, sequential one-pot cascade reaction where both transketolase substrates are generated *in situ* in a convergent fashion. The nucleophilic donor substrate hydroxypyruvate was obtained from L-serine and pyruvate by a transaminase-catalyzed reaction. In parallel, three different (2*S*)-α-hydroxylated aldehydes, L-glyceraldehyde, D-threose, and L-erythrose, were generated as electrophilic acceptors from simple achiral compounds glycolaldehyde and formaldehyde by D-fructose-6-phosphate aldolase catalysis. The compatibility of the three enzymes was studied in terms of temperature, enzyme ratio and substrate concentration. The efficiency of the process relied on the irreversibility of the transketolase reaction, driving a shift of the reversible transamination reaction and securing the complete conversion of all substrates. Three valuable (3*S*,4*S*)-ketoses, L-ribulose, D-tagatose, and L-psicose were obtained in good yields with high diastereoselectivity.

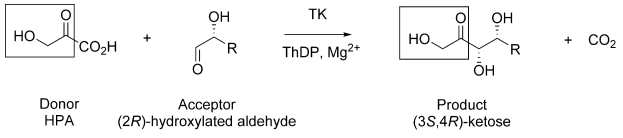
Introduction

The need for greener and more sustainable chemical production[1] has seen enzymatic strategies to emerge as a powerful approach to the eco-friendly, highly selective synthesis of valuable chiral compounds. To compete with the productivity of traditional methods, the use of two or even more enzymes in cascade can considerably improve the efficiency of a multistage synthesis by obviating the isolation of intermediates, thus saving time, resources, reagents and energy, while reducing waste.[2–4] Cascade reactions can be performed along a simultaneous one-pot strategy when all the enzyme requirements are compatible. To meet limitations, such as substrate/product/reagent inhibition or incompatibility of reaction conditions (pH, temperature), a telescoped, sequential one-pot procedure can be used.[2]

Chiral polyols, regarded as highly valuable compounds in various fields, can be obtained by stereoselective carbon-carbon bond formation.[5,6] Especially, transketolase (TK, EC 2.2.1.1) is a powerful thiamine diphosphate-dependent biocatalyst that allows a two-carbon chain elongation by transferring an α-hydroxy carbonyl (ketol) group from a ketone donor to an aldehyde acceptor, to produce a corresponding C_{n+2} ketose (Scheme 1).

In the pentose phosphate metabolism, TK catalyzes a ketol transfer reaction for the reversible equilibration of ketoses and aldoses carrying a terminal phosphate ester group. Previous *in vitro* studies showed that non-phosphorylated aldose compounds can also be used as TK substrates, yielding the equivalent free ketoses in one step. In particular, irreversible release of carbon dioxide from hydroxypyruvate (HPA) as donor kinetically drives the conversion, rendering TK a powerful tool for the asymmetric synthesis of ketoses and related acyloin compounds.[7,8]

The studies on TK acceptor substrate specificity revealed that non-phosphorylated α-hydroxylated aldehydes with short carbon chains (C_2–C_3) gave the highest TK activities. The new asymmetric center at C3 of the C_{n+2} ketose product is formed



Scheme 1. Irreversible reaction catalyzed by TK using hydroxypyruvate (HPA) as donor substrate with (2*R*)-hydroxyaldehydes as acceptor substrates.

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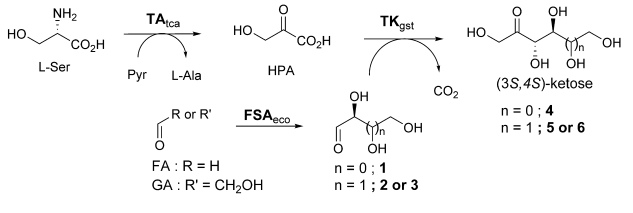
with high stereoselectivity for an *(S)* configuration. In addition, the enzyme is highly enantioselective with chiral α-hydroxy aldehydes, and converts the (2*R*)-epimers with high preference, thus leading to (3*S*,4*R*)-configured ketoses. For biocatalytic applications, mesophilic TKs from *Saccharomyces cerevisiae* and from *Escherichia coli* have been largely used and optimized by mutagenesis.[9,10] We have identified the first thermostable TK from *Geobacillus stearothermophilus* (TK_{gst}) that offers significantly improved stability at high temperature,[11] more robustness towards non-usual reaction conditions[12] and a broadened substrate spectrum obtained by *in vitro* evolution towards (2*S*)-hydroxylated,[13] aliphatic[14] and aromatic aldehydes[15] and also towards HPA analogs as new donor substrates.[16]

The purpose of this study was to optimize the synthesis of highly valuable natural (3*S*,4*S*)-ketoses from (2*S*)-hydroxyaldehydes. While the synthesis of such compounds is inaccessible using mesophilic TKs, we could recently show that TK_{gst} catalysis enabled best yields upon reaction at 60 °C in a reasonable time frame (8–24 h).[11,13] To make this TK_{gst}-catalyzed process greener and more sustainable, a major challenge was to generate the donor HPA as well as the acceptor substrates *in situ* in a one-pot procedure, not only to avoid the costly purchase or chemical synthesis of these compounds, but also to counteract their limited stability at 60 °C (Scheme 2). HPA is particularly unstable at high temperature but can be produced *in situ* from L-serine (L-Ser) by transamination to a ketoacid as amino group acceptor.[17] For this purpose, we recently introduced the thermostable L-α-transaminase from *Thermosinus carboxydivorans* (TA_{tca}), which was identified, characterized and coupled with thermostable TK_{gst}.[18] The targeted (2*S*) hydroxyaldehydes – L-glyceraldehyde **1**, D-threose **2**, and L-erythrose **3** – can be synthesized from cheap non-chiral starting materials such as glycolaldehyde (GA) and formaldehyde (FA) by enzymatic aldol reactions catalyzed by D-fructose-6-phosphate aldolase from *Escherichia coli* (FSA_{eco}; wild-type or variants).[19] The compatibility of FSA and TK-catalyzed reactions has been previously demonstrated for the synthesis of phosphorylated sugars.[20] Here, we have investigated the best conditions (*i.e.*, temperature, enzyme ratios, substrate concentrations) to combine the three individual reactions catalyzed by TA, FSA and TK into a convergent one-pot procedure. This procedure was then applied to the synthesis of three rare (3*S*,4*S*) ketoses, namely L-ribulose **4**,[21] D-tagatose **5**,[22] and L-psicose **6**,[23] which are highly valuable compounds in the pharmaceutical and food sectors.



Scheme 2. One-pot cascade reaction for the synthesis of ketoses **4**–**6** by TK_{gst} with convergent *in situ* generation of HPA using TA_{tca} and aldehydes **1**–**3** using FSA_{eco}.

Results and Discussion

The convergent reaction strategy illustrated in Scheme 2 entails the practical selectivity problems that the substrates FA and/or GA involved in the FSA reaction for the generation of L-glyceraldehyde **1**, D-threose **2** and L-erythrose **3** are also good substrates of TK_{gst}, and could also be consumed by undesired transamination from L-Ser. Therefore, the aldolase catalyzed synthesis of TK_{gst} acceptors **1**–**3** in the first stage had to be uncoupled from the remaining steps. Then, in a telescoped second stage, all other enzymes and substrates could be added simultaneously for *in situ* generation of HPA donor and for the final carboligation leading to L-ribulose **4**, D-tagatose **5** and L-psicose **6**.

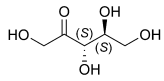
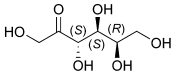
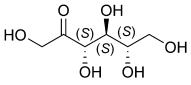
In situ Generation of Aldehydes 1–3 by FSA_{eco}

FSA_{eco}[24] belongs to the class I aldolases and catalyzes *in vivo* the reversible stereoselective addition of dihydroxyacetone to D-glyceraldehyde-3-phosphate to form D-fructose-6-phosphate. The aldolase showed a remarkable tolerance to a large panel of nucleophilic substrates, such as non-phosphorylated ketones, and aldehydes such as FA, acetaldehyde and GA.[25] In addition, efficient FSA_{eco} variants could be obtained by enzyme engineering to broaden the nucleophile scope.[26] The enzymatic syntheses of **1**, **2** and **3** have been recently achieved with wild-type and variants of FSA_{eco} using cheap, achiral aldehydes FA and GA.[19] The original procedures had to be slightly modified for the purpose of this work (Table 1). The three FSA-catalyzed reactions were monitored by HPLC and by quantitative ^1H NMR analysis to observe the conversion of aldehydes and the simultaneous formation of the ketose products (ESI).

The synthesis of L-glyceraldehyde **1** from FA and GA was catalyzed by wild-type-FSA_{eco}.[19] To prevent the self-condensation of GA leading to the production of D-threose **2**, the strategy required an excess of FA (100 mM) over GA (50 mM), which resulted in a 60% conversion of GA after 24 h at 25 °C. Because the wild-type FSA_{eco} is a thermostable enzyme (half-life 8 days, 30 h and 16 h, at 55 °C, 65 °C and 75 °C, respectively), we tested if reactions could be promoted at higher temperatures. Indeed, reactions carried out at 50 °C led to a complete conversion of GA within 19 h.

D-Threose (2*S*,3*R*) **2** or L-erythrose (2*S*,3*S*) **3** were both obtained by the condensation of two GA molecules with catalysis by two different FSA_{eco} variants, A129G or A165G/S166P, respectively, which control an opposite stereoselectivity at C3.[19] The synthesis of **2** was fast, but compromised by (i) the retroaldolization of **2** releasing GA, and (ii) the condensation of **2** to GA yielding D-idose. To avoid these undesired complications, use of GA at 200 mM with 4 mg of A129G at 25 °C led to clean formation of D-threose **2** with 98% conversion of GA in 24 h, without formation of D-idose as shown by NMR analysis of the reaction mixture. To reduce the reaction time, we also tested elevated temperatures, but this caused the denaturation of the FSA_{eco} variant. Aldehyde **3** was obtained from the self-addition of GA catalyzed by FSA_{eco} variant A165G/S166P

Table 1. Conditions and results of one-pot cascade reactions for ketoses **4**–**6** synthesis from aldehydes **1**–**3** by coupling TA_{tca} and TK_{gst}.

Ser mM	Pyr mM	Aldehyde (mM)[a]	TA_{tca}/TK_{gs} (mg/mg)	Ketose product	Reaction time (h)	Aldehyde Conversion rate %[a]	Isolated yield (%)	*de* (%)[b]
150	50	**1**–(50)	4.8/6	 L-ribulose **4**	24	>95	53	>95
150	100	**2**–(50)	4.8/10	 D-tagatose **5**	96	>95	55	>95
150	100	**3**–(50)	4.8/10	 L-psicose **6**	96	>95	49	>95

[a] Reaction mixtures obtained previously with FSA_{eco}, aldehyde concentrations were quantified by HPLC and quantitative ^{1}H NMR, [b] Determined using quantitative ^{1}H NMR relative to 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSPd4) as internal standard [b] Diastereoisomeric excess (*de*) determined by ^{1}H and ^{13}C NMR[13b]

allowing the formation of the (2*S*,3*S*) configuration, which is inaccessible with WT FSA_{eco}. The affinity of this variant for GA being very low, about six times more enzyme than in the previous reaction (25 mg) was required to convert 96% of GA (200 mM) in 10 days at 25 °C.[19] Again, a higher temperature was tried to shorten reaction time but also caused enzyme precipitation.

Finally, HPLC and NMR analysis validated that the three aldehydes **1**, **2** and **3** were obtained with excellent yield and with high diastereoselectivity (>95%). Subsequently, the reaction mixtures obtained were subjected directly without purification to the subsequent synthesis of the corresponding ketoses **3**, **4**, and **5**.

Synthesis of Ketoses 4–6 from Aldehydes 1–3 and *in situ* Generated HPA by TA_{tca} Coupled withTK_{gst}

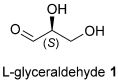
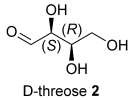
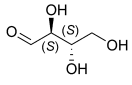
Aldehydes **1**–**3** present a (2*S*)-configuration, which is opposite to the strongly preferred natural (2*R*)-substrates of WT TK_{gst}. Previously, we showed that increased conversion of non-natural (2*S*)-substrates could be promoted by high temperature (60 °C), however, which was also resulting in HPA degradation over time.[11,13a] To overcome this problem, we found an efficient alternative to using synthetic HPA, consisting of the generation of HPA *in situ* by using a thermostable transaminase from *Thermosinus carboxydivorans* (TA_{tca}). The thermostability of both TA_{tca} and TK_{gst} at 60 °C and the irreversibility of the TK reaction, allowing an equilibrium shift of the TA reaction towards HPA, are of particular interest. Thus, TA_{tca} with L-Ser and pyruvate (Pyr) as substrates, pyridoxal phosphate (PLP) as cofactor, and TK_{gst}, with both its cofactors thiamine diphosphate (ThDP) and $MgCl_2$, were simultaneously added to the aldol reaction mixture containing aldehydes **1**–**3**, obtained with FSA_{eco} in the first stage. We observed that all substrates and reagents assayed at 60 °C were found to be stable except Pyr, which slowly decomposed with a half-life of 4 days.[18]

In order to study the influence of different enzyme quantities and substrate concentrations, small scale reaction mixtures were monitored *in situ* by ^{1}H NMR analysis against 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP-d_4) as an internal standard. Thereby, we could quantify the individual conversion rates of aldehydes and Pyr along with the formation of ketose products. The reaction mixtures containing aldehydes **1**, **2** and **3** obtained by FSA_{eco} catalysis were diluted to a final concentration of 50 mM. The ratio TA_{tca}/TK_{gst} was adjusted to avoid an accumulation of HPA while allowing its gradual conversion into ketoses **4**–**6**. The best conversion rates of aldehydes were reached by using 1.5 equivalents of L-Ser in combination with 1 equivalent of Pyr. In the case of D-threose **2** and L-erythrose **3**, for which TK_{gst} shows lower activities, a corresponding larger quantity of TK_{gst} (4.8 mg TA_{tca}/10 mg TK_{gst}) had to be used. To avoid the known inhibition of TA_{tca} by Pyr at concentrations above 50 mM,[18] this substrate was added in two portions, the second after complete disappearance of the first fraction.

Under such adjusted conditions, we observed the total conversion of aldehydes **1**–**3** and simultaneously the quantitative formation of the corresponding ketose products (Table 2). Finally, L-ribulose **4**, D-tagatose **5** and L-psicose **6** were isolated in good yields of 53%, 55% and 49%, respectively. It is noteworthy that the isolated yields of compounds **4**–**6** obtained by following the novel cascade process were 4–5 times higher than those obtained with synthetic Li-HPA and commercially available substrates **1**–**3**, which were reported as 11%,[13a] 13%,[13b] and 10%,[13b] respectively. The three L-*erythro* (3*S*,4*S*) ketoses were obtained in optically pure form with high diastereoselectivity (*de* >95%). The absolute configurations of **4**–**6** were further confirmed by comparing their optical rotations with those reported in the literature. These characterization data confirmed that the three TK_{gst}-catalyzed reactions each led

to a single ketose with the expected L-*erythro* (3*S*,4*S*) configuration, consistent with the stereoselectivity observed with other aldehyde acceptors by TK_{gst} catalysis at elevated temperatures.[10,12,13]

Table 2. Conditions and results of FSA_{eco} catalyzed reactions for the synthesis of aldehydes **1**–**3**.

GA [mM]	FA [mM]	FSA_{eco} [mg]	*T* [°C]	Reaction time	Product	GA conversion [%][d]
50	100	4[a]	50	9 h	 L-glyceraldehyde **1**	100
200	–	4[b]	25	24 h	 D-threose **2**	98
200	–	25[c]	25	10 days	 L-erythrose **3**	96

[a] Wild-type FSA_{eco}, [b] Variant FSA_{eco} A129G, [c] Variant FSA_{eco} A165G/S166P, [d] Determined by HPLC and quantitative ^{1}H NMR relative to 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP-d_4) as internal standard.

Conclusions

The synthesis of three (3*S*,4*S*)-ketoses, L-ribulose **4**, D-tagatose **5** and L-psicose **6**, which are highly valuable compounds, was performed from *in situ* generated (2*S*)-α-hydroxy aldehydes **1**–**3** and HPA using an efficient sequential one-pot, three-step cascade sequence catalyzed by FSA_{eco} and thermostable TA_{tca} and TK_{gst}. This convergent procedure allowed circumventing the instability of HPA and the cost of expensive aldehyde precursors. The efficiency of this one-pot, three-step cascade process relied on the demonstrated compatibility of the three enzyme types. It allowed for an *in situ* generation of aldehydes catalyzed by FSA_{eco} variants from achiral, cheap aldehydes GA and FA. Moreover, also the sensitive HPA could be generated by TA_{tca} from L-Ser coupled with its irreversible consumption in a TK_{gst} reaction, thus avoiding its accumulation and decomposition. In addition, the thermostability of TA_{tca} and TK_{gst}, enabled the process to be performed at 60 °C, resulting in increased activities towards the (2*S*)-configured α-hydroxyaldehydes, which are poor TK_{gst} substrates at 25 °C. Consequently, excellent conversion rates in a reasonable time (24–96 h) were attained. This environmentally friendly, straightforward approach offers a useful alternative to the conventional chemical synthesis of these compounds, and seems applicable to a wider scope of related products, using other wild-type or variant carboligation catalysts.

Experimental Section

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. Sodium chloride was from Roth. L-Ser, Pyr, imidazole, ammonium sulphate and potassium chloride were purchased from Alfa Aesar. Pyridoxal 5-phosphate monohydrate (PLP) was from Acros Organics. L-Glyceraldehyde was from Molekula. Ni-NTA resin was from Qiagen. The 96-well microplates were shaken and incubated in a Titramax 1000 incubator (Heidolph). Lyophilization was carried out with a Triad Labconco dryer. UV-visible absorbances were measured using a Safire II-Basic plate reader from Tecan and a Perkin Elmer Lambda 25 spectrophotometer enabling Peltier-effect temperature control. Macherey-Nagel GmbH & Co KG 60 F254 silica gel TLC plates and Macherey-Nagel GmbH & Co KG 60/40–63 mesh silica gel for liquid flash chromatography were used. NMR spectra were recorded in D_2O and CD_3OD on a Bruker Avance 400 spectrometer. Optical rotation was determined with a P-2000 JASCO PTC-262 polarimeter at the given temperature and wavelength (Na-D-line: $\lambda_D = 589$ nm) in a cell 10 cm long. Optical rotations ($[\alpha]_D$) values are given in $dm^{-1}.g^{-1}.cm^3$. HPLC analyses were performed on an Acclaim™ 120 C18 5 μm 120 Å (4.6×250 nm) column from VWR (USA).

***In situ* ^{1}H NMR measurements**. Reactions were monitored using quantitative *in situ* ^{1}H NMR relative to 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP-d_4) as internal standard. Aliquots of reaction mixtures (450 μL) were mixed with 50 μL of TSP-d_4 (50 mM).

HPLC measurements. HPLC analyses and derivatisation were performed according to literature [1]. Samples (30 μL) were injected and eluted in the following conditions: solvent system (A) 0.1 % (v/v) aqueous trifluoroacetic acid (TFA) and (B) 0.1 % (v/v) TFA in CH_3CN/H_2O (4:1), gradient elution 10–95 % B in 30 minutes, flow rate 1 mL min^{-1}, detection at 215 nm, column temperature 30 C. Reaction samples (10 μL) were mixed with 50 μL of a solution of *O*-benzylhydroxylamine hydrochloride (21.1 mg mL^{-1}; 0.14 mmol mL^{-1}) in pyridine:methanol:water (33:15:2). Samples were then diluted in methanol (500 μL), centrifuged (5 min., 14,500 rpm, 25 °C) and the supernatant directly analysed by HPLC.

Preparative-Scale Enzymatic Cascade Synthesis

Wild-type and variants of L-fructose-6-phosphate aldolase from *E. coli* (FSA_{eco})[19] wild-type L-α-transaminase from *Thermosinus carboxydivorans* (TA_{tca})[18] and wild-type TK from *Geobacillus stearothermophilus* (TK_{gst})[11] were produced from recombinant cells and purified as previously described (SI).

Synthesis of L-glyceraldehyde 1. GA (50 mM) and FA (100 mM) were dissolved in H_2O (20 mL), and the pH was adjusted to 8 with 0.1 M NaOH. Wild-type FSA_{eco} (8 mg) was added to initiate the reaction, and the mixture was stirred at 50 C, 100 rpm. The reaction was monitored by *in situ* ^{1}H NMR and HPLC. After complete disappearance of GA (100 % conversion, 19 h), the reaction was stopped and the purity of L-glyceraldehyde **1** was analysed by ^{1}H NMR and HPLC.

(2*S*)-2,3-dihydroxy-propionaldehyde (L-glyceraldehyde) 1. The title compound was obtained in solution in unbuffered water R_f 0.82 (CH_2Cl_2:CH_3OH, 80:20 v:v). HPLC: $t_r = 15.78$ min. NMR data were identical to those previously reported.[27] ^{1}H NMR (400 MHz, D_2O): δ (ppm) (hydrate form): 4.92 (d, $J = 5.2$ Hz, 1H, C-1), 3.73 (dd, $J = 11.1$, 2.7 Hz, 1H, H-3), 3.61 (m, 1H, H-3′), 3.56 (m, 1H, H-2). ^{13}C NMR (101 MHz, D_2O): δ (ppm) (hydrate form): 98.7 (C-1), 74.0 (C-2), 61,6 (C-3).

Synthesis of D-threose 2. GA (200 mM) was dissolved in H_2O (5 mL) and the pH was adjusted to 8 with 0.1 M NaOH. FSA_{eco} A129G mutant (2 mg) was added to initiate the reaction, and the mixture was stirred at 25 C, 900 rpm. The reaction was monitored by *in situ* ^{1}H NMR and HPLC. After quasi-complete disappearance of GA (98% conversion, 24 h), the reaction was stopped and the purity of D-threose **3** was analysed by ^{1}H NMR and HPLC.

(2*S*,3*R*)-2,3,4-trihydroxy-butyraldehyde (D-threose) 2. The title compound was obtained in solution in unbuffered water,. R_f 0.36 (EtOAc:CH_3OH, 95:5 v:v). HPLC: $t_r = 13.96$ min. NMR data were identical to those previously reported[28] (ratio α-furanose/β-furanose/hydrate linear form = 52/38/10; lit. ratio α-anomer/β-anomer/linear hydrate form = 52/38/10).[29] ^{1}H NMR (400 MHz, D_2O): δ (ppm) (hydrate linear form): 5.02 (d, $J = 6.3$ Hz, 1H, H-1), 3.88 (ddd, $J = 7.5$, 5.0, 2.7 Hz, 1H, H-3), 3.65 (m, 1H, H-4), 3.63 ppm (m, 1H, H-4′), 3.45 ppm (dd, $J = 6.2$, 2.7 Hz, 1H, H-2); (α-D-*threo*-1,4-furanose): 5.40 (d, $J = 4.2$ Hz, 1H, H-1), 4.30 (m, 1H, H-3), 4.17 (m, 1H, H-4), 4.05 (m, 1H, H-2); (β-D-*threo*-1,4-furanose): 5.24 (d, $J = 1.2$ Hz, 1H, H-1), 4.23 (m, 1H, H-3), 4.21 (m, 1H, H-4′), 4.03 (m, 1H, H-2), 3.94 (dd, $J = 9.2$, 2.7 Hz, 1H, H-4). ^{13}C NMR (101 MHz, D_2O): δ (ppm) (α-D-*threo*-1,4-furanose): 103.4 (C-1), 81;9 (C-2), 76.4 (C-3), 74.3 (C-4); (β-D-*threo*-1,4-furanose): 97.9 (C-1), 77.4 (C-2), 76.1 (C-3), 71.8 (C-4); (hydrate linear form): 91.1 (C-1), 74.5 (C-2), 72.1 (C-3), 64.2 (C-4).

Synthesis of L-erythrose 3. GA (200 mM) was dissolved in H_2O (5 mL) and the pH was adjusted to 8 with 0.1 M NaOH. FSA_{eco} A165G/S166P mutant (5 mg) was added to initiate the reaction, and the mixture was stirred at 25 C, 900 rpm. After 2, 4, 6 and 8 days, FSA_{eco} mutant A165G/S166P (5 mg) was added. The reaction was monitored by *in situ* ^{1}H NMR and HPLC. After quasi-complete disappearance of GA (96%, 11 days), the reaction was stopped and the purity of L-erythrose 3 was analysed by ^{1}H NMR and HPLC.

(2*S*,3*S*)-2,3,4-trihydroxy-butyraldehyde (L-erythrose) 3. The title compound was obtained in solution in unbuffered water, R_f 0.32 (CH_2Cl_2:CH_3OH, 90:10 v:v). HPLC: $t_r = 13{,}74$ min. NMR data were identical to those previously reported[27,28] (ratio α-L-*erythro*-1,4-furanose/β-L-*erythro*-1,4-furanose/hydrate linear form = 62/27/11); lit. ratio α-L-*erythro*-1,4-furanose/β-L-*erythro*-1,4-furanose/linear hydrate form = 63/20/10).[9] ^{13}C NMR (101 MHz, D_2O) δ (ppm) (β-L-*erythro*-1,4-furanose): 102.4 (C-1), 77.6 (C-2), 72.5 (C-4), 71.7 (C-3); δ (α-L-*erythro*-1,4-furanose): 96.8 (C-1), 72,9 (C-4), 72,4 (C-2), 70.6 (C-3); δ (ppm) (hydrate linear form): 90.7 (C-1), 74.8 (C-2), 73.0 (C-3), 63.8 (C-4).

General procedure for ketose 4–6 synthesis. ThDP (0.1 mM), $MgCl_2 \cdot 6\ H_2O$ (1 mM), PLP (0.2 mM) and Pyr (50 mM) were dissolved in H_2O 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; 10 mg in the case of D-threose and L-erythrose) and TA_{tca} (4.8 mg), and the mixture was stirred for 20 minutes at 60 °C. In another flask, the reaction mixture containing (2*S*) α-hydroxy aldehydes **1**, **2** or **3** obtained previously with FSA_{eco} was diluted to obtain a concentration of 50 mM (except for L-glyceraldehyde) and L-Ser (150 mM) were mixed and the pH adjusted to 7 with 0.1 M NaOH. After pre-incubation of enzymes, cofactors and Pyr, L-Ser and (2*S*) α-hydroxy aldehydes (50–100 mM) in the reaction mixture were added, and the mixture was stirred at 60 °C. The final volume was 20 mL. The pH was maintained at 7 by adding 0.1 M HCl using a pH stat (Radiometer Analytical). The reaction was monitored by measuring Pyr and (2*S*) α-hydroxy aldehydes consumption by *in situ* ^{1}H NMR. In the case of D-threose and L-erythrose, the complete disappearance of Pyr was observed after 48 h and a second portion of Pyr (50 mM) was added. After total conversion of Pyr and (2*S*)-α-hydroxy aldehydes (24–96 h), silica was added to the solution, and the suspension was concentrated to dryness under reduced pressure and loaded onto a flash silica column. After silica gel chromatography using CH_2Cl_2:CH_3OH v:v 90:10 as eluent (for L-ribulose **4**) and EtOAc:CH_3OH v:v 100:0–90:10 as eluent (for D-tagatose **5** and L-psicose **6**), compounds **4**, **5** and **6** were isolated.

(3*S*,4*S*)-1,3,4,5-tetrahydroxypentan-2-one (L-ribulose) 4. The title compound was isolated as a yellow oil; yield: 84 mg, 56%. R_f 0.43 (DCM/CH_3OH, 8:2). $[\alpha]_D^{20} = +15.8$ (c 0.1, H_2O) lit. $[\alpha]_D^{20} = +16.5 \pm 1.5$ (c 0.1 H_2O). NMR data were identical to those previously described[30] (ratio α-anomer/β-anomer/linear form = 63/23/14; lit. ratio α-anomer/β-anomer/linear form = 58/24/18).[30] ^{13}C NMR (101 MHz, D_2O): δ (ppm) (linear form) 213.9 (C-2), 76.8 (C-3), 74.0 (C-4), 67.9 (C-1), 62.7 (C-5); δ (ppm) (α-anomer) 104.0 (C-2), 72.9 (C-5), 71.9 (C-3), 64.2 (C-4), 71.6 (C-1); δ (ppm) (β-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, $C_5H_{10}O_5Cl$ requires 185.0217.

(3*S*,4*S*,5*R*)-1,3,4,5,6-pentahydroxyhexan-2-one (D-tagatose) 5. The title compound was isolated as a white powder; yield: 93 mg, 52%. R_f 0.31 (DCM/CH_3OH, 8:2). $[\alpha]_D^{25} = -2.9$ (c 2, H_2O) lit. $[\alpha]_D^{20} = -2.5$.[31] NMR data were identical to those previously described[32] (ratio α-D-tagato-2.6-pyranose/β-D-tagato-2.6-pyranose/α-D-tagato-2.5-furanose/β-D-tagato-2.6-furanose = 72/22/2/4.; lit. ratio α-D-tagato-2.6-pyranose/β-D-tagato-2.6-pyranose/α-D-tagato-2.5-furanose/β-D-tagato-2.6-furanose = 79/14/2/5).[33] ^{13}C NMR (101 MHz, D_2O) δ (ppm) (α-D-tagato-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); δ (ppm) (β-D-tagato-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); δ (ppm) (α-D-tagato-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); δ (ppm) (β-D-tagato-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, $C_6H_{12}O_6Cl$ requires 215.0322.

(3*S*,4*S*,5*S*)-1,3,4,5,6-pentahydroxyhexan-2-one (L-psicose) 6. The title compound was isolated as a white powder; yield: 92 mg, 51%. R_f 0.31 (DCM/CH_3OH, 8:2). $[\alpha]_D^{20} = -2.3$ (c 0.1 H_2O) lit. $[\alpha]_D^{20} = -2.4$.[34] Data for D-psicose: +3.02 ($c = 1.16$ in H_2O)[44], +3.1 ($c = 1.62$ in H_2O).[35] NMR data were identical to those previously described for the D enantiomer:[32,36] (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). ^{1}H NMR (400 MHz, D_2O) δ (ppm) 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). ^{13}C NMR (101 MHz, D_2O) δ (ppm) (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 (ppm) (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 (ppm) (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 (ppm) (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 $[M+Cl]^-$ 215.0318, $C_6H_{12}O_6Cl$ requires 215.0322.

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Conflict of Interest

The authors declare no conflict of interest.

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