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## Synthesis of Deoxysugars from Aliphatic $\alpha$ -Ketoacids and Aldoses Catalysed by Thermostable Transketolase Variants from *Geobacillus stearothermophilus*

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**Abstract.** We described a strategy for the enzymatic synthesis of 1-deoxy and 1,2 deoxyketoses from the aliphatic  $\alpha$ -ketoacids, pyruvate and 2-oxobutyrate, as donors, and natural aldoses of variable chain length as acceptors, catalyzed by thermostable transketolase variants from *Geobacillus stearothermophilus* (TK<sub>gst</sub>). Analytical studies have been carried out on a panel of TK<sub>gst</sub> variants with the appropriate substrates allowing to select the best combinations and to apply it to the preparative scale synthesis of 1-deoxy and 1,2 deoxy ketoses obtained with good to excellent isolated yields (61%-86%). To optimize the strategy, and as a proof of principle, the  $\alpha$ -ketoacids pyruvate and 2-oxobutyrate were generated *in situ* from the corresponding D-aminoacids D-alanine and D-homoalanine respectively, using a thermostable D-aminoacid oxidase DAAO4536 that was selected from a screening of 55 putative DAAOs provided by Prozomix Limited.

Hence, a one-pot one step procedure was performed at 50 °C by coupling DAAO4536 and the best TK<sub>gst</sub> variant H102L/L118I/H474S in the presence of D-alanine or D-homoalanine as  $\alpha$ -ketoacids precursors and D-erythrose as acceptor substrate. The corresponding 1-deoxy and 1,2-dideoxydeoxyketoses were isolated with good yields (64% and 72% respectively, out of two steps).

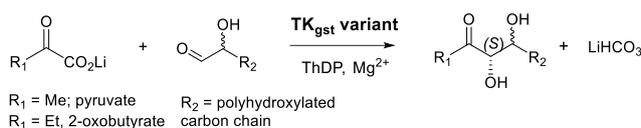
**Keywords:** biocatalysis; transketolase; D-amino acid oxidase; hydroxypyruvate; deoxyketoses; stereoselectivity.

## Introduction

Deoxysugars are components of many bioactive molecules such as antibiotics, antimicrobials and various other therapeutic agents.<sup>1</sup> The absence of hydroxyl functions in certain positions often modifies the binding to receptors by improving efficiency or making it possible to reach new targets.<sup>2</sup> The synthesis of new deoxysugars allows to diversify the toolbox used to obtain glycoconjugates. The common ways are total synthesis or regioselective deoxygenation of natural monosaccharides requiring multistep strategies due to the need for protective group.<sup>3</sup> An alternative is the use of biocatalytic ways particularly carboligases which directly generates deoxygenated sites by the

choice of suitable enzyme and substrates. This strategy avoids the protection of alcohol functions and requires mild conditions. Among carboligases, aldolases such as dihydroxyacetone phosphate (DHAP)-dependent aldolase,<sup>4</sup> fructose 6-phosphate aldolase (FSA) from *E. coli*,<sup>5</sup> transaldolase B from *E. coli* (TalB)<sup>6</sup> were largely used for asymmetric synthesis by the preparation of a large set of stereochemically pure deoxysugars from simple precursors. Another enzyme belonging to carboligase family, Transketolase (TK), catalyzes the synthesis of a wide range of ketoses including deoxyketoses by stereoselective C-C bond formation in the presence of thiamine diphosphate (ThDP) and Mg<sup>2+</sup> as cofactors.<sup>7</sup> Indeed, wild type TK transfers a ketol unit from 3-hydroxypyruvate (HPA), which is the most commonly used synthetic donor substrate, to

an aldehyde as acceptor substrate. The reaction is rendered irreversible by the release of carbon dioxide. The product of the reaction is a (3*S*)-ketose (or an analog) depending on the acceptor substrates. Recent studies showed that aliphatic  $\alpha$ -ketoacids can be recognized as donor substrates by specially designed TK variants.<sup>8</sup> Particularly, the thermostable TK from *Geobacillus stearothermophilus*<sup>9</sup> has been engineered to accept pyruvate, 2-oxobutyrate, 3-methyl-2-oxobutyrate as donors in place of HPA (scheme 1). Preparative scale syntheses were performed in the presence of glycolaldehyde and D-glyceraldehyde as acceptors leading to the corresponding deoxysugars.<sup>10</sup>



**Scheme 1.** Synthesis of chiral 1- and 1,2-deoxyketoses from aliphatic  $\alpha$ -ketoacids as donors and natural aldoses as acceptors.

The purpose of this study was to improve and to extend the product scope of TK<sub>gst</sub> with new efficient variants in the presence of  $\alpha$ -ketoacids, pyruvate and 2-oxobutyrate as donors, and aldoses with increased carbon chain length as acceptors, such as glycolaldehyde, D-glyceraldehyde, L-glyceraldehyde D-erythrose, D-ribose and D-xylose. Analytical studies were first conducted with different TK<sub>gst</sub> variants and substrates to determine the relative activities of each enzyme. The best combinations were selected and applied at preparative scale to obtain the corresponding deoxyketoses. To optimize the strategy, the aliphatic  $\alpha$ -ketoacids pyruvate and 2-oxobutyrate were generated *in situ* from the corresponding D-aminoacids, D-alanine and D-homoalanine respectively, using a thermostable D-aminoacid oxidase (DAAO) that was selected from a panel of 55 putative DAAOs. As a proof of principle, a one-pot one step procedure was performed, by coupling DAAO4536 and the best TK<sub>gst</sub> variant H102L/L118I/H474S for D-erythrose. Some of the targeted 1- or 1,2-deoxyketoses, display interesting biological properties. For instance, 1,2-dideoxy-D-threo-3-hexulose **8** was used as a synthon to obtain (2*R*,3*R*,4*R*)-2-(hydroxymethyl)pyrrolidine-3,4-diol as a potent inhibitor of  $\beta$ -mannosidase<sup>11</sup> and 1-deoxy-D-fructose **14** can serve as a potential metabolic inhibitor and an antimetabolite.<sup>12</sup>

## Results and Discussion

### Selection of TK<sub>gst</sub> variants for the targeted substrates

Recently, we reported on the triple TK<sub>gst</sub> variants H102L/H474 (S, G, and A)/L118I<sup>10b</sup> that were found to cleave ( $\pm$ )-propionin in the presence of glycolaldehyde as acceptor substrate, leading to the concomitant release of propanal and 1,2-dihydroxypentan-3-one, with 82 and 79% yields respectively, thus proving the transfer of the acyl group on glycolaldehyde. In this reaction, the L118I mutation played a key role offering novel potentiality of TK<sub>gst</sub> catalyzed-reaction. This mutation was never investigated before within the frame of the synthesis of valuable ketose analogs by stereoselective carbon-carbon bond formation from aliphatic donor and aldoses as acceptor. Following the principle of the microreversibility of TK<sub>gst</sub> reaction, we expected that the combination of L118I with the single position H102L<sup>10a</sup> or with the double-site mutation H102L/H474 (S, G)<sup>10a</sup> could lead to the desired products with high efficiency.

### Screening of TK<sub>gst</sub> variants toward donor and acceptor substrates

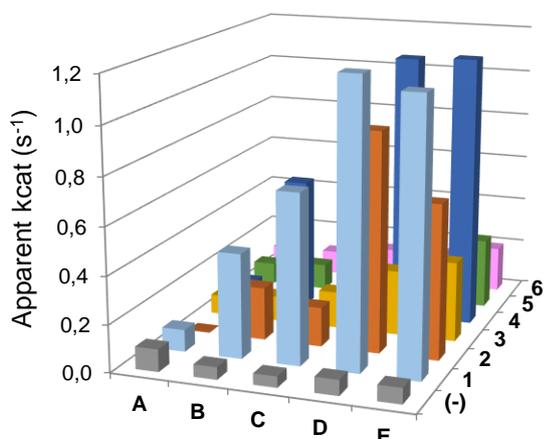
TK<sub>gst</sub> variants were expressed in *E. coli* BL21(DE3)pLysS strain and purified by Ni<sup>2+</sup> chelating affinity column chromatography. Their specific activities with aldoses as acceptors coupled with pyruvate or 2-oxobutyrate as donors were determined and compared to H102L/H474S and wild-type TK<sub>gst</sub> (Figure 1). To measure specific activities, we used an efficient, direct, quantitative high-throughput colorimetric assay developed earlier and based on pH changes caused upon TK catalyzed  $\alpha$ -ketoacid decarboxylation.<sup>13</sup> Phenol red was used as the pH indicator and absorbance variation was measured at 620 nm, at 37 °C.

In the presence of 2-oxobutyrate (Figure 1a), the results showed that both triple variants H102L/L118I/H474S and H102L/L118I/H474G led to the best improvement compared to the double variant H102L/H474S already described. The  $k_{cat}$  were particularly increased for H102L/L118I/H474S in the presence of glycolaldehyde **1**, D-glyceraldehyde **2** and D-erythrose **4** as acceptors (giving 2.5, 4.2 and 2 fold higher  $k_{cat}$  respectively compared to those obtained with H102L/H474S). The lower improvement obtained with the double variant H102L/L118I showed the importance of the replacement of His474 by Ser.

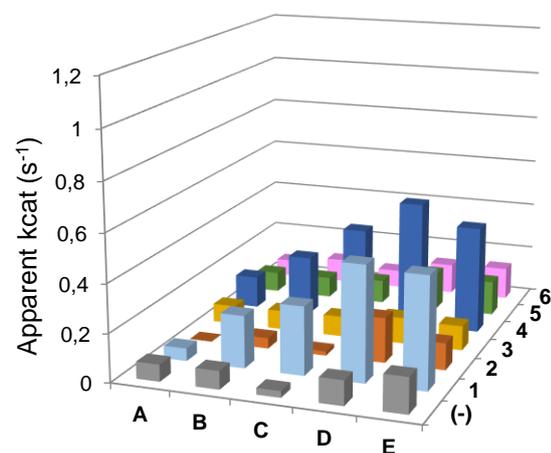
The results obtained in the presence of pyruvate as donor (Figure 1b) gave also the best  $k_{cat}$  with H102L/L118I/H474S particularly toward glycolaldehyde **1**, D-glyceraldehyde **2** and D-erythrose **4** as previously observed (2.2, 4, and 1.25 fold

improvement respectively compared to H102L/H474S) even if the  $k_{cat}$  were overall lower than those obtained with 2-oxobutyrate.

a)



b)



**Figure 1.** Three-dimensional illustration of the relative apparent  $k_{cat}$  upon catalysis by wild type  $TK_{gst}$  enzyme and some variants for the array of aldoses as acceptors and using either 2-oxobutyrate as donor a) or pyruvate b). Reaction were carried out in 200  $\mu$ L wells in 2 mM TEA buffer pH 6.8, containing ThDP 0.2 mM,  $MgCl_2$ , 1mM, Phenol Red 84  $\mu$ M, in the presence of both donors and acceptors at 50 mM, using 8  $\mu$ g of pure enzyme at 37 °C. Acceptor substrates: glycolaldehyde **1**; D-glyceraldehyde **2**; L-glyceraldehyde **3**; D-erythrose **4**; D-ribose **5**; D-xylose **6**.  $TK_{gst}$  variants used as biocatalysts: wt enzyme **A**; H102L/H474S **B**; H102L/L118I **C**; H102L/L118I/H474S **D**; H102L/L118I/H474G **E**.

### Synthesis of deoxyketoses with $TK_{gst}$ variants

Considering the results previously obtained at analytical scale, the same variants and substrates were used to perform the syntheses at 50 micromole scale, at 50 °C, in the presence of 1 mg  $TK_{gst}$  variant in 1 mL phosphate buffer adjusted at pH 7.0. At this temperature,  $TK_{gst}$  activity was 7 times higher than at 20 °C, thus allowing significantly shorter reaction times.<sup>[14]</sup> The thermostability study of the  $TK_{gst}$

variants showed a total retention of enzymatic activity after 5 days at 50 °C. The progress of the reaction was followed by TLC on silica plates after 24 h of reaction time allowing to visually estimate the conversion (Table 1).

In a general way, the best conversions were obtained using 2-oxobutyrate compared to pyruvate and particularly with the variants **C** H102L/L118I **D** H102L/L118I/H474S and **E** H102L/L118I/H474G compared to wild type enzyme and to **B** H102L/H474S variant previously described in the literature, showing the importance of the L118I mutation. Similarly, with 2-oxobutyrate as donor substrate assayed, all variants that possess the L118I mutation (**C**, **D**, **E**) improved the conversion toward aldoses with increase long carbon chain aldoses (**4**, **5**, **6**) compared to the **B** H102L/H474S variant under the same conditions. Except with **1**, the presence of H474S (and G) combined with H102L/L118I greatly increased the conversion of aldoses bearing a shorter carbon chain (**2**, **3**) compared to the double variants **B** H102L/H474S and **C** H102L/L118I. By using pyruvate as a donor, the improvements relating to substrate conversion rates remain lower. We note that the presence of H474S (or G) in the double and triple variants (**B**, **D**, **E**) were required to enhance the conversion particularly toward **2** and **4** in comparison with the results obtained with **C** H102L/L118I.

		2-oxobutyrate				
Electrophile		<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>
<b>1</b>		-	++	+	++++	+
<b>2</b>		-	++	+	++++	++++
<b>3</b>		-	++	++	+++	++
<b>4</b>		-	++	++++	++++	+++
<b>5</b>		-	+	+++	++	++
<b>6</b>		-	-	++	+	+

		Pyruvate				
Electrophile		<b>A</b>	<b>B</b>	<b>C</b>	<b>D</b>	<b>E</b>
<b>1</b>		-	++	++	++	-
<b>2</b>		-	++	+	+++	++
<b>3</b>		-	+	+	-	+
<b>4</b>		-	+++	+	+++	++
<b>5</b>		-	+	-	+	++
<b>6</b>		-	+	-	+	+

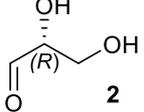
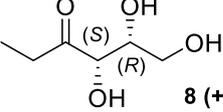
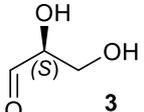
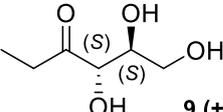
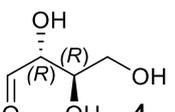
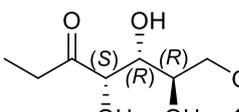
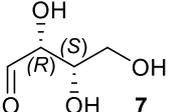
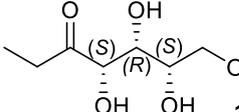
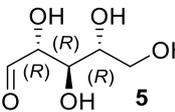
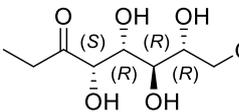
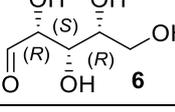
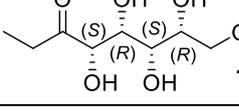
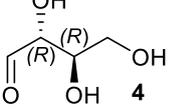
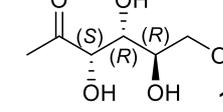
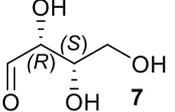
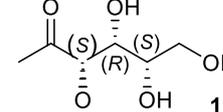
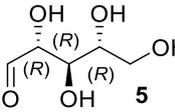
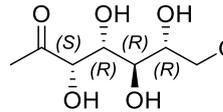
**Table 1.** Classification of  $TK_{gst}$  variant substrate quality: +++++; full conversion, +++; good conversion >75%, ++; moderate conversion 25-75%, +; fair conversion <25%, or poor-; no product detectable by TLC. Conversion was determined by TLC analysis after 24h of reaction time at 50 °C (50 mM acceptor and 50 mM donor, 1 mg  $TK_{gst}$  variant/mL of reaction, phosphate buffer 50 mM pH 7.0). Acceptor substrates: **1**, glycolaldehyde; **2**, D-glyceraldehyde; **3**, L-glyceraldehyde; **4**, D-erythrose; **5**, D-ribose; **6**, D-xylose. wt  $TK_{gst}$  **A** and variants H102L/H474S

**B;** H102L/L118I **C;** H102L/L118I/H474S **D;** H102L/L118I/H474G **E.**

Then, the syntheses were performed at 1 millimole preparative scale at 50 °C in the presence of 1 mg TK<sub>gst</sub> variant per mL in a final volume of 20 mL. The pH was kept constant at 7 by pH-stat control. The substrates and products were quantified by *in situ* <sup>1</sup>H NMR analysis of aliquots taken from the reaction mixtures upon time, allowing the measurement of the

final conversion levels (SI). The products were purified by silica gel chromatography and characterized by NMR.

Apart from **16**, all other 1- and 1,2-deoxy ketoses were obtained with good to excellent *in situ* and isolated yields (Table 2).

Donor	Acceptor	Product	Time (h)	TK <sub>gst</sub> variant	TK <sub>gst</sub> mg/mL	<i>In situ</i> Yield <sup>a</sup> (%)	Isolated Yield (%)
2-oxobutyrate			8	H102L/L118I/H474S	0.5	>95	81 ( <b>8</b> , 75) ( <b>9</b> , 6)
			36	H102L/L118I/H474S	1	71	63 ( <b>9</b> , 37) ( <b>8</b> , 26)
			24	H102L/L118I	0.5	85	71
			6	H102L/L118I/H474S	0.5	89	86
			72	H102L/L118I	3	68	68
			48	H102L/L118I	3	82	61
Pyruvate			24	H102L/L118I/H474S	1	74	68
			24	H102L/L118I/H474S	1	81	60
			36	H102L/L118I/H474G	3	55	n.d

<sup>a</sup>Determined by *in situ* <sup>1</sup>H NMR using TSP-d<sup>4</sup> as an internal standard and calculated on the basis of *in situ* product formation.

**Table 2:** Synthesis of deoxyketoses at 50 °C using TK<sub>gst</sub> variants in the presence of 2-oxobutyrate and pyruvate as donors and aldoses as acceptors (**2**, D-glyceraldehyde; **3**, L-glyceraldehyde; **4**, D-erythrose; **5**, D-ribose; **6**, D-xylose; **7**, L-threose).

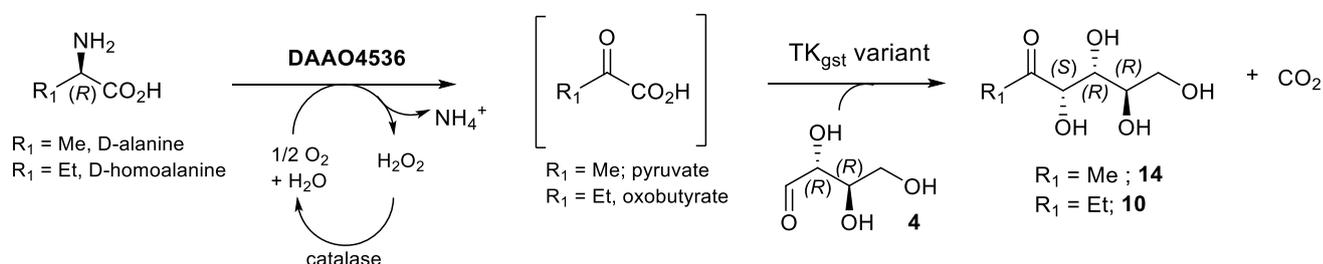
The 1,2-deoxyketoses prepared from 2-oxobutyrate **8-13** (table 2) were synthesized using the best variants H102L/L118I/ H474S identified previously (table 1) toward short carbon chain (**2** and **3**) and with H102L/L118I showing higher activities with longer carbon chain aldoses **4, 5** and **6**. The 1,2-deoxyketoses **10-13** were obtained in pure form, while the syntheses with H102L/L118I/H474S variant carried out with both enantiomers of glyceraldehyde, *i.e.* D-glyceraldehyde **2** and L-glyceraldehyde **3** led to a mixture of epimers (**8+9**), which could not be separated each other by chromatography on silica gel. Those results were possibly due to the slight epimerization of **2** in **3** and *vice et versa*, within the reaction medium, at 50 °C. Thus, product **8** was obtained with 75% yield from **2**, along with compound **9**, that was isolated in 6% yield. Starting from L-glyceraldehyde **3**, the expected product **9** was isolated in 37% yield in a mixture with epimer **8** (26% yield). To note, L-glyceraldehyde **3** is a poorer substrate than D-glyceraldehyde **2** (see Table 1, entries 1 and 2; reaction time of 36 h for the synthesis of **9** vs 8h for **8** and use of twice as much enzyme), which may explain the product ratios obtained for these two syntheses, *i.e.* 92/8 in favor of the desired epimer **8** (vs **9**) in the case of the synthesis carried out from **2**, whereas it is a ratio of 58/42 in favor of the desired epimer **9** in the case of the synthesis carried out from **3**.

H102L/L118I/ H474S variant was selected for the syntheses of 1-deoxyketoses **14** and **15**, in the presence of pyruvate as donor and aldoses D-erythrose **4**, and L-

threose **7** (C3 epimers), as acceptors. We note that both products **14** and **15** were obtained in pure forms with similar good yields showing that the configuration on C3 did not affect the enzymatic activity. Taking into account the previous results at analytical scale (Figure 1b), H102L/L118I/ H474G was used to convert aldose **5** but the product **16** was not recovered in pure form after chromatography because it was co-eluted with the remaining acceptor substrate **5**.

### Synthesis of deoxyketoses by coupling TK<sub>gst</sub> variants with a thermostable D-aminoacid oxidase

Previous studies developed by our group have already described the *in situ* generation of the common TK<sub>gst</sub> α-ketoacid donor, 3-hydroxypyruvate (HPA) from D-serine<sup>14</sup> using a DAAO from *Rhodotorula gracilis* yeast (DAAO<sub>Rg</sub>).<sup>15</sup> DAAOs (EC 1.4.3.3) are flavin adenine dinucleotide (FAD)-containing flavoenzymes that catalyze the oxidation of D-amino acids to their imino acid counterparts with concomitant reduction of FAD which is subsequently re-oxidized by molecular oxygen with generation of hydrogen peroxide. The released iminoacid spontaneously hydrolyses to the corresponding α-ketoacid and ammonia. Since the accumulation of hydrogen peroxide can lead to an enzyme inhibition, addition of catalase is required for the dismutation of hydrogen peroxide to water and oxygen (Scheme 2).



**Scheme 2.** Simultaneous cascade synthesis catalysed by coupling TK<sub>gst</sub> variant with a thermostable D-aminoacid oxidase (DAAO4536).

D-amino acid	Acceptor	Product	Time (h)	TK <sub>gst</sub> variant	<i>In situ</i> Yield <sup>a</sup> (%)	Isolated Yield (%)
			24	H102L/ L118I	92	64
			24	H102L/ L118I/ H474S	88	72

<sup>a</sup>) Determined by *in situ* <sup>1</sup>H NMR using TSP-d<sup>4</sup> as an internal standard and calculated on the basis of *in situ* product formation

**Table 3:** Simultaneous cascade synthesis catalysed by TK<sub>gst</sub> and DAAO4536.

As DAAO<sub>Rg</sub> is not thermostable, it cannot be used at 50 °C in a one pot cascade reaction with thermostable TK<sub>gst</sub>. We recently found a thermostable DAAO named DAAO4536 from a speculative panel of 55 putative DAAOs coming from a metagenomic library provided by Prozomix Limited. This DAAO accepts a wide range of D-aminoacids as substrates including D-alanine and D-homoalanine, precursors of the corresponding  $\alpha$ -ketoacids, pyruvate and 2-oxobutyrate respectively (SI). At 50 °C, DAAO4536 kept all its activity after 4 hours and retained 40% after 20 hours, while DAAO<sub>Rg</sub> was almost inactive after 4 hours (SI). To optimize the strategy, and as a proof of principle, the aliphatic  $\alpha$ -ketoacid pyruvate and 2-oxobutyrate were *in situ* generated from the corresponding D-alanine and D-homoalanine respectively using DAAO4536 (Scheme 2).

Hence, an efficient one-pot one step procedure was performed at 50 °C by coupling of DAAO4536 in the presence of D-alanine or D-homoalanine and the best TK<sub>gst</sub> variant H102L/L118I/H474S for D-erythrose **4** (Table 3). The corresponding 1,2-dideoxyketose **10** and 1-deoxy-ketose **14** were obtained with comparable conversion yields and slightly different isolated yields (64% and 72% against 86% and 60% respectively), compared to the results obtained previously from the commercial pyruvate and 2-oxobutyrate.

## Conclusion

This work showed firstly at analytical scale that the mutation L118I in TK<sub>gst</sub> combined with H102L or with H102L and H474S increased particularly TK<sub>gst</sub> activities toward aliphatic donor 2-oxobutyrate, and to a lesser extent, toward pyruvate, when coupled with long carbon chain aldoses as acceptors. The TK<sub>gst</sub> variants H102L/L118I and H102L/L118I/H474S were applied at 1 millimole scale for the preparative synthesis of eight 1- and 1,2-deoxyketoses obtained with good to excellent isolated yields (60%-86%). In addition, following a one-pot one step procedure at 50 °C, we investigated the simultaneous coupling of DAAO4536 in the presence of D-alanine or D-homoalanine precursors of the  $\alpha$ -ketoacids, pyruvate and 2-oxobutyrate respectively, and H102L/L118I/H474S TK<sub>gst</sub> variant that displayed the best activity for D-erythrose as acceptor. The corresponding 1-deoxy **10** and 1,2-dideoxydeoxyketose **14** were isolated with good yields (64% and 72%). By following this enzymatic strategy, it is possible to efficiently synthesize new 1- and 1,2-deoxysugars, as components of bioactive molecules. More generally, this approach constitutes a tool of choice for obtaining a variety of ketose analogues using natural or non-natural  $\alpha$ -ketoacids *in situ* generated from the corresponding aminoacids.

## Experimental Section

**General.** All chemicals were purchased from Sigma-Aldrich, Alfa-Aesar TCI chemicals and CarboSynth. Bradford reagent was from Bio-Rad. Ni-NTA resin was obtained from QIAGEN. Lyophilisation was carried out with Triad LABCONCO dryer. UV-visible absorbance was measured using a Spark control 10 microplate reader from TECAN and an Agilent Technologies. MARCHEREY-NAGEL GmbH & Co KG 60/40-63 mesh silica gel for Liquid Flash Chromatography and MARCHEREY-NAGEL GmbH & Co KG 60 F254 silica gel TLC plates with anisaldehyde stain for detection were used. Reaction pH for preparative synthesis was maintained using a TitroLine®7000 autotitrator. NMR spectra were recorded in D<sub>2</sub>O on a 400MHz Bruker Avance III HD and 500MHz Avance III HD spectrometers. Chemical shifts are referenced to the residual solvent peak. The following multiplicity abbreviations are used: (s) singlet, (d) doublet, (t) triplet, (m) multiplet.

**Expression of TK<sub>gst</sub> and DAAO<sub>Rg</sub>.** Expression was carried out in *E. coli* BL21(DE3)pLysS strain. This strain was transformed by heat shock with the following TK<sub>gst</sub> variants: artificial wild type TK<sub>gst</sub>,<sup>9</sup> H102L/H474S,<sup>10a</sup> H102L/L118I/H474S,<sup>10b</sup> H102L/L118I/H474G,<sup>10b</sup> and H102L/L118I.<sup>10b</sup> DAAO<sub>Rg</sub> was overexpressed<sup>16</sup> with the plasmid pT7. The strains were stored at -80 °C in glycerol 60% (10% final). A 100  $\mu$ l culture aliquot of each of the clones was transferred into 50 ml liquid LB medium containing kanamycin (30  $\mu$ g.mL<sup>-1</sup>) and grown at 27 °C, 200 rpm for 12 h. The pre-culture (20 ml) was used to inoculate 1 liter of culture medium containing kanamycin (30  $\mu$ g.mL<sup>-1</sup>) and grown at 37 °C, 200 rpm. Isopropyl  $\beta$ -D-1-thiogalactopyranoside (IPTG) at 0.5 mM was added when the OD<sub>600nm</sub> range reached 0.6 - 0.8 A.U. Then, cells were grown at 30 °C, 200 rpm, overnight. After that, cells were recovered by centrifugation (8,000 rpm, 4 °C for 15 min), washed twice with phosphate buffer NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O (50 mM), NaCl (300 mM) at pH 8.0 and finally harvested (4,000 rpm, 4 °C for 15 min). The culture medium was removed and the bacterial pellets were stored at -25 °C ( $\approx$  5 g of wet bacterial pellet•L<sup>-1</sup> of biomass for TKs).

### Expression of DAAO4536

Expression of DAAO4536 was carried out in *E. coli* BL21(DE3) competent cells. The strain was transformed by heat shock and plated on to 2% LB agar plates containing kanamycin (35  $\mu$ g.mL<sup>-1</sup>) and incubated at 37°C for 16 h. A pre-culture (10 ml) was used to inoculate 0.8 liter of auto-induction culture medium containing kanamycin (35  $\mu$ g.mL<sup>-1</sup>) and grown at 25°C, 130 rpm for 24 h. Cells were recovered by centrifugation (8,000 rpm, 4°C for 5 min) and washed with sodium phosphate buffer (50 mM, pH 7.5). Cell lysis was performed via sonication and finally harvested (10,000 rpm, 4°C for 30 min). The supernatant was stored at -20°C before lyophilization by freeze-drying at 10<sup>-1</sup> mbar.

**Purification of TK<sub>gst</sub> variants and DAAO<sub>Rg</sub>.** Harvested recombinant cells from 1 liter of culture were resuspended in 35 mL of phosphate buffer (50 mM) containing NaCl

(300 mM) at pH 8.0 for TKs and 14 mL of phosphate buffer (50 mM) containing 2-mercaptoethanol (5 mM) and FAD (0.1 mM) at pH 7.2 for DAAO. The cells were disrupted by sonication on ice for 30 min and the insoluble pellets were discarded after centrifugation at 8000 rpm for 15 min at 4 °C. Crude extracts were applied to a Ni-NTA column equilibrated with phosphate buffer for TKs and with phosphate buffer (50 mM) containing NaCl (1 M), imidazole (20 mM) and glycerol 5% at pH 7.2 for DAAO. After washing each column with the same buffer respectively for TKs and DAAO<sub>Rg</sub>, the His6-tagged TKs or DAAO<sub>Rg</sub> were finally eluted with phosphate buffer (50 mM) containing NaCl (300 mM) and imidazole (500 mM) at pH 8.0 for TKs and phosphate buffer (50 mM) containing glycerol (5%) imidazole (50 mM) at pH 7.2 for DAAO<sub>Rg</sub>. The fractions containing the eluted proteins were collected and dialyzed against triethanolamine buffer (2 mM, pH 7.5) and then against water (pH 7.5) through dialysis tubing (cut-off 14 kDa) at 4 °C for TKs and twice against water (pH 7.5) through dialysis tubing at 4 °C for DAAO<sub>Rg</sub>. Then, these protein solutions were lyophilized. Protein concentration was determined by the Bradford method and bovine serum albumin (BSA) was used as the standard.

**Determination of wild-type TK<sub>gst</sub> or variant TK<sub>gst</sub> activity.** It was achieved using the pH based assay for transketolase as reported on earlier.<sup>13</sup> One unit of TK<sub>gst</sub> activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of ketose product per minute at 37 °C in TEA buffer (2 mM, pH 6.8). TK<sub>gst</sub> enzymatic assay was performed in the presence in 96-well plates at 37 °C (200 μL per well). The assay mixture contained TK<sub>gst</sub> (8 μg), acceptor (50 mM), ThDP (0.1 mM), MgCl<sub>2</sub> (1 mM), TEA (2 mM, pH 7.0) and phenol red (0.084 mM). α-Ketoacid (50 mM) was added to start the reaction. Absorbance was measured at 620 nm using a microplate reader. Absorbance data were converted into concentrations of HCO<sub>3</sub><sup>-</sup> according to the standard curve of the assay that was determined using a series of NaHCO<sub>3</sub> concentrations (0–0.5 mM) in a 96-well plate.

**Determination of DAAO activity.** DAAO activities were determined using an enzymatic assay reported on in the literature<sup>17</sup> and based on the quantification of hydrogen peroxide using horseradish peroxidase as an auxiliary enzyme in the presence of 4-aminoantipyrin as the chromogenic dye. One unit of DAAO<sub>Rg</sub> activity was defined as the amount of enzyme that catalyzes the formation of 1 μmol of ketoacid product per minute at 25 °C in Tris-HCl buffer (50 mM, pH 7.5). The DAAO enzymatic assay was performed in 96-well plates at 25 °C (200 μL per well). The assay mixture contained DAAO (0.1–1 μg), FAD (0.01 mM), HRP (2.5 units, 10 μg) D-aminoacid (5–50 mM), 4-aminoantipyrin (3 mM), vanillic acid (4.75 mM). D-amino acid (5–50 mM) was added to start the reaction. Absorbance was measured at 498 nm using a microplate reader. DAAO activities were extrapolated from Lambert-Beer law ( $\epsilon_{498} = 5100 \text{ M}^{-1} \cdot \text{cm}^{-1}$ ).

**Determination of DAAO thermostability.** DAAO enzyme at 5 mg.mL<sup>-1</sup> was incubated in 1 mL Tris-HCl buffer (50 mM, pH 7.5) at 50 °C in the presence of FAD (0.01mM). 20μL aliquots were taken out from this reaction mixture and

residual DAAO activity was determined following the HRP-linked spectrophotometric assay used for the determination of DAAO activity.

**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)propionic-2,2,3,3-d<sub>4</sub> acid (TSP-d<sub>4</sub>) as internal standard. Aliquots of the reaction mixture were removed overtime (450 μL) and mixed with 50 μL of TSP-d<sub>4</sub> (50 mM in D<sub>2</sub>O).

**General procedure of TK<sub>gst</sub> catalyzed reaction.** In a 100 mL, three-neck, round bottom flask, ThDP (1 mg, 0.1 mM) and MgCl<sub>2</sub>·6H<sub>2</sub>O (4 mg, 1 mM) were dissolved in H<sub>2</sub>O (20 mL, total volume) and the pH adjusted to 7.0 using 0.1 M NaOH. To this solution, the lyophilized TK<sub>gst</sub> (H102L/H474S, H102L/H474S/L118I, H102L/H474G/L118I or H102L/L118I) enzyme (0.5–3 mg.mL<sup>-1</sup>) was added and the mixture stirred at 150 rpm at 50 °C. The reaction was initiated by donor addition (sodium 2-oxobutyrate, 149 mg, 60 mM, 1.2eq. or sodium pyruvate, 110 mg, 50 mM, 1eq.). The pH of the reaction mixture was automatically maintained at 7 throughout time by addition of 0.1 M HCl using a pH autotitrator. The reaction progress was followed by measuring ketoacids and aldehydes consumption by *in situ* <sup>1</sup>H NMR and TLC. After total substrates conversion (8–72 hours), TK enzyme were discarded from the solution by precipitation with 15 ml of methanol followed by centrifugation at 4000 rpm for 10 min at 20 °C. Then, 1 mL of 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 purification stage was performed by using ethyl acetate/MeOH/H<sub>2</sub>O 95/2.5/2.5 eluent for products **12,14** and **15**, ethyl acetate/MeOH 95/5 for products **8,9** and **10** and ethyl acetate/MeOH/H<sub>2</sub>O 90/5/5 for products **11–13**.

**General procedure of simultaneous synthesis catalyzed by TK<sub>gst</sub> and DAAO.** For simultaneous cascade synthesis, ThDP (1 mg, 0.1 mM) and MgCl<sub>2</sub>·6H<sub>2</sub>O (4 mg, 1 mM) D-erythrose (120 mg, 50 mM) were dissolved in H<sub>2</sub>O (20 mL, total volume) and the pH adjusted to 7.0 using 0.1 M NaOH. To this stirred solution were added catalase (1 mg/2h, 1998-4995 U/2h), TK<sub>gst</sub> H102L/L118I (0.5 mg.mL<sup>-1</sup>) or TK<sub>gst</sub>H102L/L118I/H474S (1 mg.mL<sup>-1</sup>) variants and DAAO (0.25 mg/mL) giving a final volume of 20 mL. Oxygen was bubbled into the reactor (10 mL.min<sup>-1</sup> at atmospheric pressure). The reaction was initiated by donor addition (D-alanine, 60 mM, 1.2eq. or D-homoalanine, 110 mg, 50 mM, 1eq.). The reaction mixture was stirred (100 rpm) at 50 °C and the pH was automatically maintained at 7 by adding 0.1 M HCl using a pH stat. The reaction progress was followed by measuring D-aminoacid and D-erythrose consumption by *in situ* <sup>1</sup>H NMR and TLC. After total conversion (24 h) the enzymes were discarded from the solution by precipitation with 15 ml of methanol followed by centrifugation at 4000 rpm for 10 min at 20 °C. Then, 1 mL of 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

purification stage was performed by using ethyl acetate/MeOH 95/5 for product **10** and ethyl acetate/MeOH/H<sub>2</sub>O 95/2.5/2.5 eluent for product **14**.

#### 1,2-dideoxy-D-*threo*-hex-3-ulose **8**

**8** was isolated as a white powder (111 mg) in 75% yield (along with 6% for **9**; 9 mg) with TK<sub>gst</sub> variant H102L/L/118I/H474S; TLC: R<sub>f</sub> 0.41 (Methanol/ethyl acetate, 0.5/9.5 v:v) product ratio : α-furanose anomer (6%) : β furanose anomer (14%) : open chain (80%) ; open chain : <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 1.05 (t, 3H, J = 7.2 Hz, H-1), 2.67 (m, 2H, H-2), 3.64 (dd, 1H, J = 7.1 and 11.4 Hz, H-6a), 3.72 (dd, 1H, J = 5.8 and 11.4 Hz, H-6b), 4.16 (m, 1H, H-5), 4.41 (d, J = 2.1 Hz, H-4) ; <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 6.7 (C-1), 31.9 (C-2), 62.2 (C-6), 71.7 (C-5), 76.7 (C-4), 215.7 (C-3) ; β furanose anomer : <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 0.96 (t, 3H, J = 7.5 Hz, H-1), 1.76 (m, 2H, H-2), 3.60 (dd, 1H, J = 4.8 and 9.7 Hz, H-6a), 3.90 (d, 1H, J = 5.4 Hz, H-4), 4.16 (m, 1H, H-6b), 4.34 (ddd, 1H, J = 1.1, 5.4, and 10.9 Hz, H-5) ; <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 6.9 (C-1), 30.1 (C-2), 69.4 (C-6), 75.2 (C-5), 78.8 (C-4), 104.5 (C-3) ; α-furanose anomer : <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 0.99 (t, 3H, J = 7.6 Hz, H-1), 1.76 (m, 2H, H-2), 3.86 (dd, 1H, J = 6.3 and 12.9 Hz, H-6a), 3.99 (d, 1H, J = 1.0 Hz, H-4), 4.24 (m, 2H, H-5 and H-6b) ; <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 7.2 (C-1), 26.8 (C-2), 72.0 (C-6), 76.3 (C-5), 79.6 (C-4), 108.2 (C-3); m/z HRMS ESI-MS calcd for C<sub>6</sub>H<sub>12</sub>O<sub>4</sub>Na : 171.0633 ; found [M+Na]<sup>+</sup> : 171.0627

#### 1,2-dideoxy-L-*erythro*-hex-3-ulose **9**

**9** was isolated as a white powder (49 mg) in 37% yield (along with 26% for **8**; 35 mg) with TK<sub>gst</sub> variant H102L/L/118I/H474S ; TLC: R<sub>f</sub> 0.41 (Methanol/ethyl acetate, 0.5/9.5 v:v) product ratio : β furanose anomer (8%) : α-furanose anomer (14%) : open chain (78%) ; open chain : <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 1.03 (t, 3H, J = 7.2 Hz, H-1), 2.69 (m, 2H, H-2), 3.61 (dd, 1H, J = 4.0 and 9.2 Hz, H-6a), 3.65 (dd, 1H, J = 3.1 and 9.2 Hz, H-6b) 4.03 (m, 1H, H-5), 4.35 (d, J = 5.0 Hz, H-4); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 6.6 (C-1), 33.0 (C-2), 61.2 (C-6), 72.4 (C-5), 77.2 (C-4), 215.9 (C-3) ; α-furanose anomer : <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 6.9 (C-1), 29.8 (C-2), 62.5 (C-6), 70.1 (C-4 or C-5), 70.9 (C-4 or C-5), 104.1 (C-3) ; β furanose anomer : <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): 7.1 (C-1), 27.3 (C-2), 69.8, 70.3, 74.2, 108.5 (C-3) ; m/z HRMS ESI-MS calcd for C<sub>6</sub>H<sub>12</sub>O<sub>4</sub>Na : 171.0633 ; found [M+Na]<sup>+</sup> : 171.0628

#### 1,2-dideoxy-D-*arabino*-hept-3-ulose **10**

**10** was isolated as a pale yellow oil (126 mg) in 71% yield with TK<sub>gst</sub> variant H102L/L/118I following the general procedure of TK<sub>gst</sub> reaction. The yield in product **10** was 64% for the synthesis with TK<sub>gst</sub> variant H102L/L/118I and DAAO4536 from D-homoalanine. TLC: R<sub>f</sub> 0.44 (Methanol/ethyl acetate, 1/9 v:v) product ratio : open chain (7%) : α-pyranose anomer (21%) : β pyranose anomer (58%) ; β pyranose: <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 0.84 (m, 3H, H-1), 1.78 (m, 2H, H-2), 3.56 (dd, 1H, J = 2 Hz and 12.2 Hz, H-7a), 3.60 (d, J = 10.0 Hz, H-4), 3.77 (dd, 1H, J = 3.6 Hz and 10.0 Hz, H-5), 3.87-3.93 (m, 2H, H-6 and H-7b) ; <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 4.6 (C-1), 28.4 (C-2), 61.4 (C-7), 67.3 (C-6), 67.6 (C-4), 67.8 (C-5), 97.8 (C-3); α

pyranose anomer : <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 0.86 (t, 3H, J = 7.2 Hz, H-1), 1.68 (m, 2H, H-2), 3.66 (m, 1H, H-7a), 3.73-3.82 (m, 2H, H-6 and H-7b), 3.85 (d, J = 8 Hz, 1H, H-4), 3.97 (m, 1H, H-5) ; <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 5.0 (C-1), 28.3 (C-2), 60.6 (C-7), 72.9 (C-5), 76.0 (C-4), 78.5 (C-6), 101.3 (C-3); open form : <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): 0.96 (t, 3H, J = 7.2 Hz, H-1), 2.56 (m, 2H, H-2), 3.62-3.65 (m, 1H, H-7a), 3.7-3.8 (m, 1H, H-6), 3.81-3.85 (m, 1H, H-7b), 4.0-4.05 (m, 1H, H-5), 4.5 (d, J = 1.3 Hz, H-4); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 5.0 (C-1), 30.0 (C-2), 61.0 (C-7), 68.8 (C-6), 69.2 (C-5), 74.5 (C-4), 214.4 (C-3); m/z HRMS ESI-MS calcd for C<sub>8</sub>H<sub>15</sub>O<sub>7</sub> : 223.0823 ; found [M+HCOO]<sup>-</sup> : 223.0819

#### 1,2-dideoxy-L-*xylo*-hept-3-ulose **11**

**11** was isolated as a white powder (153 mg, yield 86% with TK<sub>gst</sub> variant H102L/L/118I/H474S; TLC: R<sub>f</sub> 0.40 (Methanol/ethyl acetate, 1/9 v:v) product ratio : α-pyranose anomer (90%) : other forms (10%); α pyranose anomer: <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 0.91 (t, 3H, J = 7.6 Hz, H-1), 1.78 (q, 2H, J = 7.6 Hz, H-2), 3.36 (d, 1H, J = 9.2 Hz, H-4), 3.53-3.71 (m, 4H, H-5, H-6, H-7<sub>a,b</sub>); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 6.3 (C-1), 30.1 (C-2), 61.6 (C-7), 69.5 (C-5 or C-6), 72.6 (C-4), 73.8 (C-5 or C-6), 99.2 (C-3). m/z HRMS ESI-MS calcd for C<sub>7</sub>H<sub>14</sub>O<sub>5</sub>Na : 201.0739 ; found [M+Na]<sup>+</sup> : 201.0729

#### 1,2-dideoxy-D-*altro*-oct-3-ulose **12**

**12** was isolated as a pale yellow oil (142 mg, yield 68% with TK<sub>gst</sub> variant H102L/L/118I; TLC: R<sub>f</sub> 0.54 (Methanol/ethyl acetate, 2/8 v:v) product ratio : open chain (8%); other cyclic forms (34%), β pyranose anomer (58%); β pyranose anomer: <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O): δ = 0.95 (m, 3H, H-1), 1.78 (m, 2H, H-2), 3.60 (dd, 1H, J = 7 and 12 Hz, H-8a), 3.73 (t, 1H, J = 6.2 Hz, H-6), 3.77 (dd, 1H, J = 3.2 and 12 Hz, H-8b), 3.84 (m, 1H, H-7), 3.93 (d, 1H, J = 7.1 Hz, H-4), 4.28 (t, J = 7 Hz, 1H, H-5); <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O): δ = 6.8 (C-1), 29.9 (C-2), 62.5 (C-8), 72.8 (C-7), 75.7 (C-5), 78.2 (C-4), 80.0 (C-6), 103.4 (C-3); open chain : <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O): δ = 56.7 (C-1), 31.8 (C-2), 61.9 (C-8), 71.2 (C-5, C-6 or C-7), 71.5 (C-5, C-6 or C-7), 72.9 (C-5, C-6 or C-7), 76.5 (C-4), 216.1 (C-3); m/z HRMS ESI-MS calcd for C<sub>8</sub>H<sub>15</sub>O<sub>6</sub> : 207.0874; found [M-H]<sup>-</sup> : 207.0864

#### 1,2-dideoxy-D-*ido*-oct-3-ulose **13**

**13** was isolated as a pale yellow oil (127 mg, Yield 61% with TK<sub>gst</sub> variant H102L/L/118I; TLC: R<sub>f</sub> 0.52 (Methanol/ethyl acetate, 2/8 v:v) product ratio : other cyclic forms (22%) ; α pyranose anomer (32%) ; open chain (46%); open chain: <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O): δ = 1.07 (t, 3H, J = 7.2 Hz, H-1), 2.70 (m, 2H, H-2), 3.71 (m, 2H, H-8), 3.78 (dd, J = 2.6 and 7.1 Hz, 1H, H-6), 3.83 (m, 1H, H-7), 4.21 (dd, J = 2.2 and 7.1 Hz, 1H, H-5), 4.35 (d, J = 2.2 Hz, 1H, H-4); <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O): δ = 6.8 (C-1), 31.9 (C-2), 62.9 (C-8), 71.0 (C-7), 71.2 (C-6), 71.2 (C-5), 76.7 (C-4); α pyranose anomer: <sup>1</sup>H NMR (500 MHz, D<sub>2</sub>O): δ = 0.98 (m, 3H, H-1), 1.78 (m, 2H, H-2), 3.65 (m, 2H, H-8), 3.92 (m, 1H, H-7), 4.02 (d, 1H, J = 5.7 Hz, H-4), 4.15 (dd, J = 4.6 and 5.7 Hz, 1H, H-6), 4.35 (t, J = 6 Hz, 1H, H-5); <sup>13</sup>C NMR (125 MHz, D<sub>2</sub>O): δ = 7.0 (C-1), 30.3 (C-2), 62.6 (C-8), 70.1 (C-

7), 75.8 (C-5), 76.6 (C-6), 78.9 (C-4), 103.1 (C-3); m/z HRMS ESI-MS calcd for C<sub>8</sub>H<sub>15</sub>O<sub>6</sub>: 207.0874; found [M-H]<sup>-</sup>: 207.0866

#### 1-deoxy-D-fructose **14**

**14** was isolated as a pale yellow oil (111 mg) in 68% yield with TK<sub>gst</sub> variant H102L/L/118I/H474S following the general procedure of TK<sub>gst</sub> reaction. The yield in product **14** was 72% for the synthesis with TK<sub>gst</sub> variant H102L/L/118I/H474S and DAAO4536 from D-alanine. TLC: Rf 0.41 (Methanol/ethyl acetate, 1/9 v:v). NMR data for **14** were identical to those previously described.<sup>1</sup> β pyranose: <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 1.47 (s, 3H, H-1), 3.59 (d, 1H, J = 10.0 Hz, H-3), 3.64 (dd, 2H, J = 1.9 and 12.7 Hz, H-4, H-5, H-6a), 3.82 (dd, 1H, J = 3.5 and 10.0 Hz, H-4), 3.99 (m, 1H, H-5), 4.01 (dd, 1H, J = 1.3 and 12.7 Hz, H-6b); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 24.5 (C-1), 63.2 (C-6), 69.2 (C-5), 69.4 (C-4), 71.9 (C-3), 98.1 (C-2). m/z HRMS ESI-MS calcd for C<sub>6</sub>H<sub>12</sub>O<sub>5</sub>Na: 187.0582; found [M+Na]<sup>+</sup>: 187.0587

#### 1-deoxy-L-sorbose **15**

**15** was isolated as a white powder (98 mg), in 60% yield with TK<sub>gst</sub> variant H102L/L/118I/H474S. TLC: Rf 0.39 (Methanol/ethyl acetate, 1/9 v:v). NMR data for **15** were identical to those previously described.<sup>6c</sup> α pyranose: <sup>1</sup>H NMR (400 MHz, D<sub>2</sub>O): δ = 1.45 (s, 3H, H-1), 2.67 (d, 1H, H-2), 3.27 (d, 1H, J = 9.4 Hz, H-3), 3.72 (m, 4H, H-4, H-5, H-6a, H-6b); <sup>13</sup>C NMR (101 MHz, D<sub>2</sub>O): δ = 24.4 (C-1), 61.6 (C-6), 69.6 (C-5), 73.6 (C-4), 75.0 (C-3), 97.7 (C-2). m/z HRMS ESI-MS calcd for C<sub>6</sub>H<sub>12</sub>O<sub>5</sub>Na: 187.0582; found [M+Na]<sup>+</sup>: 187.0587

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