



Enzymatic Synthesis of "Natural-labeled" 6-deoxy-L-sorbose Precursor of an Important Food Flavor

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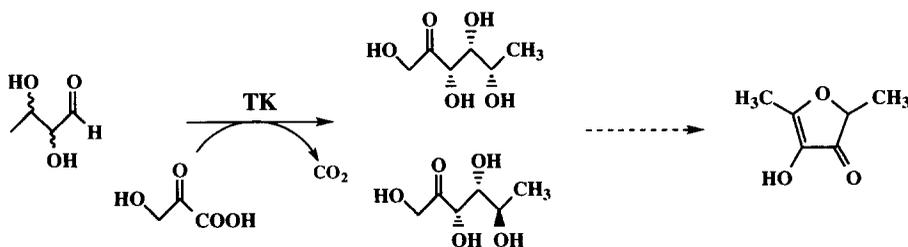
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Abstract : A biological route to natural 6-deoxy-L-sorbose is described. This method is based on the production of natural hydroxypyruvate and 4-deoxy-L-threose and their conversion into 6-deoxy-L-sorbose: hydroxypyruvate is obtained from L-serine by serine : glyoxylate aminotransferase catalysis, 4-deoxy-L-threose is obtained by microbial isomerization of 4-deoxy-L-erythrulose, this last being obtained from acetaldehyde (a naturally-available compound) and hydroxypyruvate by transketolase catalysis. Copyright © 1996 Elsevier Science Ltd

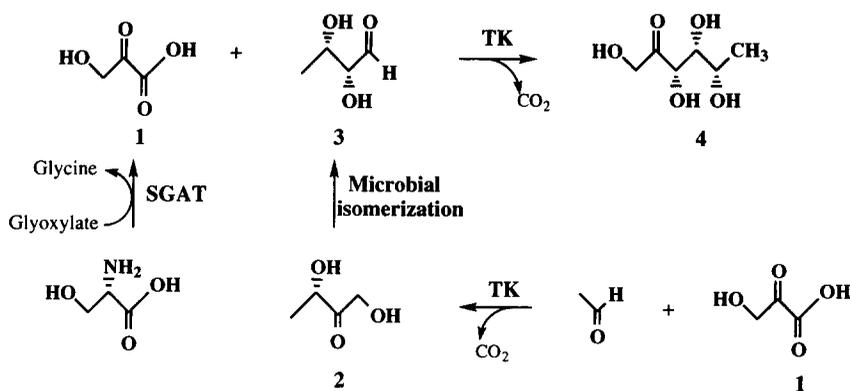
It has been shown that 6-deoxy-L-sorbose and 6-deoxy-D-fructose can serve as precursors of furaneol (2,5-dimethyl-4-hydroxy-3(2H)-furanone)¹. Furaneol is an important industrial aromatic product with caramel-like flavor used in the food industry. Although furaneol is a naturally-occurring compound^{2, 3, 4} it is difficult to extract in large amounts, and synthetic furaneol is generally employed. However, the use of synthetic products as additives in food is not always well accepted by consumers, so that procedures to obtain such compounds with a "natural" label are of economic interest. This is a challenge for chemists since a "natural" label implies that all reactions must be catalyzed by "natural" catalysts and that each precursor has a "natural" origin.

We have previously described a chemoenzymatic route to 6-deoxy-(D-fructose and L-sorbose)^{5, 6} based on the action of spinach leaf transketolase (TK) on racemic 2, 3-dihydroxybutyaldehyde (Scheme 1).



Scheme 1

This paper described a method that can be applied to the synthesis of natural-labeled 6-desoxyhexuloses if both substrates of the transketolase are of natural origin. We describe the production of natural hydroxypyruvate **1** and 4-deoxy-L-threose **3** and their conversion into 6-deoxy-L-sorbose **4**: hydroxypyruvate is obtained from L-serine by serine : glyoxylate aminotransferase (SGAT) catalysis, 4-deoxy-L-threose is obtained by microbial isomerization of 4-deoxy-L-erythrulose **2**, this last being obtained from acetaldehyde (a natural available compound) and hydroxypyruvate by transketolase (TK) catalysis (Scheme 2).



Scheme 2

Results and discussion

1. Production of hydroxypruvate 1 from L-serine

We have already described an enzymatic method to produce hydroxypruvate by oxidation of D-serine catalyzed by D-aminoacid oxidase⁷. However, this method could not be used here because D-serine is not a common natural compound. We therefore turned to serine glyoxylate aminotransferase (SGAT). This enzyme has been isolated from various sources⁸. In plants it is especially active and plays an important role in the photorespiratory glycolate pathway^{8d,e}.

The enzyme, previously extracted from spinach leaf, is described elsewhere⁹. It has been shown to catalyze the irreversible transamination between L-serine and glyoxylate leading to glycine and hydroxypruvate. However, this reaction has not been used for preparative scale production of hydroxypruvate.

We prepared a partially purified extract of SGAT by precipitation with increasing concentrations of ammonium sulfate and elution from Sephadex G75. From 300 g of spinach, we obtained a total of 140 units with a specific activity of 0.54 U/mg of protein. We measured the kinetic constants for the transamination reaction, the results of which are reported in Table 1 along with the literature values.

Table 1.
Kinetic constants of SGAT

	Km (mM)	Km (mM) ^{8d}
Glyoxylate	0.7	0.37
L-serine	7	2.7

GAT was assayed in a spectrophotometer cell containing 70 μ moles of HEPES buffer 0.1 M pH 7.6, 0-200 mM of acetaldehyde, 20 μ moles of L-serine, 1 μ moles of glyoxylate, 0.17 μ moles of NADH, 0.1 μ moles of pyridoxal-5-phosphate, 0.05 U of glyoxylate reductase and 10 μ L of GAT test sample. The optical density of the sample was measured every minute at 340nm.

No inhibition was observed at concentrations up to 250 mM for serine, 100 mM for glyoxylate, and 100 mM for glycine. In preparative scale transamination, we have calculated the concentration of

hydroxypyruvate produced by SGAT from increasing concentrations of glyoxylate and with a serine concentration of 100 mM during 24 hours. The higher concentration of hydroxypyruvate produced was 20 mM whatever the glyoxylate concentration.

Hydroxypyruvate was purified with a yield of 18% by passage of the solution through ion exchange resin (H⁺ form), neutralization with lithium hydroxide and lyophilization. However, it was more convenient to use the solution from the transamination reaction directly for the next step or carry out both steps in a "one-pot" procedure, as we did previously for the synthesis of labeled D-xylulose⁷. This procedure also circumvents the inhibition.

2. Synthesis of natural-labeled 4-deoxy-L-erythrulose 2

We have already described the synthesis of this compound¹⁰ by the reaction of hydroxypyruvate and acetaldehyde catalyzed by TK extracted from spinach leaf^{5,7}. This synthesis can be applied here since natural-labeled acetaldehyde is available.

We first performed a more precise study of the reaction of transketolase with acetaldehyde. We measured for this substrate a K_m of 80 mM with a V_{max} of 0.14 U/mg (45 % of glycolaldehyde activity). Despite this low activity the synthesis is possible on large scale provided that a large concentration of acetaldehyde can be used.

As it is known that the aldehydes can have a deactivating effect on enzymes, we checked the influence of increasing concentrations of aldehyde on TK and SGAT stabilities. 100 units of TK and SGAT were dissolved in 40 mL of Hepes buffer 0.1 M pH 7.5 containing increasing concentrations of aldehyde, the activities of TK and SGAT were measured after 10 hours of incubation. The results are reported in Table 2.

Table 2.
Influence of increasing concentration of acetaldehyde on activity of TK and SGAT

Remaining activity after 10 hours (%)	Acetaldehyde			
	0	100 mM	150 mM	200 mM
TK	80	42	38	22
GAT	84	45	40	35

GAT activity test. GAT was assayed in a spectrophotometer cell containing 70 μ moles of HEPES buffer 0.1 M pH 7.6, 0-200 mM of acetaldehyde, 20 μ moles of L-serine, 1 μ moles of glyoxylate, 0.17 μ moles of NADH, 0.1 μ moles of pyridoxal-5-phosphate, 0.05 U of glyoxylate reductase and 10 μ L of GAT test sample. The optical density of the sample was measured every minute at 340nm.

Transketolase activity test. 1 ml of 0.1 M glycylglycine buffer, pH =7.5, 10 μ l of transketolase test sample, 50 μ l of D-xylulose-5-phosphate (5 mg/ml; 21 μ mol/ml), 50 μ l of D-ribose-5-phosphate (25 mg/ml; 10 μ mol/ml), 10 μ l of thiamine phosphate (10 mg/ml; 21 μ mol/ml), MgCl₂ (1 mg/ml ; 10 mM) 10 μ l of NADH (10 mg/ml; 14 μ mol/ml), one unit of triose phosphate isomerase and 10 units of glycerophosphate dehydrogenase were placed in a spectrophotometer cell at 25°C. The optical density of the sample was measured every minute at 340 nm.

The decreases of TK and SGAT activities are very important. After 10 hours, with 200 mM of acetaldehyde, 22% and 35% of initial activities of TK and SGAT remained. We decided to use 100 mM acetaldehyde for the synthesis, although the initial activity of TK at this concentration was only about half of the V_{max} .

We previously observed that glyoxylate is a substrate of TK and can replace hydroxypyruvate, allowing the transfer of a formyl anion equivalent to aldoses¹¹. It can also replace acetaldehyde although with a high K_m . To avoid these unwanted reactions, we added glyoxylate to the reaction medium stepwise to minimize its concentration.

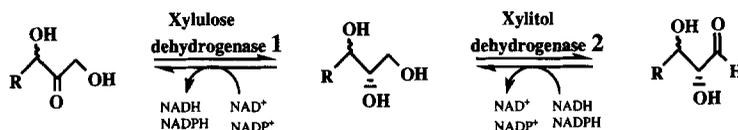
The reaction was carried out on a 500 mg scale and was monitored by HPLC. After 24 hours, methanol was added to precipitate the proteins and the solution was concentrated under vacuum. The residue was purified by chromatography leading to 4-deoxy-L-erythrulose with a 30 % yield, calculated from L-serine. 4-deoxy-L-erythrulose **2** was unambiguously identified by comparing this ^{13}C and ^1H spectra with those reported in the literature^{10,12}.

3. Synthesis of 4-deoxy-L-threose **3**

In this step, 4-deoxy-L-erythrulose had to be isomerized into an aldose with 2R configuration since TK only accepts (2R)- α -hydroxyaldehyde. This aldose must be 4-deoxy-L-threose. We have already prepared this compound in a racemic form^{5,19}.

Aldose-ketose isomerases, often called glucose isomerases, are present in numerous microorganisms. They are actually xylose isomerases and are known to be active on sugars with 5 or 6 carbons but not on C4 compounds like erythrose or threose. We tried unsuccessfully to isomerase L-erythrulose and racemic 4-deoxyerythrulose chemically prepared¹².

Some microorganisms devoid of xylose isomerase are still able to grow with xylose as a carbon source using another route to convert it into xylulose. Xylose is first reduced to xylitol by a NADP-dependant xylose reductase, and then xylitol is oxidized to xylulose by a NAD-dependant xylitol dehydrogenase. This metabolic pathway has been studied on *Candida albicans*¹³, *Corynebacterium equi*¹⁴, *Serratia liquefaciens*¹⁵ and *Penicillium chrysogenum*¹⁶. The reversibility of these reactions has been demonstrated in some cases and some activity with erythritol has been observed. We used this approach to perform the required isomerization (Scheme 3).



Scheme 3

a. Isomerization by resting cells (Analytical study)

We studied the isomerization of various C₄ ketoses and aldoses by resting cells of various bacteria, yeasts and fungi. The reactions were monitored by HPLC. The results are reported in Table 3.

Table 3.

Analytical study with *Corynebacterium equi*, *Serratia liquefaciens* or *Candida utilis* resting cells.

Starting compound	Aldose/ketose ratio at equilibrium		
	<i>Corynebacterium equi</i>	<i>Serratia liquefaciens</i>	<i>Candida utilis</i>
L-erythrulose	70/30	50/50	25/75
4-deoxy-L-erythrulose	70/30	50/50	18/82
4-deoxy-DL-erythrulose	53/47	40/60	12/88
L-threose	55/45	70/30	85/15
4-deoxy-tetrose	60/40	72/28	90/10

3 mL of Tris buffer (0.05 M pH 7,5) containing 40 mg of resting cells and 15 mg of substrate were placed in a 5 mL round-bottomed flask. After 24 hours at 25°C the medium was centrifuged 5 min. at 10,000 g. The supernatant was analyzed by HPLC.

We can observe that 4-deoxyaldoses or ketoses are substrates as well as natural tetroses. The configuration of the C₃ has no significant effect, as racemic 4-deoxyerythrulose gives a mixture of 4-deoxy-L-threose and 4-deoxy-L-erythrose.

It might be expected that a single equilibrium value would be reached independently of the microorganism used, but we observed that for isomerization of 4-deoxy-L-erythrulose, *Corynebacterium equi* leads to a higher proportion of aldose.

b. Enzymatic study

To confirm the mechanism of the isomerization, we measured the dehydrogenase activities on crude enzymatic extracts of *Corynebacterium equi* and *Serratia liquefaciens*. The results are reported in Table 4.

Table 4.
Analytical study by cell-free extracts

Substrate	Reaction type	NAD or NADH (U/mg)		NADP or NADPH (U/mg)	
		<i>Serratia</i>	<i>Corynebacterium</i>	<i>Serratia</i>	<i>Corynebacterium</i>
		<i>liquefaciens</i>	<i>equi</i>	<i>liquefaciens</i>	<i>equi</i>
L-erythrulose	Reduction (1)	0.102	0.05		
4-deoxy-DL-erythrulose	Reduction (1)	0.054	0.02		
4-deoxy-L-erythrulose	Reduction (1)	0.074	0.04		
D-xylose	Reduction (1)	0.5		0.30	0.032
D-erythrose	Reduction (1)	0.09		0.20	0.021
L-threose	Reduction (1)	0.2		0.35	0.05
4-deoxy-tetrose	Reduction (1)	0.15		0.30	0.04
erythritol	Oxidation (1 or 2)	0.014		0.05	
Threitol	Oxidation (1 or 2)	0.017		0.064	

The reaction mixture contained 1mL of Tris buffer 0.5 M pH 7.5, 200 μ L of cell-free extracts, 10 μ L of NADH, NADPH, NAD, or NADP. Tetrose reduction or polyol oxidation was measured spectrophotometrically by following the oxidation of NADPH/NADH or reduction of NAD/NADP at 340 nm.

We studied the reduction pathway for both enzymes independently. In the oxidation pathway both enzymes can act, probably depending on the cofactor used. We can observe that enzymes involved in these reactions are very active in these microorganisms since high specific activities are found in crude extracts.

For reaction 1, highest activities were observed with *Serratia liquefaciens*. A hydroxyl group in position 4 is not necessary; 4-deoxy-L-erythrulose shows a high activity.

For reaction 2, although activities are higher with NADP as a cofactor, the reaction remains possible with NAD. As usually observed for dehydrogenases initial velocities are higher for the reduction pathway. Aldoses with four carbons are as good substrates as xylose. L-threose, which has the same configuration on C₃ as D-xylose, is a better substrate than D-threose. Chemically⁵ obtained 4-deoxy-tetrose (in fact a mixture of racemic 4-deoxyerythrose and 4-deoxythreose) evidently contains at least one good substrate (probably 4-deoxy-L-threose and 4-deoxy-D-threose).

c. Isomerization on a preparative scale

We carried out the isomerization with *Corynebacterium equi* and *Serratia liquefaciens* as catalysts on 400 mg of 4-deoxy-L-erythrose at concentrations of 200 mM. However, in these conditions after 24 hours the equilibrium position observed previously was not reached; a ratio of only 20 % of aldose for *Corynebacterium equi* and 15 % for *Serratia liquefaciens* was determined by HPLC with chemically prepared racemic compound^{5,19}. A possible explanation of these poor yields is a product inhibition. No purification of the aldose was attempted.

4. Synthesis of 6-deoxy-L-sorbose 4

Having both natural-labeled hydroxypyruvate and natural-labeled 4-deoxy-L-threose at our disposal, it was possible to perform the last step to produce natural-labeled 4-deoxy-L-sorbose.

We first used the solution obtained previously, containing 40 mM 4-deoxy-L-threose and 160 mM 4-deoxy-L-erythrose. Hydroxypyruvate and transketolase were added and the reaction monitored by enzymatic determination of hydroxypyruvate. After 20 hours the hydroxypyruvate consumed corresponds to the amount of aldose present. The reaction was stopped and 6-deoxy-L-sorbose purified as previously described and isolated in 15 % yield. Given that microbial isomerization yield could be increased if the aldose could be removed from the solution, we coupled the isomerization and transketolase reactions.

Starting with 4-deoxy-L-erythrose (100 mM) in the presence of 1g of cells, hydroxypyruvate (100 mM) and 200 units of transketolase, after 48 hours of reaction, 6-deoxy-L-sorbose was isolated with 35 % yield. The limiting factor was the isomerization step and at the end of the reaction there still remained 60 % of the initial ketose as indicated by HPLC.

In both cases 6-deoxy-L-sorbose was identified by comparing its ¹³C and ¹H spectra with those reported in the literature^{1,5}. Moreover, we prepared peracetylated 6-deoxy-L-sorbose, which was carefully purified by chromatography and identified by comparison with authentic sample⁵.

An attempt was made to perform the four steps in the same vessel: production of hydroxypyruvate from serine, production of 4-deoxy-L-erythrose from acetaldehyde, isomerization and synthesis of 6-deoxy-L-sorbose. However, this failed due to L-serine metabolism by microorganisms. This difficulty could be overcome by using enzymatic extracts instead of whole cells; since both enzymes accept the same cofactor (NADP) no cofactor regeneration system would be needed.

Conclusion

This study enabled us to synthesize the desired 6-deoxy-L-sorbose according to a procedure leading to a compound with "natural" label. Four steps were necessary, three of them being carried out in the same vessel. The biological catalysts were inexpensive. The most important feature of this method is that 6-deoxy-L-sorbose could be used as a precursor to make "natural" furaneol, an important industrial aromatic product with a caramel-like flavor used in the food industry.

Experimental

Chemicals. All chemical reagents were purchased from Sigma and Aldrich. The organic solvents were obtained from Carlo-Erba. The products utilized for microbiological media were obtained from Difco Laboratories.

General. $^1\text{H-NMR}$ and $^{13}\text{C-NMR}$ spectra were determined on a Brüker 400 MHz instrument. High pressure liquid chromatography (HPLC) was performed on a Waters 600 E System controller fitted with a PPC-257 Polypore calcium (250 x 70 mm-10 microns ; H_2O eluant). The samples for HPLC analysis were centrifuged at 5000xg for 10 min and filtered on a 0.45 μm membrane filtered and directly analyzed by HPLC. The sugars were detected by refractometry. 10 μl of the sample for analysis was injected with a flow rate of 1 ml/min. for 1000 psi. The cell was heated to 40°C. The column was placed in a water bath heated to 80°C. Gas chromatography (GC) was performed on a Delsi Nermag instrument fitted with a Carbowax 20 M(50 m x 0.32 mm). capillary column Oven temperature was 120°C for 5 min, then 120°C to 150°C at 3°C/min and 150°C for 5 min at 150°C. Column chromatography was carried out on Merck Kieselgel 60 (70-230 mesh). Eluents varied and are indicated in each case. All enzymatic reactions were carried out on a Beckman DU-8 Spectrophotometer at 340 nm. Proteins were assayed by the method of Bradford¹⁷. 0.1 ml of a solution containing 10 to 100 μg of protein and 5 ml of Bradford's reagent was placed in a test tube. The mixture was thoroughly shaken for one minute. The optical density was measured at 595 nm (the absorbance was stable for one hour). A calibration curve was plotted with 1 mg/ml solution of albumin as a standard.

Production of hydroxypyruvate 1 from L-serine.

Purification and assay of SGAT. Spinach leaves (300 g from the local market) were homogenized at 0°C in 0.01 M K_2HPO_4 buffer (500 mL pH 9), then centrifuged for 10 min. at 10,000 g. The supernatant solution was filtered and $(\text{NH}_4)_2\text{SO}_4$ (89.5 g) was added. The mixture was stirred and then centrifuged to remove the precipitate. The operation was repeated with 70 g of $(\text{NH}_4)_2\text{SO}_4$. The resulting precipitate was removed with 400 mL of acetone at 40°C and filtered. The powder obtained was homogenized at 0°C in 0.01 M K_2HPO_4 buffer (400 mL pH 9). 50g of $(\text{NH}_4)_2\text{SO}_4$ was added and the mixture was stirred and then centrifuged. The operation was repeated with further portions (11 g and 10 g) and the resulting precipitate was separated by centrifugation and eluted from Sephadex G75. This last was calibrated beforehand with dextran blue. 20 mL fractions were collected. Before purification the specific activity was 0.1 U/mg of protein and total units of GAT was 130 U. After purification the specific activity was 0.5 U/mg and total units was 120 U. The purity factor was 5 and yield of GAT was 82%.

GAT activity test. The GAT assay was performed as described by Rehfeld and Tolbert. This method required glyoxylate reductase to convert hydroxypyruvate to glycerate with NADH. The rate of disappearance of NADH was measured spectrophotometrically at 340 nm (the molar extinction of NADH, $\epsilon=6.22 \mu\text{M}^{-1}\text{cm}^{-1}$). GAT was assayed in a spectrophotometer cell containing HEPES buffer 0.1 M pH 7.6, 50 μL (20 mg/mL) of L-serine, 10 μL of glyoxylate (10 mg/mL), 20 μL of NADH (10 mg/mL), 10 μL of pyridoxal-5-phosphate (10 mg/mL), 0.05 U of glyoxylate reductase and 10 μL of GAT test sample. The optical density of the sample was measured every minute at 340 nm. The activity was determined using the following equations : $\text{U/mL} = \mu\text{moles of substrate formed/min./mL} = \Delta \text{ absorbance}/\Delta t(\text{min}) \times \text{cell volume}/\epsilon(6.22) \times \text{light path} (1 \text{ cm}) \times \text{GAT sample volume}$, $\text{U/mg} = \text{mmoles of substrate formed/min./mg of proteins} = (\text{U/mL}) / (\text{mg of protein}) / \text{mL}$

Synthesis of hydroxypyruvate 1 from L-serine. 40 mL of enzyme extract containing 100 U of GAT, 0.1 M of HEPES buffer, 100 mM of L-serine, i.e. 372 mg (4mmol), 2 mM of pyridoxal-5-phosphate, i.e. 9.52 mg (0.05mmol) were placed in a round-bottomed flask at 30°C. The pH was adjusted to 7.5. Five portions of 20 mM glyoxylate, i.e. 280 mg (4 mmol) dissolved in 2 mL of HEPES buffer pH 7.5 were introduced stepwise at regular intervals. The reaction was monitored by enzymatic assay. The appearance of hydroxypyruvate was

followed with lactate dehydrogenase and NADH at 340 nm. 20 μ l of NADH (10 mg/mL), 10 U of LDH, 20 μ l of sample synthesis were introduced in 1 mL of triethanolamine buffer 0.1 M pH 7.5. The rate of disappearance of NADH was proportional to the rate of appearance of hydroxypyruvate. The concentration of hydroxypyruvate was determined using the following equation: $\mu\text{moles of hydroxypyruvate/mL} = \Delta \text{ absorbance} \times \text{cell volume} / \epsilon(6.22) \times \text{light path (1cm)} \times \text{synthesis sample volume}$. After 24 hours reaction time, the concentration of hydroxypyruvate was stable and the proteins were precipitated out with 0.1N HCl and centrifuged. The supernatant was purified by chromatography on cation exchange resin (Dowex-50W). Hydroxypyruvate was eluted with water. L-serine and glycine were removed. Fractions containing hydroxypyruvate were pooled and lyophilized. The yield was determined by spectrophotometric assay with LDH and NADH and was 20% from L-serine initial, i.e. 80 mg (0.8 mmol).

Synthesis of "natural" 4-deoxy-L-erythrulose 2¹⁰.

45 mL of enzyme extract containing 300 U of TK and 100 U of GAT, 0.1 M Hepes buffer pH 7.5, 100 mM L-serine, i.e. 410 mg (4.5 mmol), 5x20 mM of glyoxylate, i.e. 220 mg (4.5 mmol) dissolved in 2 mL of HEPES buffer introduced at regular intervals, 2 mM phosphate pyridoxal, i.e. 40 mg (0.09 mmol) cofactors vital for the activity of GAT, 3 mM of MgCl₂, i.e. 26 mg (0.13 mmol) and 2 mM of thiamine pyrophosphate, i.e. 46 mg (0.09 mmol) cofactors vital for the activity of TK were placed in a 100mL round-bottomed flask. The pH was adjusted to 7.5. The solution was deoxygenated with a stream of argon and then 100 mM acetaldehyde, i.e. 198 mg (4.5 mmol) was introduced with a syringe. The flask was placed in a water bath stirred at 30°C away from light. The reaction was monitored by HPLC. After 24 hours the amount of 4-deoxy-L-erythrulose was stable. The proteins were precipitated out with methanol and the concentrated solution was purified by chromatography on proton exchange resin (Dowex-50W) to eliminate buffer and ions. Fractions containing 4-deoxy-L-erythrulose were pooled. The pH was adjusted to 6.5 with proton exchange resin (IRA-93). The solution was concentrated and purified by chromatography on silica gel. The yield was 37% calculated from L-serine, i.e. 160mg (1.6 mmol). $[\alpha]_{\text{D}}^{25} = +4^{\circ}$ (c= 0.3 in methanol) Lit. $[\alpha]_{\text{D}}^{25} = +4^{\circ}$ (c= 0.59 in methanol). ¹H NMR (300 MHz), CD₃OD δ : 1.3 (d, J = 4.8 Hz, 1 H); 4.24 (q, J = 4.8 Hz, 3 H); 4.41 (s, 2 H). ¹³C NMR (300 MHz), CD₃OD δ : 21.0 ; 67.1 ; 73.5 ; 215.3.

Synthesis of "natural" 4-deoxy-L-threose 3.

Micro-organisms. The strains used were *Corynebacterium equi*, IFO 3730 and *Serratia liquefaciens*, CIP 103 328 T.

Medium for Corynebacterium equi. Potassium gluconate (9.6%), (NH₄)₂SO₄ (0.6%), yeast extract (0.6%), KH₂PO₄ (0.1%), MgSO₄ (0.05%), pH 6.5, 30°C. This medium was incubated 2 days at 30°C and 5 days in aerobic conditions. This medium was inoculated with cell suspension This starter culture was incubated at 30°C for 2 days. One hundred microliters from the starter culture were dispensed into this medium and incubated for 5 days at 30°C.

Medium for Serratia liquefaciens. This medium contained D-xylose (2%), (NH₄)₂SO₄ (0.6%), KH₂PO₄ (0.1%), K₂HPO₄ (0.3%), MgSO₄ (0.05%), yeast extract (0.6%), CaCO₃ (1%) the pH was adjusted to 6.5. This medium was inoculated with cell suspension This starter culture was incubated at 30°C for 2 days. One hundred microliters from the starter culture were dispensed into this medium and incubated for 2 days at 30°C.

Analytical study with Corynebacterium equi or Serratia liquefaciens. resting cells. After 2 days for *Corynebacterium equi* and 5 days for *Serratia liquefaciens*, the cells were harvested by centrifugation at 1500xg for 15 min and washed three times with NaCl (1 M). After the last centrifugation the cells were kept at -30°C or utilized directly. 3mL of Tris buffer (0.05 M pH 7.5) containing 40 mg of resting cells and 15 mg of substrate were placed in a 5 mL round-bottomed flask. After 24 hours at 25°C the medium was centrifuged 5 min. at 10,000g. The supernatant was filtered and analyzed by HPLC.

Analytical study by cell-free extracts. Preparation of cell-free extracts. Method for serratia liquefaciens. Cells were disrupted in an ultrasonic disintegrator (MSE model 60 W) by treating a suspension of 1g wet wt of organisms in 20 mL of Tris buffer (0.01 M, pH 7.5) containing 2-mercaptoethanol (2 mM) for 5x1 min. at 0°C (power output 60 W at 25 Hz). Extract were used after centrifugation at 15000 g for 15 min. Method for Corynebactrium equi. Cells of *Corynebacterium equi* were disrupted by adding to the culture medium at the end of exponential phase of growth 10^6 U of penicillin G, 300 mg of lysozyme, 50 mg of EDTA and 50 U of mutanolysine. This mixture was stored at 37°C overnight. After centrifugation at 15.000 g the supernatant was dialyzed against the same buffer 0.01M for 24 hours at 0°C. Enzyme assays. The reaction mixture contained 1 mL of Tris buffer 0.5 M pH7.5, 200 μ L of cell-free extracts, 20 μ L of NADH, NADPH, NAD, or NADP (10 mg/mL) and 10 μ L of substrates (100 mg/mL). The disappearance or apparence of NADH or NADPH were monitored spectrophotometrically at 340 nm. Amount of NADH or NADPH was proportional to the substrate oxidized or reduced.

Synthesis of 4-deoxy-L-threose 3. 20mL of Tris buffer (0.05 M pH 7.5) containing 200 mM of 4-deoxy-L-erythrulose **2** (obtained as previously), i.e.400mg (4 mmol) and 1 g or 2 g of resting cells were placed in a 50 mL round-bottomed flask. After 24 hours at 25°C equilibrium was reached. The amount of 4-deoxy-L-threose was determined by HPLC with standard^{5,19} and was 40 mM (20%), i.e. 80mg (0.8 mmol). The mixture was centrifuged and the supernatant directly utilized for the following synthesis.

Synthesis of 6-deoxy-L-sorbose **4**^{1,5}

Method 1. 20mL of Tris buffer (0.05 M, pH 7.5) containing the solution of 4-deoxy-L-threose **3** obtained previously, 100mM of hydroxypyruvate **1**, i.e. 220 mg (2 mmol), 2 mM of thiamine pyrophosphate i.e. 20.4 mg (0.04 mmol), 3 mM of MgCl₂, i.e. 11.5 mg (0.057 mmol) and the TK extract containing 200 U were placed in a 100mL round-bottomed flask. The pH was adjusted to 7.5. The flask was placed in a water bath stirred at 30°C away from light. The reaction was monitored by HPLC and after 20 hours the hydroxypyruvate consumed corresponded to the amount of the 4-deoxy-L-threose. The reaction was stopped by adding methanol. After centrifugation and concentration of supernatant 6-deoxy-L-sorbose was purified as previously described and isolated with 15% yield, i.e. 19.7 mg (0.12 mmol). It was characterized by its optical rotation and ¹³C spectrum. $[\alpha]_D^{25} = -25^\circ$ (c=0.01, in H₂O). Lit¹⁸ $[\alpha]_D^{25} = -27.7^\circ$ (c=0.01, in H₂O). ¹³C NMR (300 MHz), CD₃OD δ : 17.60; 65.91; 78.2; 80.10; 82.30; 107.10.

Method 2. 40 mL of Tris buffer (0.05 M pH 7.5) containing 100 mM of 4-deoxy-L-erythrulose **2** (previously obtained), i.e. 400 mg (4 mmol), 100 mM of hydrxypyruvate **1**, i.e. 410 mg (4 mmol), 2mM of MgCl₂, i.e. 23 mg (0.11 mmol), 2 mM of thiamine pyrophosphate, i.e. 40.8 mg (0.08 mmol), TK extract containing 200 U, 1 g of resting cells were placed in a round-bottomed flask. The reaction was monitored by HPLC and after 48 hours the reaction was stable. At the end of the reaction 60% of the 4-deoxy-L-erythrulose still remained. The mixture was centrifuged and concentrated. The 6-deoxy-L-sorbose was purified, isolated with 35% yield, i.e. 230 mg (1.4 mmol) and characterized as previously described.

*Synthesis of peracetylated 6-deoxy-L-sorbose*⁵. 6-deoxy-L-sorbose **4** was dissolved in acetic anhydride and pyridine at 0°C on an ice water bath. The mixture was left to return to ambient temperature. The excess acetic anhydride was neutralized by adding sodium bicarbonate. The solution obtained was extracted three times with dichloromethane. The organic phases were combined, washed and dried. The dichloromethane was evaporated to dryness after adding toluene. A yellow oil was obtained. The acetylated product was purified on a silica column and eluted with a pentane/ether mixture (2/1). Peracetylated 6-deoxy-L-sorbose: ¹H NMR, (300 MHz), CDCl₃, δ (ppm): 1.48 (d, 3 H); 2.25 (m, 12 H); 4.12 (m, H); 4.25 (m, 2 H); 4.75 (m, H); 5.30 (d, H).

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