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Chemoenzymatic Synthesis of 6-Deoxy-D-Fructose and 6-Deoxy-L-Sorbose using Transketolase

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Abstract : A chemoenzymatic route to 6-deoxy (D-fructose **5b** and L-sorbose **5a**) is described.This method is based on conversion of racemic 2,3-dihydroxybutyraldehyde to **5a** and **5b** catalyzed by spinach leaves Transketolase. Only the (R,R) and (R,S) isomers of 2,3-dihydroxybutyraldehyde react, yielding 6-deoxy-D-fructose and 6-deoxy-L-sorbose.

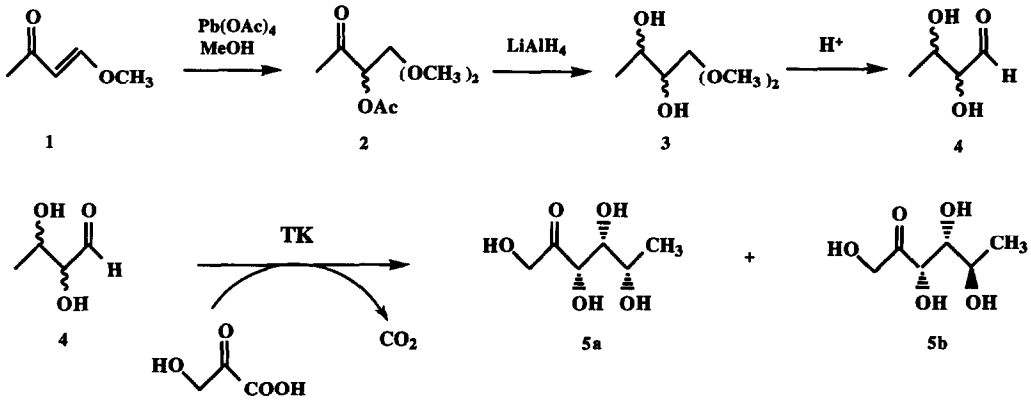
Deoxysugars have recently been found to play an important role not only in cell adhesion processes such as the inflammatory response in infections, but also in the reperfusion tissue injuries that often occur after organ transplants[1]. It has recently been demonstrated that deoxysugars are located on various cancer cells suggesting that they play an important role in the metastasis of human cancer[2]. While naturally-occurring 6-deoxyaldoses, i.e. 6-deoxyglucose (quinovose), 6-deoxygalactose (fucose) and 6-deoxymannose (rhamnose) are abundant, 6-deoxyhexuloses and especially 6-deoxy-D-fructose and 6-deoxy-L-sorbose are much scarcer. In addition, it has been shown that 6-deoxy-L-sorbose and 6-deoxy-D-fructose can serve as precursors of a valuable aromatic compound, 2,5-dimethyl-4-hydroxyfurane-3-one (furaneol)[3]. This prompted us to develop a method to obtain these ketose analogues. The chemical syntheses of 6-deoxy-L-sorbose[4] and 6-deoxy-D-fructose[5] have been described. An enzymatic synthesis involving the condensation of dihydroxyacetone-phosphate and lactaldehyde catalyzed by an aldolase has been developed[3]. This synthesis yields 6-deoxy-L-sorbose-1-phosphate and 6-deoxy-D-fructose-1-phosphate. More recently[6], a chemoenzymatic synthesis of 6-deoxy-L-sorbose has been performed by the condensation of dihydroxyacetone-phosphate and L-lactaldehyde catalyzed by fructose-1,6 biphosphate aldolase from *Staphylococcus carnosus*.

In the course of a study on the enzymatic synthesis of furaneol from natural sources[7], we developed a new synthesis of a mixture of 6-deoxy-hexuloses by transketolase (TK) catalyzed reactions. It appeared to us that this way constituted an interesting opening to 6-desoxy-D-fructose **5a** and 6-deoxy-L-sorbose **5b** from easily available precursors. In previous work[8-10], we showed that the reaction catalyzed by TK extracted from spinach leaves afforded numerous unphosphorylated sugar analogues. TK catalyzes the irreversible transfer of a hydroxyacetyl group from hydroxypyruvate onto an aldose acceptor, yielding a ketose with a 3S configuration. We and others[10,11] have shown that this enzyme accepts a large number of α-

hydroxylated aldehydes with a 2R configuration, yielding ketoses with a *threo* configuration (3S,4R). Therefore, 2,3-dihydroxybutyraldehyde should lead to **5a** and **5b**. We describe here the activity of TK for this substrate, a study of the conditions for the enzymatic synthesis and the synthesis itself.

Results and discussion

The synthesis of 6-deoxy-L-sorbose and 6-deoxy-D-fructose was carried out according to scheme 1. 2,3-dihydroxybutyraldehyde dimethylacetal **3** was obtained from 4-methoxy-3-butene-2-one **1**[12] in a 50/50 ratio of the racemic *erythro* and *threo* forms which were separated by chromatography for analytical characterisation. The presence of the various groups was unambiguously demonstrated by ^1H NMR spectra. No attempt was made to separate *erythro* and *threo* isomers for synthetic purpose. The deprotection of **3** to **4** was carried out by warming with H^+ resin in water. The concentration of **4** was determinated by enzymatic assay using alcohol dehydrogenase[13]. The yield of the deprotection step was 85 %. The hydroxyaldehyde **4** could not be extracted from the aqueous phase and so it was directly used in the enzymatic step.



Scheme 1

Study of experimental conditions for the enzymatic step :

Activity of TK towards 2,3-dihydroxybutyraldehyde 4. We measured the activity of TK towards the aldehyde substrate at different concentrations. This activity was determined by spectrophotometric assay of the residual hydroxypyruvate with L-lactate dehydrogenase (L-LDH). The maximum specific activity was obtained at 200 mM (concentration of the mixture of all four isomers). This value is 35% of that for glycolaldehyde, which is the best unphosphorylated substrate for transketolase.

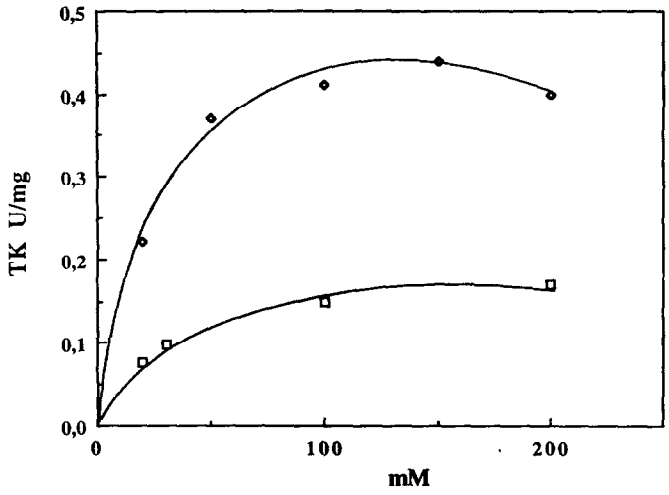


Fig.1. Activity of TK in the presence of glycolaldehyde and 2,3-dihydroxybutyraldehyde **4**. ◆, Glycolaldehyde mM; □, 2,3-Dihydroxybutyraldehyde mM. The tests were carried out in the presence of one unit of transketolase, 2,3-dihydroxybutyraldehyde at different concentrations, and hydroxypyruvate at a concentration of 10 mM, in 1 ml of glycylglycine buffer, at pH 7.5 and 25°C.

The enzyme is known to be enantioselective for α-hydroxyaldehydes, and so only diastereoisomers (2R,3R) and (2R,3S) would be expected to be substrates in this reaction. We did not observe substrate inhibition even at concentrations in dihydroxybutyraldehyde as hight as 200 mM so that the synthesis can be performed at this concentration for which the reaction rate is maximal.

Aldehydes often have an desactivating effect on enzymes due to their possible reaction with lysine residues. We checked that the presence of 2,3-dihydroxybutyraldehyde for 24 h at different concentrations up to 200 mM did not affect the stability of the transketolase. Moreover, since hydroxypyruvate is an unstable compound[14], its stability was checked under our reaction conditions (HEPES buffer; 0.2 M; pH 7.5, 25°C, presence of enzyme extract). After 24 hours, 70 % of the initial concentration remained.The synthesis was therefore carried out on 200 mM 2,3-dihydroxybutyraldehyde and 200 mM hydroxypyruvate in the presence of 200 units of transketolase in 50 ml of HEPES buffer. The cofactors vital for the activity of the enzyme were added to the reaction mixture, i.e. 2 mM $MgCl_2$ and 3mM thiamine pyrophosphate. The reaction was monitored by enzymatic assay and by HPLC. After 12 hours reaction time, the deoxyfructose **5b**/deoxysorbose **5a** ratio determined by HPLC was 0.62, indicating that the (2R,3S) isomer was the better of the two substrates. However, after 24 h the two hexuloses were obtained in equal amounts. At the end of the reaction, the proteins were precipitated out with methanol, and the concentrated solution was purified by chromatography on proton exchange resin to eliminate remaining hydroxypyruvate, buffer and ions. A second chromatography on silica gel separated the two remaining isomers of 2,3-dihydroxybutyraldehyde from the

two isomeric 6-deoxyhexuloses, 6-deoxy-D-fructose **5b** and 6-deoxy-L-sorbose **5a**, unambiguously identified by comparing their ^{13}C and ^{1}H NMR spectra with those reported in the literature[3]. The yield was 50 % after purification: 24 % for 6-deoxy-D-fructose and 26 % for 6-deoxy-L-sorbose. The optical rotations of the two compounds measured in water were close to literature values: **5a** $[\alpha]_D^{25}$ = - 5 (c = 0.03 in H_2O); lit[4] $[\alpha]_D^{25}$ = -7 (c = 0.01 in H_2O); **5b** $[\alpha]_D^{25}$ = - 25 (c = 0.03 in H_2O); lit[4] $[\alpha]_D^{25}$ = - 27.7 (c = 0.01 in H_2O).

Conclusion

TK enabled us to synthesize with a good yield the two desired 6-deoxy-hexuloses **5a** and **5b** which are easily separated by chromatography. Each compound could be obtained independently starting from diastereoisomerically pure dihydroxybutyraldehyde **4**. This study confirms that the (2R) α-hydroxyaldehydes are better TK substrates than the (2S) isomers since **5a** and **5b** were the unique hexuloses formed. The enantioselectivity of TK allowed us to run the synthesis with racemic substrates, so that the optical resolution of **4** was unnecessary. Also this method using transketolase could afford ^{13}C or ^{14}C labelled 6-deoxyhexuloses using the procedure described by us[8].

Experimental

General. ^{1}H-NMR and ^{13}C-NMR spectra were determined on a Brüker 400MHz instrument. High pressure liquid chromatography (HPLC) was performed on a Waters 600E System controller fitted with a PPC-257 Polypore calcium (250 x 70 mm-10 microns; H_2O eluant). The samples for HPLC analysis were ultrafiltered at 5000 g. 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 capillary column Carbowax 20 M(50 m x 0,32 mm). 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 were 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 Bradford[15]. 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 a standard 1 mg/ml solution of albumin.

Extraction of the transketolase. 300 g of fresh spinach leaves were finely blended in the presence of 400 ml of phophate buffer (0.01 M K_2HPO_4 pH 9). Insoluble plant matter was removed by centrifuging followed by filtration. 17.9 g of $(NH_4)_2SO_4$/100 ml (32 % saturation) was then added to the filtrate. The precipitate formed was removed by centrifuging. 14 g of $(NH_4)_2SO_4$/100 ml (53 % saturation) was added to the supernatant, the precipitate was discarded and the supernatant was treated again with 6.5 g of $(NH_4)_2SO_4$/100 ml (61 % saturation) and centrifuged. The precipitate was removed and 12.2 g of

$(NH_4)_2SO_4$/100 ml (78 % saturation) was added to the supernatant. The mixture was centrifuged once more. The pellet contained the transketolase. This pellet was resuspended in 10 ml of glycylglycine buffer and then chromatographed on a column of Sephadex G75 gel, eluting with buffer. The dead volume was determined beforehand with dextran blue. After running off the dead volume, 20 ml fractions were collected. Before purification the specific activity was 0.25 U/mg of protein and total units of TK was 420. After purification the specific activity was 1U/mg of protein and total units of TK was 380. The purity factor was 4 and yield of TK was 90 %.

Transketolase activity test. The TK assay in which ribose-5-phosphate was the acceptor and xylulose-5-phosphate was the donor was performed as described by J.J. Willafranca and B. Axelrod[16].

xylulose-5-phosphate + ribose -5-phosphate ⟶ sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate

This method required triose phosphate isomerase to convert glyceraldehyde-3-phosphate to dihydroxy-acetone-phosphate. The oxidation of dihydroxyacetone-phosphate was catalyzed by α-glycerophosphate dehydrogenase in the presence of NADH. The rate of disappearance of NADH was measured spectrophotometrically at 340 nm (the molar extinction of NADH, $\varepsilon = 6.22\ mM^{-1}\ cm^{-1}$). 1 ml of 0.1 M glycylglycine buffer, pH = 7.5, 10 µl of transketolase test sample, 50 µl of D-xylulose-5-phosphate (5 mg/ml; 21µmol/ml), 50 µl of D-ribose-5-phosphate (25 mg/ml; 10µmol/ml), 10 µl of thiamine phosphate (10 mg/ml; 21µmol/ml), $MgCl_2$ (1 mg/ml ; 10 mM) 10 µl of NADH (10 mg/ml; 14µmol/ml), one unit of triose phosphate isomerase and 10 units of glycerophosphate dehydrogenase both purchased from Sigma were placed in a spectrophotometer cell at 25°C. The optical density of the sample was measured every minute at 340 nm; the Δ absorbance/$\Delta t_{(min.)}$ slope was determined and the activity of the enzyme per ml of protein was determined using the following equations: U/ml = µmoles of substrate formed/min./ml = (Δ absorbance/Δt) x cell volume/6.22 x light path (1 cm) x TK sample volume; U/mg = µmoles of substrate formed/min./mg proteins = (U/ml)/(mg protein/ml)

Synthesis of α-4,4-dimethoxy-3-acetoxybutan-2-one(2). $Pb(OAc)_4$ (O.1 mole, 45 g) and 140 ml of pure methanol were placed in a 3-necked round-bottomed flask fitted with a $CaCl_2$ tube and a thermometer. The mixture was kept at 0 - 5°C. 3-methoxy-2-butene-1-one (0.1 mole, 10 g) was added dropwise. The mixture was left to return to ambient temperature. Reaction was taken as complete when a sample of the reaction mixture no longer produced a brown colour in the presence of water. The solution was evaporated and the $Pb(OAc)_2$ precipitate was washed 3 to 4 times with ether. The ether was evaporated and the residue was vacuum-distilled (b.p. = 100-120°C/12 mm Hg). The yield of the reaction was 70 %. ^1H NMR , 400 MHz, $CDCl_3$, δ(ppm) : 2.09 (s, 3H, -CO-C<u>H</u>$_3$) ; 2.16 (s, 3H, -O-CO-C<u>H</u>$_3$) ; 3.34 (s, 3H, -0-C<u>H</u>$_3$) ; 3.37 (s, 3H, -0-C<u>H</u>$_3$) ; 4.45 (d, H, -CO-C<u>H</u>-OAc) ; 5.0 (d, H, -C<u>H</u>$(OCH_3)_2$). ^{13}C NMR, 400 MHz, $CDCl_3$, δ(ppm) : 20.23 ; 28.16 ; 54.80 ; 55.97 ; 77.34 ; 103.46 ; 169.80 ; 202.63 .

Synthesis of 1,1-dimethoxy-2,3-dihydroxybutane(3) (dimethylacetal of 4). 6 g of $LiAlH_4$ and 1 ml of dry ether were placed in a 500 ml 2-necked round-bottomed flask fitted with a condenser. 10 g of α-acetoxy-β-ketobutyraldehyde dimethyl acetal previously synthesized dissolved in 100 ml of dry ether was added dropwise. The mixture was stirred for 3 to 5 hours. The excess $LiAlH_4$ was destroyed with a minimum

amount of water. A white-yellow precipitate was formed. The mixture was evaporated to dryness and the precipitate taken up 3 to 4 times in ether. The organic phase was dried and evaporated, yielding a yellow oil.The yield of the reaction was 80 % with a ratio of 54/46 for the two diastereoisomers as determined by GC. Retention time of 1,1-dimethoxy-2,3-dihydroxybutane : 540s (RR/SS or RS/SR), 50.02 % ; 780s (RR/SS or RS/SR), 41.82 %. The two diastereoisomers were separated by column chromatography on analytical scale. They gave two distinct NMR spectra. RR/SS or RS/SR: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.25 (3H, d, J=6Hz) , 2.37 (1H, d, J=4Hz), 2.63 (1H, d, J=4Hz), 3.47 (6H, d, J=7Hz), 3.53 (1H, m) ; 3.90 (1H,m), 4.37 (1H,d, J=6Hz). ^{13}C NMR (400 MHz, $CDCl_3$) δ 18.36; 54.81 ; 55.23; 68.06; 73.60; 105.24. RR/SS or RS/SR: ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.26 (3H, d, J=6Hz), 2.46 (1H, d, J=4Hz), 2.50 (1H, d, J=4Hz), 3.38 (1H, m), 3.49 (6H, d, J=7Hz) ; 4.00 (1H,m), 4.39 (1H,d, J=6Hz). ^{13}C NMR (400 MHz, $CDCl_3$) δ 19.81; 55.39 ; 56.70; 66.25; 73.74; 106.05.

2,3-dihydroxybutyraldehyde(4). The acetal was dissolved in water. A proton exchange resin H^+ form (Dowex 50Wx8) with HCl was added. The mixture was stirred in a closed tube at 80°C. The deprotection was monitored on TLC plates. The eluant was chloroform/methanol (9/1). The mixture was then filtered to remove the resin. The filtrate contained the dihydroxybutyraldehyde; after enzymatic assay, the yield of the deprotection was 85 %.

Enzymatic assay of 2,3-dihydroxybutyraldehyde. The assay was performed with alcohol dehydrogenase. 1 ml of TRIS buffer (0.1 M, pH = 7.5), 20 µl of NADH (10 mg/ml) and 20 µl of solution for assay were placed in a spectrophotometer cell. The disappearance of NADH was monitored spectrophotometrically at 340 nm.

Acitivity test of 2,3-dihydroxybutyraldehyde with TK. In 1 ml of glycylglycine buffer (0.1 M pH = 7.5) were added one unit of transketolase, 50 µl of hydroxypyruvate (20 mg/ml; 192 µmol/ml), varying amounts of 2,3-dihydroxybutyraldehyde, 10 µl of thiamine pyrophosphate (10 mg/ml; 181 µmol/ml) and 10 µl of $MgCl_2$ (10 mg/ml; 106 µmol/ml). 10 µl of the reaction mixture was immediately removed to determine the initial hydroxypyruvate concentration. After 5 min., a further 10 µl was sampled. To assay the hydroxypyruvate, 1 ml of triethanolamine buffer (0.1 M pH = 7.5), 20 µl of NADH (10 mg/ml; 14 µmol/ml), 10 units of L-LDH and 10 µl of the reaction mixture were placed in a spectrophotometer cell at 25°C, and the disappearance of NADH was monitored at 340 nm. The initial hydroxypyruvate concentration and that remaining after 5 min. were determined using the following equation: Hydroxypyruvate µmoles/ml = Δabsorbance x cell volume/6.22 x sample volume. The activity of the transketolase is given by the following equation: U/ml = µmoles hydroxypyruvate lost/min./ml; U/mg = µmoles hydroxypyruvate lost/min./mg proteins = (U/ml)/(mg proteins/ml).

Synthesis of 6-deoxy-L-sorbose and 6-deoxy-D-fructose. 40 ml of enzyme extract containing 200 U of TK, 0.1 M HEPES buffer, 200 mM 2,3-dihydroxybutyraldehyde, i.e. 832 mg (8mmol) dissolved in 5 ml of water, 200 mM commercial hydroxypyruvate, i.e. 832 mg (8mmol), 2 mM thiamine pyrophosphate, i.e. 40 mg (0,42mmol), and 3 mM $MgCl_2$, i.e. 11 mg (0,1mmol) were placed in a 100 ml 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. After 24 hours reaction, all the hydroxypyruvate had been consumed. The reaction mixture was

poured into 120 ml of methanol and centrifuged. The supernatant was concentrated and chromatographed on cation exchange resin (Dowex-50W). The fractions containing 6-deoxy-D-fructose and 6-deoxy-L-sorbose were combined and their pH adjusted to 6.5 with weakly basic Amberlite anion exchange resin (IRA-93). The solution was concentrated and chromatographed on a silica column to separate the two diastereoisomers. The eluant was a mixture of chloroform and methanol (4/1). The yields obtained were 23 % for 6-deoxy-L-sorbose, i.e. 150 mg (0,9mmol), and 25 % for 6-deoxy-D-fructose, i.e. 170 mg (1mmol). They were characterised by their optical rotations and ^{13}C NMR spectra. 6-deoxy-D-fructose: $[\alpha]_J^{25} = -5°$ (c = 0.03, in H_2O). Lit[4] $[\alpha]_J^{25} = -7°$ (c = 0.01, in H_2O); ^{13}C NMR (300 MHz), CD_3OD δ: 20.05; 68.75; 77.80; 78.20; 82.50; 103.30. 6-deoxy-L-sorbose: $[\alpha]_J^{25} = -25°$ (c = 0.03, in H_2O). Lit[4] $[\alpha]_J^{25} = -27.7°$ (c = 0.01, in H_2O).^{13}C NMR (300 MHz), CD_3OD δ: 17.60; 65,91; 78.22; 80.10; 82,30; 107,10.

Synthesis of peracetylated 6-deoxy-L-sorbose and 6-deoxy-D-fructose. 6-deoxy-L-sorbose and 6-deoxy-D-fructose were 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 neutralised 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, $CDCL_3$, δ (ppm): 1.48 (d, 3H); 2.25 (m, 12H); 4.12 (m, H); 4.25 (m, 2H); 4.75 (m, H); 5.30 (d, H). Peracetylated 6-deoxy-D-fructose: ^1H NMR, 300 MHz, $CDCL_3$, δ (ppm): 1.42 (d, 3H); 2.15 (m, 12H); 4.28 (m, H); 4.42 (m, 2H); 4.65 (m, H); 5.43 (d, H).

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