Transketolase-Mediated Synthesis of 4-Deoxy-D-fructose 6-Phosphate by Epoxide Hydrolase-Catalysed Resolution of 1,1-Diethoxy-3,4-epoxybutane

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4-Deoxy-D-fructose 6-phosphate is synthesised from nonnatural sources in four steps, including two enzymatic reactions. (3*S*)-1,1-Diethoxy-3,4-epoxybutane is first obtained by epoxide hydrolase-catalysed resolution. Opening of this epoxide by inorganic phosphate leads to 2-deoxy-D-erythrose 4-phosphate. In the last step, transketolase transfers a hydroxyacetyl group from L-erythrulose onto this aldehyde, controlling the second asymmetric center.

Introduction

The synthesis of 4-deoxyketoses in a free or phosphorylated form has received little attention in spite of the fact that these compounds can serve as valuable probes for enzymatic mechanism studies and can also strongly interfere with sugar metabolism. 4-Deoxy-D-fructose was first obtained by microbial oxidation of a mixture of 3-deoxy-D-*arabino*-hexitol and 3-deoxy-D-*lixo*-hexitol[1] and then by isomerisation of 4-deoxy-D-glucose,[2] but it was not isolated nor fully characterised in either case. More recently, Enders et al.[3] published a synthesis of various protected deoxyketoses by alkylation of protected dihydroxyacetone with the "SAMP" method, and we applied this strategy to the synthesis of 4-deoxy-D-fructose.[4] We showed that this compound was a substrate for hexokinase, leading to 4-deoxy-D-fructose 6-phosphate (**1**) as indicated by an enzymatic assay. No other mention of 4-deoxy-D-fructose or of its derivative **1** (phosphorylated in position 6) was found in the literature. This last compound, however, appears to be a very useful tool to explore the metabolism of fructose. We already know that the first two enzymes of this compound act on the 4-deoxy analogs and could allow the synthesis of 4-deoxy-D-glucose 6-phosphate or 4-deoxyphosphogluconic acid.[4] An efficient synthesis of 4-deoxy-D-fructose 6-phosphate (**1**) is therefore of obvious interest.

Some of us have explored the potential of transketolase (TK) in the synthesis of ketoses[5] and, more recently, of 4-deoxyketoses.[6] We found that TK, which catalyses the condensation of a hydroxyacetyl group from a ketose or from hydroxypyruvate onto aldehydes, accepts racemic (2-hydroxy-4-oxobutyl) phosphate (**2**) (2-deoxy-D,L-erythrose 4-phosphate) as the aldehydic substrate with conveniently high activity, so providing access to 4-deoxy-D-fructose 6-phosphate (**1**)[6] (Scheme 1). However, due to the lack of availability of **2** in its optically pure form, this could only be achieved starting from racemic **2**. Furthermore, although TK catalysis appeared to be quite effective, both enantiomers of **2** are probably substrates of this enzyme, thus leading to a mixture of products [i.e. 4-deoxy-D-fructose 6-phosphate (**1**) and 4-deoxy-L-sorbose 6-phosphate] from which it was not possible to isolate the desired **1** in its pure form. In addition, an inhibition of the reaction was observed at concentrations higher than 15 mM but, as the assays were carried out with racemic material, it was not possible to ascertain the real reason for this inhibition. Therefore, a very important improvement on this strategy would clearly be to achieve this condensation using enantiopure **2** as a building block. The problem was to synthesize the intermediate 1,1-diethoxy-3,4-epoxybutane (**3**) in its optically pure form.

Owing to the very active development of the use of "new" cofactor-independent enzymatic catalysts, i.e. microbial epoxide hydrolases, that enable the efficient achievement of the resolution of various racemic epoxides,[7] it appeared that one possible solution to the problem discussed above was to perform the resolution of *rac*-**3** using an appropriate epoxide hydrolase. In this paper, we describe the successful preparation of (*S*)-**3** using this methodology, and the subsequent use of this chiron to achieve the preparation of pure 4-deoxy-D-fructose 6-phosphate (**1**).

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Results and Discussion

Resolution of Racemic 1,1-Diethoxy-3,4-epoxybutane (3)

We had previously described the synthesis (in 40% yield) of racemic 2-deoxyerythrose 4-phosphate based on the opening of (*R,S*)-1,1-diethoxy-3,4-epoxybutane (**3**) with in-

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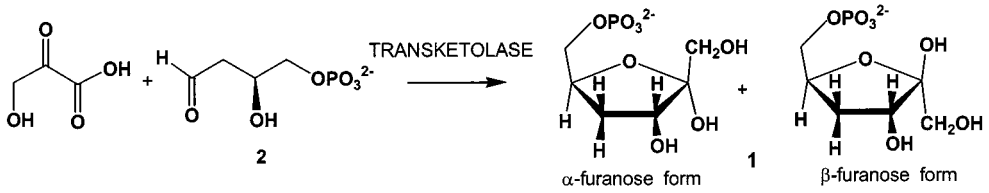
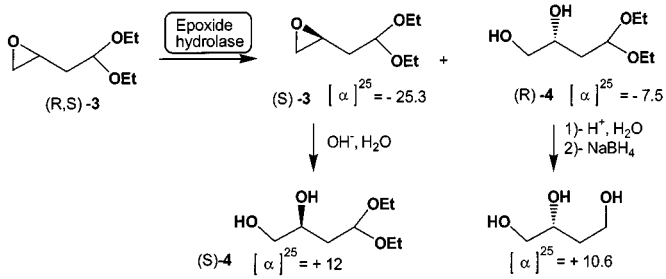


Scheme 1. Transketolase mediated synthesis of 4-deoxy-D-fructose-6-phosphate from hydroxypyruvic acid

organic phosphate in basic conditions.[6] In order to prepare an optically pure enantiomer of **3**, the epoxide hydrolase resolution of racemic **3** was explored.

Soluble epoxide hydrolases of various origins[7d] including fungi (*Aspergillus niger*, *Syncephalastrum racemosum*, *Aspergillus terreus*, *Beauveria sulfurescens*, *Chaetomium globosum*), mammals (rat and human) and plant (potato) were tested at 27°C. The best results were obtained using the *Aspergillus niger* epoxide hydrolase, which showed an *E* value of about 15. The enantioselectivity was unchanged when the reaction was performed at 4°C and was not affected by varying the substrate concentration up to 1 M. Therefore, large-scale resolution was carried out using 15 g of racemic **3** at a concentration of 0.3 M in phosphate buffer (pH 8) at 4°C. Under these conditions the reaction rate was slow enough to allow monitoring of the resolution by chiral GC. The hydrolysis was stopped after 30 hours when the epoxide enantiomeric excess was superior to 98%. After workup, 4.5 g of nearly enantiopure epoxide **3** was obtained, as well as 9 g of diol **4** with an enantiomeric excess of 47% (Scheme 2).



Scheme 2. Epoxide hydrolase resolution of 1,1-diethoxy-3,4-epoxybutane and attribution of the absolute configuration of the products

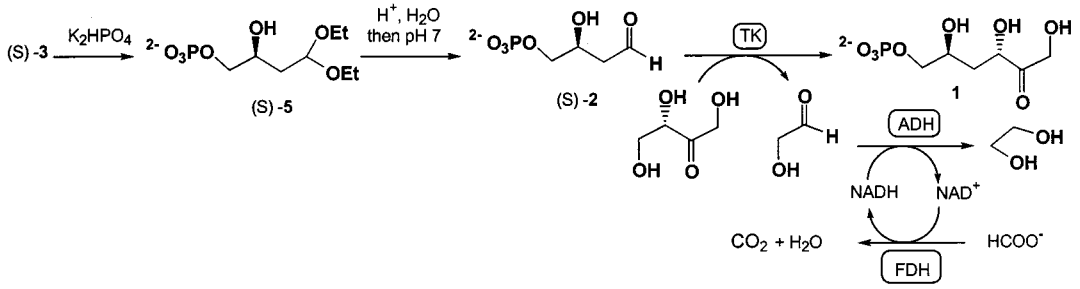
The absolute configurations of the obtained epoxide and diol were determined by chemical correlation as indicated in Scheme 2. Diethyl ketal **4** $\{[\alpha]_D^{25} = -7.5$ ($c = 2.0$, $CHCl_3)\}$ was hydrolysed under acidic conditions and the aldehyde obtained was reduced with $NaBH_4$, leading to 1,2,4-butanetriol (73% isolated yield). This compound showed a positive optical rotation $\{[\alpha]_D^{25} = +10.6$ ($c = 2.16$, MeOH)}, which allowed the attribution of the (*R*) configuration to this enantiomer of **4** by comparison with the sign previously assigned in the literature $\{[\alpha]_D^{25} = +26.9$ ($c = 10$, MeOH)}.[8] As a consequence, the absolute configuration of the remaining (unchanged) epoxide **3** enantiomer could be deduced as being (*S*), assuming that no inversion of configuration occurred during the enzymatic hydrolysis, i.e. considering that the enzymatic attack took place at the less-substituted carbon atom of the oxirane ring. In order to confirm this hypothesis, the remaining optically active epoxide **3** $\{[\alpha]_D^{25} = -25.3$; ($c = 3.4$, $CHCl_3)\}$ was hydrolysed under basic conditions. This led to the corresponding diol **4**, which displayed a positive optical rotation of $[\alpha]_D^{25} = +12.0$ ($c = 0.6$, $CHCl_3$) opposite to the one obtained for diol **4** produced by the epoxide hydrolase hydrolysis. This result therefore confirms the (*S*) absolute configuration attributed to the remaining epoxide **3**.

Synthesis of 4-Deoxy-D-fructose 6-Phosphate (1)

Further synthesis of 4-deoxy-D-fructose 6-phosphate (**1**) was achieved as shown in Scheme 3.

Opening of (*S*)-**3** by inorganic phosphate in basic conditions led to **5**. Acidic hydrolysis of the acetal moiety led to enantiopure (*S*)-2-hydroxy-4-oxobutyl 1-phosphate (**2**) (2-deoxy-D-erythrose 4-phosphate), which was tested as a substrate for the transketolase. Compound (*S*)-**2** proved to be a reasonably good substrate for the enzyme, showing a V_{max} of 71% relative to ribose 5-phosphate and a K_m of 20 mM. These values are in the same range as those measured previously for racemic **2** ($K_m = 3.8$ mM, $V_{max} = 61\%$). However, the slightly higher K_m suggests that the L isomer should be a better substrate than its D antipode.

Examination of the literature shows that, as a general approach, hydroxypyruvate has been used as the hydroxyacetyl group donor in most, if not all, transketolase-catalysed syntheses described to date.[9] This is essentially due to the fact that, in this case, the second product of the reaction is carbon dioxide instead of an aldehyde, so that the reaction is irreversible and is driven to completion. However, in our experimental conditions (in the presence of phosphate anions), we observed the formation of a significant amount of polar by-products, which proved to be difficult to separate from the desired **1**. Therefore, the same transketolase-catalysed reaction was checked by using L-erythrulose instead of hydroxypyruvate. It is known that L-erythrulose is a good substrate for transketolase, with a K_m of 4.9 mM as opposed to 33 mM for hydroxypyruvate,[10] but the drawback is that one of the reaction products, glycolaldehyde, is also a substrate of transketolase. Therefore, in order to shift the equilibrium to the synthesis of the new ketose, the glycolaldehyde was reduced *in situ* into glycol in the presence of yeast alcohol dehydrogenase and a catalytic amount of NADH. This cofactor was recycled following the classical closed-loop process, using the formate/formate dehydrogenase system. Although this method might seem somewhat complicated, it was actually very convenient and easy to perform, at least for small-scale synthesis. Indeed,



Scheme 3. Transketolase mediated synthesis of 4-deoxy-D-fructose-6-phosphate from 2-deoxy-D-erythrose-4-phosphate and L-erythrulose

glycolaldehyde is a good substrate for alcohol dehydrogenase[10] (whereas 2-deoxy-D-erythrose phosphate is not), and formate dehydrogenase is commercially available. This new procedure is also convenient from an economical point of view on comparing the costs of the necessary reagents with that of the hydroxypyruvate acid that is generally used. Using this procedure, a preparative scale synthesis of enantiopure 4-deoxy-D-fructose 6-phosphate (**1**) was carried out on a 1 mmol scale. Thus, 0.57 mmol of **2** (130 mg) was condensed with 1 mmol of L-erythrulose in the presence of 2×30 units of transketolase. The reaction was monitored by TLC and, after completion, addition of methanol to the reaction mixture led to the precipitation of the desired 4-deoxy-D-fructose 6-phosphate (**1**) (85 mg sodium salt, 52% yield) as a pale yellow solid, which showed a purity higher than 90% according to NMR spectra. The optical rotation measured for **1** was $[\alpha]_D^{25} = +5.9$ ($c = 7.8$, H_2O). This compound exists in solution as the two anomeric forms α and β in the ratio 40/60, which were identified on the basis of the chemical shift of C-2 in the ^{13}C-NMR spectrum; $\delta =$ 109.2 for the α anomer and 105.1 for the β anomer.[11] The two forms are well characterised by interpretation of the NMR spectra: the protons at C-4 are well differentiated at $\delta = 1.98$ and 2.45 for the major form, as well as the 3-CH and 5-CH at $\delta = 4.36$ and 4.30, respectively. The protons at C-1 and C-6 give a broad unresolved signal, so H−P coupling constants are not available. C−P coupling constants appear in the ^{13}C-NMR spectrum with ^3J values of 8.5 and 8.0 Hz for the C-5 signal of the β and α forms and a ^2J value in the range of 4 Hz for the poorly resolved signals of C-6.

Conclusion

In the course of this study we have described a new methodology to achieve the synthesis of 4-deoxy-D-fructose 6-phosphate (**1**) by following a route combining three different biocatalytic reactions. These involve (a) the epoxide hydrolase-catalysed resolution of the key epoxide **3** [and the subsequent synthesis of 2-deoxy-D-erythrose 4-phosphate (**2**) from this synthon], (b) the transketolase-catalysed reaction between this enantiomer of **2** and L-erythrulose, and (c) recycling of the NADH cofactor necessary for this condensation reaction using formate dehydrogenase. This strategy illustrates the possible fruitful use of enzymatic reactions for fine organic synthesis

Experimental Section

General: All chemical reagents were purchased from Sigma−Aldrich. The products used for microbiological media were obtained from Difco. − ^{1}H NMR and ^{13}C NMR spectra were obtained using a Bruker 400 MHz instrument. − UV/Vis absorptions were determined on a Secomam 1000 spectrophotometer.

Transketolase Expression and Purification: The expression plasmid pTKL1 and the transketolase-deficient yeast strain H402 have been described earlier.[12] The yeast cells carrying plasmids were cultured in a leucine-deficient medium to obtain a high copy number of the plasmid. Culture conditions were as described previously.[13] The protocol for protein purification developed by Wikner et al.[13] was used to purify transketolase. During the purification procedure the specific activity of transketolase was measured spectrophotometrically. One unit is defined as the formation of 1 μmmol of glyceraldehyde 3-phosphate per minute. The reaction was followed by the rate of NADH oxidation in a coupled system with triose phosphate isomerase and glycerol 3-phosphate dehydrogenase.[14] Protein concentrations were determined using the extinction coefficient $E^{1\%}_{280\ cm} = 14.5$. The specific activity of purified transketolase was about 37 units/mg at 25°C. The purification protocol resulted in about 60−80 mg of pure transketolase from 5 L of cells culture.

Epoxide Hydrolase Screening: 10 mg of enzymatic extract was dissolved in 1 mL phosphate buffer (pH 8) at 27°C. 5 μL of a 1.5 M solution of racemic **3** dissolved in DMF was added. The reaction was monitored by GC: the conversion ratio was obtained using an Optima 5 column (150°C, 2 bars) and tetradecane as internal standard, the *ee*'s of the remaining epoxide and formed diol were determined, respectively, with chiral Lipodex E [70°C, 1.5 bar; (*S*) $t_R = 27.4$ min, (*R*) $t_R = 28.3$ min] and Chirasil-dex CB [115°C, 1.5 bar; (*S*) $t_R = 26.3$ min, (*R*) $t_R = 27.3$ min] columns.

Resolution of 1,1-Diethoxy-3,4-epoxybutane: Racemic 1,1-diethoxy-3,4-epoxybutane (**3**) (15 g, 93 mmol) was dissolved in 300 mL of phosphate buffer (pH 8). The temperature was decreased to 4°C and 1.2 g of crude epoxide hydrolase from *Aspergillus niger* was added.[15] After 30 h, the reaction mixture was extracted with pentane until complete extraction of epoxide was achieved (checked by GC), the organic phases were then combined and concentrated under reduced pressure. The oil obtained was purified by bulb-to-bulb distillation to give 4.5 g (30% yield) of (*S*)-1,1-diethoxy-3,4-epoxybutane (**3**). $[\alpha]_D^{25} = -27.5$ ($c = 3.4$, $CHCl_3$), *ee* >98%. The aqueous phase was continuously extracted with CH_2Cl_2 and led, after concentration, to 9 g (54% yield) of (*R*)-1,1-diethoxy-3,4-dihydroxybutane (**4**). (*R*)-1,1-Diethoxy-3,4-dihydroxybutane (**4**). $[\alpha]_D^{25} = -7.5$ ($c = 2.0$, $CHCl_3$), *ee* = 47%.

(2*S*)-4,4-Diethoxy-2-hydroxybutyl Phosphate (5), Sodium Salt: To a solution of K_2HPO_4 (7.4 g, 42 mmol) in 60 mL of water was added

(*S*)-1,1-diethoxy-3,4-epoxybutane (**3**)[6] (2.6 g, 16 mmol). The mixture was heated under reflux for 24 h. During this time the mixture turned dark yellow. The solution was extracted with 3 × 30 mL of ether. To the aqueous layer was added a solution of barium acetate (15 g, 55 mmol) in 20 mL of water. The pH of the mixture was adjusted to 8 and the precipitate formed was removed by centrifugation (4000 g). Four volumes of ethanol were added and the resulting solution was kept at 4°C overnight. The precipitate formed was recovered by centrifugation and dried in vacuo to obtain the barium salt of (2*S*)-4,4-diethoxy-2-hydroxybutyl phosphate as a yellow solid. To a solution of this barium salt was added Dowex 50Wx8 (Na^+ form). The mixture was stirred for 1 h. The resin was separated by filtration and the aqueous solution was concentrated. A yellow solid was obtained (3.9 g, 39% yield). − ^{1}H NMR (400 MHz, D_2O): δ = 1.30 (m, 6 H, 2 CH_3), 1.90 (m, 2 H, CH_2), 3.60−3.90 (m, 7 H, 2 CH_2, $CH_2OPO_3Na_2$, CHOH), 4.00 (m, 1 H, CH). − ^{13}C NMR (100 MHz, D_2O): δ = 17.1 (2 CH_3), 39.5 (CH_2), 65.8 (CH_2), 70.3 (d, *J* = 7.0 Hz, CHOH), 70.9 (d, *J* = 4.8 Hz, $CH_2OPO_3Na_2$), 103.7 (CH).

(2*S*)-2-Hydroxy-4-oxobutyl Phosphate (2), Sodium Salt: To a solution of (2*S*)-4,4-diethoxy-2-hydroxybutyl 1-phosphate (**5**) (3.9 g; 6.3 mmol) in 20 mL water was added Dowex resin 50Wx8 (H^+ form). The mixture was stirred for 30 min. The resin was separated by filtration. The solution and acetic acid formed were concentrated. The residue was diluted with water and Dowex 50Wx8 (Na^+ form) was added. The resin was separated by filtration. Acetone (500 mL) was added to the filtrate and the precipitate was collected by filtration to give 1.10 g of sodium salt as a yellow solid (76% yield). − ^{1}H NMR (400 MHz, D_2O): δ = 1.85 (m, 2 H, CH_2), 4.8 (m, 3 H, $CH_2OPO_3Na_2$, CHOH), 5.30 [m, 1 H, $CH(OH)_2$]. − ^{13}C NMR (100 MHz, D_2O): δ = 41.0 (C-3), 67.6 (d, *J* = 7.3 Hz, C-2), 69.4 (d, *J* = 4.2 Hz, C-1), 89.4 (C-4).

4-Deoxy-D-fructose 6-Phosphate (1): To 30 mL of water were added (2*S*)-hydroxy-4-oxobutyl phosphate (130 mg, 5.7 × 10^{-4} mol), L-erythrulose (120 mg, 10^{-3} mol), $MgCl_2$ (16 mg, 7.9 × 10^{-3} mol), thiamine pyrophosphate (2 mg, 4.3 × 10^{-6} mol), sodium formate (50 mg) and NADH (10 mg, 1.4 × 10^{-3}mol). The mixture was adjusted to pH 7.5 with 1 M NaOH. The final volume was adjusted to 40 mL. 10 units of formate dehydrogenase (FDH), 30 units of transketolase (TK) and 150 units of alcohol dehydrogenase (ADH) were added. The progress of the reaction was followed by TLC. After 12 h, 5 units of FDH, 30 units of TK and 50 units of ADH were added. The mixture was stirred for 12 h. Water was partially removed under reduced pressure. Methanol was added and the precipitate was filtered off. The filtrate was concentrated to give a yellow solid (85 mg, 52% yield). − $[\alpha]_D^{25}$ = +5.9 (*c* = 7.8%, H_2O). − **β-Furanose (Major Form):** ^{1}H NMR (400 MHz, D_2O): δ = 1.98 (td, 1 H, *J* = 9.6 Hz, *J* = 12.3 Hz, 4-CH), 2.45 (td, 1 H, *J* = 7.1 Hz, *J* = 12.3 Hz, 4-CH), 3.60−4.0 (m, 4 H), 4.36 (dd, 1 H, *J* = 7.1 Hz, *J* = 9.6 Hz, 3-C), 4.28−4.32 (m, 1 H, 5-CH). − ^{13}C NMR (100 MHz, D_2O): δ = 34.7 (C-4), 65.2 (C-1), 69.3 (d, *J* = 4 Hz, C-6), 72.9 (d, *J* = 8.5 Hz, C-5), 78.9 (C-3), 105.1 (C-2). − **α-Furanose (Minor Form):** ^{1}H NMR (400 MHz, D_2O): δ = 1.88 (ddd, 1 H, *J* = 14.1 Hz, *J* = 4.9 Hz, *J* = 2.1 Hz, 4-CH), 2.65 (ddd, 1 H, *J* = 6.2 Hz, *J* = 8.6 Hz, *J* = 14.1 Hz, 4-CH), 3.70−4.00 (m, 4 H), 4.24 (dd, 1 H, *J* = 2 Hz, *J* = 6.2 Hz, 3-CH), 4.52−4.58 (m, 1 H, 5-CH). − ^{13}C NMR (100 Mhz, D_2O): δ = 36.8 (C-4), 65.1 (C-1), 68.6 (C-1), 77.6 (d, *J* = 8.0 Hz, C-5), 80.1 (C-3), 109.3 (C-2).

Acknowledgments

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