Preparative scale enzymatic synthesis of d-sedoheptulose-7-phosphate from β-hydroxypyruvate and d-ribose-5-phosphate

Franck Charmantray[a], Virgil Hélaine[a], Bertrand Legeret[a] and Laurence Hecquet [a,]

[a]Université Blaise Pascal, laboratoire de Synthèse et Etude de Systèmes à Intérêt Biologique, UMR CNRS 6504, 63177 Aubière Cedex, France

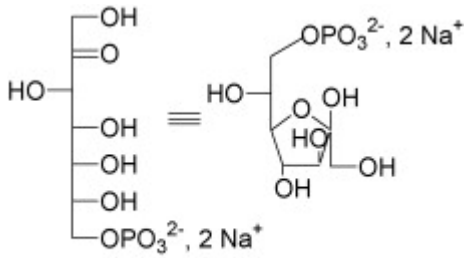
Abstract

An enzymatic method for ready access to D-sedoheptulose-7-phosphate on a preparative scale was developed, based on the irreversible transketolase-catalyzed reaction: β-hydroxypyruvate + D-ribose-5-phosphate → D-sedoheptulose-7-phosphate. D-Sedoheptulose-7-phosphate disodium salt was obtained in 81% overall yield determined using a standard curve obtained by LC/MS/MS.

Keywords: d-Sedoheptulose-7-phosphate; Biocatalysis; Transketolase

1. Introduction

D-Sedoheptulose-7-phosphate (Scheme 1) is an extremely important compound that is of great interest in fields such as metabolism studies and the synthesis of compounds with biological properties. It is a well-known key metabolite in the non-oxidative pentose phosphate pathway and in the Calvin cycle.



D-sedoheptulose-7-phosphate

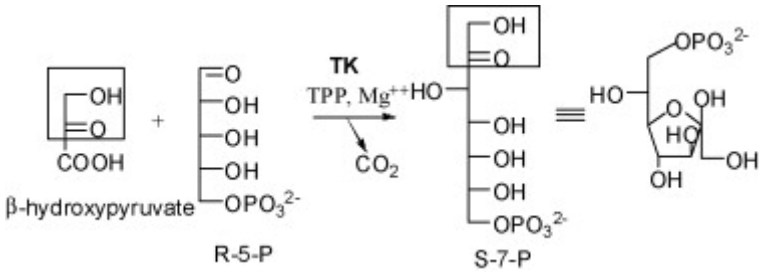
Scheme 1

Recently, Mahmud and co-workers reported that d-sedoheptulose-7-phosphate was the only precursor of the *m*C_7N units of both the α-glucosidase inhibitor acarbose [1] used for the treatment of type II insulin-independent diabetes, and validamycin A [2] an antibiotic used to control sheath blight, a phytopathogenic disease of rice plants. Also, jointly with the Naganawa group, the same authors reported on the biosynthetic pathway to the generic family of pyralomycin antibiotics [3] from D-sedoheptulose-7-phosphate.

The role of D-sedoheptulose-7-phosphate as the precursor of the l-glycero-D-manno-heptose (L,D hep) segment in the lipopolysaccharides (LPS) of Gram-negative bacteria such as *E. coli*, *Salmonella*, and *Shigella* is also of particular interest [4].

Studies in progress in all these fields prompted researchers to seek a practical route for the synthesis of this phosphorylated sugar. Thiem and co-workers explored the hexokinase-mediated phosphorylation of D-sedoheptulose [5], but surprisingly, they recovered only the secondary phosphate, D-sedoheptulose-6-phosphate. Floss and co-workers, who were interested in elucidating the biosynthetic pathways that lead to the aminocyclitol moieties of acarbose, validamycin, and pyralomycin, proposed an enzymatic synthesis of isotopically labelled D-sedoheptulose-7-phosphate. This was obtained using a multi-enzymatic system with alanine racemase, D-amino acid oxidase, catalase and transketolase [6], from unlabeled D-ribose-5-phosphate and l-[3-^{13}C]serine *via* β-hydroxypyruvate as the C2 fragment donor. This multi-step synthesis afforded labelled d-sedoheptulose-7-phosphate with 69% yield based on its radioactivity.

Our goal was to develop an efficient, scalable synthesis of D-sedoheptulose-7-phosphate. We were also interested in finding a reliable method to determine its purity. One approach was to take advantage of the irreversible reaction catalyzed by transketolase (TK: EC 2.2.1.1). When β-hydroxypyruvate (HPA) was used as the donor substrate, the reaction went to completion owing to the release of carbon dioxide (Scheme 2).



Scheme 2.

TK is thus a useful catalyst to obtain D-*threo* ketoses, and its synthetic potential has been largely studied with various aldehydes [7]. In our group, we used a *Saccharomyces cerevisiae* recombinant strain to overproduce TK enzyme [8].

Here we describe a method to obtain, in only one step and on a preparative scale (multigram synthesis), highly pure D-sedoheptulose-7-phosphate, quantified by the calibration curve obtained by LC/MS/MS.

2. Experimental

2.1. Materials

2.1.1. Chemistry

Chemicals and solvents were purchased from Aldrich and Acros and were reagent grade. All solvents for analysis were LC/MS grade (Riedel de Haen, Sophyc-Socolab, France). ^{1}H, ^{31}P and ^{13}C NMR spectra were recorded on a Bruker Avance 400 spectrometer in D_2O; δ values are given in ppm and J values in Hertz. For ^{31}P NMR we used H_3PO_4 (5%) as the external standard in a sealed capillary. For the quantification of D-sedoheptulose-7-phosphate by ^{1}H NMR, we used TSP-d_4 as an internal standard.

2.1.2. LC–MS and LC–MS/MS (ESI) analyses

The LC/MS system consisted of a Waters® Alliance® 2695 high-performance liquid chromatography (HPLC) set-up (Waters SA, St-Quentin-en-Yvelines, France) coupled to a quadrupole time-of-flight (micro-QTOF) mass spectrometer (Micromass, Manchester, UK). This was fitted with a Z-spray source operating in negative ionization mode ESI(−)). Source and desolvation temperatures were set to 110 and 350 °C, respectively. Gaseous N_2 was used as both nebulizer gas (35 L h^{-1}) and desolvation gas (350 L h^{-1}). The voltages were 2.1 kV for the stainless-steel capillary and 35 V for the sample cone. For MS–MS experiments, the collision energy used depended on the analyte assayed. It was set at 12 eV for D-ribose-5-phosphate and 17 eV for D-sedoheptulose-7-phosphate. Analyses were performed as follows: 20 μL aliquots from reaction mixtures were quenched with 1% formic acid in water (180 μL) and acetonitrile (800 μL). LC analyses of D-ribose-5-phosphate and D-sedoheptulose-7-phosphate were carried out using an analytical zwitterionic and hydrophilic phase (Zic®-Hilic column, 2.1 mm × 100 mm; with 5.0 μm particle size from SeQuant AB, Sweden) at a flow rate of 0.2 mL min^{-1}. The mobile phase was composed of (A) acetonitrile and (B) 2 mM ammonium formate pH 3.5. Isocratic elution at A/B, 80/20, v/v was performed for 10 min. The injection volume was 5 μL.

For MS/MS experiments transition settings for the different sugar phosphates were:

l-glycerol-3-phosphate (r.t: 3.9 min), *m*/*z* −169/−97,
D-ribose-5-phosphate (r.t: 4.7 min), *m*/*z* −229/−97, and
D-sedoheptulose-7-phosphate (r.t: 6.0 min), *m*/*z* −289/−97.

Data were acquired and processed using MassLynx™ software. For the sugar phosphates, the loss of the sugar moiety in the collision cell was unambiguously detected by the *m*/*z* −97 fragment (phosphate group).

2.2. Expression and purification of transketolase

Transketolase from *S. cerevisiae* was produced from *S. cerevisiae* strain H402xpTKL1 [8]. TK crude extract was obtained following our own procedure. The cells were collected by centrifugation at 3000 × *g* for 4 min. 10 g of cells were resuspended in 100 mL of Tris buffer (0.1 M) at pH 7.4 and placed in a one-shot cell disrupter at 2 kBar. The solution was centrifuged at 15,000 × *g* for 20 min. Once the precipitate was discarded, the pH of the supernatant was adjusted to 7.4 and the TK crude extract was kept at −20 °C until use. TK activity was determined thanks to a spectrophotometric assay as described earlier [9].

2.3. Determination of β-hydroxypyruvate content

The initial concentration of β-hydroxypyruvate was enzymatically assayed by spectrophotometry using NADH-dependent lactate dehydrogenase [10].

2.4. Determination of the standard curves for the phosphorylated sugars by LC/MS/MS

The D-ribose-5-phosphate standard curve was obtained from diluted solutions of commercial D-ribose-5-phosphate (■, Fig. 1) in the 0–200 μM concentration range (l-glycerol-3-phosphate at 20 μM was used as the internal standard (IS). In the conditions tested, the detection limit was found to be 5 μM (*i.e.*, 5 pmol, injection volume 5 μL).

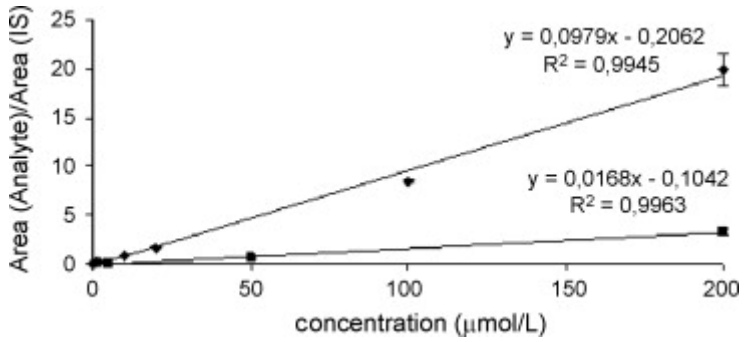


Fig. 1. D-Ribose-5-phosphate (■) and d-sedoheptulose-7-phosphate (♦) standard curves.

As D-sedoheptulose-7-phosphate was not commercially available, we decided to generate it *in situ* from d-ribose-5-phosphate by TK-catalyzed reaction. It was possible to plot the D-sedoheptulose-7-phosphate standard curve only under two conditions. First, the reaction had to be quantitative. Second, the reaction had to be one-to-one (no side reactions). The reaction was carried out as follows: D-ribose-5-phosphate in the 0–10 mM range reacted with a large excess of HPA (165 mM; K_M = 33 mM) in the presence of TK for 2 h.

We observed a total conversion of D-ribose-5-phosphate as evidenced by LC/MS/MS (data not shown). Similar slopes were observed for D-ribose-5-phosphate disappearance (♦, Fig. 2) and D-sedoheptulose-7-phosphate appearance (■, Fig. 2). Our approach thus provided a very accurate D-sedoheptulose-7-phosphate standard curve.

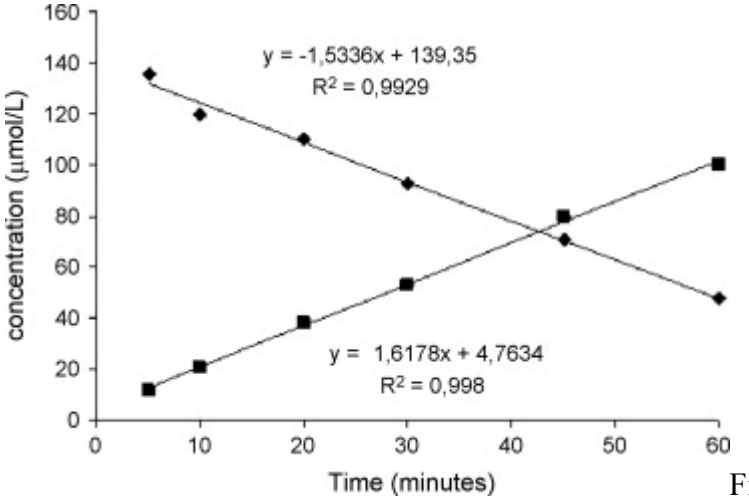


Fig. 2. TK-catalyzed reaction causing a fall in D-ribose-5-phosphate (♦) and the formation of D-sedoheptulose-7-phosphate (■) in time.

After sampling as previously described (dilution factor: 50), the D-sedoheptulose-7-phosphate standard curve was plotted from the corresponding means and standard deviations (S.D.) from five measurements for each concentration of D-sedoheptulose-7-phosphate studied (♦, Fig. 1). Under the conditions tested, the detection limit was found to be 3 µM (*i.e.*, 15 pmol, injection volume 5 µL).

3. Results

3.1. Principle

In the reaction with transketolase, the C1–C2 ketol unit was transferred from β-hydroxypyruvate to D-ribose-5-phosphate in the presence of thiamine pyrophosphate (ThDP) and divalent cation (Mg^{2+}) to give D-sedoheptulose-7-phosphate *via* an irreversible reaction.

3.2. Synthesis of sedoheptulose-7-phosphate barium salt

D-ribose-5-phosphate as its disodium salt (15.1 g, 55 mmol) was dissolved in distilled water (230 mL, 240 mM). ThDP (200 mg, 0.44 mmol, 2 mM) and $MgCl_2$ (63 mg, 0.66 mmol, 3 mM) were then added. After adjustment of the pH to 7.5 with 1 N NaOH, β-hydroxypyruvate [11] (60% pure) as its lithium salt (12.1 g, 66 mmol, 285 mM) was added. The reaction was initiated by addition of TK (300 units) and the pH was maintained at 7.5 by addition of a 0.5 N HCl solution (pH stat apparatus). The reaction progress was monitored by determining β-hydroxypyruvate content (see Section 2.3). As shown in Fig. 3, the control without TK exhibited an intrinsic slight degradation of β-hydroxypyruvate in the conditions of the reaction. This degradation was estimated to be 20% of the starting material in 8 h. This problem was circumvented by adding a slight excess (1.2 equivalent) of β-hydroxypyruvate *versus* D-ribose-5-phosphate. After 8 h under the conditions tested, β-hydroxypyruvate consumption was 4 times higher in the reaction (with TK) than in the blank (without TK).

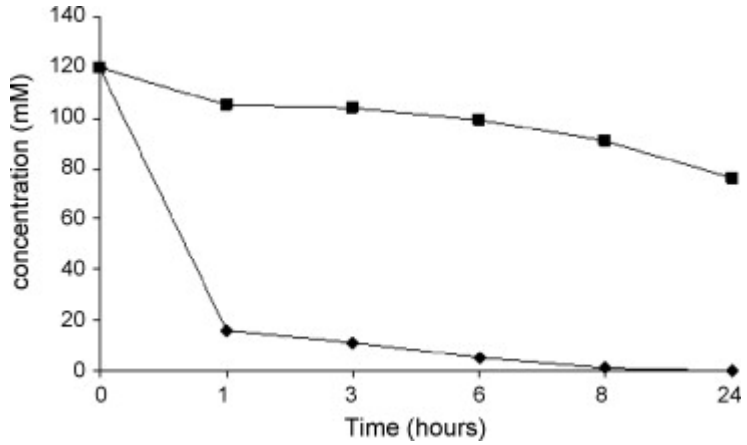


Fig. 3. Consumption and stability of β-hydroxypyruvate in time at pH 7.5, 25 °C: HPA, D-ribose-5-phosphate, ThDP, $MgCl_2$ (with TK) (♦); *versus* blank reaction: HPA, D-ribose-5-phosphate, ThDP, $MgCl_2$ (without TK) (■).

After complete reaction of β-hydroxypyruvate and D-ribose-5-phosphate the mixture was cooled in ice. Three volumes of MeOH were then added and the mixture was stirred. The suspension was then centrifuged at 4500 rpm for 15 min. The precipitate was discarded and the supernatant collected. Aqueous $BaCl_2$ solution (75 mmol, 50 mL) at pH 6.0 was poured in with stirring and the solution cooled in an ice bath. The precipitate formed was discarded after centrifugation. Five volumes of ethanol were gently added to the supernatant with continued stirring. The precipitate formed was washed twice with ethanol and once with diethyl ether, and air-dried. D-Sedoheptulose-7-phosphate (29.9 g) was isolated as its Ba salt. Its organic purity was high, neither β-hydroxypyruvate nor D-ribose-5-phosphate being detected by ^{1}H, ^{13}C or ^{31}P NMR experiments. ^{31}P and ^{13}C NMR data fitted with those published earlier [12] and [13].

Due to slight excess in $BaCl_2$ used (1.35 equivalent) to achieve the precipitation of D-sedoheptulose-7-phosphate as its Ba salt, the sample purity was 65% [14] from the D-sedoheptulose-7-phosphate standard curve obtained by LC/MS/MS (see Section 2.4). Presumably, the sample was contaminated by barium hydroxide. The reaction yield was 90% based on d-ribose-5-phosphate.

3.3. Conversion to sodium d-sedoheptulose-7-phosphate

A sample salt of D-sedoheptulose-7-phosphate isolated as its barium salt (500 mg, 0.8 mmol) was converted into its disodium salt with gentle stirring with a Dowex 50 × 8 H^+ resin. After filtration, the supernatant was adjusted to pH 7.0 using a 1 N NaOH solution, followed by lyophilization. D-Sedoheptulose-7-phosphate as its disodium salt was recovered in 90% yield (251 mg, 0.72 mmol).

4. Conclusion

We describe a one-pot, straightforward, multigram-scale synthesis giving D-sedoheptulose-7-phosphate, isolated as its disodium salt, in high overall yield (81%). Considering more general aspects about carbohydrate synthesis, our approach highlights enzymatic carbon–carbon bond formation is an attractive alternative to conventional chemical methods. It offers stereochemical control, mild conditions and needs no protecting group.

Supplementary data

^{1}H, ^{31}P and ^{13}C NMR data for D-sedoheptulose-7-phosphate synthesized are available as supplementary data.

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[14] Crude d-sedoheptulose-7-phosphate (consisted of a mixture of β-furanose, α-furanose and α-pyranose) was found to be 65% pure using TSP-d4 as an internal standard in ^{1}H NMR (see Supplementary data).