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Enzymatic synthesis of [7-^{14}C, 7-^{3}H]- and [1-^{13}C]sedoheptulose 7-phosphate and [1-^{13}C]*ido*-heptulose 7-phosphate [1]

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Abstract

The enzymatic synthesis of isotopically labeled D-sedoheptulose 7-phosphate from either labeled D-glucose or labeled L-serine is described. [7-^{14}C, 7-^{3}H]Sedoheptulose 7-phosphate is prepared with transketolase from xylulose 5-phosphate, as C_2 donor, and D-[5-^{14}C, 5-^{3}H]ribose 5-phosphate, which in turn is generated from D-[6-^{14}C, 6-^{3}H]glucose with the glycolytic enzymes. [1-^{13}C]Sedoheptulose 7-phosphate is obtained in a one-pot reaction in 69% yield from unlabeled ribose 5-phosphate and L-[3-^{13}C]serine via hydroxypyruvate as the C_2 donor using the enzymes alanine racemase, D-amino acid oxidase, catalase and transketolase. By the latter route labeled [1-^{13}C]*ido*-heptulose 7-phosphate was also prepared when xylose 5-phosphate was substituted for the ribose 5-phosphate.

Keywords: Enzymatic synthesis; Labeled compounds; Sedoheptulose 7-phosphate; *ido*-Heptulose 7-phosphate; Transketolase; Hydroxypyruvate

1. Introduction

Isotopically labeled compounds are very useful to study the biosynthetic pathways of natural products. Many labeled compounds are commercially available but frequently one needs to synthesize specifically labeled biosynthetic intermediates from commercially available starting materials. In this paper we report enzymatic syntheses of differently labeled sedoheptulose 7-phosphate and *ido*-heptulose 7-phosphate samples, compounds which are suspected to be a precursor of the mC_7N units of acarbose and validamycin A, from labeled glucose or labeled serine as starting material.

2. Results and discussion

Acarbose (**1**) [1] (Fig. 1) isolated from *Actinoplanes* sp. is an α-glucosidase inhibitor and a clinically useful drug for the treatment of type II insulin-independent diabetes. Validamycin A (**2**) [2] is an important antibiotic from *Streptomyces hygroscopicus* var. *limoneus*

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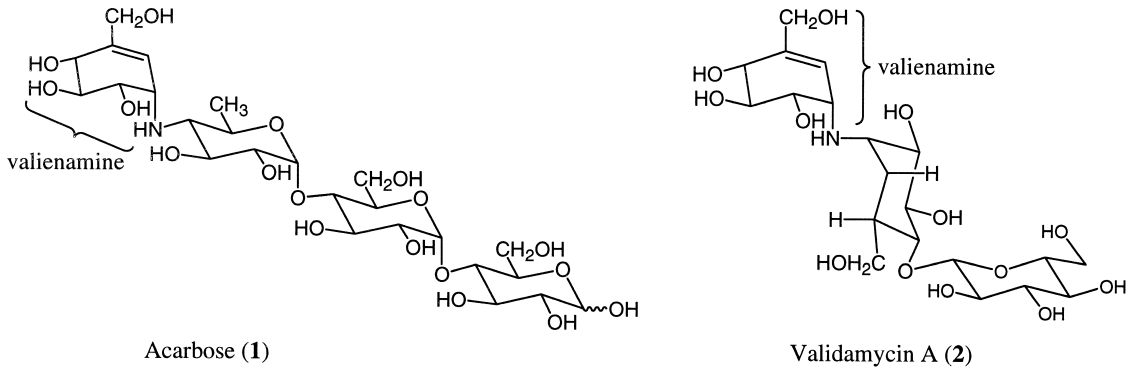

Fig. 1. Structures of acarbose and validamycin A.

which is used in the Orient to control Blight Sheath, a disease of rice plants caused by the phytopathogen, *Pellicularia sasakii*. Both compounds contain an aminocyclitol, the valienamine moiety, which can be considered an aliphatic analog of the mC_7N units found in the ansamycin and mitomycin antibiotics [3]. The latter are synthesized via a new branch of the shikimate pathway [4]. However, experiments with stable isotope-labeled precursors have shown that the mC_7N units of acarbose [5] and validamycin A [6] are derived from the pentose phosphate pathway. Specifically, it was proposed that either sedoheptulose 7-phosphate or *ido*-heptulose 7-phosphate is a precursor of these cyclitols. We needed to prepare these compounds in isotopically labeled form in order to evaluate this suggestion.

The mechanism of the cyclization of a heptulose 7-phosphate to a cyclitol may either be similar to that of 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAHP) to dehydroquinate (DHQ) [7] or, less likely, to that of glucose 6-phosphate to *myo*-inositol 1-phosphate [8]. The far more plausible first mechanism (Fig. 2), which is supported by genetic data [9], would likely involve sedoheptulose 7-phosphate as the substrate and would produce either valiolone or 2-*epi*-valiolone, depending on whether the configuration at C-5 of the substrate is retained or inverted during the cyclization. The two mechanisms can be distinguished based on the fate of the hydrogens at C-7 of the substrate; in a DHQ synthase-like mechanism both C-7 hydrogens would be retained in the resulting (*epi*-)valiolone, and one in the valienamine moiety of **1**, whereas in the *myo*-inositol 1-phosphate synthase-like mechanism which would produce either 2-*epi*-valienone or valienone the two hydrogens would be lost (Fig. 2).

To probe the cyclization mechanism, specifically radiolabeled D-[7-^{14}C, 7-^{3}H]sedoheptulose 7-phosphate was prepared from D-[6-^{14}C, 6-^{3}H]glucose by the sequence of enzymatic reactions shown in Fig. 3. The double-labeled glucose was first activated with hexokinase to give D-glucose 6-phosphate, which was isolated by paper chromatography in 80% radiochemical yield. The D-glucose 6-phosphate was then converted in a single reaction mixture into D-[5-^{14}C, 5-^{3}H]ribose 5-phosphate with glucose 6-phosphate dehydrogenase, phosphogluconate dehydrogenase and phosphoribose isomerase in the presence of a NADP-regenerating system. After purification by paper chromatography the labeled ribose 5-phosphate, isolated in 59% yield, was converted into the final product, D-[7-^{14}C, 7-^{3}H]sedoheptulose 7-phosphate, with transketolase and xylulose 5-phosphate in the presence of a trapping system for the phosphoglyceraldehyde generated in order to make the reaction irreversible. The product was isolated by paper chromatography in 61% yield, giving an overall

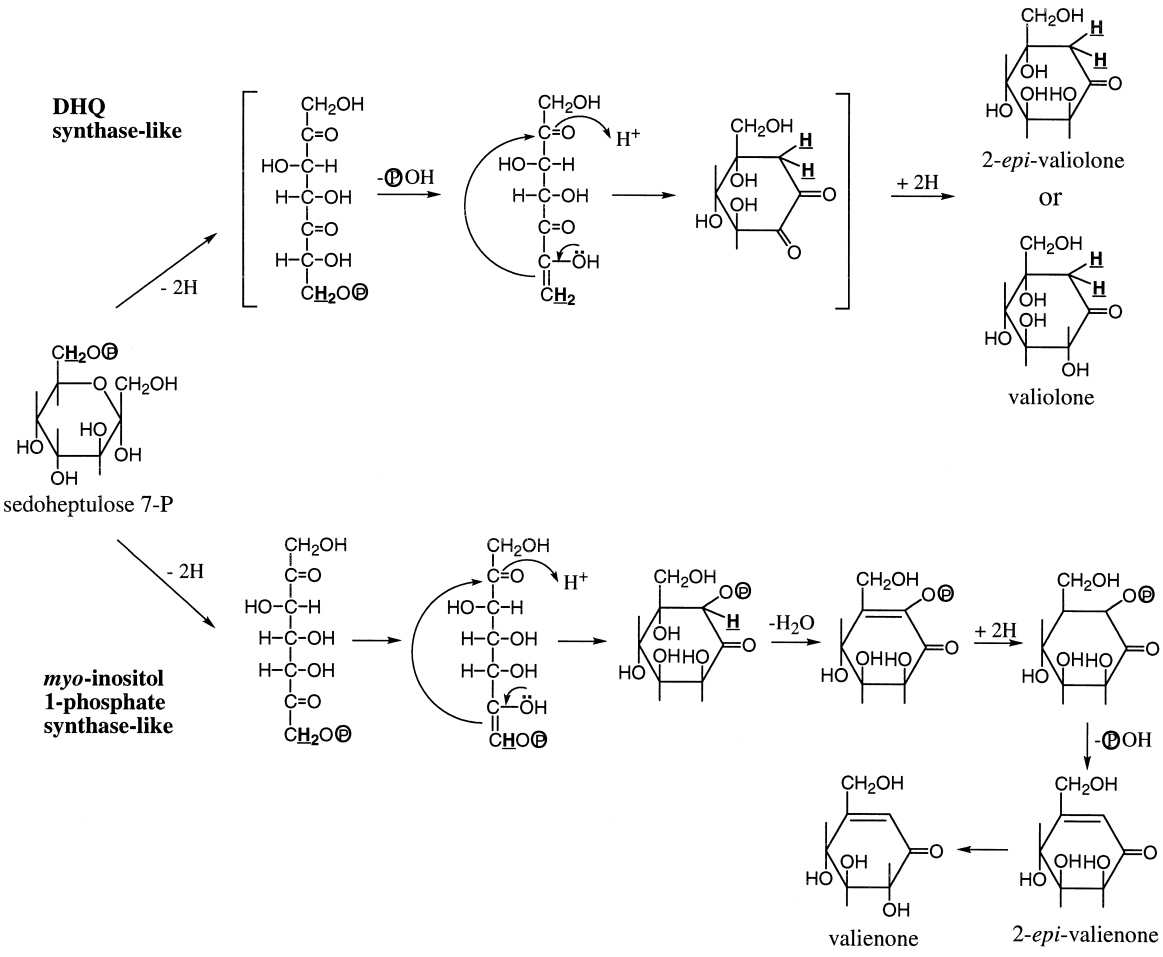

Fig. 2. Two possible mechanisms for the cyclization of sedoheptulose 7-phosphate to cyclitols.

radiochemical yield from glucose of 29%. Within error the $^{3}H/^{14}C$ ratio of the product (10.1) was the same as that of the starting glucose (10.4).

We also needed ^{13}C-labeled sedo- and *ido*-heptulose 7-phosphate to generate the enzymatic cyclization product in labeled form and feed it to the acarbose and validamycin A fermentations in order to establish its role as a true biosynthetic intermediate. The same final enzymatic reactions as employed above can also be used to synthesize *ido*-heptulose 7-phosphate, simply by replacing the ribose 5-phosphate with xylose 5-phosphate. However, the preparation of labeled material by this route would be difficult because the conversion of labeled glucose into xylose 5-phosphate requires additional enzymes which are not readily available and/or have unfavorable equilibria. A more practical approach to prepare sedoheptulose 7-phosphate carrying a ^{13}C label, which is also applicable to the *ido* isomer, involves labeling the 'top' part of the molecule in the transketolase reaction. In principle, this can be done by using D-[1-^{13}C]xylulose 5-phosphate as the second substrate, which can be prepared from commercially available D-[2-^{13}C]glucose via ribulose 5-phosphate and its epimerization with phosphoribose-3-epimerase. However, a much better alternative is the use of 3-hydroxypyruvate as the C_2 donor in the transketolase reaction [10]. This simple substrate has the advantage of making

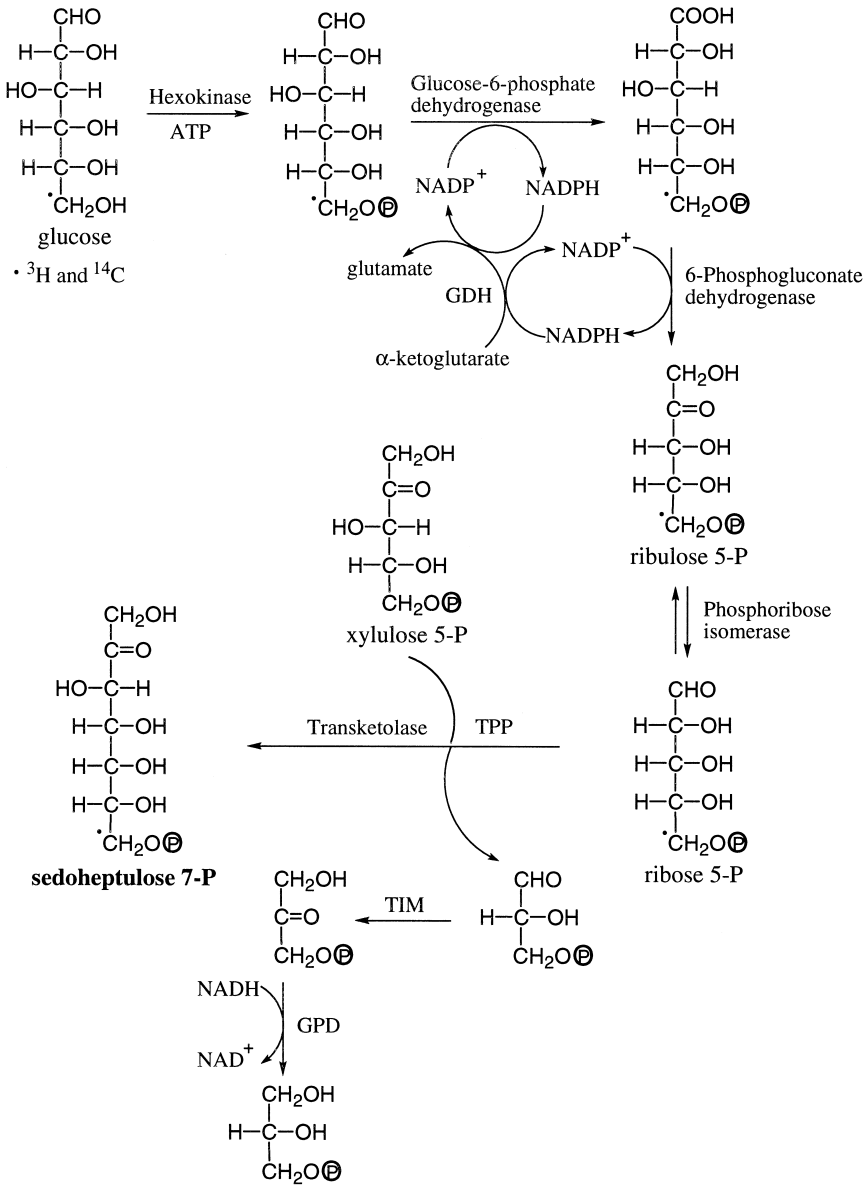

Fig. 3. Synthesis of [7-^{14}C, 7-^{3}H]sedoheptulose 7-phosphate.

the reaction irreversible by generating CO_2 as the second product.

Labeled 3-hydroxypyruvate is not commercially available. The task was therefore to develop a convenient method for producing this compound in labeled form from a readily available starting material. The obvious substrate of choice is serine, which could be converted into 3-hydroxypyruvate by the action of transaminases [11], amino acid oxidases [12] or alanine dehydrogenase [13]. Two additional constraints are (i) the fact that only L-serine and to a lesser extent the racemate, but not the D isomer, are commercially available with radioactive or stable isotope labels in the 2- and/or 3-position and (ii) the need to generate the stable isotope-labeled compounds in quantities in the tens to hundreds of milligrams, which necessitates the

use of efficient and readily available enzymes. The use of transaminases was tested first, but with the five different enzymes tried very little 3-hydroxypyruvate was produced, suggesting that this was not a particularly useful route. Some of these were commercial enzymes from thermophiles, and one problem here was the limited stability of the product under the reaction conditions. It was found that 15% of 10 mM 3-hydroxypyruvate decomposed within 1.5 h at 70°C in 50 mM Tris buffer, pH 7.5.

The second method tried was the use of the thermostable enzyme L-alanine dehydrogenase from *Bacillus stearothermophilus* [13]. The enzyme is much less reactive with serine (0.5 μmol mg^{-1} min^{-1}) than with alanine (1000 μmol mg^{-1} min^{-1}), but one can compensate for this by using more enzyme. In addition, the optimum pH for the oxidation reaction is 10.5, a pH at which transketolase is inactive, but that for the reductive reaction is 8.2 [14]. Therefore, it is necessary to run the oxidation reaction at an

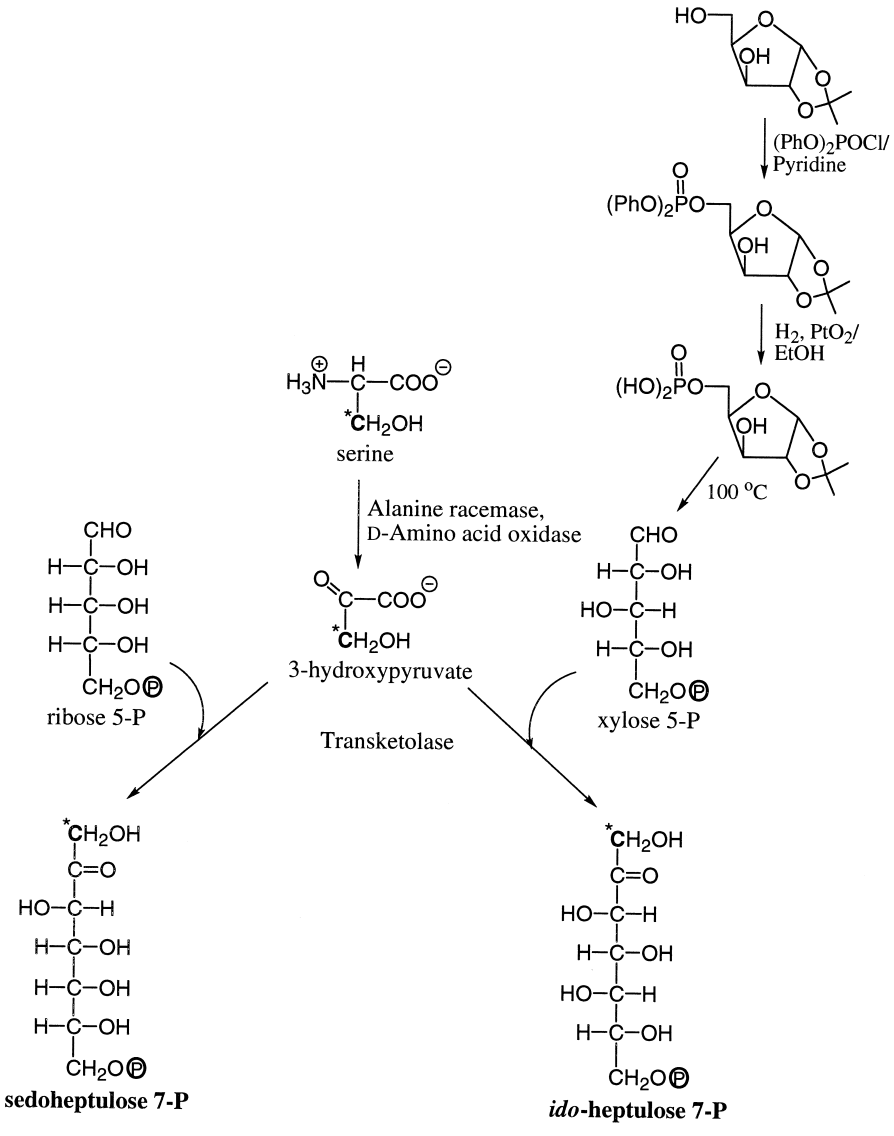

Fig. 4. Synthesis of ^{13}C-labeled sedo- and *ido*-heptulose 7-phosphate.

alkaline pH and then change the pH to 7.5 for the transketolase reaction. However, this created another problem, as became evident when 50 mM glycine/NaOH, pH 10.0, and 300 U of alanine dehydrogenase were used for the oxidation of serine (10 mM) and then the pH was changed to 7.5–8.0 with an equal volume of 100 mM Tris buffer, pH 7.5, for the transketolase (20 U) reaction with ribose 5-phosphate. Most of the serine (95%) was recovered, because 3-hydroxypyruvate is an even better substrate than pyruvate (1.2 fold at pH 8.5) for the reverse reaction, the reductive amination by alanine dehydrogenase [14], which therefore outcompeted the transketolase reaction. This problem could be partially overcome by reoxidizing NADH to NAD^+ and consuming ammonium ion with glutamate dehydrogenase. Thus, sedoheptulose 7-phosphate was obtained in 30% yield when the reaction mixture containing 50 mM glycylglycine buffer, pH 8.5, and 20 mM serine in a total volume of 0.5 ml was incubated with glutamate dehydrogenase (4 U) and α-ketoglutarate (20 mM) in addition to alanine dehydrogenase (75 U), transketolase (5 U) and ribose 5-phosphate (20 mM).

The third method (Fig. 4) to convert serine to 3-hydroxypyruvate is to use an amino acid oxidase [12]. Unfortunately, L-serine is a poor substrate for L-amino acid oxidase [15], whereas D-amino acid oxidase reacts at a reasonable rate with D-serine [16]. Since we needed to start from L-serine as the available labeled substrate we employed a thermostable alanine racemase from *B. stearothermophilus* [17] to convert the labeled L-serine into the D isomer in situ. The reaction rates favor the D to L conversion and, again, the enzyme has a strong preference for alanine (1000 μmol mg^{-1} min^{-1}) over serine (0.5 μmol mg^{-1} min^{-1}) as substrate, which can be compensated by using more enzyme. Importantly, the reaction can be run under the same conditions, i.e., simultaneous with, the transketolase reaction. In this way we prepared D-[1-^{13}C]sedoheptulose 7-phosphate in 69% yield from L-[3-^{13}C]serine, containing a trace of L-[3-^{14}C]serine to guide the work-up, in a single incubation with alanine racemase, D-amino acid oxidase, catalase and transketolase under oxygen gas and with ribose 5-phosphate as second substrate.

The same method of using D-amino acid oxidase coupled with alanine racemase was employed to prepare [1-^{13}C]*ido*-heptulose 7-phosphate from L-[3-^{13}C]serine and unlabeled D-xylose 5-phosphate. Since unlabeled xylose 5-phosphate is not commercially available, the xylose 5-phosphate was synthesized from 1,2-*O*-isopropylidene-α-D-xylofuranose following a previously published procedure [18].

3. Experimental

3.1. General

[6-^{3}H]Glucose and [6-^{14}C]glucose were purchased from Amersham. L-[3-^{14}C]Serine was obtained from ICN and L-[3-^{13}C]serine (90.0% ^{13}C enrichment) from the Los Alamos Stable Isotope Resource. Whatman 3MM paper for paper chromatography was purchased from Baxter, chemicals and enzymes from Sigma, cation exchange resin Dowex 50×8 (H^+ form) from Aldrich and anion exchange resin AG-1×8 (formate form) from BioRad. The transaminases were a gift from Dr. Michael Breuer, BASF, Germany. Alanine dehydrogenase and alanine racemase were gifts from Professor K. Soda, Kansai University and Professor N. Esaki, Kyoto University, respectively, Japan. Radioactivity was determined by scintillation counting (Beckman LS 1801) using Bio-Safe II biodegradable scintillation cocktail (Research Products International). The solvent system for paper chromatography was *n*-butanol:acetic acid:water = 2:1:1; the unlabeled phosphate esters were detected on the paper chromatograms by spraying with Hanes reagent and exposure to UV light for 15 min to locate the adjacent bands of the corresponding labeled phosphate esters [19].

3.2. Synthesis of [7-^{14}C, 7-^{3}H]sedoheptulose 7-phosphate from [6-^{14}C, 6-^{3}H]glucose

3.2.1. [6-^{14}C, 6-^{3}H]Glucose 6-phosphate

To the reaction mixture containing 20 mM $MgCl_2$, 1 mM DTT, 15 mM ATP and 50 mM Tris buffer, pH 7.5, was added a mixture of [6-^{3}H]- and [6-^{14}C]Glucose (total 500 μCi ^{3}H, $^{3}H/^{14}C$ ratio 10.4) specific radioact: 5 Ci/mmol for ^{3}H, 10 mCi/mmol for ^{14}C) and hexokinase (5 U) in a total volume of 500 μl. The reaction mixture was incubated at room temperature for 30 min, boiled for 2 min to remove protein, lyophilized, and chromatographed on paper. After drying the paper, the areas containing glucose 6-phosphate and fructose 6-phosphate (due to contaminating phosphoglucose isomerase) (R_f 0.27 and 0.37) were eluted with water to give 236 and 164 μCi of ^{3}H (80% combined radiochemical yield).

3.2.2. [5-^{14}C, 5-^{3}H]Ribose 5-phosphate

The labeled glucose 6-phosphate (236 μCi) and fructose 6-phosphate (164 μCi) were separately added each to a reaction mixture containing 75 mM Tris buffer, pH 7.9, 5 mM ammonium chloride, 5 mM α-ketoglutarate, 1 mM $MgCl_2$, 0.5 mM NADP, glucose 6-phosphate dehydrogenase (3 U), 6-phosphogluconate dehydrogenase (2 U) and glutamate dehydrogenase (2 U) in a total volume of 550 μl. The enzymes again contain enough contaminating phosphoglucose isomerase to allow for the conversion of the fructose- to the glucose 6-phosphate. After incubation at room temperature for 1 h, phosphoribose isomerase (3 U) was added and the reaction mixture was incubated for another 1 h and then chromatographed on paper to give a total of 236 μCi of ribose 5-phosphate (R_f 0.64, 59% radiochemical yield).

3.2.3. [7-^{14}C, 7-^{3}H]Sedoheptulose 7-phosphate

The labeled ribose 5-phosphate was added to a reaction mixture containing 90 mM glycylglycin buffer, pH 7.4, 6 mM $MgCl_2$, 0.3 mM NADH, 0.1 mM TPP, phosphoglyceraldehyde dehydrogenase (5 U), triosephosphate isomerase (5 U) and transketolase (10 U) in a total volume of 850 μl. The reaction was followed by monitoring the absorbance at 340 nm. After 30 min, the reaction mixture was boiled for 2 min to remove proteins and lyophilized. The final product, sedoheptulose 7-phosphate was purified by paper chromatography (R_f 0.46) to give a total of 145 μCi ^{3}H (61% radiochemical yield, $^{3}H/^{14}C$ ratio 10.1). The overall radiochemical yield from glucose was 29%.

3.3. Synthesis of [1-^{13}C]sedoheptulose 7-phosphate from L-[3-^{13}C]serine

The reaction mixture containing 25 mM Tris buffer, pH 8.0, 100 mM L-[3-^{13}C]serine containing 8 μCi of L-[3-^{14}C]serine, 35 mM ribose 5-phosphate, 1 mM TPP, 3 mM $MgCl_2$, D-amino acid oxidase (15 U), catalase (70 U) and transketolase (9 U) in a total volume of 2.5 ml was placed in a 25 ml two-neck round-bottom flask. The flask was filled with oxygen through a needle connected to an oxygen balloon. The reaction was started by addition of alanine racemase (350 μl, 5 mg) and the mixture was stirred at room temperature. Catalase (100 U) was added every 3 h and ribose 5-phosphate (90 μmol) was added at 3 h and 6 h. More alanine racemase (100 μl), D-amino acid oxidase (8 U) and transketolase (10 U) were added at 6 h. After 14 h, the enzymes were removed by passage through a PD-10 column and the reaction mixture was applied to an AG-1 × 8 (formate form, 2.5 × 16 cm) column. The column was washed with water (50 ml) and eluted with a gradient (400 ml water vs. 400 ml 0.4 N formic acid/0.1 N ammonium formate). The fractions containing the sedoheptulose 7-phosphate, as determined by radioactivity, were pooled (120 ml) and concentrated to 5 ml in vacuo. The remaining ammonium ions were removed by passage through a Dowex 50 (H^+ form, 1.5 × 3 cm) column and the flow-through solution was

neutralized with 4 N NaOH. Barium chloride (0.6 mmol) was then added to the solution, followed by ethanol to a final concentration of 80% (v/v, total 80 ml). After standing at 4°C for 3 h, the precipitated Ba salt of sedoheptulose 7-phosphate was collected by centrifugation and washed with 25 ml of 80% ethanol. The Ba salt was dried in a vacuum to give 80 mg of barium sedoheptulose 7-phosphate (75%). The Ba salt was dissolved in water (11 ml) with the aid of 1 N HCl (100 μl) and 250 μl of 1 N Na_2SO_4 was added. After neutralization with NaOH, the $BaSO_4$ was removed by centrifugation and the supernatant was lyophilized. Yield 69% based on radioactivity, ^{13}C NMR: enriched signals at 63.5 (highest), 63.8 (α and β anomers of pyranose form) and 65.1 ppm (furanose form), ^{13}C enrichment 80%, based on ES-MS.

3.4. Synthesis of xylose 5-phosphate [18]

3.4.1. 1,2-O-Isopropylidine-α-D-xylofuranose 5-O-diphenylphosphate

To a solution of 1,2-*O*-isopropylidene-α-D-xylofuranose (3.75 g, 19.7 mmol) in dry pyridine (20 ml) at 0°C was added diphenyl chlorophosphate (4.4 ml, 21.2 mmol) dropwise under N_2 over 15 min. After stirring at 0°C for 5 h and then at room temperature for 4 h, the reaction mixture was treated with 150 ml $CHCl_3$, washed with water (3×50 ml) and brine (25 ml), and dried over Na_2SO_4. After filtration, the filtrate was concentrated to dryness under reduced pressure and the residue was recrystallized from a $CHCl_3$ and hexane mixture. The crystals were collected, washed with hexane and dried under reduced pressure to give product (7.5 g, 90.1%): R_f 0.8 ($CHCl_3$:MeOH = 8:1); ^1H NMR (CD_3OD) δ 1.29 (s, 3H), 1.41 (s, 3H), 4.1–4.5 (m, 5H), 5.91 (d, $J = 4.2$ Hz, 1H), 7.2–7.5 (m, 10H).

3.4.2. 1,2-O-Isopropylidine-α-D-xylofuranose 5-hydrogenphosphate

The reaction flask containing 1,2-*O*-isopropylidine-α-D-xylofuranose 5-*O*-diphenylphosphate (680 mg, 1.65 mmol) and PtO_2 (250 mg) in ethanol (6 ml) was evacuated and filled with hydrogen through a needle connected to a hydrogen balloon. The reaction mixture was stirred at room temperature for 2 days (only one phenyl group was removed, R_f 0.67 MeOH:$CHCl_3$:pyridine = 10:5:1). After filtering off the Pt, fresh PtO_2 was added (250 mg) and the reaction mixture was stirred for another 2 days under hydrogen to complete the reaction. The catalyst was filtered off through Celite and washed with ethanol. The filtrate was concentrated under reduced pressure to yield the product (403 mg, 92.6%): R_f 0.36 (MeOH:$CHCl_3$:pyridine = 10:5:1).

3.4.3. Dilithium D-xylose 5-phosphate

The solution containing 1,2-*O*-isopropylidine-α-D-xylofuranose 5-hydrogenphosphate (350 mg, 1.30 mmol) and water (1 ml) was refluxed under N_2 for 70 min. The light-tan solution was diluted with water (20 ml) and treated with active charcoal. The suspension was filtered through Celite and the solution was refiltered through an ultramembrane to remove trace amounts of charcoal and catalyst. After neutralizing the filtrate with 1 N lithium hydroxide, the solution was concentrated to 3 ml and treated with ethanol (30 ml) to induce precipitation. The precipitate was collected by centrifugation and washed with 80% ethanol. After centrifugation the residue was dried under reduced pressure to give D-xylose 5-phosphate as the lithium salt (240 mg, 75.6%). The purity of the D-xylose 5-phosphate was checked using the transketolase reaction with xylulose 5-phosphate, comparing to ribose 5-phosphate.

3.4.4. Synthesis of [1-^{13}C]ido-heptulose 7-phosphate from L-[3-^{13}C]serine

The reaction mixture containing 50 mM Tris buffer, pH 8.0, 125 mM L-[3-^{13}C]serine containing 8 μCi of L-[3-^{14}C]serine, 42 mM xylose 5-phosphate, 1 mM TPP, 3 mM $MgCl_2$, D-amino acid oxidase (15 U), catalase (100 U) and trans-

ketolase (15 U) in a total volume of 2.0 ml was placed in a 25 ml two-neck round-bottom flask. The flask was filled with oxygen through a needle connected to an oxygen balloon. The reaction was started by addition of alanine racemase (300 μl) and the mixture was stirred at room temperature. Catalase (100 U) was added every 3 h and xylose 5-phosphate (84 μmol) was added at 3 h and 6.5 h. Alanine racemase (200 μl) and transketolase (20 U) were again added at 6.5 h. After 15 h, the enzymes were removed by passage through a PD-10 column and [1-^{13}C]*ido*-heptulose 7-phosphate was purified following the same procedure used for the purification of [1-^{13}C]sedoheptulose 7-phosphate. Yield 70% based on radioactivity, ^{13}C NMR: enriched signals at 63.5, 64.4 (highest) (α and β anomers of pyranose form) and 64.9 ppm and (furanose form), ^{13}C enrichment 82%, based on ES-MS.

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