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Efficient multi-enzymatic synthesis of D-xylulose 5-phosphate

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Abstract

Pure D-*threo*-2-pentulose 5-phosphate, a valuable substrate required for enzymatic assays, is prepared in gram-quantities (82% overall yield) by an enzymatic one-pot procedure starting from the readily available precursors hydroxypyruvate and fructose 1,6-bisphosphate. The procedure is based on a stereospecific C–C bond formation catalyzed by the recombinant transketolase A from *Escherichia coli*. © 1999 Elsevier Science Ltd. All rights reserved.

For complex, multifunctional structures that are difficult to prepare by conventional means, asymmetric framework construction by enzymatic carbon–carbon bond formation is an attractive alternative to standard chemical methods due to an often complete stereochemical control, mild reaction conditions, and no requirement for protecting group chemistry.[1] In particular, a number of aldolases have proved invaluable for the preparation of complex carbohydrates and related targets.[2] Recently, the transketolase A from *Escherichia coli* (TK_{eco}; EC 2.2.1.1) has become available on a large scale by recombinant expression[3,4] which has stimulated investigations towards the development of practical procedures that use this enzyme as a catalyst in asymmetric synthesis.[5,6]

In vivo, transketolase catalyzes the stereospecific transfer of a hydroxyacetyl nucleophile between various sugar phosphates in the presence of a thiamine diphosphate cofactor and divalent cations. For a spectrophotometric determination of the substrate specificity of native transketolase, or for the kinetic evaluation of site-directed mutant enzymes, the C_2-donor component D-xylulose 5-phosphate (D-*threo*-2-pentulose 5-phosphate, **1**) is the assay substrate of choice because of its superior kinetic constants (K_m 160 μM).[4] D-Xylulose 5-phosphate has also been implicated to serve as a second messenger in liver tissue for the stimulation of glycolysis by activating a regulatory protein phosphatase (PP2A).[7] However, commercial **1** is only ~80% pure and very expensive. Preparation by phosphorylation of the free ketose requires the isolation of a non-commercial kinase,[8] while published protocols for the synthesis of **1** from

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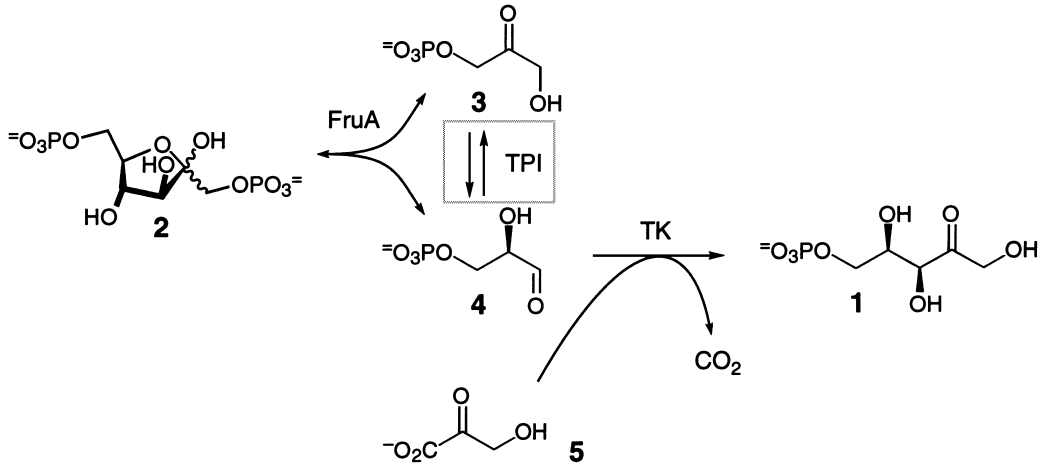
other phosphorylated precursors are tedious, micro-scale and/or low-yielding.[9–12] Another difficulty is posed by the propensity of **1** towards chemical or enzymatic C-3 epimerization or ketol isomerization, each of which gives rise to a product that is a known transketolase inhibitor.[4]

Based on the stereospecific transketolase-catalyzed ketol transfer from hydroxypyruvate **5** to D-glyceraldehyde 3-phosphate **4**,[10–12] we have developed a practical and efficient procedure for the preparation of **1** on a gram-scale. Retro-aldolization of D-fructose 1,6-bisphosphate **2** in the presence of D-fructose 1,6-bisphosphate aldolase (FruA, EC 4.1.2.13) is used as an in situ source of the triose phosphate **4** (Scheme 1). This is an adaption of a prior strategy originally proposed by Racker et al. who dispensed with the **3**↔**4** interconversion.[9,10] In our view, clear disadvantages of their procedure — and likely cause for low overall yields and difficulties upon product purification — are: (1) that **4** is highly unstable to phosphate elimination in solution but is produced at high stationary concentrations; (2) that only one equivalent of triose phosphates (i.e., **4**) is utilized; and (3) that the concomitant steady build-up of dihydroxyacetone phosphate (DHAP, **3**) during the course of the synthesis diminishes the effective concentration of **4** attainable by the aldolase equilibrium.



Scheme 1. One-pot enzymatic synthesis of D-xylulose 5-phosphate from hydroxypyruvate and D-fructose 1,6-bisphosphate

In contrast to the procedure introduced by Racker et al.,[9] the addition of triosephosphate isomerase (TPI, EC 5.3.1.1) would allow the equilibration of unused **3** into a second equivalent of aldehyde substrate **4**. As a consequence, the effective concentration of **4** would be lowered, initially due to the adverse TPI equilibrium constant which strongly favors the ketone **3** (K_{eq} ~20).[13] However, reaction rates should still be sufficient for practical purposes owing to the affinity of transketolase for **4** (K_m 2.1 mM);[4] in addition, the overall synthetic reaction is rendered irreversible by the release of CO_2 from hydroxypyruvate.[14] The concomitant increase in pH, which must be compensated by controlled addition of mineral acid (i.e., autotitration), can be utilized to monitor the course of the reaction (Fig. 1). Indeed, a steady consumption of **2** occurred which was almost complete after 48 h, as verified by enzymatic analysis for remaining starting material. The product was isolated by anion exchange chromatography using formic acid as eluent to provide gram-amounts of **1** in 82% overall yield (based on **2** consumed).[15]

Previous protocols for preparation of **1** suffer from the need for laborious separation of contaminant epimer **6** arising from a common contamination of transketolase with ribulose 5-phosphate 3-epimerase which is hard to eliminate.[10,11] Indeed, although the *E. coli* transketolase has often been applied as a rather crude preparation,[5] it proved essential to utilize an enzyme additionally purified by tentacle-type chromatography (Fractogel, EMD DEAE-650) to obtain diastereomerically pure compound **1** devoid of

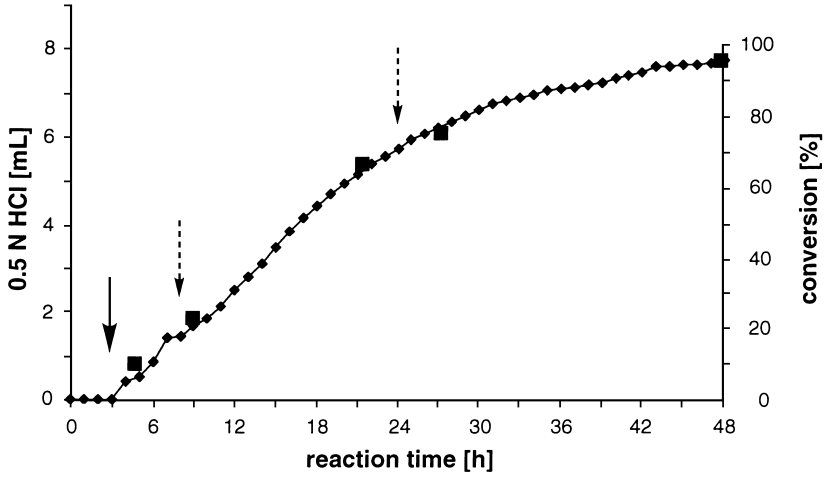


Figure 1. Monitoring of conversion by acid consumption (◆) and by enzymatic assay for remaining D-fructose 1,6-bisphosphate (■). Reaction started after addition of enzymes at the time indicated by solid arrow; further substrate and enzymes were added at times indicated by broken arrows[15]

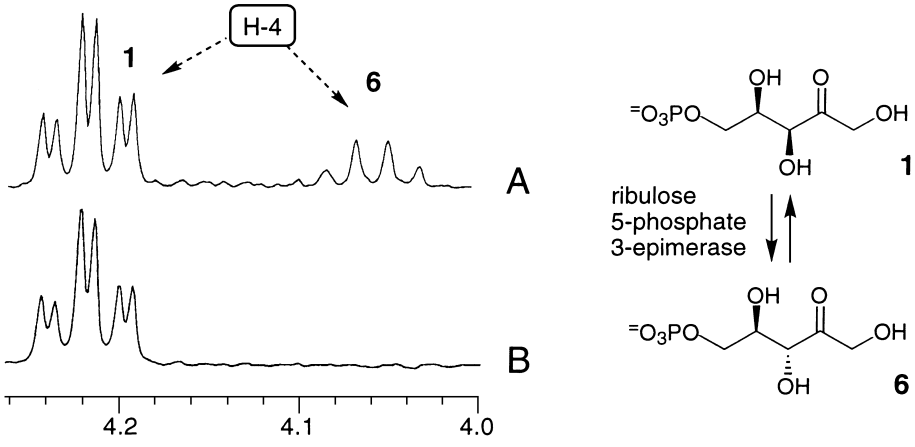


Figure 2. Epimerization of **1** by contaminating ribulose 5-phosphate 3-epimerase. Determination of diastereomer composition by ^{1}H NMR analysis on key signals for H-4. (A) Product obtained using transketolase of standard purity; (B) product from reaction with transketolase purified by additional tentacle chromatography

its epimer. The presence of **6** can be easily assessed by ^{1}H NMR analysis since signals for H-4 are well resolved (Fig. 2, traces A versus B).

In summary, we have demonstrated that the valuable ketosugar phosphate **1** can be efficiently prepared on a gram-scale by a simple one-pot procedure utilizing the recombinant transketolase from *E. coli*. This multi-enzymatic synthesis is highly practical for the multi-gram synthesis of the phosphosugar in very good yield.

Acknowledgements

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14. Dickens, F.; Williamson, D. H. *Biochem. J*. **1958**, *68*, 74–81. Although the use of **5** results in irreversible conversion to products, and hence is preferred to other C-2 ketol donors, its expense and exacting preparation currently pose a limitation on the scale-up of the reaction. The synthesis of Li-**5** from bromopyruvic acid suffers from poor overall yield (23% on 2.5 g scale). In our hands, slight modification of this procedure and omission of the final recrystallization step yielded lithium hydroxypyruvate sufficiently pure for synthesis (>95%) reproducibly in ≥55% isolated yield on a ca. 20 g scale.
15. D-*threo*-2-Pentulose 5-phosphate, Na-salt **1**: an aqueous solution (250 mL) containing Na_3 D-fructose 1,6-bisphosphate (**2**; 1.015 g, 2.5 mmol), Li hydroxypyruvate (**5**; 550 mg, 5.0 mmol), $MgCl_2(H_2O)_6$ (76.2 mg, 0.38 mmol), thiamine diphosphate (86.4 mg, 0.19 mmol) was adjusted to pH 7.0 using a pH controller (SCHOTT TitroLine) with 0.5 M HCl. The reaction was started by adding $FruA_{rab}$ (72 U), TPI (125 U) and TK_{eco} (300 U). After 6 h, additional FruA (36 U), TPI (65 U) and TK (200 U), after 8 h additional TK (150 U) and Li-**5** (330 mg, 3.0 mmol), and after 24 h additional TK (200 U) and Li-**5** (330 mg, 3.0 mmol) were added. The reaction was monitored by the consumption of acid and by enzymatic assay for remaining DHAP equivalents. After 48 h, the reaction was terminated by addition of cation exchange resin (20 g, Dowex AG 50W-X8, H^+-form). After dilution with water (250 mL) and degassing, the solution was passed through an anion exchange column (60 mL, AG 1-X8, formate form). Product was eluted with 1.3 M formic acid (flow rate 2 mL min^{-1}, fraction size 13 mL). Fractions 56–100 containing **1** were combined and evaporated three times. The concentrate was taken up in water and the pH adjusted to 6.95 with 0.5 M NaOH. Final concentration yielded 1.117 g (4.08 mmol, 82% based on **2**) of **1** as a pale yellow syrup. ^{1}H NMR (300 MHz; D_2O): δ 4.69 (d, 1-H_a), 4.55 (d, 1-H_b), 4.55 (d, 3-H), 4.22 (dt, 4-H), 3.90–3.82 (m, 2H, 5-$H_{a,b}$), $J_{1a,1b}$=19.5, $J_{3,4}$=2.1, $J_{4,5a}$=$J_{4,5b}$=6.4 Hz; ^{13}C NMR (75.4 MHz; D_2O): δ 213.7 (s, C-2), 75.8 (s, C-3), 71.8 (d, C-4), 66.6 (s, C-1), 64.5 (d, C-5), $J_{C\text{-}4,P}$=7.3, $J_{C\text{-}5,P}$=4.9 Hz; $[\alpha]_D^{25}$=−6.5 (*c* 1.0, H_2O).