Tetrahedron Letters,Vol.29,No.4,pp 427-430,1988
Printed in Great Britain

0040-4039/88 $3.00 + .00
Pergamon Journals Ltd.

Synthesis of 3-Deoxy-D-manno-2-octulosonate-8-phosphate (KDO-8-P) from D-Arabinose: Generation of D-Arabinose-5-Phosphate using Hexokinase[1]

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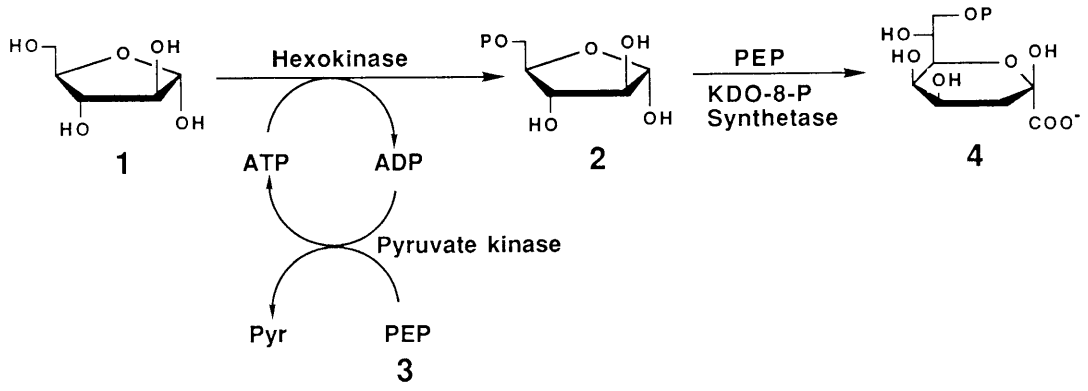
Summary. Three enzymes (used in soluble form enclosed in a dialysis membrane) efficiently produce 3-deoxy-D-manno-2-octulosonate-8-phosphate (KDO-8-P, **4**) from D-arabinose (Ara, **1**) and phosphoenolpyruvate (PEP **3**); production of D-Ara-5-P **2** from Ara using hexokinase is the key step in the synthesis.

This letter reports a multi-gram enzymatic synthesis of 3-deoxy-D-manno-2-octulosonate-8-phosphate[6] (KDO-8-P, **4**) from D-arabinose (Ara, **1**) and phosphoenolpyruvate (PEP, **3**). The key step in this synthesis is the production of the expensive intermediate D-arabinose-5-phosphate (Ara-5-P, **2**, ~ 10^4/mole) from D-arabinose (Ara, **1**) using hexokinase.[7] KDO-8-P is a key intermediate in the biosynthesis of the lipopolysaccharide (LPS) region of gram-negative bacteria.[8,9] Inhibitors of LPS biosynthesis are candidates in the design of antimicrobial pharmaceuticals.[10,11] The availability of gram quantities of KDO-8-P may facilitate the search for new inhibitors. This synthesis may also be applicable to the synthesis of analogs of KDO-8-P.



Production of Ara–5-P **2** relies on two enzymes: hexokinase (E.C. 2.7.1.1) and pyruvate kinase (E.C. 2.7.1.40). Hexokinase catalyzes the generation of **2** and ADP from Ara **1** and a catalytic amount of ATP. In a coupled system, pyruvate kinase regenerates ATP from ADP, while converting the readily available PEP to pyruvate.[12] Aldol condensation of **2** with additional PEP using KDO-8-phosphate synthetase (E.C. 4.1.3.3), isolated from E. coli B, produces **1**.[13] The enclosure of all three enzymes in a single dialysis membrane facilitates separation of the reaction products from protein and allows reuse of the catalysts.[14] In our hands the enzymatic production of Ara-5-P is superior to the method based upon the reaction between D-glucosamine-6-phosphate and ninhydrin.[15]

Isolation of **4** is straightforward. Addition of barium chloride to the crude reaction mixture precipitates a mixture of barium salts containing primarily KDO-8-P and inorganic phosphate (P_i). Stirring the mixture with ion exchange resin (Dowex 50W, H^+ form), followed by neutralization with LiOH and filtration, removes P_i as its insoluble lithium salt. Lyophilization of the filtrate followed by further purification by gel filtration (Biogel P-2, eluant: water) provides 90% pure **4** in 63% yield; PEP and KDO (trace) are also present. Ion exchange chromatography (AG 1-X2, eluant: 0 to 1.3 M $HCO_3^-NH_4^+$) removed residual PEP to give **4** in >95% purity (42% yield). Experimental procedures are provided below.

KDO-8-phosphate (4) from Ara (1). To a solution of PEP^-K^+ [12] **3** (15.25 g, 74 mmol; pH 7) in water (250 mL) were added Ara **1** (9.00 g, 60 mmol) and ATP (3.68 g, 6 mmol) and the solution was adjusted to pH 7.6 by addition of 1 N NaOH. After addition of $MgCl_2 \cdot 6H_2O$ (1.76 g, 8.7 mmol) and 2-mercaptoethanol (0.1 mL), the reaction solution was placed in a 500-mL graduated cylinder containing a magnetic stirring bar, and was degassed with N_2 for 30 min. KDO-8-phosphate synthetase[13] (50 U, 1 U = 1 μmol $min^{-1}mg^{-1}$), pyruvate kinase (160 U) and hexokinase (16,000 U) were dissolved in 13 mL of the reaction solution, placed in a dialysis membrane (Spectrapor 2, MW cutoff 12,000-14,000, 25 mm) and added to the reaction vessel; N_2 was continously bubbled through the solution. After 4 days, the enzyme-containing bag was removed and dialyzed twice against 50 mL of water. The combined reaction mixture and dialysates (350 mL) were transfered to a 2-L Erlenmeyer flask and $BaCl_2 \cdot 2H_2O$ (20 g, 82 mmol) was added, followed by 700 mL of acetone. The solution was stirred for 2 h and left overnight at 4 °C. Filtration of the resulting barium precipitate (which consisted primarily of P_i and KDO–8–P) yielded 26.4 g of a brown solid. Water (500 mL) was added to this solid followed by ion exchange resin (Dowex 50W, H^+, 17 g) and the solution was stirred for 72 h. Neutralization of the filtrate with LiOH precipitated P_i. The filtrate was concentrated in vacuo to approximately 100 mL, and this solution was passed through a column of Biogel P–2 (100 x 4.5 cm, eluant: water). Fractions containing **4** were pooled[16] and concentrated in vacuo to give 8.2 g of **4** (90% purity by ^1H NMR spectroscopy; 63% yield); PEP (10%) and KDO (trace) were also present. Ion exchange chromatography (AG 1-X2, eluant: 0 to 1.3 M $HCO_3^-NH_4^+$) removed residual PEP. The fractions containing KDO-8-P were pooled and brought to pH 6 by

addition of Dowex 50W (H+ form) and the solution was filtered and concentrated in vacuo to give 5.41 g of **4** (>95% purity, 42% yield). The product was characterized by ^{1}H NMR, ^{13}C NMR and by assaying for release of phosphate according to the procedure of Ray;[6] treatment of **4** with acid phosphatase (E.C. 3.1.3.2) yielded KDO .

Arabinose-5-phosphate (2). To a solution of $PEP^{-}K^{+}$ **3** (11.0 g, 91%, 48 mmol; pH 7) in water (75 mL) in a 250-mL Erlenmeyer flask containing a magnetic stirring bar were added: Ara **1** (10.9 g, 72.8 mmol), ATP (1.29 g, 2.16 mmol) and $MgCl_2 \cdot 6H_2O$ (2.2 g, 10.8 mmol). The pH was adjusted to pH 7.6 with 1 N NaOH, the solution was degassed with N_2 for 30 min and 0.1 mL of 2-mercaptoethanol was added. Hexokinase (10,000 U) and pyruvate kinase (500 U) were dissolved in 4 mL of the reaction mixture and the enzyme solution was placed in a section of dialysis tubing . The enzyme-containing bag was added to the reaction flask which was then stoppered and agitated at 150 rpm on an orbital shaker at room temperature. Analysis by ^{31}P NMR spectroscopy monitored the formation of **2**. After 4 days, 10,000 U of additional hexokinase in a dialysis membrane were added to the reaction mixture. After an additional 3 days, 10,000 U of hexokinase, 500 U of pyruvate kinase and 850 mg (4.2 mmol) of $MgCl_2 \cdot 6H_2O$ were added. A white precipitate, presumably the magnesium salt of P_i, formed during the reaction. After an additional 5 days, the enzyme-containing bags were removed and dialyzed twice against 50 mL quantities of water. To the combined reaction and dialysates was added 4 g of $BaCl_2 \cdot 2H_2O$ (in 1 g portions dissolved in approximately 10 mL of water) to precipitate remaining ATP and P_i. After each addition, the mixture was filtered through Celite and each filter cake was washed with approximately 30 mL of water. To the combined filtrates (300 mL) was added $BaCl_2 \cdot 2H_2O$ (13 g, 53 mmol) and then 900 mL of EtOH and the mixture was left at 4 °C overnight. The precipitate was collected by filtration, washed with 100 mL of EtOH:water (3:1, v:v) and dried in vacuo over P_2O_5 to give 18.5 g of the barium salt of **2** (85% purity, 89% yield). The spectral data matched that of a sample obtained from Sigma; PEP (5%) and dipyruvate (10%) were also present. Anal: Calcd. for $C_5H_9O_8PBa$: C, 16.43; H, 2.48; Ba, 37.58; Found: C, 16.33; H, 2.45; Ba, 37.17.

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1. Supported by NIH Grant GM 30367.
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(Received in USA 5 November 1987)