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CATALYTIC MECHANISM OF KDO8P SYNTHASE: SYNTHESIS AND EVALUATION OF A PUTATIVE REACTION INTERMEDIATE

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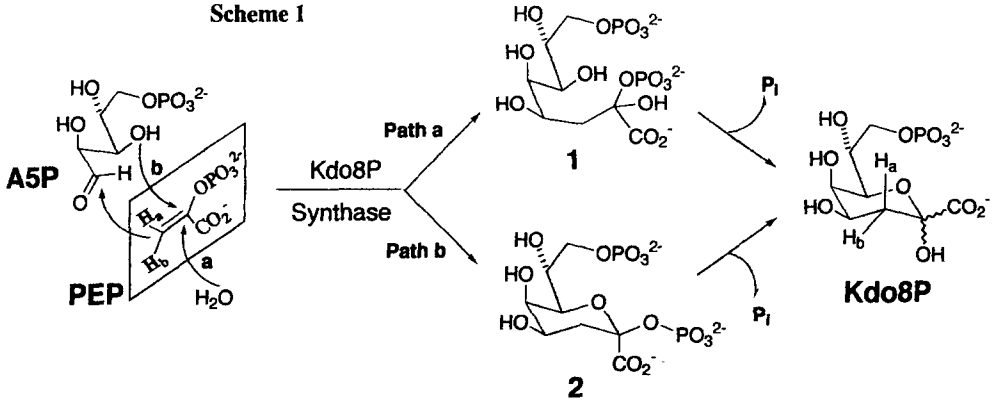
Abstract: The catalytic mechanism of 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase from *Escherichia coli* was investigated on the basis of synthesis and evaluation of the putative cyclic intermediate **2**.

3-Deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase (EC 4.1.2.16) is a key enzyme that controls the carbon flow in the biosynthetic formation of 3-deoxy-D-*manno*-2-octulosonate (Kdo),[1] a constituent of the lipopolysaccharide of most Gram-negative bacteria.[2] The enzyme catalyzes the unusual condensation of phosphoenolpyruvate (PEP) with D-arabinose-5-phosphate (A5P) to produce Kdo8P and inorganic phosphate (P_i).[3] Interestingly, a similar reaction between PEP and erythrose-4-phosphate to produce 3-deoxy-D-*arabino*-heptulosonate-7-phosphate (DAHP) is catalyzed by the shikimate pathway enzyme DAHP synthase.[4] The mechanisms of these reactions are still unknown. Earlier studies by Abeles,[5] and recently by Woodard,[6] have established that the Kdo8P synthase reaction is essentially irreversible and proceeds through the C−O bond cleavage of PEP. We have recently shown[7] that the enzyme acts upon the acyclic form of A5P, and have demonstrated an ordered sequence of substrate binding (PEP followed by A5P) and product release (P_i prior to Kdo8P). By using stereospecifically labeled 3-deuterio and 3-fluoro analogues of PEP as alternative substrates of the synthase, it has been shown that the condensation step is stereospecific, involving the attachment of the *si* face of PEP to the *re* face of the carbonyl of A5P.[8] Based on these mechanistic data, in combination with the results accumulated through the synthesis and examination of various analogues of A5P,[7,9] analogues of PEP[8,10] and of the product Kdo8P[11,12] as mechanistic probes, two distinct pathways have been proposed for the reaction of Kdo8P synthase. These pathways, as illustrated in Scheme 1, involve the formation of either the acyclic intermediate **1** (path a)[5] or the cyclic intermediate **2** (path b).[6,11] Although these proposals for the formation of **1** and **2** are based on reasonable chemical grounds, no distinction, to date, between these two mechanistic pathways is available. After extending our studies on Kdo8P synthase mechanism, we are able to describe here the first chemical synthesis of the putative intermediate **2** and its evaluation with Kdo8P synthase.

Compound **2** was synthesized (Scheme 2) from the previously reported benzyl β-glycoside **3**.[13] Treatment of **3** with *t*-butyldimethylsilyl chloride resulted in selective silylation of the primary hydroxyl group. This material was then peracetylated to give triacetate **4**. Desilylation was performed, followed by phosphorylation of the primary hydroxyl group with diphenylphosphoryl chloride, to afford compound **5**.

Selective hydrogenolysis of the benzyl group with Pd/carbon gave the anomeric alcohol **6**, which was converted to the chloride **7** by using Vilsmeier reagent.[14] Preliminary experiments have shown that the phosphorylation of **7** with silver dibenzyl phosphate[15] in dry benzene at reflux provides a mixture of anomeric phosphates (**8** and **9**), predominantly in the form of α-phosphate (**8**:**9** = 1:17). Although in this procedure the desired β-phosphate (**8**) was obtained as a byproduct, upon close investigation, we found that by using one equivalent of the reagent for short periods and under kinetic control, a predominance of β-phosphate **8** is formed. However, prolongation of the reaction time resulted in an almost complete inversion of the anomeric center, and the thermodynamically more stable α-anomer **9** was exclusively obtained.

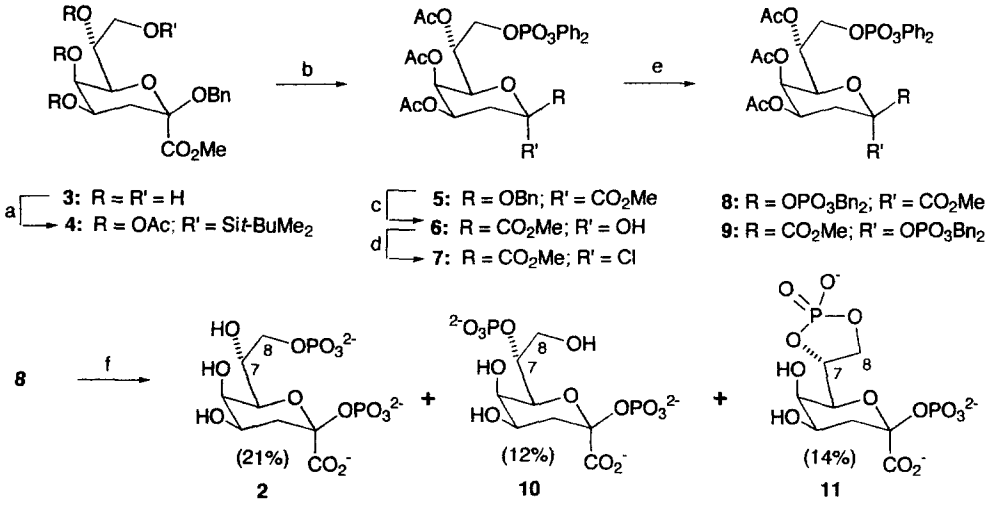
Scheme 1



The anomeric configuration of the phosphate linkage in **8** and **9** was determined by a combination of ^{1}H, ^{13}C, and ^{31}P NMR analysis. The results were in agreement with the previously reported analysis of Kdo-2-phosphates.[12] At this point, we decided to focus upon the deprotection of **8**, because this compound has the β-configuration proposed for the putative intermediate.[11] Deprotection of **8** was a major task in our synthetic plan. Lithium-ammonia reduction, which had been previously employed[16] for the simultaneous deprotection of benzyl and phenyl phosphates, resulted in extensive decomposition. Attempted purification of the reaction products, either by anion-exchange chromatography on AG1X8 (HCO_3^- form) or by gel-permeation chromatography (Bio-Gel P2 column), gave only monophosphorylated products. Finally, we found that **8** could be deprotected with reasonable efficiency by the following three-step procedure (Scheme 2). Hydrogenolysis with palladium catalyst in the presence of 2 mol equiv of triethylamine smoothly removed the benzyl protecting groups within 2 h. The following removal of phenyl esters by hydrogenolysis with PtO_2 catalyst (pH 8) proceeded very slowly, and it appeared that mostly only one phenyl ester was released (^{1}H and ^{31}P NMR evidence). The observed crude product was saponified with LiOH (pH 12) to give a mixture of three bisphosphates as judged from ^{31}P NMR. Separation of this mixture was performed by anion-exchange chromatography (Neobar AQ column, eluting with a linear gradient of 0–0.5 M ammonium bicarbonate salt) followed by passing the pooled fractions through a column of Dowex 50 W (K^+ form) to afford the following pure compounds as potassium salts: β-2,8-bisphospahte **2** (21%, for last three steps), β-2,7-bisphosphate **10** (12%) and cyclic bisphosphate **11** (14%). The formation of by-products **10** and **11** is a result of a side reaction observed on the intermediate monophenylphosphate ester after base treatment, a problem reported previously in a related systems.[17] Thus, on LiOH treatment the adjacent C-7 hydroxyl undergoes a nucleophilic attack

onto monophenylphosphate ester to give the cyclic phosphate **11**. This in turn, on a prolonged treatment with base, undergoes ring opening to give the mixture of 2,7- and 2,8-bisphosphates (**10** and **2**, respectively). This result was further corroborated by performing the base treatment (2.0 M LiOH) on the chemically pure **11**. The mixture of β-2,8-bisphosphate **2** and β-2,7-bisphosphate **10** was afforded, which was separated by anion-exchange chromatography.

Scheme 2[a]



[a]Key: (*a*) *t*-Butyldimethylsilyl chloride, Py, DMAP, 78%; Ac_2O, Py, 85%; (*b*) H_2SO_4, MeOH; $(PhO)_2P(O)Cl$, CH_2Cl_2, Et_3N, 81%; (*c*) Pd/C, MeOH, 91%; (*d*) CH_2Cl_2, $ClCH{=}N(CH_3)_2Cl$, 75%; (*e*) $AgOPO_3Bn_2$, C_6H_6, 36% to 63%; (*f*) H_2, Pd/C, THF, Et_3N; H_2, PtO_2, THF/H_2O; LiOH, H_2O.

The purity and structure of each product were established by a combination of ^1H, ^{13}C, and ^{31}P NMR analysis.[18] The proton coupled ^{31}P NMR spectrum of **2** showed the C-8 phosphate signal as a triplet (J = 8.3 Hz), whereas the same spectrum of **10** displayed a doublet (J = 10.1 Hz) for the C-7 phosphate. In contrast, the proton coupled ^{31}P NMR spectrum of **11** exhibited very down field signal for the second phosphate (18.15 ppm), which was split as a doublet of triplets (J_1 = 7.3, J_2 = 10.7 Hz). Such a down field shift of the phosphate signal is very characteristic of the cyclic phosphate linkages and has been reported previously[19] for similar structures. Therefore, the observed chemical shift and the multiplicity in **11** are only consistent with the 7,8-cyclic phosphate structure. Further confirmation of the desired β-orientation of the anomeric phosphate in target bisphosphate **2** was derived from the three-bond heteronuclear coupling constant between C-1 and phosphorus ($^3J_{P\text{-}C1}$ = 9.7 Hz), as has been reported for β-Kdo-2-phosphate.[12]

Compound **2** was first evaluated as a substrate of Kdo8P synthase. We expected that if **2** were a true intermediate in the Kdo8P-synthase-catalyzed reaction, then the enzyme should be capable of catalyzing its conversion to Kdo8P and inorganic phosphate. Since anomeric phosphate linkages are very sensitive under either thiobarbituric acid assay[3] or inorganic phosphate assay[20] conditions, we used the proton decoupled ^{31}P NMR assay in which the appearance of inorganic phosphate could be clearly monitored. Compound **2** was incubated (0.1 M Tris-HCl buffer, pD 7.0) with a 1000-fold higher concentration of the enzyme[21] than is

typically introduced into an assay experiment, and the reaction progress was monitored by ^{31}P NMR over a 24-h period. Unfortunately, no significant difference in anomeric phosphate release was detected versus the blank experiments (same conditions, but without enzyme) either at room temperature (25 °C) or at 37 °C. Further increase in the enzyme concentration (up to 8 mg enzyme per experiment), yielded similar results (i.e., no enzyme-catalyzed acceleration in the anomeric phosphate hydrolysis was detected). The half-lives for the hydrolysis of **2** (pD 7.0) at 25 °C and 37 °C were estimated as 4.7 h and 2.5 h, respectively, which are similar to that previously reported for β-Kdo-2-phosphate.[12]

Compound **2** was next evaluated as an inhibitor of the synthase.[22] The kinetic behavior of **2** reveals it as competitive with respect to PEP binding (Fig. 1). The observed competitive inhibition versus PEP was anticipated from the kinetic mechanism described by Kohen et al.[7] This work has demonstrated that the enzyme adopts an ordered sequential kinetic pattern with PEP as a first substrate. Therefore, it was expected that compound **2**, which combines the structural determinants of both substrates, should compete with PEP in the same, free enzyme form. The inhibition constant (K_i) could be measured from initial velocity studies and was estimated as 35 μM.

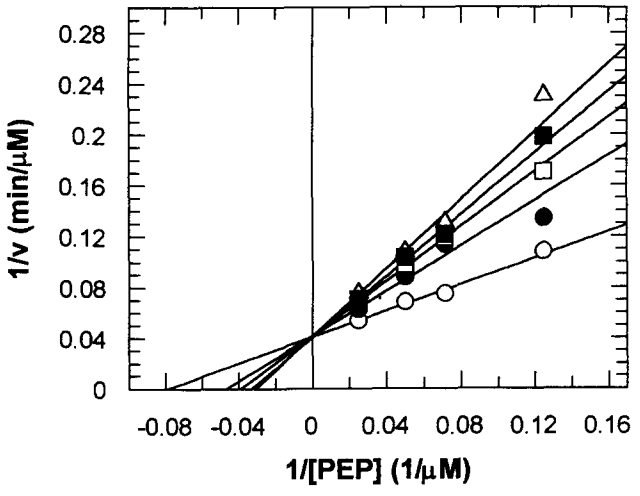


Figure 1: Inhibition of Kdo8P synthase by **2**. Double-reciprocal plots of initial velocities are given as a function of PEP, when the A5P concentration was 0.5 mM and the inhibitor concentrations were none (○), 21 μM (●), 42 μM (□), 63 μM (■) and 84 μM (Δ). The assays were carried out at 37 °C, pH 7.3 (Tris-acetate buffer, 0.1 M), including 38 milliunits of homogeneous Kdo8P synthase, PEP, A5P, and inhibitor (**2**) in a final volume of 1 mL. All samples were assayed in triplicate and analogous results were obtained in 2-4 different experiments.

The catalytic mechanism of Kdo8P synthase has been the subject of considerable controversy. Plausible explanations have been proposed for the enzyme-catalyzed reaction, but there is no direct experimental evidence to support them or to demonstrate their advantage over possible alternatives. The suggestion that **2** is a possible intermediate of Kdo8P-synthase-catalyzed reaction was based on reasonable chemical grounds and results accumulated through the synthesis and examination of various analogues of substrates, product and possible intermediate mimics.[6,11,12] However, all these data were only a poor and indirect support to the cyclic intermediate mechanism and, therefore, with some limitation could be well accommodated with both cyclic

(Scheme 1, path b) and acyclic (Scheme 1, path a) intermediate mechanisms. Indeed, the most potent inhibitor that was found to date for the enzyme (the isosteric phosphonate analogue of **2**, in which the bridged oxygen of anomeric phosphate of **2** is replaced by a bridged methylene group) displayed only moderate inhibition (K_i = 4.9 μM).[11] Although this inhibitor was designed to fit well within the topological and electrostatic properties of **2**, the observed binding affinity was considered weak for a reaction intermediate analogue, and raised doubts about the validity of **2** as a correct intermediate structure. The results of the present experiments demonstrate that cyclic bisphosphate **2** is neither a substrate nor a potent inhibitor for the synthase. Therefore, we conclude that this structure cannot be an intermediate in the normal reaction pathway catalyzed by the enzyme. This leaves us with the mechanism that involves the formation of acyclic hemiketal phosphate **1** as a reaction intermediate (Scheme 1, path a), originally proposed by Hedstrom and Abeles.[5] The moderate inhibitory activity (K_i = 35 μM), observed with **2**, strongly supports that the conformational flexibility of **1** is possibly a key component for the recognition with the enzyme active site. The compact cyclic structures, such as **2** and its isosteric phosphonate analogue, do not fit well the flexible spatial orientation imposed by **1** into the enzyme binding site. These results appear consistent with the proposal that structure **1** is the correct intermediate structure handled by the enzyme. However, we should note here that to date there is neither evidence nor strong indirect support that demonstrates the validity of **1** as a true intermediate structure. More studies are underway to resolve the mechanistic puzzles of this enzyme.

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18. Data for **2**: ^{1}H NMR (D_2O, 400 MHz) δ 3.72-3.96 (m, 3H, H-4, H-7 and H-8), 3.76 (br s, 1H, H-5), 3.66 (td, 1H, $J_{8',7} = J_{8',P} = 7.2$ Hz, $J_{8',8} = 11.6$ Hz, H-8'), 3.26 (d, 1H, $J_{6,7} = 7.6$ Hz, H-6), 2.41 (dd, 1H, $J_{3e,3a} = 12.0$ and $J_{3e,4} = 4.8$ Hz, H-3e), 2.20 (t, 1H, $J_{3e,4} = 12.0$ Hz, H-3a); ^{13}C NMR (D_2O, 100.6 MHz) δ 177.6 (d, $J = 9.7$ Hz, C1), 102.2 (d, $J = 6.2$ Hz, C2), 76.1 (C6), 72.4 (C7), 70.6 (C8), 69.0 (C5), 68.2 (C4), 36.3 (C3); proton coupled ^{31}P NMR (D_2O, 81 MHz) δ 5.34 (t, $J = 8.3$ Hz, 8-P), -0.37 (s, 2-P); FAB mass spectrum *m/z* 588.8 (MH^+, $C_8H_{11}O_{14}P_2K_5$ requires 588.6).

Data for **10**: ^{1}H NMR (D_2O, 400 MHz) δ 4.03 (m, 1H, $J_{7,6} = 8.8$, $J_{7,P} = 10.1$ and $J_{7,8} = J_{7,8'} = 3.2$ Hz, H-7), 3.83 (d, 1H, $J_{5,4} = 3.2$ Hz, H-5), 3.78 (ddd, 1H, $J_{4,3e} = 4.4$ and $J_{4,3a} = 12.4$ Hz, H-4), 3.73 (m, 2H, H-8,8'), 3.31 (d, 1H, H-6), 2.43 (dd, 1H, $J_{3e,3a} = 12.0$ Hz, H-3e), 2.07 (t, 1H, H-3a); ^{13}C NMR (D_2O, 50.3 MHz) δ 177.8 (d, $J = 6.5$ Hz, C1), 102.4 (d, $J = 6.7$ Hz, C2), 76.2 (d, $J = 6.4$ Hz, C6), 74.3 (d, $J = 4.6$ Hz, C7), 70.1 (C8), 68.0 (C5), 65.9 (C4), 36.6 (C3); proton coupled ^{31}P NMR (D_2O, 81 MHz) δ 5.03 (d, $J = 10.1$ Hz, 7-P), -2.05 (s, 2-P); FAB mass spectrum *m/z* 588.8 (MH^+, $C_8H_{11}O_{14}P_2K_5$ requires 588.6).

Data for **11**: ^{1}H NMR (D_2O, 400 MHz) δ 4.41 (quintet, 1H, $J = 6.8$ Hz, H-7), 4.13-4.26 (m, 2H, H-8 and H-8'), 3.73 (ddd, 1H, $J_{4,3a} = 12.4$, $J_{4,3e} = 4.8$ and $J_{4,5} = 2.8$ Hz, H-4), 3.64 (d, 1H, H-5), 3.50 (d, 1H, $J_{6,7} = 7.2$ Hz, H-6), 2.36 (dd, 1H, $J_{3e,3a} = 12.6$ Hz, H-3e), 2.11 (t, 1H, H-3a); ^{13}C NMR (D_2O, 100.6 MHz) δ 177.4 (C1), 102.2 (d, $J = 8.1$ Hz, C2), 76.7 (C6), 76.6 (C7), 70.0 (C8), 69.5 (C5), 69.0 (C4), 36.4 (C3); proton coupled ^{31}P NMR (D_2O, 81 MHz) δ 18.13 (dt, $J_1 = 7.3$, and $J_2 = 10.7$ Hz, 7,8-P), -0.36 (s, 2-P); FAB mass spectrum *m/z* 532.8 (MH^+, $C_8H_{10}O_{13}P_2K_4$ requires 532.5).

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21. The homogeneous enzyme (specific activity 9 units/mg) was isolated from the overproducing strain *E. coli* DH5α (pJU1), as previously reported.[11]

22. The enzyme activity was assayed spectrophotometrically (232 nm) as described previously.[7] The data were fitted to the competitive model using the equation: $Y = V[S]/(K(1+[I]/K_i) + [S])$, employing the commercial software GraFit (Leatherbarrow, R. J. GraFit Version 2.0, Erithacus Software Ltd., Staines, U.K., 1990) IBM-PC program. The K_i value was calculated either from the above treatment, or from the secondary replots of the slopes from initial double-reciprocal plots ($1/v$ vs $1/[S]$) versus inhibitor concentration (Segel, I. H. *Biochemical Calculations* 2nd ed; Wiley, New York, 1976).

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