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# SYNTHESIS AND EVALUATION OF PUTATIVE OXOCARBENIUM INTERMEDIATE MIMIC IN THE KDO8P SYNTHASE-CATALYZED REACTION AS A TOOL FOR THE DESIGN OF POTENT INHIBITORS FOR LIPOPOLYSACCHARIDE BIOSYNTHESIS

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**Abstract**: The new amino phosphonate **4**, which mimics the ionic and steric properties of putative oxocarbenium intermediate **3**, was synthesized from D-mannose and evaluated as inhibitor of Kdo8P synthase. It was found that **4** is the most potent inhibitor of the enzyme yet tested, with a $K_i$ value of 3.3 μM.

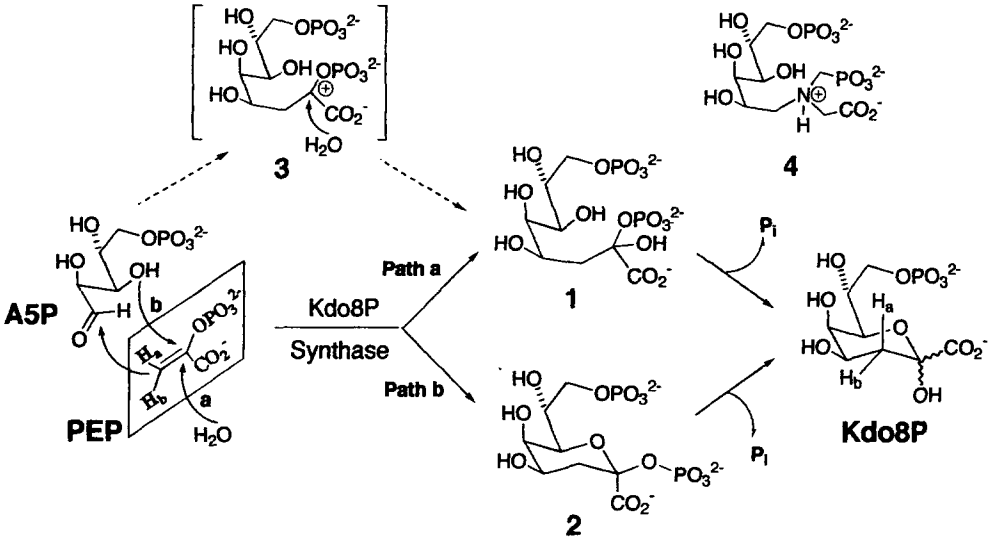

3-Deoxy-D-*manno*-2-octulosonic acid (Kdo) is a site-specific constituent of the lipopolysaccharide of most Gram-negative bacteria, providing the link between lipid A and the growing polysaccharide chain.[1,2] Since the synthesis and activation of Kdo is a vital part of the assembly process of lipopolysaccharides, several groups have recently pursued the inhibition of Kdo metabolism as a strategy for the development of novel antiinfective agents.[3] For this purpose, we have selected 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase [EC 4.1.2.16], which catalyzes the condensation of phosphoenolpyruvate (PEP) with D-arabinose 5-phosphate (A5P) to produce Kdo8P and inorganic phosphate ($P_i$).[4] The catalytic mechanism of this enzyme has been the subject of considerable controversy. Earlier studies by Hedstrom and Abeles[5] demonstrated that the Kdo8P-synthase-catalyzed reaction proceeds through the C–O bond cleavage of PEP and postulated a mechanism involving the formation of acyclic intermediate **1** (Scheme 1, path a). Recently,[6,7] an alternative pathway in which the formation of cyclic bisphosphate **2** (Scheme 1, path b) as a reaction intermediate has been proposed. This proposal was mainly based on the results accumulated through the synthesis and evaluation of various analogues of the substrates[8,9] and product,[6,10] as mechanistic probes of the synthase.

Although the above proposals were based on reasonable chemical grounds, the distinction between these two mechanistic pathways and the detailed insight into the nature of the reaction intermediates awaited the application of pre-steady-state kinetic measurements, using rapid-quench techniques,[11] in combination with the chemical studies.[12] A very recent chemical synthesis of the proposed cyclic intermediate **2** and its evaluation as a substrate of Kdo8P synthase, clearly demonstrated[12] that the reaction does not operate through the formation of **2** as a reaction intermediate. This observation left us with an acyclic intermediate mechanism (Scheme 1, path a), although, to date, there is no evidence available for the existence of **1** as a true intermediate. In addition, the timing of the condensation step is an unresolved issue and, therefore, both a synchronous generation of new C–C and C–O bonds, or a stepwise process (through the formation of the

transient oxocarbenium intermediate **3**), can be suggested as possible mechanisms that may lead to the formation of **1**.

Since many mechanistic questions for the action of the synthase are still unresolved, the rational design of potent inhibitors of this enzyme is a difficult target. On the other hand, it is noteworthy that all the above mechanistic proposals are largely based on the results accumulated through the evaluation of various synthetic compounds as mechanistic probes of the enzyme. Therefore, in keeping with the possibility that the condensation process in Scheme 1 might be a stepwise process, we should expect the stable analogues of cation **3** to serve as potent inhibitors of the enzyme. Close structural analogues of **3** not only incorporate the binding determinants of both substrates, but may also take advantage of any stabilization that **3** could enjoy in the active site. In this communication, we present evidence that amino phosphonate analogue **4**, which mimics the structural and electrostatic properties of the putative intermediate **3**, is a potent inhibitor of the enzyme.

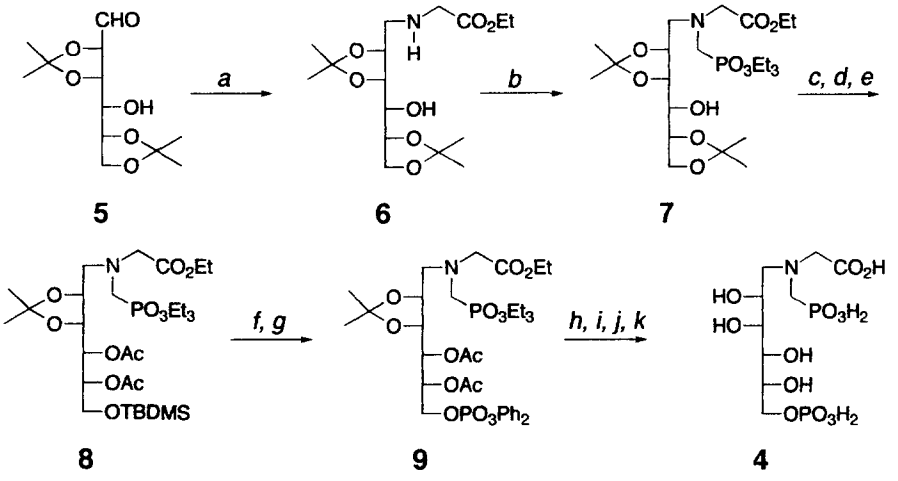
**Scheme 1**




Analogue **4** was successfully synthesized (Scheme 2) from D-mannose via the known acetonide **5**.[13] Direct reductive amination of **5** ($NaBH_3CN$, MeOH) with glycine ethyl ester resulted in acyclic secondary amine **6** in 56% isolated yield. Subsequent treatment of **6** with diethyl phosphonomethyltriflate[14] to introduce the protected phosphonate functionality gave the phosphonate **7** in 65% yield. A variety of methods were examined to effect the deprotection and selective phosphorylation of **7** at C-6. Most were plagued by multiple side products and/or product decomposition. These problems were circumvented by using the following five step procedure. Treatment of **7** with mild acidic condition [0.16 M TFA in MeOH:$H_2O$ (5:3)] caused selective deprotection of 5:6-*O*-isopropylidene moiety to afford the corresponding triol as a major product. Treatment of this triol with *t*-butyldimethylsilyl chloride resulted in the primary silylated product, which was isolated as its diacetate **8** (30% yield for three steps). Desilylation in mild acidic condition ($H_2SO_4$ in MeOH) and then phosphorylation with diphenylphosphochloridate afforded compound **9** in 48% yield. Deprotection of the phosphonate (TMSBr, $CH_3CN$, $Et_3N$, 0–25 °C) was followed by hydrogenolysis (MeOH, HOAc, pH~3, over

$PtO_2$) and then by sequential treatment with acid (Dowex $H^+$, $H_2O$) and aqueous base (1 M KOH, $H_2O$, 25 °C) to provide the target compound **4** (35% yield from **9**). The spectral characterization[15] of purified **4** using 2D-COSY, $^1H$, $^{13}C$, $^{31}P$ NMR, and mass spectra were all consistent with this structure.

**Scheme 2**[a]




[a]Key: (*a*) $H_2NCH_2CO_2Et$, $NaBH_3CN$, MeOH; (*b*) $TfOCH_2PO_3Et_2$, sat. $NaHCO_3$, $CH_2Cl_2$; (*c*) TFA, MeOH/$H_2O$; (*d*) *t*-butyldimethylsilyl (TBDMS) chloride, Py, DMAP; (*e*) $Ac_2O$, Py; (*f*) $H_2SO_4$, MeOH; (*g*) $(PhO)_2P(O)Cl$, Py, DMAP; (*h*) TMSBr, $CH_3CN$, $Et_3N$; (*i*) $H_2$, $PtO_2$, MeOH, HOAc; (*j*) Dowex ($H^+$), $H_2O$; (*k*) KOH, $H_2O$.

Analogue **4** was evaluated as an inhibitor of homogeneous Kdo8P synthase from *Escherichia coli*.[16] Competition experiments were performed[17] by varying PEP at saturating concentrations of A5P (0.5 mM), and the inhibition constant ($K_i$) was estimated to be 3.3 ± 0.3 μM. The kinetic behavior of **4** reveals it as competitive with respect to PEP, as is expected from the kinetic mechanism described by Kohen et al.[9]

With a $K_i$ of 3.3 μM, compound **4** represents the most potent inhibitor of the enzyme yet reported.[6] Furthermore, **4** is the first bisubstrate inhibitor of the enzyme that combines key features of A5P and PEP into a single molecule and has an *acyclic* structure. Therefore, this result provides the first indirect support to the concept of a mechanism which proceeds through the formation of an acyclic intermediate **1** (Scheme 1). Intuitively, one can now assume that the observed inhibition with **4** is the result of its considerable fit to putative oxocarbenium intermediate **3**. Alternatively, the tetrahedral geometry at the ammonium center in **4** and its acyclic structure, also suggests that the enzyme•**4** binary complex may actually mimic the enzyme-bound acyclic intermediate (enzyme•**1**). At this stage of investigation, however, it is difficult to provide a clear distinction between these two possibilities. Nevertheless, either scenario implies that **4** has direct interaction with those Kdo8P synthase amino acid residues intimately involved in binding of both substrates and in catalysis. Therefore, we believe that the observed result with **4** bears significance in the important question of the mechanism of this unusual enzyme transformation, permitting the design of new mechanism-based Kdo8P synthase inhibitors, which may ultimately serve as antibiotics acting on lipopolysaccharide biosynthesis.[3] The in depth study of this enzyme mechanism, including a detailed description of the inhibition by **4** (especially the

factor of pH and the possibility of slow binding) and its application to the kinetic mechanism, is underway. Also in progress is research into more advanced synthetic analogues of the putative intermediate **1**. These studies will be reported in due course.

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15. Analytically pure **4** was prepared by using ion-exchange chromatography on AG 1X8 (100-200 mesh, $HCO_3^-$ form) eluted with the linear gradient (0.1-1.0 M) of triethylammonium bicarbonate, pH 7.5, followed by Dowex 50W ($K^+$). $^1H$ NMR ( $D_2O$, pD=13.0, 400 MHz, referenced to HOD at 4.63 ppm): δ 2.53 ( d, 1H, $J$ = 10.2 Hz, C*H*H-P), 2.532 ( d, 1H, $J$ = 11.8 Hz, CH*H*-P), 2.618 ( dd, 1H, $J$ = 13.5 and 7.6 Hz, $C_1$-H), 2.850 ( dd, 1H, $J$ =13.5 and 4.0 Hz, $C_1$-H'), 3.182 (d, 1H, $J$ = 16.4 Hz, N-C*H*H-$CO_2^-$), 3.290 (d, 1H, $J$ = 16.4 Hz, N-CH*H*-$CO_2^-$), 3.610 ( dd, 1H, $J$ = 8.3 and 0.9 Hz, $C_3$-H), 3.630 ( ddd, 1H, $J$ = 8.0, 4.8 and 2.2 Hz, $C_5$-H), 3.676 ( dd, 1H, $J$ = 8.0 and 0.9 Hz, $C_4$-H), 3.69 ( ddd, 1H, $J$ = 8.3, 7.6 and 4.0 Hz, $C_2$-H), 3.759 ( ddd, 1H, $J$ = 11.5, 7.4 and 4.8 Hz, $C_6$-H), 3.847 ( ddd, 1H, J = 11.5, 6.5, 2.2 Hz, $C_6$-H'). $^{13}C$ NMR ($D_2O$, 100 MHz, referenced to DSS at 0.0 ppm) δ 56.80 ( d, $J$ = 141.8 Hz, N-*C*$H_2$-P), 61.77 ( d, $J$ = 7.45, C1), 63.14 ( d, $J$ = 5.57 Hz, N-*C*$H_2$-$CO_2^-$), 68.17 ( d, $J$ = 5.59 Hz, C6 ), 70.417 ( C2), 71.70 (C4), 72.93 (d, $J$ = 5.5 Hz, C5), 74.33 (C3), 182.76 ($CO_2^-$). Proton coupled $^{31}P$ NMR ( $D_2O$, 81.03 MHz, referenced to 200 mM $D_3PO_4$ at 0 ppm) δ 5.42 ( dd, $J$ = 6.5 and 7.4 Hz, $CH_2OP$), 16.94 ( dd, $J$ = 10.2 and 11.8 Hz, $CH_2P$).
16. The homogeneous enzyme (specific activity 9 units / mg) was isolated from the overproducing strain *E. coli* DH5α (pJU1), as previously reported.[6]
17. The enzyme activity was assayed spectrophotometrically (232 nm) as described previously.[9] 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) PC-IBM 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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