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Towards the Synthesis of the Putative Reaction Intermediate in the Kdo8P Synthase-Catalyzed Reaction. Synthesis and Evaluation of 3-Deoxy-D-*manno*-2-octulosonate-2-phosphate

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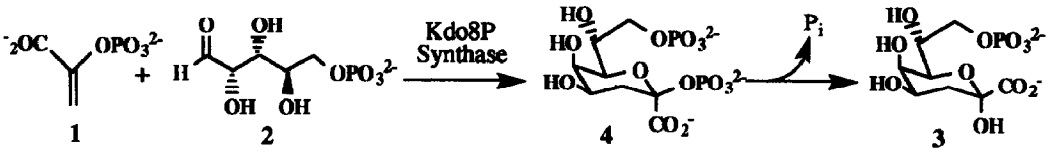
Abstract: The new compounds α- and β-Kdo 2-phosphate (8 and 9) were synthesized in order to probe the introduction of the phosphate group at the anomeric center of Kdo. This method was used towards the synthesis of the putative bisphosphate intermediate 4 in Kdo8P synthase-catalyzed reaction.

3-Deoxy-D-*manno*-2-octulosonic acid (Kdo) is a site-specific constituent of the lipopolysaccharide of most Gram-negative bacteria, and provides 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 the 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase [EC 4.1.2.16] which catalyzes the condensation of phosphoenolpyruvate (1) with D-arabinose 5-phosphate (2) to produce Kdo8P (3) and inorganic phosphate (P_i). In our recent mechanistic studies of this enzyme-catalyzed reaction, we proposed[4] the formation of bisphosphate 4 as a reaction intermediate. To further support the above mechanism, we aimed to examine the chemical synthesis of the putative intermediate 4 and then to evaluate its kinetic competence.[5] This manuscript describes the successful synthetic methodology for the introduction of the phosphate group at the anomeric carbon (C-2) of Kdo molecule in both the α- and β-configuration. The short and very efficient chemical-enzymatic strategy towards the synthesis of 4 is also described.



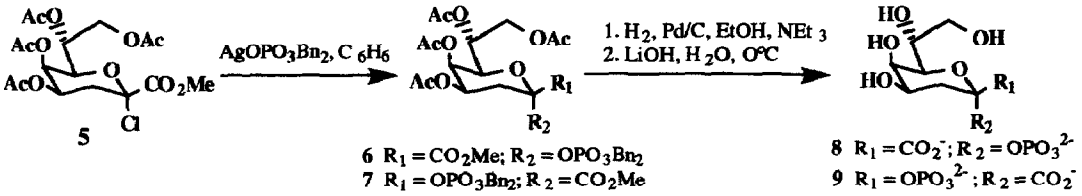
1 2 4 3

Prior to embarking on the multistep synthesis of target bisphosphate 4, we elaborated the suitable methodology for the preparation of α- and β-Kdo 2-phosphate (8 and 9), two simplified analogues of 4 lacking the C-8 phosphate substituent. No Kdo 2-phosphates have been previously reported.

Many new and elegant methods for the synthesis of glycosyl phosphates, either enzymatic[6] or chemical,[7] have been developed. However, since the selectively protected Kdo molecule predominantly exists with its anomeric hydroxyl in the α-configuration,[8] synthetic methods that use the anomeric hydroxyl as a nucleophile and various electrophiles as phosphate donors,[7] could be unsuited for the preparation of β-

Kdo 2-phosphate. In addition, since Kdo is a 3-deoxy sugar, all stereoselective methods of phosphorylation that use the anchimeric assistance of the neighboring groups[9] may be inadequate. Therefore, we turned our attention towards the direct methods of phosphorylation employing glycosyl halides as glycosyl donors. Instead of highly unstable 2-deoxy-glycosyl bromides, we chose the known[10] chloride **5** as a glycosyl donor and silver dibenzyl phosphate[11] as a nucleophile.

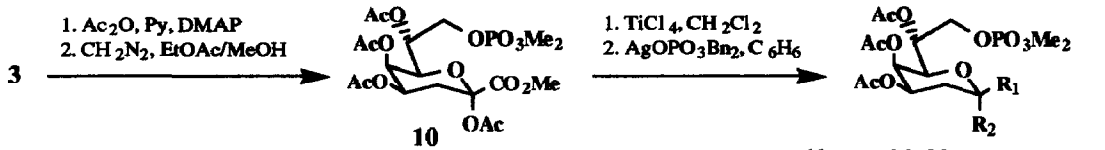
Phosphorylation of **5** was successfully accomplished in dry benzene at reflux to afford the mixture of **6** and **7** in 91% yield, predominantly as the α-phosphate (**6**:**7** = 20:1). Although in this procedure the desired β-phosphate (**7**) was obtained as a by-product, it should be mentioned that in the recently reported synthesis of 2-deoxyglucose 1-phosphate,[12] by use of a similar procedure, the formation of only the α-phosphate in moderate yield was noted. Therefore, upon close investigation of the above procedure we found that by using a two mole-equivalent of the reagent, for short times, the β-phosphate **7** is formed predominantly, under kinetic control. Prolongation of this phosphorylation process resulted in an almost complete inversion of the anomeric center, and the thermodynamically more stable α-phosphate **6** was exclusively obtained.



Due to their enhanced hydrolytic instability, the unmasking of protecting groups from anomeric phosphates has proved problematic and in many cases led to a loss of the anomeric phosphate group.[9] We found that the removal of benzyl groups in **6** and **7** could be accomplished in quantitave yield and without any loss of anomeric phosphate by hydrogenolysis in the presence of two mole-equivalents of dry triethylamine. Saponification of the crude reaction mixtures then afforded the desired α- and β-2-deoxy Kdo phosphate in 90% and 70% isolated yields (for **8** and **9**, respectively) for each of the last two steps. The anomeric configuration of phosphate linkage was determined by a combination of ^{1}H, ^{13}C and ^{31}P-NMR analysis.[13] The observed 3-bond ($^{3}J_{P\text{-}C3}$ = 8.8 Hz) and 2-bond ($^{3}J_{P\text{-}C2}$ = 7.1 Hz) coupling in the proton decoupled ^{13}C-NMR spectrum of **8**, along with 4-bond coupling ($^{4}J_{H3a\text{-}P}$ = 4.5 Hz) between phosphorus and axial H-3, indicates the axial orientation of the phosphate group in **8**. However, no such 4-bond coupling was recorded for **9**. In addition, the lack of 3-bond coupling between C-3 and phosphorus in **9**, along with the other 3-bond carbon-phosphorus coupling constant ($^{3}J_{P\text{-}C1}$ = 7.95 Hz) is only consistent with an *anti*-staggered conformation between carboxylate carbon and phosphorus,[14] and thus establishes the β-phosphate configuration in **9**. These spectral data are in accordance with the earlier assignment[15] of the configuration of CMP-Kdo and thus provide further evidence that Kdo retains the β-configuration when linked in CMP-Kdo.

The presence of a phosphate functionality at the anomeric center in **4** represents a significant problem as such a phosphate or protected phosphoryl group might be too unstable to be carried through a synthetic sequence. Therefore, we elected to insert the anomeric phosphate at the penultimate step, followed by the removal of suitable protecting groups. In an attempt to check the usefulness of the above procedure for the preparation of target bisphosphate **4**, the peracetylated **10** was synthesized utilizing a combined chemical-enzymatic method. Since the introduction of the C-8 phosphate in Kdo is rather tedious and requires a large

number of chemical steps,[4d] we used Kdo8P (3) as a starting material. The compound 3 was prepared enzymatically in multigram quantities according to the procedure of Whitesides.[16] Acetylation of 3 was followed by methylation to afford compound 10 as a single stereoisomer. Treatment of 10 with titanium tetrachloride gave the anomeric chloride which was phosphorylated according to the above procedure to obtain the mixture of 11 and 12 (1:1) in 75% isolated yield. The diastereomerically pure 11 and 12 could be isolated by chromatography on a silica gel column. The configuration of the anomeric phosphate was diagnosed through the coupling constant of the phosphorus in proton-coupled ^{31}P-NMR.[17] A very characteristic 4-bond coupling constant (^4J = 4.0 Hz) between phosphorus and H_{3ax} is only consistent with the *trans*-diaxial (H_{3ax}, phosphate) orientation, and thus establishes the α-configuration of the anomeric phosphate in 12. Studies of the deprotection of 11 and 12 are underway and will be reported in due course.



11 $R_1 = CO_2Me$; $R_2 = OPO_3Bn_2$
12 $R_1 = OPO_3Bn_2$; $R_2 = CO_2Me$

Compounds 8 and 9 were evaluated as substrates and as inhibitors of Kdo8P synthase. No release of inorganic phosphate was detected after prolonged incubation of 8 or 9 with high quantities of the enzyme. Only weak competitive inhibition (K_i =1.3 and 0.16 mM, respectively) was observed following testing of these compounds under standard kinetic assay conditions.[4d] The results clearly demonstrate that the C-8 phosphate is crucial for the binding and the catalysis of the anomeric phosphate hydrolysis. In addition to the above studies, the structures 8 and 9 represent very good models for the investigation of anomeric phosphate hydrolysis in solution. Thus, despite the extensive endeavors to understand the mechanism of glycosyl phosphate hydrolysis,[18] very little has been reported on the hydrolytic properties of such systems when the anomeric carbon of sugar links to both the phosphate and carboxylate groups.[19] Such studies could shed additional light on the Kdo8P synthase catalyzed reaction, and on other enzymes handling similar structures during their catalytic process. The in depth study of the hydrolysis of 8 and 9, especially the possible assistance of the anomeric carboxyl group, is underway.

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13. (a) Spectral data for the lithium salt of **8** in D_2O, pD=10.0: ^{1}H-NMR (500 MHz, referenced to HOD at 4.63 ppm) δ 1.48 (1H, ddd, $J_{3a\text{-}3eq}$=12.8 Hz, $J_{3a\text{-}4}$=12.2 Hz, $J_{3a\text{-}p}$=4.5 Hz, 3-H_a), 1.84 (1H, dd, $J_{3a\text{-}3eq}$=12.8 Hz, $J_{3eq\text{-}4}$=4.3, 3-H_{eq}), 3.31 (1H, dd, *J*=12.0 and 2.5 Hz, 8-H), 3.58 (1H, ddd, *J*=12.0, 2.4 and1.0 Hz, 8'-H), 3.80 (1H, d, *J*=3.0 Hz, 5-H), 3.82 (1H, ddd, *J*=2.5, 2.4 and 9.5 Hz, 7-H), 3.87 (1H, d, *J*=9.5 Hz, 6-H), 3.91 (1H, ddd, *J*=3.0, 4.3 and 12.2 Hz, 4-H); ^{13}C-NMR δ 63.9 (C-8), 71.1 (C-7), 73.4 (C-6), 68.4 (C-5), 68.6 (C-4), 38.0 (d, *J*=8.8 Hz, C-3), 101.0 (d, *J*=7.1 Hz, C-2), 178.8 (s, C-1); proton coupled ^{31}P-NMR δ 0.1 (d, $J_{H3a\text{-}P}$=4.5 Hz).

    (b) Spectral data for the lithium salt of **9** in D_2O, pD=10.0: ^{1}H-NMR (500 MHz, referenced to HOD at 4.63 ppm) δ 2.13 (1H, dd, $J_{3a\text{-}3eq}$=12.4 Hz, $J_{3a\text{-}4}$=12.1 Hz, 3-H_a), 2.32 (1H, dd, $J_{3a\text{-}3eq}$=12.4 Hz, $J_{3eq\text{-}4}$=3.8, 3-H_{eq}), 3.12 (1H, dd, *J* = 8.4 and 3.0 Hz, 8-H), 3.56 (1H, dd, *J*=8.4, 3.7 Hz, 8'-H), 3.57 (1H, d, *J*=3.5 Hz, 5-H), 3.60 (1H, ddd, *J*=3.5, 3.8 and 12.4 Hz, 4-H), 3.61 (1H, d, *J*=4.0 Hz, 6-H), 3.68 (1H, ddd, *J*=3.0, 3.7 and 4.0 Hz, 7-H); ^{13}C-NMR δ 66.3 (C-8), 71.8 (C-7), 76.5 (C-6), 68.8 (C-5), 70.8 (C-4), 36.3 (s, C-3), 102.1 (d, *J*=7.9 Hz, C-2), 178.0 (d, *J*=7.95 Hz C-1); proton coupled ^{31}P-NMR δ -0.1.
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17. Proton coupled ^{31}P-NMR spectra ($CDCl_3$) for **11**: δ -0.68 (m, *J*=7.0 and 3.8 Hz, C8-P), -8.18 (dq, *J*=4.0 and 8.7 Hz, C2-P); for **12**: δ -0.71 (m, *J*=7.0 and 3.9 Hz, C8-P), -8.85 (q, *J*=7.9 Hz, C2-P).
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