Synthesis, Inhibition, and Acid-Catalyzed Hydrolysis Studies of Model Compounds of the Proposed Intermediate in the Kdo8P-Synthase-Catalyzed Reaction

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Abstract: The proposed mechanistic pathway for the reaction catalyzed by 3-deoxy-D-*manno*-2-octulosonate-8-phosphate synthase was examined in terms of structure and stability of the putative bisphosphate intermediate. Two simplified analogues of the proposed intermediate (**3**), possessing the stereochemistry of either the β-phosphate (compound **5**) or the α-phosphate (compound **6**), were synthesized and probed as inhibitors for the enzyme. It was found that both analogues bind to the enzyme and are competitive inhibitors with respect to phosphoenolpyruvate binding, having K_i values of 160 and 1300 μM, respectively. This uncertain stereochemical preference exhibited by the enzyme for the stereoisomers at the anomeric carbon suggests that the binding subsites of the phosphate and carboxylate moieties are not absolute in their specificity and may interchange, while the observed one order magnitude preference of the β-phosphate **5** supports the proposed β-phosphate configuration in the putative bisphosphate intermediate. To test the stability of this intermediate structure, the acid-catalyzed phosphate hydrolysis of the analogues **5** and **6** and the carboxylic acid methyl ester **7** were examined at various pH values, and rate profiles were constructed from the data obtained. These data indicate that anomeric carboxylate affords catalysis and is more effective than identically oriented carboxylic acid or ester groups by a factor of at least two orders of magnitude. These kinetic results are inconsistent for two potential roles of C1 carboxylate function in anomeric phosphate hydrolysis: the intramolecular general acid catalysis and anchimeric assistance, but are consistent with the inductive stabilizing effect that the carboxylate is expected to have on the positive charge being generated on the transiently formed oxocarbenium ion intermediate in the rate-determining process. The applications of these results to Kdo8P-synthase-catalyzed reaction are discussed.

Introduction

The rapid spread of antibiotic resistance in Gram-negative bacteria has prompted a continuing search for new agents with antibacterial activity against this important class of bacterial pathogen.[1] Because the biosynthesis of lipopolysaccharide is unique to Gram-negative bacteria[2] and required by them for growth and virulence,[3] attempts have been made to discover antibacterial agents acting at this site. However, since the biosynthesis of lipopolysaccharide accommodates a rather large number of enzymatic steps,[4] the site-specific constituent of this macromolecule—the unusual eight-carbon sugar 3-deoxy-D-*manno*-2-octulosonic acid (Kdo)—has been identified and most efforts to rationally design synthetic inhibitors have been aimed towards the inhibition of this sugar biosynthesis.[5] Indeed, some of the potent inhibitors of CMP-Kdo synthetase have shown *in vivo* antibacterial activity.[6] These results have prompted us to a further design of synthetic molecules exhibiting selective activity against Gram-negative bacterial cells.

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 unusual condensation of phosphoenolpyruvate (PEP) with D-arabinose 5-phosphate (Ara5P) to produce Kdo8P and inorganic phosphate (P_i).[7] The available evidence supports the following overall reaction:

$$\text{Ara5P} + \text{PEP} \rightarrow \text{Kdo8P} + P_i$$

Our selection of Kdo8P synthase was encouraged by two major points: (i) A *Salmonella typhimirium* mutant with a temperature sensitive lesion in Kdo8P synthase was isolated and has shown that lipopolysaccharide synthesis stops immediately at nonpermissive temperatures, followed by cessation or protein, DNA, and RNA synthesis.[3c] These results indicate that Kdo8P synthase is required for cell growth and makes this enzyme an attractive target for the development of antibiotics.[5] (ii) Although the catalytic mechanism of this enzyme has not yet been established, from the available data it is clear that the stable analogues of the reaction intermediate should incorporate the binding determinants of both substrates (PEP and Ara5P) and therefore might serve as potent inhibitors of the enzyme. Thus, in contrast to the previous study of CMP-Kdo synthetase

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(6) (a) Hammond, S. M.; Claesson, A.; Jannson, A. M.; Larsson, L. G.; Brian, G. P.; Town, C. M.; Ekstrom, B. *Nature* **1987**, *327*, 730−732. (b) Goldman, R.; Kohlbrenner, W.; Lartey, P.; Pernet, A. *Nature* **1987**, *329*, 162−164.

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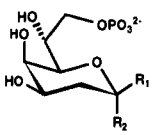
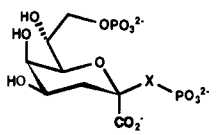
inhibition, which was largely concerned with the design of substrate (Kdo) or product (CMP-Kdo) analogues rather than the reaction transition-state mimics,[6,8] the detailed investigation of the Kdo8P synthase catalytic mechanism promises to help us toward our planned goal, as stated above.

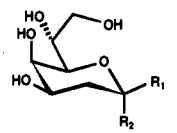
While earlier studies of this enzyme mechanism have established exclusively that the reaction is essentially irreversible and proceeds through the C−O bond cleavage of PEP,[9] the elementary steps of this transformation have remained unresolved. Recently, we have shown that the enzyme acts upon the acyclic form of Ara5P and have demonstrated an ordered sequence of substrate binding (PEP followed by Ara5P) and product release (P_i prior to Kdo8P).[10] 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 Ara5P.[11] Based on the available data pertaining to this enzyme mechanism, in conjunction with our recent results obtained from the evaluation of 2-deoxy analogues (**1** and **2**) of the product Kdo8P as inhibitors, we have proposed[12] a new mechanistic pathway for the Kdo8P-synthase-catalyzed reaction as illustrated in Scheme 1. In this sequence, the formation of cyclic bisphosphate **3** as a reaction intermediate was suggested. This intermediate may be formed either by a synchronous (path a) or by a stepwise (path b, through the formation of transient intermediate I) generation of new C−C and C−O bonds, while the question of which of these pathways is true remains to be determined conclusively. The intermediate **3** is expected to decompose rapidly by releasing inorganic phosphate through the C−O bond cleavage, producing an oxocarbenium ion intermediate (II), which in turn undergoes a rapid hydrolysis to produce the product Kdo8P. The β-configuration of anomeric phosphate in **3** was derived through the stereochemical studies of the condensation step[11a] and was further supported by the synthesis and evaluation of the stable isosteric phosphonate analogue **4**.[12] This structure closely mimics the topological and electrostatic properties of the proposed intermediate **3** and was found to be the most potent inhibitor of the enzyme with a K_i value of 5 μM.



1: R_1 = H; R_2 = CO_2^-
2: R_1 = CO_2^-; R_2 = H



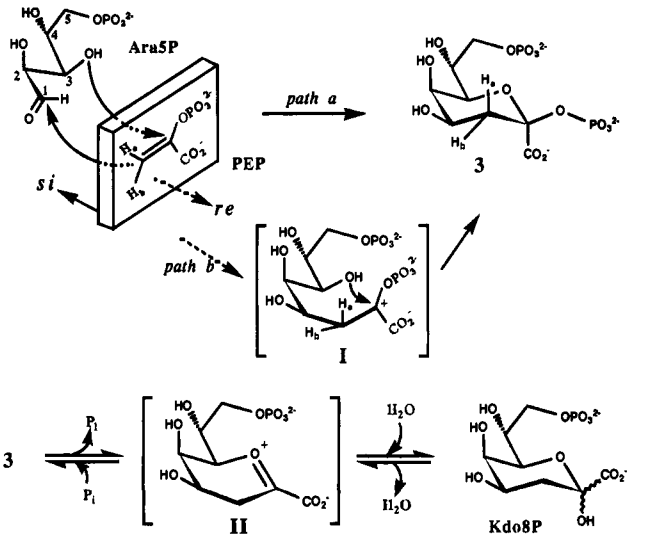
3: X = O
4: X = CH_2



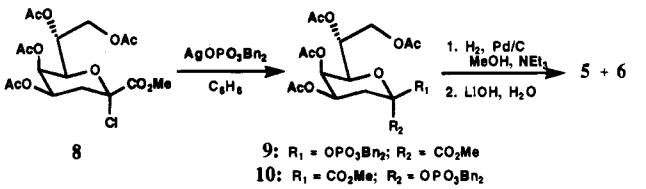
5: R_1 = OPO_3^{2-}; R_2 = CO_2^-
6: R_1 = CO_2^-; R_2 = OPO_3^{2-}
7: R_1 = CO_2Me; R_2 = OPO_3^{2-}

Taking into account all the above observations, many questions on the enzyme mechanism still remain unresolved. Firstly, the observed binding affinity of the analogue **4** (based on a comparison of its K_i value with the K_m for PEP) is still considered weak for a reaction intermediate analogue, raising doubts as to the anomeric phosphate configuration in the proposed structure of **3**. These are compounded by the fact that the opposite diastereomer at C2 of the analogue **4** was not examined. Secondly, the proposed steps for the hydrolysis of anomeric phosphate in **3** (Scheme 1) are based on the earlier

Scheme 1



Scheme 2



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9: R_1 = OPO_3Bn_2; R_2 = CO_2Me
10: R_1 = CO_2Me; R_2 = OPO_3Bn_2

observations of Abeles[9] which demonstrated that the reaction proceeds through the C−O bond cleavage of PEP. However, no results regarding the hydrolytic properties of such structures have been previously reported.

Our extended studies of the catalytic mechanism of Kdo8P synthase, encompassed several goals regarding the structure of the putative reaction intermediate **3**: to devise synthetic methodology for the introduction of labile ketal phosphate moiety at the anomeric carbon; to scrutinize the anomeric phosphate configuration; and to investigate the hydrolytic properties of this unique phosphate linkage. In the following account we describe the synthesis, inhibition, and acid-catalyzed hydrolysis studies of β- and α-Kdo 2-phosphates (**5** and **6**), two simplified analogues of **3** lacking the C-8 phosphate.[13]

Results

Synthesis of Kdo-2-phosphates. The anomeric phosphates **5** and **6** were synthesized as shown in Scheme 2. As a starting material we selected the anomeric chloride **8**, which was

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(12) (a) Sheffer-Dee-Noor, S.; Belakhov, V.; Baasov, T. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1583−1588. (b) Baasov, T.; Sheffer-Dee-Noor, S.; Kohen, A.; Jakob, A.; Belakhov, V. *Eur. J. Biochem.* **1993**, *217*, 991−999.

(13) The preliminary results on the synthesis of **5** and **6** have recently published as a communication: Kohen, A.; Belakhov, V.; Baasov, T. *Tetrahedron Lett.* **1994**, *35*, 3179−3182.

Table 1. Selected NMR Data of Compounds **5** and **6**

	^{1}H-NMR		^{13}C-NMR			^{31}P-NMR
	H-3_{ax}	H-3_{eq}	C1	C2	C3	
5	2.13 (dd) $J_{3a-3e} = 12.4$; $J_{3a-4} = 12.1$	2.32 (dd) $J_{3a-3e} = 12.4$; $J_{3e-4} = 3.8$	178.0 (d) $J_{C-P} = 7.95$	102.1 (d) $J_{C-P} = 7.9$	36.3 (s)	−0.1 (s)
6	1.48 (ddd) $J_{3a-3e} = 12.8$; $J_{3a-4} = 12.2$ $J_{3a-P} = 4.5$	1.84 (dd) $J_{3a-3e} = 12.8$; $J_{3a-4} = 4.3$	179.7 (s)	100.9 (d) $J_{C-P} = 7.1$	38.5 (d) $J_{C-P} = 8.8$	0.1 (d) $J_{3a-P} = 4.5$

prepared in three steps from the ammonium salt of Kdo[14] following the previously reported procedure.[15] Preliminary experiments have shown that the reaction of **8** with silver dibenzyl phosphate[16] in dry benzene at reflux provides a mixture of **9** and **10**, predominantly in the form of the α-phosphate (**9**: **10** = 1:20). Although in this procedure the desired β-phosphate (**9**) was obtained as a byproduct, it should be mentioned that in the recently reported synthesis of 2-deoxyglucose 1-phosphate,[17] through the 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 it was found that by using an excess of dry reagent, for short periods and under kinetic control, a predominance of β-phosphate **9** is formed. The mixture of anomers may be separated by chromatography to provide pure **9**. However, prolongation of this phosphorylation process resulted in an almost complete inversion of the anomeric center, and the thermodynamically more stable α-phosphate **10** was exclusively obtained.

The second key step in this synthesis was the deprotection of **9** and **10**. Indeed, due to their enhanced hydrolytic instability, the unmasking of protective groups from anomeric phosphates has proved problematic and in many cases has led to a loss of the anomeric phosphate group.[18] We found that the removal of benzyl groups in **9** and **10** could be accomplished, in almost quantitative yield and without significant loss of anomeric phosphate, by hydrogenolysis (Pd/C, MeOH) in the presence of 2 mole-equiv of dry triethylamine. Saponification of the crude reaction mixtures then afforded the desired β- and α-2-deoxy Kdo phosphate in 70% and 90% isolated yields (for **5** and **6**, respectively) for each of the last two steps.

The methyl ester **7** was derived from the protected α-phosphate **10**. Hydrogenolysis (Pd/C, MeOH) was followed by the basic hydrolysis (NaOMe/MeOH) to give the desired carboxylic ester **7**.

Determination of Anomeric Configuration. The anomeric configuration of phosphate linkage was determined by a combination of ^{1}H, ^{13}C, and ^{31}P-NMR analysis (Table 1). The comparison of ^{1}H-NMR spectra of **5** and **6** in the region of geminal C3 protons already enabled the discrimination between these two diastereomers. Thus, the observed four-bond (W) coupling ($^4J_{H3a-P} = 4.5$ Hz) between phosphorus and the axial C3 proton in **6** is only consistent with a trans orientation of this proton and the anomeric phosphate. The same ^{1}H−P coupling ($^4J_{P-H3a} = 4.5$ Hz) was also observed in proton coupled ^{31}P-NMR of **6**. However, no such four-bond coupling was

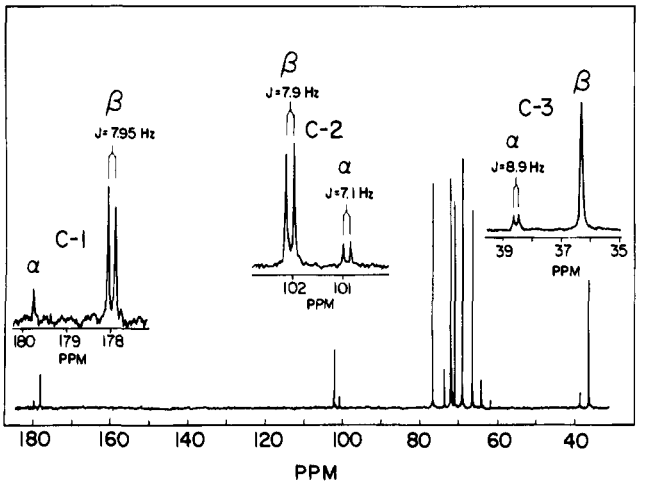
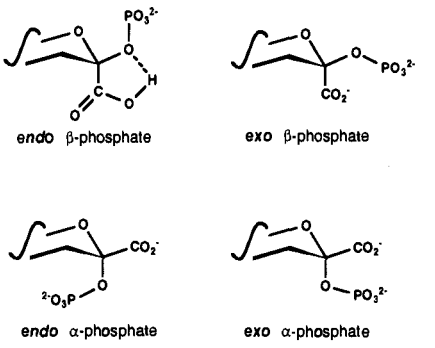


Figure 1. Proton decoupled ^{13}C-NMR spectrum of the mixture of Kdo-β-2-phosphate **5** (88%) and Kdo-α-2-phosphate **6** (12%).

Chart 1



recorded for **5**, indicating the β-anomeric configuration of the phosphate linkage in this diastereomer.

The magnitude of three-bond carbon-phosphorus coupling constants, observed in the proton decoupled ^{13}C-NMR of **5** and **6**, provide additional structural information (Figure 1, Table 1). It has been reported that these couplings are dihedral angle-dependent.[19] Large couplings of 8−10 Hz have been observed for trans orientation of carbon and phosphate groups, while smaller couplings (2−4 Hz) have been observed for gauche orientations. The 7.95 Hz coupling between C1 and phosphorus in **5** suggests an *anti*-staggered conformation between carboxylate carbon and phosphorus. This indicates that the phosphate group adopts an *endo* conformation (Chart 1). Such an arrangement easily occurs when the anomeric phosphate possesses a β-pyranose configuration and the sugar ring is in the $^5C_2$ conformation. An opposite, $^2C_5$ ring conformation would not be expected to result in a coupling constant of the above magnitude. Therefore, these spectral data are in accordance with

(14) Hershberger, C. S.; Davis, M.; Binkley, S. B. *J. Biol. Chem.* **1968**, *243*, 1585−1588.

(15) Lutham, K.; Orbe, M.; Waglund, T.; Claesson, A. *J. Org. Chem.* **1987**, *52*, 3777−3784.

(16) The silver salt was prepared from pure, crystalline dibenzyl phosphate, commercially available by Aldrich, according to Sheehan, J. C.; Frank, V. S. *J. Am. Chem. Soc.* **1950**, *72*, 1312−1316. We found it necessary to prepare this reagent with the exclusion of light and to keep the dry product excluded from both light and moisture.

(17) Niggemann, J.; Lindhorst, T. K.; Walfort, M.; Laupichler, L.; Sajus, H.; Thiem, J. *Carbohydr. Res.* **1993**, *246*, 173−183.

(18) Marianoff, B. E.; Reitz, A. B.; Tutwiler, G. F.; Benkovic, S. J.; Benkovic, P. A.; Pilkis, S. J. *J. Am. Chem. Soc.* **1984**, *106*, 7851−7853.

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Table 2. Estimated Kinetic Constants for Kdo8P Synthase Inhibition by the Analogues of the Putative Reaction Intermediate[a]

compd	K_i (μM) vs PEP
Kdo8P[b]	590 ± 10
1[b]	303 ± 5
2[b]	470 ± 11
4[b]	4.9 ± 0.5
5	160 ± 8
6	1300 ± 25

[a] Determined at 37 °C in Tris-acetate buffer, pH 7.3. [b] Reference 12b.

the earlier assignment[20] of the configuration of CMP-Kdo and thus provide further evidence that Kdo retains the β-configuration when linked in CMP-Kdo.

In contrast to **5**, no coupling between C1 and phosphorus was detected in compound **6**. Rather, the other three-bond coupling, between C3 and phosphorus ($^3J_{P-C3}$ = 8.8 Hz), was noted (Figure 1), indicating an *anti*-staggered conformation of the phosphate group with respect to C3. This result, along with four-bond coupling ($^4J_{H3a-P}$ = 4.5 Hz) between phosphorus and axial H-3, suggests that the α-phosphate group in **6** extends away from the sugar ring and assumes an *exo* conformation. Thus, the observed spectral data not only define the absolute configuration at the anomeric carbon of **5** and **6** but also account for the predominant conformation of the phosphate linkage toward the sugar ring: the α-phosphate in **6** adopts an *exo* conformation, while the β-phosphate in **5** adopts an *endo* conformation (Chart 1). Such conformational preference of phosphate moiety in solution might be very important for the hydrolysis process of these compounds.[21] It should be specifically noted that the preferred *endo* conformation of the β-phosphate in **5** allows the formation of a stable five-membered ring between the carboxylic acid and the bridged oxygen of phosphate and raises the possibility of an intramolecular general acid catalysis. This possibility does not exist for the *exo* phosphate conformation in **6**. This issue is discussed in the section of acid-catalyzed hydrolysis studies of **5** and **6**.

Interaction of Kdo-2-phosphates with the Enzyme. The compounds **5** and **6** were evaluated as inhibitors of homogeneous Kdo8P synthase from *Escherichia coli* and found to be inhibitors for the PEP binding (Table 2). The inhibition constants were able to be measured from initial velocity studies, and inhibition patterns were found to be competitive (Figure 2). The observed competitive inhibition versus PEP was anticipated from the kinetic mechanism described by Kohen et al.[10] Thus, since it has been demonstrated that the enzyme adopts an ordered sequential kinetic pattern with PEP as a first substrate, it was expected that the analogues **5** and **6**, which combine the structural determinants of both substrates, should compete with PEP in the same, free enzyme form. The K_i values for **5** and **6** were measured as 160 and 1300 μM, respectively. This result clearly demonstrates that the β-phosphate moiety of compound **5** confers a factor of approximately 10 in specificity to that to the α-phosphate in compound **6**. This specificity gives an advantage of 1.23 kcal/mol in the binding energy of **5** to that of **6**.

Since at the anomeric carbon moiety—the reaction center, compound **5** possesses a high similarity to that of the putative intermediate **3**, one could logically assume that the enzyme is able to accept it as a substrate, i.e., the enzyme may catalyze

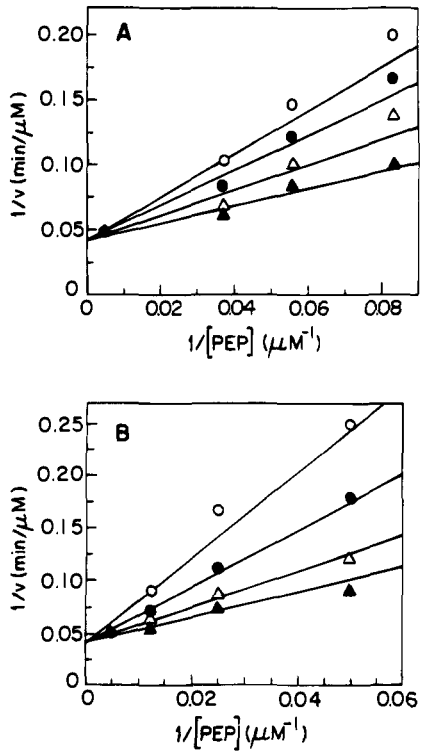


Figure 2. Inhibition of Kdo8P synthase by Kdo-2-phosphates **5** and **6**. Double-reciprocal plots of initial velocities are given as a function of PEP, when the Ara5P concentration was 671 μM and the inhibitor concentrations were (A) (inhibition with analogue **5**) none (▲), 85 μM (△), 190 μM (●), and 270 μM (○); (B) (inhibition with analogue **6**) none (▲), 1.16 mM (△), 3.48 mM (●), and 5.80 mM (○).

hydrolysis of the phosphate in **5**. Ideally we wanted to show the enzyme discriminating between β- and α-phosphates in such an enzyme-catalyzed hydrolysis process. To carry out this aim, the compounds **5** and **6** were separately incubated (0.1 M Tris-HCl buffer, pH 7.3, 37 °C) with a 1000-fold higher concentration of the enzyme than is typically introduced into an assay experiment, and the reaction progress was monitored by ^{31}P-NMR over a 24-h period. Interestingly, although the hydrolysis rate of β-phosphate (the half-life of 2.07 h) was considerably higher to that of α-phosphate (the half-life of 12.8 h), no significant difference in phosphate release was detected in either experiment versus the blank experiments (same conditions, but without the enzyme). Further increase in the enzyme concentration (up to 15 mg of enzyme per experiment) or addition of inorganic phosphate, vanadate, or arsenate, yielded similar results,[22] i.e., no enzyme-catalyzed acceleration in the phosphate hydrolysis was detected.

Acid-Catalyzed Hydrolysis. Figure 3 displays the observed first-order rate constants for the liberation of inorganic phosphate from the anomeric phosphate monoesters **5** and **6**, along with the methyl ester **7**, measured as a function of pH at 12 °C. In all these measurements the anionic strength was maintained at 1.0 M with sodium perchlorate, and the pH was monitored continually during the entire hydrolysis process. The hydrolysis products of **5** and **6** at pH 1.0, 3.0, and 5.0 were Kdo and inorganic phosphate only. The analysis of the hydrolysis products was carried out in separate experiments by following the reaction progress simultaneously in ^{31}P and ^{1}H-NMR spectroscopy and through comparison of the recorded spectra to that of Kdo. Similar experiments with methyl ester **7** [following the changes of methyl ester resonance centered at δ (ppm) 3.55 in ^{1}H-NMR] have shown that the carboxylic acid ester group was stable under conditions examined (pH 1.5, 2.6,

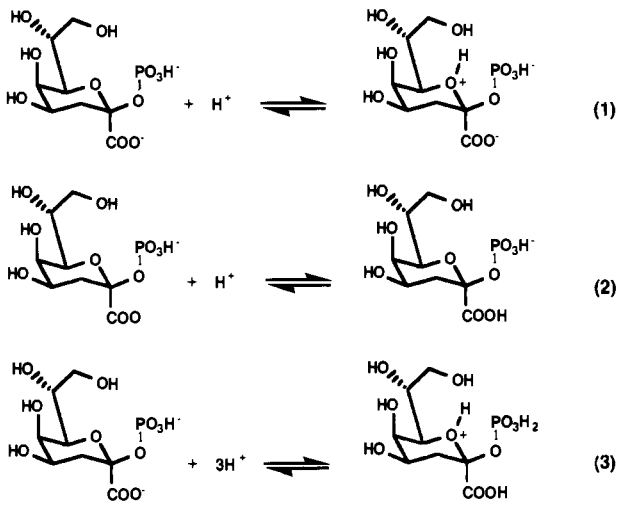
(20) Kohlbrenner, W. E.; Fesik, S. W. *J. Biol. Chem.* **1985**, *260*, 14695–14700.

(21) (a) Li, S.; Kirby, A.; Deslongchamps, P. *Tetrahedron Lett.* **1994**, *34*, 7757–7758. (b) Li, S.; Deslongchamps, P. *Tetrahedron Lett.* **1994**, *34*, 7759–7762.

and 4.1) and did not interfere with the anomeric phosphate hydrolysis studied.

First-order rate constants for the hydrolysis of **5** and **6** at pH 7.3, 37 °C, were measured as above and found to be 9.3×10^{-5} and 1.5×10^{-5} s^{-1}, respectively. In order to compare these values to those at pH 0, 37 °C, the rate constants were measured at several low temperatures, and the values for higher temperatures were determined by extrapolation using Arrhenius plot (Table 3).

As seen from the hydrolysis rate profiles of **5** and **6** in Figure 3, even though the reaction is catalyzed by acid, the observed first-order rate constants (log k_{obs}) are not a linear function of pH, and some curvature develops as the acidity increases. Such behavior could be attributed to partial conversion of the substrate into a less reactive form through rapid equilibrium protonation.[23] The most reasonable *less reactive* forms might be either the protonation of the ring oxygen (eq 1), or the protonation of the carboxylate to its carboxylic acid form (eq 2), or the simultaneous protonation of both (eq 3).



However, since the pK_a of the oxonium cation formed through eqs 1 and 3 is much lower than that of carboxylic acid, it may be logically assumed that the observed rate retardation in the hydrolysis of **5** and **6**, particularly in the pH range of 2.5−4.5, is mostly due to the formation of a neutral carboxylic acid as illustrated in eq 2. For the lower pH values, however, the additional rate retardation should be observed through the protonation on the ring oxygen (eq 3). This will, however, be largely compensated for by the simultaneous formation of the neutral phosphate (from its monoanion) as a better leaving group that in turn accelerates hydrolysis process. Therefore, the results obtained here suggest that the carboxylate group situated in the α-position of Kdo-2-phosphates fulfills an important role in tuning the rate of phosphate hydrolysis. The neutral acid form has a rate-retarding effect, while the ionization to its carboxylate form accelerates that rate of acid-catalyzed hydrolysis. In an attempt to support the above explanation, the carboxylic acid ester **7** was synthesized, and its hydrolytic properties were compared to that of a parent acid **6**. The results of this study are shown in Figure 3.

Ionization Constants. The second ionization constants (pK_{a2}) for **5** and **6** were estimated by proton decoupled ^{31}P-NMR measurements of chemical shift and found to be 6.8 (±0.05) and 7.2 (±0.05), respectively. These values were determined for a 20 mM solution of the sugar in a 0.1 M Tris-HCl buffer in the pH range of 5−11 at 37 °C. The difference observed (0.4 pK_a unit) between **5** and **6** is similar to that

Table 3. Observed First-Order Rate Constants $k_{obs} \times 10^3$ (s^{-1}) for Acid-Catalyzed Hydrolysis of Kdo-2-Phosphates in 1 M $HClO_4$ and at Various Temperatures[a]

	T (°C) =					
	1.0	5.0	8.2	13.4	17.5	37.0
5	9.0	12.6	20.4	28.0	42.0*	213.0*
6	1.6*	2.5	4.9	6.6	12.9	100.0*

[a] Values with asterisk were estimated from extrapolation using Arrhenius plot.

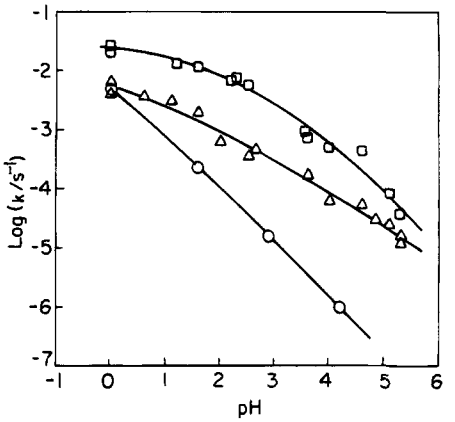


Figure 3. Rate profiles for the hydrolysis of the anomeric phosphate groups of methyl ester **7** (○), Kdo-α-2-phosphate **6** (△), and Kdo-β-2-phosphate **5** (□) in aqueous solution at 12 °C. The curves shown were drawn manually.

estimated for α- and β-glucopyranosyl phosphates.[24] The first ionization constants (pK_{a1}) could not be extracted using these measurements due to the high sensitivity of the compounds at low pH values and under the prevailing conditions.

Solvent Isotope Effects. Rates of phosphate hydrolysis for the β-phosphate **5** were measured in H_2O and D_2O solutions of perchloric acid (1 M, pH = 0) or sodium perchlorate (1 M) by adjusting the pH through the addition of appropriately diluted perchloric acid. Pairs of determinations were made at the same pH value in the two solvents, and isotope effects were calculated by dividing rate constants determined in H_2O by those determined in D_2O. The following rate constants were measured at pH values of 0, 2.3, and 3.6: $K_{H_2O} = 5.7 \times 10^{-3}$, 4.5×10^{-4}, 1.7×10^{-4} s^{-1}; $k_{D_2O} = 5.2 \times 10^{-3}$, 4.2×10^{-4}, 1.4×10^{-4} s^{-1}. The isotope effects (k_{H_2O}/k_{D_2O}) at pH values of 0, 2.3, and 3.6 were measured as 1.10, 1.07, and 1.20, respectively.

Discussion

The anomeric phosphates **5**−**7** were designed to resolve several questions concerning the proposed mechanism shown in Scheme 1.

First, from the K_i values presented in Table 2, it appears that the discrimination by the synthase between the diastereomers **5** and **6** is significantly higher than that observed earlier for the

(22) These experiments were performed according to the suggestion of one of the referees of this manuscript. The coaddition of inorganic phosphate (up to 5 mM), into the reaction mixture containing compound **5** and the enzyme, was expected to cause a noncovalent occupation of the site on the enzyme which is presumably occupied by C8 phosphate of the proposed intermediate **3** and thus may promote the enzyme catalysis. Similarly, the addition of arsenate (up to 1 mM) and of vanadate (up to 0.2 mM) was expected to form the covalent esters with C8 hydroxyl group of **5** leading to productive complexes with the enzyme, as it was demonstrated with glucose-6-phosphate dehydrogenase: *J. Biol. Chem.* **1985**, *260*, 6836−6842.

(23) Kresge, A. J.; Leibovitch, M.; Sikorski, J. A. *J. Am. Chem. Soc.* **1992**, *114*, 2618−2622.

(24) O'Connor, J. V.; Barker, R. *Carbohydr. Res.* **1979**, *73*, 227−234.

2-deoxy analogues **1** and **2**. The comparison of the above structures to that of the putative intermediate **3** reveals that in compounds **1** and **2** the anomeric phosphate is omitted and therefore the observed slight preference in binding of the α-carboxylate (**1**) over the β-carboxylate (**2**) dictates that the carboxylate moiety will be well accommodated either in its usual binding site, i.e., an axial orientation, or in the site that is normally occupied by the phosphate group, i.e., the equatorial orientation. However, as the analogues **5** and **6** incorporate a phosphate moiety to the anomeric center, at this region they are closer mimics to **3** than are the 2-deoxy analogues **1** and **2**. Therefore, the discrimination shown by the enzyme in binding to **5** and **6** is significantly increased. The observed 8-fold advantage in the relative binding capacity of the β-phosphate **5** over the α-phosphate **6** supports the postulated β-configuration in the putative bisphosphate intermediate **3** and suggests that the anomeric phosphate and carboxylate groups are capable of interchanging their binding sites. Thus, not only the carboxylate group is able to accommodate the phosphate binding site, as was revealed from the earlier studies[12] due to examination of **1** and **2**, but also the present study dictates that the phosphate group may also accommodate the carboxylate binding site, although the latter is less favorable.

Very similar phenomena to the uncertain stereochemical preference exhibited by the enzyme have recently been reported for the shikimate pathway enzyme 5-*enol*pyruvylshikimate-3-phosphate (EPSP) synthase.[25] The mechanism of this enzyme was recently confirmed by the isolation and characterization of the reaction intermediate.[26] The extracyclic stereocenter of this intermediate contains both the carboxylate and ketal phosphate moieties and therefore is very similar to that of compound **3** at the anomeric region. A number of diastereomeric pairs of side-chain analogues of the above intermediate, where the labile ketal phosphate was replaced by the phosphonate or the ketal phosphate was stabilized against ionization through the introduction of fluorine substituents, were synthesized and evaluated as inhibitors of the enzyme.[25] From the inhibition study it was concluded that the carboxylate and phosphate binding subsidies are not absolute in their specificity and may interchange without undue penalty. Furthermore, it is noteworthy that the highest difference in discrimination of this enzyme between the side-chain diastereomers was 70-fold, while in the others it was reduced to 20-fold and below. In our case, however, the observed low (only 8-fold) difference in the relative binding capacity of the β-phosphate **5** over the α-phosphate **6** might be explained as due to an *incomplete mimic* by these analogues of the proposed intermediate **3**. Indeed, the observed high K_i values (Table 2) of these analogues and their inert nature as substrate further support this explanation. Thus, the absence of C-8 phosphate in **5** and **6** results in a weak binding to the enzyme active site. This allows the inhibitor more freedom at the binding site and therefore substantially lowers the ability of the enzyme to control the specificity. The observed results suggest that the C-8 phosphate group of the proposed intermediate **3** should be critical for both the binding and the catalytic network of the enzyme.[27]

Acid-Catalyzed Hydrolysis. The acid-catalyzed hydrolysis of glycosidic phosphates has been shown, by a series of elegant studies, to proceed *via* a mechanism involving a rate-determining heterolysis of the glycosidic C–O bond to generate a glycosyl-oxonium ion intermediate or at least a transition state having a substantial oxonium-ion character.[28] However, despite extensive studies of the hydrolysis of various glucopyranosyl phosphates,[29] very little mechanistic attention has been paid to the hydrolysis of anomeric phosphate esters of Kdo (e.g., Kdo-2-phosphate, CMP-Kdo) or those of sialic acids 2-phosphates, which have the same structural motif as Kdo and are very important in many aspects of cell–cell and cell–protein recognition.[30] In this family of compounds the anomeric center possesses a carboxylate group in place of the anomeric hydrogen and the adjacent position is not oxygenated. The unique structure of these compounds may result in unusual hydrolytic properties. Indeed, in recent studies of the hydrolysis of arylglycosides of *N*-acetylneuraminic acid it has been suggested that the transiently generated oxocarbenium ion intermediate could be stabilized by the nucleophilic assistance of the α-carboxylate ion, despite the resulting strained α-lactone ring.[31] Since it has been shown that the hydrolysis of glucopyranosyl phosphates proceeds by a similar mechanism as that of parent arylglucopyranosydes,[29a] then it is reasonable to extrapolate the above mechanism suggested for the acid-catalyzed hydrolysis of arylglycosydes of *N*-acetylneuraminic acid to the subsequent pyranosyl phosphates. However, no results have been previously published on the rates of acid-catalyzed hydrolysis of Kdo-2-phosphates or those of sialic acids 2-phosphates, nor has there been direct, experimental proof of the similarities of the transition states for the acid-catalyzed hydrolysis of Kdo glycosides and of Kdo glycosyl phosphates. The present investigation, which was originally stimulated by our desire to understand the catalytic mechanism of Kdo8P synthase, addresses these questions.

As seen from Figure 3, the rates of hydrolysis of all three substrates increase with acidity. The ratio of hydrolysis rates between two anomers of Kdo-2 phosphate [β(**5**):α(**6**)] vary between 3.3 at pH 5.1, to 4.2 at pH 0, and in the middle it reaching even higher values. These ratios are very similar to those measured previously[28b] for β- and α-glucopyranosyl phosphate (β:α = 2.7:1) and for a variety of other hexopyranosyl phosphates.[29a] Thus, substitution of the carboxylic group at the anomeric carbon in **5** and **6** does not significantly affect this value. This suggests relatively similar ground-state energies of the anomeric phosphates with or without the carboxylic group. Furthermore, since the above ratio is not significantly affected by the pH, the observed data rules out the participation of the carboxylic acid in the β-phosphate (**5**) as an intramolecular general acid. Thus, as we suggested in the previous discussion, the preferred *endo* conformation of the β-phosphate in **5** could

(25) Alberg, D. G.; Lauhon, C. T.; Nyfeler, R.; Fassler, A.; Bartlett, P. A. *J. Am. Chem. Soc.* **1992**, *114*, 3535–3546.

(26) (a) Anderson, K. S.; Sikorski, J. A.; Benesi, A. J.; Johnson, K. A. *J. Am. Chem. Soc.* **1988**, *110*, 6577–6579. (b) Anderson, K. S.; Sikorski, J. A.; Johnson, K. A. *Biochemistry* **1988**, *27*, 1604–1610. (c) Anderson, K. S.; Sikorski, J. A.; Johnson, K. A. *Biochemistry* **1988**, *27*, 7395–7406.

(27) It is noteworthy that we also could not detect any catalytic activity when, instead of Ara5P, the [1-^{13}C]-D-(−)-arabinose was examined as an alternative substrate by ^{13}C-NMR assay. There is no discernible formation of either the expected condensation product [4-^{13}C]Kdo-2-phosphate, or the [4-^{13}C]Kdo over a 52-h period in the presence of the enzyme, under conditions where 0.1% of the expected product could have been detected. Furthermore, the coaddition of inorganic phosphate or vanadate yielded similar results (see note 22). The observed inert nature of this analogue, coupled with the earlier observation of Ray[5b] that the shortened phosphonate analogue of Ara5P was only a moderate inhibitor (I_{50} = 17.1 mM) while the isosteric phosphonate analogue functioned as a substrate, is consistent with the above suggestion that indeed the side-chain phosphate group may play a very crucial role in this enzyme catalysis.

(28) (a) Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Vernon, C. A. *J. Chem. Soc. C* **1958**, 3574–3587. (b) Bunton, C. A.; Llewellyn, D. R.; Oldham, K. G.; Vernon, C. A. *J. Chem. Soc. C* **1958**, 3588–3594. (c) Bunton, C. A.; Humeres, E. *J. Org. Chem.* **1969**, *34*, 572–576.

(29) (a) Withers, S. G.; MacLennan, D. J.; Street, I. P. *Carbohydr. Res.* **1986**, *154*, 127–144. (b) Percival, M. D.; Withers, S. G. *Can. J. Chem.* **1988**, *66*, 1970–1972. (c) Withers, S. G.; Percival, M. D.; Street, I. P. *Carbohydr. Res.* **1989**, *187*, 43–66.

(30) Rademacher, T. W.; Parekh, R. B.; Dwek, R. A. *Annu. Rev. Biochem.* **1988**, *57*, 785–838.

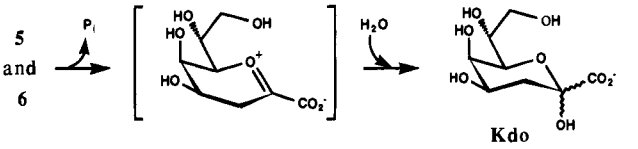
(31) Ashwell, M.; Guo, X.; Sinnott, M. L. *J. Am. Chem. Soc.* **1992**, *114*, 10158–10166.

formally allow the participation of the carboxylic acid as a general acid through the formation of a stable five-membered ring (Chart 1), while no such catalysis was expected for the α-phosphate **6** because the preferred *exo* conformation of its phosphate group. If this is true, then we would expect that the ratio of hydrolysis rates between β- and α-phosphate should increase with the increase in acidity (as below, a pK_a of carboxylic acid) because of the extra acceleration of the β-phosphate hydrolysis due to the general acid catalysis. However, the observed similar ratio (Figure 3) over a large pH range dictates against this mechanism in the hydrolysis of the β-phosphate **5**. This is further supported by the absence of an appreciable solvent isotope effect in the hydrolysis of **5** at pH values of 0, 2.3, and 3.6 (k_{H_2O}/k_{D_2O} = 1.10, 1.07, and 1.20, respectively), since the directly comparable formic acid catalyzed hydrolysis of tetrahydropyranyl *p*-nitrophenyl ether exhibits an isotopic effect of 3.4.[32] It is noteworthy that a similar conclusion against intramolecular general acid catalysis was also deduced for the acid-catalyzed hydrolysis of aryl α-glycosides of *N*-acetylneuraminic acid.[31] In this system the sugar ring adopts the preferred $^2C_5$ conformation, and the carboxylic acid was able to formally participate as an intramolecular general acid, as similarly suggested for the β-phosphate **5**. Due to the absence of solvent isotope effect (k_{H_2O}/k_{D_2O} = 0.96), this mechanism was, however, ruled out.

As shown in Figure 3, the phosphate hydrolysis of methyl ester **7** is a specific-acid-catalyzed process over the pH ranges examined (0−4.1). The slower hydrolysis of **7** as compared to that of **6** at all pH's most likely reflects the electron-withdrawing effect of the ester group in **7**, which would destabilize the oxocarbenium ion intermediate involved in acid-catalyzed phosphate hydrolysis. It is clear that the rate retardation caused by the ester group is much larger at high pH (factor of 55 at pH = 4.1), where the carboxylate of **6** most likely exists in its ionized form, than that observed at low pH values (factor of 7.4 at pH = 1.7), where we actually compare the relative effects of carboxylic ester with the corresponding carboxylic acid. The observed rate determinants support the role of carboxylic acid anion as an accelerator in the acid-catalyzed hydrolysis process of anomeric phosphates **5** and **6**.

Since the observed effect of the carboxylate appears to be similar to both the β-phosphate **5** and the α-phosphate **6** (Figure 3) and the moderately higher rate of the β-phosphate hydrolysis may be explained as due to the difference between the ground-state energy of this anomer to that of α-phosphate, it follows that the carboxylate may effect inductive stabilization rather than anchimeric assistance. Indeed, the nucleophilic assistance of the carboxylate function could not be accommodated similarly well for both anomers due to the very different geometric and stereoelectronic arrangement of this group (Chart 1). This is further supported by the preferred conformation of α-carboxylate of Kdo in aqueous solutions. Indeed, the structural analysis of several derivatives of Kdo was performed by X-ray crystallography and by conformational and metal-ion-binding analysis in solution,[33] and it was demonstrated that the carboxylate plane in β-glycosides of Kdo is orthogonal to the sugar ring and stabilized by an intramolecular hydrogen bond with the side-chain hydroxyl at C8. Such intramolecular stabilization of carboxylate in solution requires that its oxygen atoms stand off from the sugar ring in an *exo* conformation (as illustrated by the structure III discussed below). However, the side-chain conformation of the α-glycosides of Kdo was found to be different from that of the β-glycosides. Obviously, since the nucleophilic participation of carboxylate requires the precise orientation of one of its oxygens on the side opposite the leaving phosphate group, e.g., the *endo* oxygen, and the orthogonal carboxylate in β-glycosides of Kdo (the carboxylate group is α) has both oxygen atoms in the preferred *exo* conformation to the sugar ring, we may then rule out the possible role of carboxylate as a nucleophile in the hydrolysis of Kdo-2-phosphates. This conclusion is, of course, based on the assumption that the carboxylate function in **5** and **6** has a similar solution conformation as in the parent glycosides. Thus, although the nucleophilic assistance of the carboxylate function was proposed as a possible mechanism for the rate acceleration in acid-catalyzed hydrolysis of aryl α-glycosides of *N*-acetylneuraminic acid,[31] this mechanism seems to not be valid for the hydrolysis of currently examined Kdo-2-phosphates. Inductively, the carboxylate group is electron-*donating*, while the carboxylic acid and ester groups are electron-*withdrawing*.[34] Furthermore, since the inductive effect is geometry independent, then the simplest explanation for the observed rate effects of carboxylate function in hydrolysis of **5** and **6** is most likely an inductive stabilization of the transiently formed oxocarbenium ion intermediate as illustrated in Scheme 3.

Scheme 3

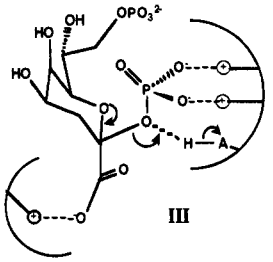


Application to Kdo8P-Synthase-Catalyzed Reaction. From the proposed mechanism in Scheme 1, it seems that the Kdo8P synthase must not only actively catalyze the formation of the putative bisphosphate intermediate **3** but also catalyze its decomposition to produce Kdo8P and inorganic phosphate. This assumption is based on the inherent stability of Kdo-2-phosphates in solution at pH ≥ 7. The first order rate constant for the hydrolysis of **5** at pH 7.3, 73 °C, was measured as 9.3 × 10^{-5} s^{-1}. Therefore, assuming that the putative intermediate has a β-phosphate configuration and that the k_{cat} of the enzyme-catalyzed reaction is 7.0 s^{-1},[10] then we can formally approximate the factor by which the enzyme accelerates the breakdown of the intermediate, to be at least 10^5-fold (7.0 s^{-1}/9.3 × 10^{-5} s^{-1}).[35] Thus, although an enzyme is most commonly considered to produce an unstable intermediate, in the case of Kdo8P synthase, and the previously well characterized EPSP synthase,[36] it seems that the enzyme forms a very stable intermediate and must then catalyze its decomposition. Now, the most important question is how the enzyme does this.

Although at this stage of investigation, all the suggestions to account what factors are really responsible in the enzyme active

(32) Fife, T. H.; Brod, L. H. *J. Am. Chem. Soc.* **1970**, *92*, 1681−1684.
(33) Lutham, K.; Claesson, A.; Kenne, L.; Csoregh, I. *Carbohydr. Res.* **1987**, *170*, 167−179.
(34) Hine, J. *Structural Effects on Equilibria in Organic Chemistry*; Robert E. Krieger Publishing Company: Huntington, NY, 1981; pp 97−99.
(35) In calculating this factor, two important points must be mentioned: (1) we assume that the lack of C8 phosphate in **5** will not significantly affect the hydrolysis rate, and therefore compound **5** is a good model for the intermediate **3**; (2) the mechanism for the hydrolysis of anomeric phosphate **5** at pH 7.3 might be different to that at lower pH values (Figure 3). Indeed, the consensus mechanism of the acid-catalyzed hydrolysis of α-D-glucopyranosyl phosphate suggests[28a,29a] that reaction through the monoanionic phosphate (at pH values >5) undergoes with P−O bond-cleavage, probably *via* the metaphosphate mechanism or some preassociative mechanism that has a transition state having considerable metaphosphate character, and, hydrolysis of neutral phosphate (primarily at pH values lying between 2 and 5) and of conjugate-acid species (below pH 1) occurs through C−O bond-cleavage, involving the formation of oxocarbenium-ion intermediate.
(36) Anderson, K. S.; Johnson, K. A. *J. Biol. Chem.* **1990**, *265*, 5567−5572.

site will be only speculation, some mechanistic pathways, supported by present investigation, could be satisfactory for the purpose. First, since at neutral pH, α-carboxylate of the intermediate **3** can itself provide a rate acceleration of up to 2 orders of magnitude, the enzyme may use this advantage without any additional involvement in this pathway. In other words, intermediate **3** might be recognized at the enzyme active site in such a way that enables it to maintain the ionized form of its carboxylate group during catalysis. Second, since at pH = 7.3 anomeric phosphate is mostly in its dianionic form (pK_{a2} of **5** was measured to be 6.8 at 37 °C), strong chelation by metal cation[37] or by the enzymic electrophiles will largely neutralize negative charges on the oxygen atoms, thereby aiding expulsion of this phosphate group as well as neutral phosphate. Indeed, the comparison of acid-catalyzed hydrolysis rate constant of **5** at 37 °C, pH 0 (213×10^{-3} s^{-1}) to that observed at 37 °C, pH 7.3 (9.3×10^{-5} s^{-1}) gives a factor of 2290. Third, the enzyme can increase very selectively the effective molarity of acid in the vicinity of the bridging oxygen,[38] while leaving the ring oxygen unprotonated, as illustrated schematically in structure III. Thus, because the Kdo8P synthase is neither a metalloenzyme, nor does it require the addition of metal cations for catalytic activity,[7] it is most likely that the suggested *chelation* of the carboxylate and phosphate groups will be achieved only peripherally by the enzymic electrophile/s. A more detailed characterization of the initial steps of this enzyme catalysis is the subject of an ongoing study.



III

Conclusions. The results we have obtained illustrate the value of organic synthesis to characterize enzymatic reaction. Mechanistic postulates of the Kdo8P-synthase-catalyzed reaction have pointed the way to the design of inhibitors, and these in turn have provided insight into details of the reaction. The stereochemical tolerance exhibited by the enzyme for the C2 diastereomerics **5** and **6** suggests that the binding subsites of the carboxylate and phosphate functions are not absolute in their specificity and may interchange, while the observed one order magnitude preference of the β-phosphate **5** supports the proposed β-phosphate configuration in the putative bisphosphate intermediate. We have shown that the nonenzymatic hydrolysis of the β-phosphate **5** in aqueous solution occurs by the well-established mechanism for anomeric phosphate hydrolysis, and, by measuring the rate of this reaction, we have provided a base against which an acceleration of 10^5 for catalysis of this reaction by Kdo8P synthase may be estimated. We also have shown that anomeric carboxylate affords catalysis and is more effective than identically oriented carboxylic acid or ester groups by a factor of at least two orders of magnitude, which suggests that the putative intermediate **3** may recognized at the enzyme active site so as to maintain the ionized form of its carboxylate group during catalysis.

Experimental Section

General Methods. Kdo8P synthase (specific catalytic activity 9 U/mg) was isolated from overproducing strain *Escherichia coli* DH5α (pJU1). The plasmid pJU1 containing the *kdsA* gene[39] was provided by Professor J. R. Knowles. The purification procedure followed the protocol of Ray[7] with some modifications as previously described.[12b] Ara5P and Kdo8P were prepared enzymatically according to the procedure of Whitesides.[40] The potassium salt of PEP was prepared in large quantities as already described.[41] Silver dibenzyl phosphate was prepared from pure, crystalline dibenzyl phosphate (Aldrich) by the published method.[16] Compound **8** was synthesized from the ammonium salt of KDO[14] by a slight modification of the published method.[15] [1−^{13}C]-D-arabinose (96% ^{13}C enrichment) was purchased from Cambridge Isotope Laboratories (Woburn, MA). All other chemicals were received from Aldrich or from Sigma and used without further purification unless noted. In all the synthetic work described, reactions were performed under dry argon atmosphere, unless otherwise noted.

Spectrophotometric measurements were made on a Hewlett-Packard 8452A diode array spectrophotometer using 1-cm path-length cells with a thermostated cell holder and circulating water bath at desired temperature. ^{1}H-NMR were recorded on a Bruker AM-200, AM-400, or AM-500 spectrometer, and chemical shifts reported (in ppm) relative to internal tetramethylsilane ($\delta = 0.0$) with $CDCl_3$ as the solvent and to HOD ($\delta = 4.63$) with D_2O as the solvent. ^{13}C-NMR were recorded on a Bruker AM-200 spectrometer at 50.3 MHz and the chemical shifts reported (in ppm) relative to external sodium 2,2-dimethyl-2-silapentane sulfonate ($\delta = 0.0$) in D_2O. ^{31}P-NMR were recorded on a Bruker AM-200 spectrometer at 81.0 MHz, and the chemical shifts reported (in ppm) relative to external orthophosphoric acid ($\delta = 0.0$) in D_2O. All the coupling constants (J) are in Hz. Mass spectra were obtained by the use of a TSQ-70B mass spectrophotometer (Finnigan Mat) under fast-atom bombardment (FAB) in the glycerol matrices.

Methyl (4,5,7,8-Tetra-*O*-acetyl-3-deoxy-β-D-*manno*-2-octulopyranosid)onate 2-(Dibenzyl phosphate) (9). To a solution of chloride **8** (1.57 g, 3.6 mmol) in freshly distilled dry benzene (5 mL) was added silver dibenzyl phosphate (0.3 g, 8.0 mmol). The resulting solution was warmed at reflux with the exclusion of light and moisture. The progress of the reaction was monitored by TLC [silica gel, EtOAc/hexane (1:1), the R_f of **9** and **10** were 0.36 and 0.40, respectively]. After approximately 60% conversion of starting material (30 min total reflux time), the reaction mixture was cooled, diluted with dry benzene, and filtered through Celite. The filtrate was rotary-evaporated to dryness and chromatographed on a silica gel column with EtOAc/hexane (1:1) as eluent, to afford **9** (0.76 g, 31% based on unreacted starting material recovered) and **10** (0.24 g, 10%) along with unreacted chloride **8** (0.628 g, 40%). Data for **9**: ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 2.00 (6H, s, 2OAc), 2.01 (3H, s, OAc), 2.04 (3H, s, OAc), 2.05 (1H, dd, $J_{3a-3eq} = 12.8$, $J_{3a-4} = 12.5$, 3-H_a), 2.44 (1H, dd, $J_{3a-3eq} = 12.8$, $J_{3eq-4} = 3.4$, 3-H_{eq}), 3.75 (s, 3H, CO_2Me), 4.04−4.16 (1H, m, 8-H), 4.30−4.43 (2H, m, 8′-H′ and 6-H), 4.92−5.16 (2H, m, 7-H and 4-H), 5.07−5.08 (4H, m, CH_2OBn), 5.29 (1H, d, $J = 1.5$, 5-H), 7.28−7.32 (10H, m, aromatic protons); proton coupled ^{31}P-NMR ($CDCl_3$, 81 MHz) δ −8.73 (quintet, $J = 8.0$).

(37) Interestingly, the hydrolysis of 1,10-phenanthroline-2-carbonyl phosphate was facilitated by a factor of $> 10^7$, due to strongly chelated metal ions such Ni^{2+}, Co^{2+}, and Zn^{2+}: Fife, T. H.; Pujari, M. *J. Am. Chem. Soc.* **1990**, *112*, 5551−5557.

(38) It should also be noted that in our measurements at pH = 0 the acid concentration in solution was 1 M, while the effective molarity of acid provided by the enzyme in the vicinity of bridging oxygen (structure III) might be in several orders of magnitude higher than that in solution (for a review on effective molarity, see: A. J. Kirby, *Adv. Phys. Org. Chem.* **1980**, *17*, 183). This would affect the factor of 2290 to reach higher values.

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Methyl (4,5,7,8-Tetra-*O*-acetyl-3-deoxy-α-D-*manno*-2-octulopyranosid)onate 2-(Dibenzyl phosphate) (10). This compound was prepared from 250 mg (0.57 mmol) of chloride **8** in the same manner as the corresponding β-anomer (**9**) with the following modification: the reagent silver dibenzyl phosphate (4.6 g, 12 mmol) was added in portions of 3 mmol every 30 min, and the reflux was continued until all the starting material was consumed (3 h). After purification by chromatography (EtOAc/hexane, 1:1) pure **10** was obtained as a clear oil (352 mg, 91%): ^{1}H-NMR ($CDCl_3$, 400 MHz) δ 1.97 (3H, s, OAc), 1.98 (3H, s, OAc), 2.05 (3H, s, OAc), 2.08 (3H, s, OAc), 2.15 (1H, ddd, J_{3a-3eq} = 13.0, J_{3a-4} = 12.8 and J_{3a-P} = 4.3, 3-H_a), 2.30 (1H, dd, J_{3a-3eq} = 13.0, J_{eq-4} = 4.8, 3-H_{eq}), 3.60 (s, 3H, CO_2Me), 4.03−4.16 (1H, m, 8-H), 4.27−4.39 (1H, m, 8′-H), 4.47 (1H, dd, J = 10.2 and 1.5, 6-H), 4.98−5.07 (4H, m, CH_2OBn), 5.22−5.29 (2H, m, 7-H and 4-H), 5.30 (1H, d, J = 1.5, 5-H), 7.23−7.32 (10H, m, aromatic protons); proton coupled ^{31}P-NMR ($CDCl_3$, 81 MHz) δ −8.12 (*doublet of quintet*, J = 4.3 and 8.5).

3-Deoxy-β-D-*manno*-2-octulopyranosylonate 2-Phosphate (5). To a solution of protected phosphate **9** (0.25 g, 0.37 mmol) in dry methanol (3 ml) was added dry Et_3N (100 μL, 0.72 mmol). To this solution was added 10% Pd/C (0.8 g), and the mixture was stirred under H_2 at 1 atm for 1 h. The catalyst was removed by filtration through Celite, and the filtrate was concentrated under reduced pressure. The residue was dissolved in cold water (2 mL), and cold aqueous 1 N LiOH (3 ml) was added. This solution was stirred for 1 h at room temperature, and then the pH was carefully adjusted to 9.0 with Dowex 50W (H^+ form). After removal of the resin by filtration, the solvent was lyophilized. A quantitative assay for phosphate esters resulted in a yield of product **5** of 0.26 mmol (70%). This product was >95% pure as judged by its spectral analysis and therefore for most purposes was used without additional purification. The analytically pure material was obtained by purification through anion-exchange chromatography on AG1X8 (HCO_3^- form), eluting with a linear gradient of triethylammonium bicarbonate buffer (0−0.8 M, pH 8.5). Fractions were analyzed for inorganic phosphate after digestion with $HClO_4$. The active fractions were then combined and concentrated till dry. The residue was dissolved in water, passed through a column of Dowex 50W (K^+ form) and concentrated again to give the highly purified phosphate **5**: ^{1}H-NMR (lithium salt, pD = 10.0, D_2O, 500 MHz) δ 2.13 (1H, dd, J_{3a-3eq} = 12.4, J_{3a-4} = 12.1, 3-H_a), 2.32 (1H, dd, J_{3a-3eq} = 12.4, J_{3eq-4} = 3.8, 3-H_{eq}), 3.12 (1H, dd, J = 8.4 and 3.0, 8-H), 3.56 (1H, dd, J = 8.4 and 3.7, 8′-H), 3.57 (1H, d, J = 3.5, 5-H), 3.60 (1H, ddd, J = 3.5, 3.8 and 12.4, 4-H), 3.61 (1H, d, J = 4.0, 6-H), 3.68 (1H, ddd, J = 3.0, 3.7 and 4.0, 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, C-2), 178.0 (d, J = 7.95 Hz C-1); proton coupled ^{31}P-NMR δ −0.1; FAB mass spectrum *m/e* 433.0 (M^+ + H, $C_8H_{12}O_{11}PK_3$ requires 433.0).

3-Deoxy-α-D-*manno*-2-octulopyranosylonate 2-Phosphate (6). In a procedure similar to that described above for the β-anomer **5**, 1.4 g of compound **10** was converted to 0.6 g of the lithium salt **6** (91%): ^{1}H-NMR (lithium salt, pD = 10.0, D_2O, 500 MHz) δ 1.48 (1H, ddd, J_{3a-3eq} = 12.8, J_{3a-4} = 12.2, J_{3a-P} = 4.5, 3-H_a), 1.84 (1H, dd, J_{3a-3eq} = 12.8, J_{3eq-4} = 4.3, 3-H_{eq}), 3.31 (1H, dd, J = 12.0 and 2.5, 8-H), 3.58 (1H, ddd, J = 12.0, 2.4 and 1.0, 8′-H), 3.80 (1H, d, J = 3.0, 5-H), 3.82 (1H, ddd, J = 2.5, 2.4 and 9.5, 7-H), 3.87 (1H, d, J = 9.5, 6-H), 3.91 (1H, ddd, J = 3.0, 4.3 and 12.2, 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), 38.5 (d, J = 8.8, C-3), 100.9 (d, J = 7.1, C-2), 179.7 (s, C-1); proton coupled ^{31}P-NMR δ 0.1 (d, J_{H3a-P} = 4.5); FAB mass spectrum *m/e* 385.0 (M^+ + H, $C_8H_{12}O_{11}PNa_3$ requires 385.0).

Methyl (3-Deoxy-α-D-*manno*-2-octulopyranosid)onate 2-Phosphate (7). The protected α-phosphate **10** (210 mg, 0.29 mmol) was dissolved in dry methanol (10 mL), and dry Et_3N (82 μL, 0.72 mmol) was added. To this solution was added 10% Pd/C (0.8 g), and the mixture was stirred under H_2 at 1 atm for 1 h. The catalyst was removed by filtration through Celite, and the filtrate was concentrated under reduced pressure to a final volume of 5 mL. The resulting solution was cooled in an ice-bath, and freshly prepared NaOMe (1 mmol) in 10 mL methanol was added dropwise. The reaction mixture was stirred for 1 h at 0 °C. The pH was then carefully adjusted to 8.5 using Dowex 50W (H^+ form), which was prepared by thorough washing with dry methanol. After removal of the resin by filtration, 10 mL of water was added, and the resulting solution was concentrated by lyophilization to give methyl ester **7** in 90% yield (0.26 mmol) as determined by quantitative analysis for inorganic phosphate after digestion with $HClO_4$: ^{1}H NMR (pD = 8.5, D_2O, 400 MHz) δ 1.55 (1H, ddd, J_{3a-3eq} = 12.6, J_{3a-4} = 12.3, J_{3a-P} = 4.5, 3-H_a), 1.88 (1H, dd, J_{3a-3eq} = 12.8, J_{3eq-4} = 4.3, 3-H_{eq}) 3.32 (1H, dd, J = 12.0 and 2.5 H, 8-H), 3.55 (s, 3H, CO_2Me), 3.58 (1H, ddd, J = 12.0, 2.4 and 1.0, 8′-H), 3.8 (1H, d, J = 3.0, 5-H), 3.82 (1H, ddd, J = 9.5, 2.5 and 2.4, 7-H), 3.8 (1H, d, J = 9.5, 6-H), 3.91 (1H, ddd, J = 12.2, 4.3 and 3.0, 4-H); proton coupled ^{31}P-NMR δ −1.2 (d, J_{H3a-P} = 4.5 Hz); FAB mass spectrum *m/e* 377.0 (M^+ + H, $C_9H_{15}O_{11}PNa_2$ requires 377.0).

Enzyme Assays. Unless otherwise stated, the enzyme activity was assayed in a 0.8 mL reaction buffer consisting of 0.1 M Tris-acetate, pH 7.3, PEP (0.2 mM, =27K_m), and Ara5P (0.5 mM, =20K_m). All solutions except the enzyme were filtered through Millipore type-HA filters (0.45 μm) before use. Following equilibration at 37 °C for 2 min, Kdo8P synthase (20 μL, at a final concentration of about 30 nM) was added, and the decrease in the absorbance difference between 232 and 350 nm (as internal reference) was monitored with time (MS-DOS UV/VIS software). This method[42] is based on the absorbance difference at 232 nm between PEP (ϵ = 2840 M^{-1} cm^{-1}) and the other substrates and products (ϵ < 60 M^{-1} cm^{-1}) under the assay conditions. The initial rate was calculated from a linear least-squares fit of the first 30 s of the progress curve. The concentrations of PEP, Ara5P, and of **5**, **6**, and **7** were determined precisely by quantitative assaying of the P_i released by alkaline phosphatase.[43] In each case to ensure complete hydrolysis of the phosphate monoester, the aliquots of the incubation mixture with alkaline phosphatase were tested by ^{31}P NMR. For the determination of Kdo-2-phosphates (**5**, **6**, and **7**) were found that treatment with alkaline phosphatase was not needed, as the acidity of the assay mixture was enough to bring about the complete hydrolysis of anomeric phosphates. Furthermore, concentrations of the substrates (PEP and Ara5P) were double checked by Kdo8P synthase using either the above procedure or a thiobarbituric acid assay[7] that had been modified so that the final volume of the assay mixture was 1.6 mL, and the entire procedure was carried out in the ependorf tube (all the solutions used here were 10.6-fold diluted, in contrast to the 94-fold dilutions used in the original procedure). Very close agreement (an approximately 2−5% difference) between these assay methods was obtained. One unit of the enzyme activity is the amount that catalyzes the consumption of 1 μmol PEP/min at 37 °C. During the enzyme purification the enzyme activity was assayed by the above modified thiobarbituric acid assay.

Inhibition Studies. In order to determine the steady-state kinetic parameters, K_m for PEP and K_i values of anomeric phosphates **5** and **6**, the reaction solutions were prepared as described above but with constant (671 μM, = 25K_m) Ara5P and variable (12−200 μM) PEP. The rate measurements were made as described above, while a 5-s delay was allowed following initiation of the reaction. The initial rate was then determined by least-squares fitting of the first 10% of the progress curve (between 20 and 80 s, depending on the initial concentration of PEP) to a straight line. Four inhibitor concentrations were examined for each compound, and for each inhibitor concentration, four concentrations of PEP (12−200 μM) were used (Figure 2). All samples were assayed in triplicate, and analogous results were obtained in 2−4 different experiments. 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 program.[44] The K_i values, given in Table 2, were 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.[45]

Examination of Substrate Activity for 5, 6, and Arabinose with Kdo8P Synthase. Since the compounds **5** and **6** are very sensitive under either thiobarbituric acid assay[7] or inorganic phosphate assay[43] conditions, we used the proton decoupled ^{31}P-NMR assay in which the appearance of inorganic phosphate could be clearly monitored. Reaction mixtures contained 0.1 M Tris-HCl buffer (prepared in D_2O, pD 7.3, 37 °C), bovine serum albumin (1.5 mg/mL, for the stabilization of the enzyme), 10 mM substrate, and 225 units (approximately 25 mg) Kdo8P synthase in a total volume of 0.6 mL. A control experiment, containing all of the above but without the enzyme, was run parallel to the above. The resonances of **5** (−1.2 ppm) and **6** (−1.4

ppm) were gradually diminished in intensity by time and were replaced by a new singlet at δ (ppm) 2.6 of inorganic phosphate, which was further identified by the addition of an authentic sample of inorganic phosphate (pH 7.3). A very similar time course for the formation of inorganic phosphate was determined in each experiment and corresponding control experiment, as judged by the integration of signals. Both **5** and **6** failed to show any detectable enzyme-catalyzed acceleration in the phosphate hydrolysis after 24 h. In this way the half-lives for the hydrolysis of **5** (2.07 h) and **6** (12.8 h) could be determined.

The experiments with arabinose were performed as follows: The reaction mixture contained 0.1 M Tris-HCl buffer (prepared in D_2O, pD 7.3), bovine serum albumin (1.5 mg/mL, for the stabilization of the enzyme), PEP (3 mM), [1-^{13}C]-D-(−)-arabinose (10 mM), and 20 units Kdo8P synthase, in a total volume of 0.6 mL. Progress of the reaction was monitored by proton decoupled ^{13}C-NMR over 52 h at 37 °C. Over this period of time there was no discernable formation of either the expected condensation product [4-^{13}C]Kdo-2-phosphate or the [4-^{13}C]Kdo. The entire region of 70.8 ppm, where we had expected to find the formation of new resonance for the C4 of the above products, remained unchanged, and only a natural abundance of ^{13}C resonances of the starting sugar without any significant change, were recorded.

Acid-Catalyzed Hydrolysis Measurements. The acid-catalyzed hydrolysis rates of various glucopyranosyl phosphates[29a] have been determined in the past by monitoring the appearance of inorganic phosphate, using the sensitive phosphate assay which had been proven suitable for analysis of phosphate levels in the presence of acid-labile phosphate esters.[46] However, we found this method unsuitable for our measurements, since there was very substantial hydrolysis of **5** during exposure to acid prior to the addition of the reducing reagent. Therefore, although some of the hydrolysis rates could be determined by ^{31}P-NMR, due to the limited quantities of **5** and **6**, a novel assay method for accurate measuring of the extent of hydrolysis as a function of time, had to be developed. This method was based upon an instantaneous quench of the hydrolysis process through the addition of cold LiOH + LiCl and the determination of the remaining substrate concentration through the use of a P_i assay of Ames.[43] This assay for the hydrolysis at pH = 0 (1 M $HClO_4$) was performed as follows: The solutions of the substrate (30 μM, pH 9, 40 μL) and of 2 M $HClO_4$ (40 μL) were incubated separately at the desired temperature for 10 min. The hydrolysis process was then initiated by mixing these solutions together. Aliquots (10 μL) were withdrawn at various time intervals and immediately quenched by addition to a cold (0 °C) mixture containing 2 N LiOH (10 μL) and 8 N LiCl (5 μL). Under these conditions, the pH of the aliquot was instantly increased which ceased the acid-catalyzed hydrolysis. All the inorganic phosphate generated during the hydrolysis process precipitates as a trilithium salt. After centrifugation in an Ependorf centrifuge (3 min), 20 μL of the resulting solution was removed, and the quantity of Kdo-2-phosphate remaining in each aliquot was readily determined by addition of freshly prepared ascorbate-molibdate reagent[43] (1 mL) and heating at 45 °C for 20 min. Under these conditions all the anomeric phosphate of substrate undergoes hydrolysis to yield inorganic phosphate, which in turn reacts with the reagent giving a very characteristic blue color. After cooling at room temperature the absorbance difference between 816 and 426 nm (as an internal reference) was measured, and the amount of phospohate was determined from a standard curve (M-DOS UV/VIS software). First-order rate constants for the hydrolysis reactions were determined by plotting ln of concentration of the remaining substrate in each time point against time.

In this manner, the time course of acid-catalyzed hydrolysis of **5** and **6** at pH = 0 (1 M $HClO_4$) was determined for various low temperatures (Table 3), and the time course for 37 °C was calculated by extrapolation of the data to 37 °C using an Arrhenius plot. For the rate measurements at higher pH values (Figure 3), the solution of substrate (30 μM, pH 9) in 1 M $NaClO_4$ (80 μL) was incubated at 12 °C, and the pH was carefully adjusted to the desired value through the addition of appropriately diluted $HClO_4$. The pH of solution was monitored continually during all the experiments, and the rates of hydrolysis were measured as described above. All pH points were assayed in triplicate and hydrolysis reactions were followed to completion (at low pH values) or at least two half-lives (at higher pH values).

In order to check the precision of the above assay method, the observed first-order rate constants for all three substrates (**5**−**7**) and at least two different pH values were also determined under similar conditions by following the hydrolysis process in ^{31}P-NMR. A typical procedure for these experiments was as follows: The reaction mixture in D_2O (total volume of 0.6 mL) containing substrate (20 mM, pH 9), trimethylphosphate (5 mM, used as an internal standard), and $NaClO_4$ (1 M) was incubated at the desired temperature, and the pH was carefully adjusted to the desired value by the addition of appropriately diluted $HClO_4$. The resulting solution was immediately transferred into an NMR-tube, and the release of phosphate was monitored over time in a proton decoupled ^{31}P-NMR at constant temperature. The extent of hydrolysis was measured by integration relative to internal trimethylphosphate (which was proven to be stable under the experimental conditions). The first-order rate constants were determined by plotting ln of relative integration versus time. Very close agreement (approximately 5−8% difference) between these two assay methods was obtained.

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