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1-(DIHYDROXYPHOSPHYNYL)VINYL PHOSPHATE: THE PHOSPHONATE ANALOGUE OF PHOSPHOENOLPYRUVATE IS A PH-DEPENDENT SUBSTRATE OF Kdo8P SYNTHASE

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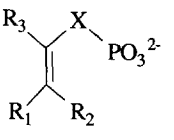
Abstract: The phosphonate analogue of PEP (structure **5**), in which the carboxylate group is replaced by an isosteric phosphonate, was synthesized and evaluated as a substrate and as an inhibitor of the Kdo8P synthase. It was demonstrated that **5** is a very slow, pH-dependent substrate of the enzyme.

Phosphoenolpyruvate (PEP, **1**) is a highly functionalized, chemically versatile molecule used at several intersections of cellular energy metabolism and biosynthesis.[1] Making slight alterations to the PEP molecule and then quantifying the effects of these changes provided interesting information about the mechanisms of enzymatic reactions. In this concern a series of analogues of PEP have previously been synthesized and investigated as substrates and as inhibitors of several PEP-utilizing enzymes.[2] Studies were recently initiated in our laboratory dealing with the mechanism of 3-deoxy-D-*manno*-2-octulosonate-8-phosphate (Kdo8P) synthase, which catalyzes the unusual condensation of PEP with arabinose-5-phosphate (Ara5P) to produce Kdo8P and inorganic phosphate (P_i).[3] Most recent mechanistic proposals for this enzyme-catalyzed reaction (Scheme 1) involve the formation of either the acyclic intermediate **II**,[4] or the cyclic intermediate **IV**,[5] while which of these pathways is true still remains to be conclusively determined. It is noteworthy, that these mechanistic proposals are largely based on the results accumulated through the synthesis and examination of various analogues of PEP,[4,6] analogues of Ara5P,[7] and of the product Kdo8P,[5,8] as mechanistic probes of Kdo8P synthase.

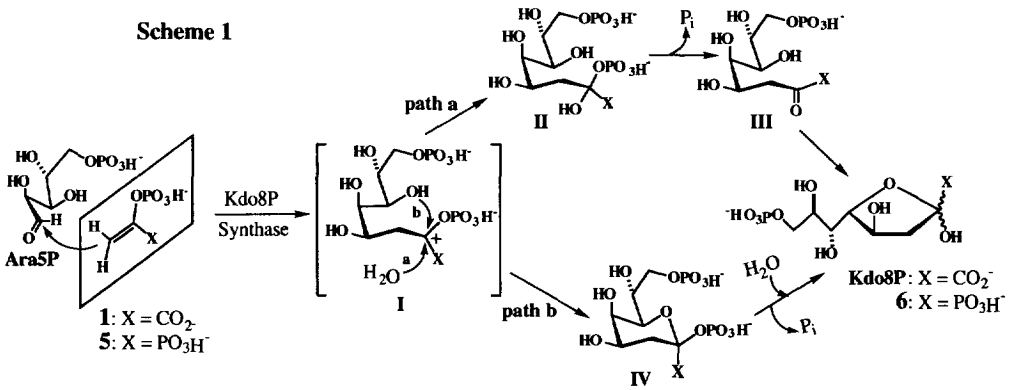


	X	R_1	R_2	R_3
1	O	H	H	CO_2^-
2	O	F	H	CO_2^-
3	O	H	F	CO_2^-
4	NH	H	H	CO_2^-
5	O	H	H	PO_3^{2-}

In particular, an earlier study[4] with [^{18}O]-PEP specifically labeled in the bridged oxygen revealed that the reaction proceeds through the C-O bond cleavage of PEP. More recently,[6a] by using (*E*)- and (*Z*)-isomers of 3-fluorophosphoenolpyruvate (structures **2** and **3**) we have demonstrated that the condensation step is stereospecific, involving the attachment of *si* face of PEP to the *re* face of Ara5P. In order to investigate the unique C-O bond cleavage mechanism of PEP, we have prepared[9] a new isosteric phosphoramidate analogue of PEP (**4**), in which the bridged oxygen is replaced by a bridged NH group and tested with the synthase. Although **4** proved to be a substrate for other PEP-utilizing enzymes, it provided neither substrate nor inhibitory activity in reaction with Kdo8P synthase. This result demonstrates that not only electronic factors, but also steric factors of the bridged oxygen moiety of PEP, are critical for the correct recognition and catalysis by the enzyme. After extending our studies on the synthase mechanism, we are able to describe here the

evaluation of phosphonate analogue **5**, in which the carboxylic acid of PEP is replaced by phosphonic acid, as a substrate and as an inhibitor of the enzyme.

Scheme 1



The selection of **5** was encouraged for the following reasons: (i) **5** is an isosteric analogue of **1**; (ii) in many enzymes,[10] as well as in Kdo8P synthase,[5b,8] the carboxylate binding site is indistinct and may well be accommodated by the isosteric phosphonate; (iii) since the carboxylate group was suggested to afford catalysis during both steps of the enzyme-catalyzed reaction[8] (the initial inductive stabilization of the transient oxocarbenium intermediate **I** and the subsequent catalysis in the hydrolysis of the phosphate group), it was very interesting to test the similar effects for the isosteric phosphonate; and (iv) if **5** would function as a substrate, then only through the path a (Scheme 1) it should unmask an electrophilic acyl phosphonate moiety in **III** capable of covalently modifying any nearby nucleophiles in the active site,[11] and thus providing a very efficient way for the differentiation between the proposed two mechanisms.

The analogue **5** was synthesized in three steps from the commercially obtained chloroacetyl chloride following the previously reported procedure.[12] Initially, the analytically pure **5** was evaluated as an alternative substrate of homogeneous Kdo8P synthase using the standard assay conditions [0.1 M Tris-acetate buffer, pH 7.2, 37 °C],[8] and the reaction progress was followed either by thiobarbituric acid assay[3] or by the proton decoupled ^{31}P NMR assay, in which the appearance of inorganic phosphate could be clearly monitored. However, no discernible conversion of **5** was detected over a 24-h period even with a 1000-fold higher concentration of the enzyme than is typically introduced into an assay experiment. The observed inert nature of the analogue **5** was very intriguing; therefore, upon closer investigation of various factors we found that the global charge difference between PEP and **5** is indeed the critical factor for the catalysis to occur. The second ionization constants (pK_{a2}) for the phosphate and phosphonate groups in **5** were estimated by proton decoupled ^{31}P NMR measurements of chemical shifts and found to be 5.8 (±0.3) for both groups. This value is very similar to that of the phosphate group in PEP (pK_{a2} = 5.75).[13] According to the data, at pH 7.2 the PEP should mostly be as a *tri*anion while **5** is a *tetra*anion, and the highest electrostatic similarity is expected only around pH 5, where both compounds should be in *di*anionic forms. Indeed, incubation of **5** with Kdo8P synthase in the presence of Ara5P at pH 5 resulted in the formation of Kdo8P-like product (**6**), which could be easily detected by the proton decoupled ^{31}P NMR assay. The activity of **5** as a substrate was measured by disappearance of the singlet at 1.8 ppm (Ara5P) and the subsequent development of new resonances at 1.6 ppm (P_i) and at 2.01 ppm (C-8 phosphate of **6**) in ^{31}P NMR (Figure 1). Thus, treatment of **5** (cyclohexylammonium salt, 20 mM) with Kdo8P synthase [7 units in the total volume of 1 mL containing

Ara5P (10 mM), bovine serum albumin (1.5 mg, for the stabilization of the enzyme), 100 mM acetate buffer, pH 5.2, 37 °C] resulted in formation of **6** ($t_{1/2}$ = 567 min) at about 0.26% of the rate observed for the PEP ($t_{1/2}$ = 10.5 min) determined under the same conditions (but with the presence of 1 unit of the enzyme). Under these conditions the substrates (**5** and Ara5P) were stable and no significant changes were observed in the control experiment, containing all of the above but without the enzyme, and was run parallel to the above.

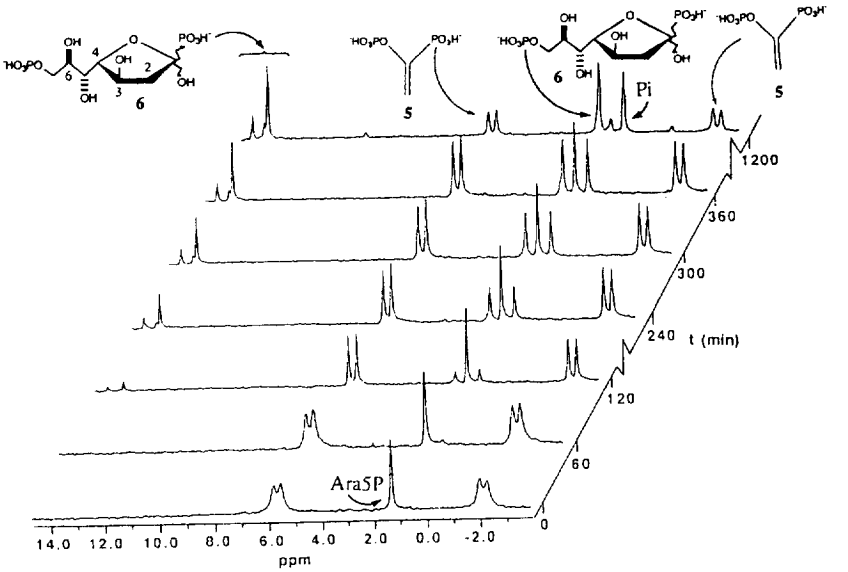


Figure 1. A time course of proton-decoupled ^{31}P NMR spectra showing Kdo8P-synthase-catalyzed conversion of phosphonate **5** to **6**. The conditions were as mentioned in the text. The first spectrum (0 time) was taken prior to the addition of the enzyme. Measurements were made on a Bruker AM-200 operating at 81.0 MHz. Typically 360 data points were collected and Fourier transformed with a line broadening of 1 Hz. Chemical shifts are relative to 200 mM D_3PO_4 referenced at 0 ppm. Inserts indicate the corresponding structural assignments.

The enzymatic reaction product **6** was purified by ion-exchange chromatography [on AG 1X8 (100-200 mesh, HCO_3^- form) eluted with a linear gradient (0-0.7 M) of triethylammonium bicarbonate, pH 7.5, 4 °C] and its structure was established by a combination of ^1H, ^{13}C, 2-D COSY, C-H correlation, and ^{31}P NMR analysis. It appears that unlike Kdo8P that exists in solution as a mixture of four anomeric forms (about 70% of α- and β-pyranose and 30% of α- and β-furanose),[14] compound **6** exists in solution mostly as an acyclic form (52.6% of hydrated form and 9.5% of free keto form) and only a relatively small part forms the furanose cyclic structures (26.6% of β-furanose and 11.3% of α-furanose).[15] The furanose structures were diagnosed from the observed large geminal coupling constants of H-2 protons ($^2J_{H2a-H2e}$~15 Hz)[6a] and from the relatively small 3-bond coupling constants between phosphorus and H-2 ($^3J_{P,H2}$).[16] Another indication relevant to the assignment of the furanose structures of **6** was obtained by the examination of the values of the coupling constants $^3J_{H2a,H3a}$. These coupling constants, in the pyranose structures of Kdo8P, are relatively large (12-13 Hz) indicating a *trans*-diaxial relationship between H-2 and H-3, while smaller couplings (2-3 Hz) have been observed for the pseudo-axial orientation of these two protons in the furanose anomers. Thus, the observed couplings of $^3J_{H2a,H3a}$ = 2.7 Hz (for β-**6**) and $^3J_{H2a,H3a}$ = 2.0 Hz (for α-**6**) are only consistent with the furanose structures of **6**. The anomeric configuration of phosphonate linkage was determined by the magnitude of $^3J_{P,H2}$ values in ^1H NMR. These coupling constants for the pseudo-axial (H-2a) proton ($^3J_{P,H2a}$)

were 2.78 Hz and 7.6 Hz for the β- and α-furanose anomers, indicating trans and cis orientation respectively, between H-2a and phosphonate group. This data agrees with the previously reported values for compounds possessing anomeric phosphoryl groups in the furanose rings.[16]

Although the observed results clearly demonstrate that the analogue **5** is a pH-dependent substrate of the enzyme, its activity is 378 times lower than that of natural substrate PEP, indicating that subtle structural alterations can have dramatic consequences in analogue processing. In order to further account for this phenomenon, the phosphonate **5** was evaluated as inhibitor of the synthase at pH 5 and 7.[17] The inhibition constants were able to be measured from initial velocity studies, and inhibition patterns were found to be competitive against PEP binding (Figure 2). The K_i values were measured as 60 (±5) μM and 360 (±10) μM at pH 5 and 7, respectively. Taking into account the K_m values of PEP at the above pH values [4 (±0.5) and 10 (±1) μM], we calculate the ratio K_m/K_i as 0.07 and 0.03, at pH 5 and 7, respectively.

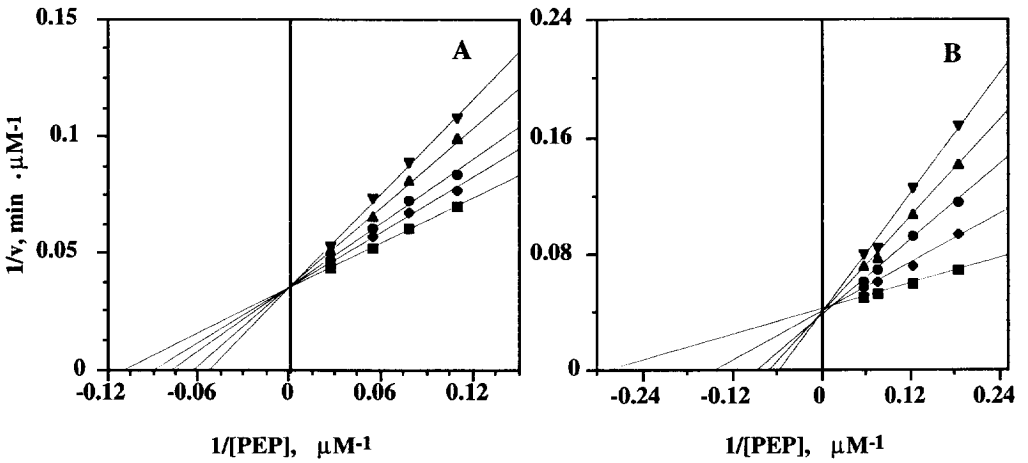


Figure 2. Inhibition of Kdo8P synthase by the analogue **5**. Double-reciprocal plots of initial velocities are given as a function of PEP at (A) pH 7.1 (Tris-acetate buffer, 0.1 M), when the Ara5P concentration was 0.5 mM and the concentrations of **5** were none (■), 100.0 (◆), 200.0 (●), 300.0 (▲) and 400.0 (▼) μM; (B) pH 5.1 (acetic acid/sodium acetate buffer, 0.1 M), when the Ara5P concentration was 0.5 mM and the concentrations of **5** were none (■), 50.0 (◆), 100.0 (●), 150.0 (▲) and 200.0 (▼) μM. The assays were carried out at 37 °C, including 25 milliunits of homogeneous Kdo8P synthase, bovine serum albumin (0.1 mg/mL), PEP (**1**), Ara5P, and inhibitor (**5**) in a final volume of 1 mL. All samples were assayed in triplicate and analogous results were obtained in 2-4 different experiments.

This data shows that the replacement of carboxylate group in PEP by isosteric phosphonate to form the analogue **5** results in a significant reduction in binding affinity at both pH values: 15-fold and 36-fold reduction at pH 5 and 7, respectively. In addition, the binding affinity of **5** is enhanced as the pH decreases. Since the phosphonate group in **5** undergoes its second ionization in the pH range studied, this trend suggests that it is the phosphonate monoanion that is bound to the enzyme. Indeed, the monoanionic phosphonate in **5** should be a closer mimic of the carboxylate of PEP; therefore, the observed binding results can explain the dramatic effect of pH on the activity of **5** to serve as a substrate, while the observed high rate-retarding effect of the phosphonate group may be due to its shape and size.[11] Further biochemical studies of this system, especially the possible formation of very unstable enzyme-bound imine derivative through the acylphosphonate **III**, as well as the experiments to trap the possible intermediates using rapid-quench techniques, are now in progress.

In addition to its mechanistic importance, the observation that **5** can be proceeded by the synthase to form the phosphonate **6** bears significance as a very short synthesis of the phosphonate analogues of Kdo and of Kdo8P,[18] which may act as potent inhibitors of Kdo biosynthesis.[19]

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15. The relative ratio of different anomers was determined by ^{1}H NMR of the purified **6** due to integration of geminal protons at C-2. The designations α and β refer to the pseudo-axial and pseudo-equatorial orientation respectively, of the anomeric hydroxyl in the furanose structures of **6**. Selected spectral data for the potassium salt of **6** in D_2O, pD 7.2, 25 °C. **^{1}H NMR** (400 MHz, referenced to HOD at 4.63 ppm) δ β-furanose: 1.80 (ddd, 1H, $J_{2a\text{-}2e}$ = 14.8 Hz, $J_{2a\text{-}3}$ = 2.7 Hz, $J_{2a\text{-}P}$ = 2.78 Hz, H_{2a}), 2.41 (ddd, 1H, $J_{2e\text{-}2a}$ = 14.6 Hz, $J_{2e\text{-}3}$ = 7.08 Hz, $J_{2e\text{-}P}$ = 7.8 Hz, H_{2e}), 4.29 (dd, 1H, $J_{3\text{-}2e}$ = 7.18 Hz, $J_{3\text{-}2a}$ = 2.66 Hz, H_3); α-furanose: 2.07 (ddd, 1H, $J_{2a\text{-}2e}$ = 14.9 Hz, $J_{2a\text{-}3}$ = 2.0 Hz, $J_{2a\text{-}P}$ = 7.6 Hz, H_{2a}), 2.39 (dd, 1H, $J_{2e\text{-}2a}$ = 14.95 Hz, $J_{2a\text{-}3}$ = 7.1 Hz, $J_{2e\text{-}P}$ < 1 Hz, H_{2e}), 4.40 (dd, 1H, $J_{3\text{-}2e}$ = 7.2 Hz, $J_{3\text{-}2a}$ = 2.11 Hz, H_3); keto form: 2.16 (dd, 1H, J = 7.5 and 2.7 Hz, H_2'), 2.14 (d, 1H, J = 7.5 Hz, H_2'');

gem-diol form: 1.73 (d, 1H, J = 9.8 Hz, H_2'), 1.72 (dd, 1H, J = 10.6 and 2.9 Hz, H_2''). H_{2a} and H_{2e} refer to the geminal H-2 protons of the furanose anomers that have similar orientation to the axial and equatorial protons, respectively, in the pyranose anomers. **^{13}C NMR** (50.30 MHz, referenced to DSS at 0.0 ppm) δ β-furanose: 107.3 (d, J = 194 Hz, C1), 45.8 (d, J = 9.0 Hz, C2), 87.67 (d, J = 10.0 Hz, C3); α-furanose: 108.1 (d, J = 194 Hz, C1), 44.44 (d, J = 9.0 Hz, C2), 88.43 (d, J = 6.5 Hz, C3); keto form: 45.22 (d, J = 10.5 Hz, C2), 75.52 (d, J = 6.0 Hz, C3); gem-diol form: 99.0 (d, J = 194 Hz, C1), 34.18 (d, J = 8.2 Hz, C2), 68.34 (d, J = 9.0 Hz, C3); proton decoupled **^{31}P NMR** (81.62 MHz, referenced to 200 mM H_3PO_4 at 0 ppm) δ 13.71 (s), 13.30 (s), 13.21 (s), 9.63 (s), 4.91 (s), 4.64 (s), 4.93 (s), 2.27 (s).

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17. The homogeneous enzyme (specific activity 9 units/mg) was isolated from the overproducing strain *E. coli* DH5α (pJU1) as previously reported.[5,8] The enzyme activity was assayed spectrophotometrically (232 nm) as described previously[5,8] and the kinetic parameters (V_{max} and K_m) were estimated by using an Enzfitter (1987) PC-IBM program.

18. In addition to the complexity of the chemical preparation of these structures, in the recently reported chemical synthesis of the similar phosphonate analogue of Kdo (Coutrot, P.; Grison, C.; Lecouvey, M. *Tetrahedron Lett.* **1996**, *37*, 1595), it was noted that the final product and its protected precursor were very unstable compounds because the high lability of the C-P bond in the reaction conditions used by these workers.

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