Probing the Stereochemistry of *E. coli* 3-Deoxy-D-*arabino*-heptulosonate 7-Phosphate Synthase (Phenylalanine-Sensitive)-Catalyzed Synthesis of KDO 8-P Analogues

Appavu K. Sundaram and Ronald W. Woodard*

Interdepartmental Program in Medicinal Chemistry, College of Pharmacy, University of Michigan, Ann Arbor, Michigan 48109-1065

rww@umich.edu

Received September 28, 1999

The five-carbon phosphorylated monosaccharide analogues, D-arabinose 5-phosphate, D-ribose 5-phosphate, and 2-deoxy-D-ribose 5-phosphate, were separately condensed with (*Z*)- and (*E*)-[3-^{2}H]-phospho*enol*pyruvate (PEP) in the presence of *Escherichia coli* 3-deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) synthase (phe) to give in the case of (*Z*)-[3-^{2}H]-PEP (3*S*)-[3-^{2}H]-3-deoxy-D-*manno*-octulosonate 8-phosphate, (3*S*)-[3-^{2}H]-3-deoxy-D-*altro*-octulosonate 8-phosphate, and (3*S*)-[3-^{2}H]-3,5-dideoxy-D-*altro*-octulosonate 8-phosphate, respectively, whereas incubation with (*E*)-[3-^{2}H]-PEP gives the corresponding (3*R*)-monosaccharides. These results are in complete agreement with the observed facial selectivity of DAH 7-P synthase for its normal substrates D-erythrose 4-phosphate and PEP and provide direct evidence that DAH 7-P synthase (phe) catalyzes the *si* face addition of the C3 of PEP to the *re* face of C1 of the phosphorylated monosaccharides tested. Products formed by DAH 7-P synthase (phe)-catalyzed condensation of (*Z*)- and (*E*)-[3-F]-PEP with E 4-P were completely characterized by ^{1}H and ^{19}F NMR analysis for the first time. Results of our studies suggest that disappearence of the double bond between C2 and C3 of PEP and formation of a bond between C3 of PEP and C1 of the phosphorylated monosaccharide tested occur in concert during the DAH 7-P synthase-catalyzed condensation reaction.

Introduction

Regio- and stereospecifically labeled substrate analogues are commonly used to probe enzyme reaction mechanisms. A number of regiospecifically labeled phospho*enol*pyruvate (PEP) analogues have been prepared and utilized to study both the stereoselectivity and the stereospecificity of several PEP-utilizing enzymes. The analogues (*Z*)- and (*E*)-[3-F]-PEP,[1−22] (*Z*)- and (*E*)-[3-methyl]-PEP,[23−39] and/or (*Z*)- and (*E*)-[3-^{2}H or 3-^{3}H]-PEP[40−50] have been used in mechanistic studies of pyruvate kinase,[1,23,24,28,36,43] PEP carboxylase,[5,35,38,43] PEP carboxy-kinase,[4,6,8,10,31,32] 5-*enol*pyruvylshikimate 3-phosphate (EPSP) synthase,[12,18] UDP-*N*-acetylglucosamine *enol*pyruvyl transferase (EPTase),[14−21,30,39] and 3-deoxy-D-*manno*-octulosonate 8-phosphate (KDO 8-P) synthase.[13,50] In the case of EPTase and EPSP synthase, stable, fluorine-containing tetrahedral intermediates of

* To whom correspondence should be addressed.

(1) Stubbe, J. A.; Kenyon, G. L. *Biochemistry* **1972**, *11*, 338−45.
(2) Blumberg, K.; Stubbe, J. *Biochim. Biophys. Acta* **1975**, *384*, 120−6.
(3) Pilch, P. F.; Somerville, R. L. *Biochemistry* **1976**, *15*, 5315−20.
(4) Hebda, C. A.; Nowak, T. *J. Biol. Chem.* **1982**, *257*, 5503−14.
(5) O'Leary, M. H. *Physiol. Veg.* **1983**, *21*, 883−88.
(6) Duffy, T. H.; Nowak, T. *Biochemistry* **1984**, *23*, 661−70.
(7) Hoving, H.; Crysell, B.; Leadlay, P. F. *Biochemistry* **1985**, *24*, 6163−9.
(8) Hwang, S. H.; Nowak, T. *Biochemistry* **1986**, *25*, 5590−5.
(9) Diaz, E.; O'Laughlin, J. T.; O'Leary, M. H. *Biochemistry* **1988**, *27*, 1336−1341.
(10) Hwang, S. H.; Nowak, T. *Arch. Biochem. Biophys.* **1989**, *269*, 646−63.
(11) Janc, J. W.; Urbauer, J. L.; O'Leary, M. H.; Cleland, W. W. *Biochemistry* **1992**, *31*, 6432−40.
(12) Walker, M. C.; Jones, C. R.; Somerville, R. L.; Sikorski, J. A. *J. Am. Chem. Soc.* **1992**, *114*, 7601−3.
(13) Kohen, A.; Berkovich, R.; Belakhov, V.; Baasov, T. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 1577−1582.
(14) Kim, D. H.; Lees, W. J.; Walsh, C. T. *J. Am. Chem. Soc.* **1994**, *116*, 6478−6479.
(15) Kim, D. H.; Lees, W. J.; Haley, T. M.; Walsh, C. T. *J. Am. Chem. Soc.* **1995**, *117*, 1494−1502.
(16) Kim, D. H.; Lees, W. J.; Walsh, C. T. *J. Am. Chem. Soc.* **1995**, *117*, 6380−6381.
(17) Li, Y.; Krekel, F.; Ramilo, C. A.; Amrhein, N.; Evans, J. N. *FEBS Lett.* **1995**, *377*, 208−12.
(18) Kim, D. H.; Tucker-Kellogg, G. W.; Lees, W. J.; Walsh, C. T. *Biochemistry* **1996**, *35*, 5435−40.
(19) Salleh, H. M.; Dotson, G. D.; Woodard, R. W. *Bioorg. Med. Chem. Lett.* **1996**, *6*, 133−138.
(20) Kim, D. H.; Lees, W. J.; Kempsell, K. E.; Lane, W. S.; Duncan, K.; Walsh, C. T. *Biochemistry* **1996**, *35*, 4923−8.
(21) Skarzynski, T.; Kim, D. H.; Lees, W. J.; Walsh, C. T.; Duncan, K. *Biochemistry* **1998**, *37*, 2572−7.
(22) Duggan, P. J.; Parker, E.; Coggins, J.; Abell, C. *Bioorg. Med. Chem. Lett.* **1995**, *5*, 2347−2352.
(23) Bondinell, W. E.; Sprinson, D. B. *Biochem. Biophys. Res. Commun.* **1970**, *40*, 1464−7.
(24) Stubbe, J. A.; Kenyon, G. L. *Biochemistry* **1971**, *10*, 2669−77.
(25) James, T. L.; Reuben, J.; Cohn, M. *J. Biol. Chem.* **1973**, *248*, 6443−9.
(26) Appelbaum, J.; Stubbe, J. A. *Biochemistry* **1975**, *14*, 3908−12.
(27) Zemell, R. I.; Anwar, R. A. *J. Biol. Chem.* **1975**, *250*, 4959−64.
(28) Adlersberg, M.; Dayan, J.; Bondinell, W. E.; Sprinson, D. B. *Biochemistry* **1977**, *16*, 4382−7.
(29) Jursinic, S. B.; Robinson, J. L. *Biochim. Biophys. Acta* **1978**, *523*, 358−67.
(30) Anwar, R. A.; Vlaovic, M. *Biochim. Biophys. Acta* **1980**, *616*, 389−94.
(31) Duffy, T. H.; Markovitz, P. J.; Chuang, D. T.; Utter, M. F.; Nowak, T. *Proc. Natl. Acad. Sci. U.S.A.* **1981**, *78*, 6680−3.
(32) Duffy, T. H.; Saz, H. J.; Nowak, T. *Biochemistry* **1982**, *21*, 132−9.
(33) Hoving, H.; Nowak, T.; Robillard, G. T. *Biochemistry* **1983**, *22*, 2832−8.

10.1021/jo991529l CCC: $19.00
Published on Web 08/22/2000

the enzyme-catalyzed reactions have been isolated and characterized using [3-F]-PEP as a pseudo-substrate.[12,14−21] The breakdown of the fluorinated tetrahedral intermediates into the final products is not observed due to the presence of the fluorine atom.[51] The PEP analogues (*Z*)- and (*E*)-[3-F]-PEP as well as (*Z*)- and (*E*)-[3-^{2}H]-PEP have been incubated with KDO 8-P synthase in the presence of arabinose 5-phosphate (A 5-P), and the labeled KDO 8-Ps obtained have been isolated and characterized by NMR analysis.[13,50] The two regioisomers of [3-F]-PEP serve as true substrates for KDO 8-P synthase, albeit at different rates.[13]

3-Deoxy-D-*arabino*-heptulosonate 7-phosphate (DAH 7-P) synthase, a pivotal enzyme in the biosynthesis of aromatic amino acids, catalyzes an aldol-type condensation reaction similar to that catalyzed by KDO 8-P synthase except between D-erythrose 4-phosphate (E 4-P) and PEP. Floss and co-workers[41,42] have utilized (*Z*)- and (*E*)-[3-^{3}H]-PEP, derived via a series of enzymatic transformations of [1-^{3}H] glucose and [1-^{3}H] mannose, respectively, to study the stereochemical course of DAH 7-P synthase. The enzymatically obtained [3-^{3}H]-DAH 7-Ps were converted to [3-^{3}H]-malates via a series of enzymatic and chemical reactions. The stereochemistry of the labeled malates was determined utilizing fumarase [EC 4.2.1.2]. On the basis of the known stereochemistry of the enzymatic and chemical reactions involved in both the synthesis of the [3-^{3}H]-PEPs, as well as the degradation of DAH 7-P, a *si* face attack of the C3 of PEP on the C1 *re* face of E 4-P was postulated. Pilch and Somerville[3] reported an apparent K_m of (*Z*)-[3-F]-PEP for DAH 7-P synthase less than twice that of PEP (65 μM versus 38 μM) and an apparent V_{max} 1% that of PEP. Although (*E*)-[3-F]-PEP was completely converted to [3-F]-DAH 7-P, the kinetics of the reaction were nonlinear. The authors report that they isolated and obtained the NMR spectra for the condensation product (3*S*)-[3-F]-DAH 7-P; however, no data or spectra were presented. Their assignment of the stereochemistry was based soley on the study described above by Floss and not on data from their own study.

Later, Duggan and co-workers[22] utilized (*Z*)- and (*E*)-[3-F]-PEP (88:12) and E 4-P as substrates for DAH 7-P synthase in the first step of a one-pot multistep enzymatic synthesis of [6-F]-shikimic acid. In this study each enzyme involved in the biosynthetic pathway was added successively to the reaction mixture and the reaction progress monitored by ^{19}F NMR. Based on the stereochemistry of the final product, [6-F]-shikimic acid, and the coupling constant values obtained from the mixture of 3*S* and 3*R* [3-F]-DAH 7-Ps, the previously reported stereochemical course of the DAH 7-P synthase catalyzed reaction appears to be correct, although the individual [3-F]-DAH 7-Ps were neither isolated nor completely characterized. Sheflyan et al. have recently shown that the five-carbon monosaccharides, A 5-P, 2-deoxy-D-ribose 5-phosphate (dR 5-P), and D-ribose 5-phosphate (R 5-P) are substrates for DAH 7-P synthase (phe).[52] Since binding of these alternate substrates (longer by one carbon atom) could potentially perturb the architecture of the enzyme active site and hence may alter the binding of PEP and/or alter the relative orientations of the two substrates in the active site, we were interested in studying the stereochemical course of the reaction using these alternate monosaccharide substrates as potential mechanistic probes.

In an effort to directly compare as well as to confirm the results from previous studies of DAH 7-P synthase to those for KDO 8-P synthase with various PEP analogues, we have chemically synthesized (*Z*)- and (*E*)-[3-F]-PEP as well as (*Z*)- and (*E*)-[3-^{2}H]-PEP and utilized them to investigate the stereoselectivity of the reaction catalyzed by DAH 7-P synthase with its natural substrate E 4-P as well as with several five-carbon analogues. The KDO8P synthase condensation reaction with A 5-P and both [3-F]-PEPs have also been repeated. The studies involved the isolation and NMR characterization of the isotopically labeled analogues obtained from the DAH 7-P synthase-catalyzed condensation reactions.

Results and Discussion

The initial stereochemical studies of DAH 7-P synthase focused on the condensation of (*Z*)-[3-F]-PEP with E 4-P. The purified reaction product obtained from incubation of (*Z*)-[3-F]-PEP and E 4-P with DAH 7-P synthase was characterized by both ^{1}H and ^{19}F NMR spectroscopy, to completely characterize, for the first time, the product. The ^{19}F NMR spectrum (Figure 1) shows one major and two minor signals (each one as a doublet of a doublet). The two-dimensional NMR spectrum (^{1}H−^{19}F HETCOR) recorded for the product shows a correlation between the major fluorine signal at $\delta = -130.2$ and the proton signal at $\delta = 4.8$ as well as a correlation between the same fluorine signal and the proton signal at $\delta = 3.9$ (data not shown). The ^{1}H NMR spectrum of the product confirms that the fluorine on C3 is coupled to the C3 proton at $\delta = 4.8$ ($J = 49.1$ Hz) as well as to the C4 proton at $\delta = 3.9$ ($J = 30.2$ Hz) (Table 1). Incubation of PEP and E 4-P, A 5-P, R 5-P, or dR 5-P with DAH 7-P synthase results in a phosphorylated monosaccharide elongated by three carbons in which the orientation of the C4 proton is axial, indicating that the stereochemistry at C4 of all the

(34) Izui, K.; Matsuda, Y.; Kameshita, I.; Katsuki, H.; Woods, A. E. *J. Biochem. (Tokyo)* **1983**, *94*, 1789−95.
(35) Fujita, N.; Izui, K.; Nishino, T.; Katsuki, H. *Biochemistry* **1984**, *23*, 1774−9.
(36) Dougherty, T. M.; Cleland, W. W. *Biochemistry* **1985**, *24*, 5875−80.
(37) Duffy, T. H.; Nowak, T. *Biochemistry* **1985**, *24*, 1152−60.
(38) Gonzalez, D. H.; Andreo, C. S. *Eur. J. Biochem.* **1988**, *173*, 339−43.
(39) Lees, W. J.; Walsh, C. T. *J. Am. Chem. Soc.* **1995**, *117*, 7329−7337.
(40) Levin, J. G.; Sprinson, D. B. *J. Biol. Chem.* **1964**, *239*, 1142−50.
(41) Onderka, D. K.; Floss, H. G. *Biochem. Biophys. Res. Commun.* **1969**, *35*, 801−4.
(42) Onderka, D. K.; Floss, H. G. *J. Am. Chem. Soc.* **1969**, *91*, 5894−6.
(43) Rose, I. A. *J. Biol. Chem.* **1970**, *245*, 6052−6.
(44) Bondinell, W. E.; Vnek, J.; Knowles, P. F.; Sprecher, M.; Sprinson, D. B. *J. Biol. Chem.* **1971**, *246*, 6191−6.
(45) Floss, H. G.; Onderka, D. K.; Carroll, M. *J. Biol. Chem.* **1972**, *247*, 736−44.
(46) Rose, I. A. *Methods Enzymol.* **1975**, *41*, 110−5.
(47) Grimshaw, C. E.; Sogo, S. G.; Knowles, J. R. *J. Biol. Chem.* **1982**, *257*, 596−8.
(48) Bartlett, P. A.; Chouinard, P. M. *J. Org. Chem.* **1983**, *48*, 3854−55.
(49) Gore, M. P.; Nanjappan, P.; Hoops, G. C.; Woodard, R. W. *J. Org. Chem.* **1990**, *55*, 758−760.
(50) Dotson, G. D.; Nanjappan, P.; Reily, M. D.; Woodard, R. W. *Biochemistry* **1993**, *32*, 12392−7.
(51) Walsh, C. T.; Benson, T. E.; Kim, D. H.; Lee, W. J. *Curr. Biol.* **1996**, *3*, 83−91.

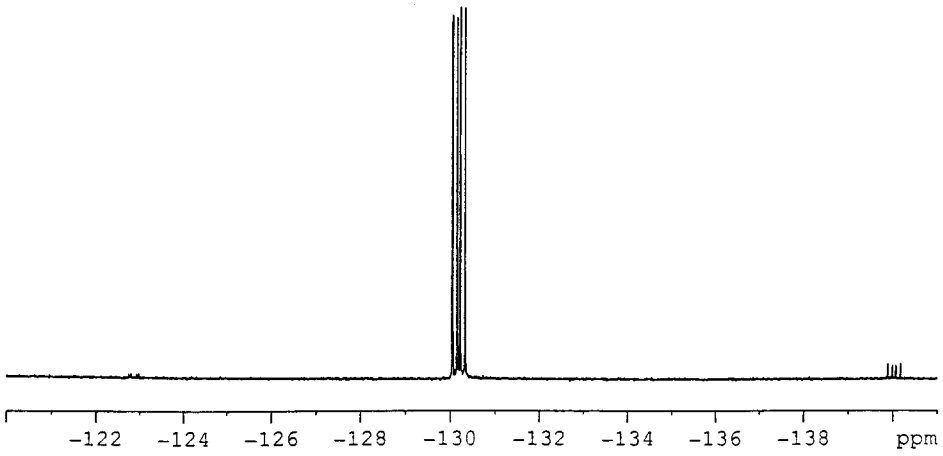


Figure 1. The ^{19}F NMR spectrum of (3*S*)-[3-F]-DAH 7-P obtained from incubation of (*Z*)-[3-F]-PEP with E 4-P in the presence of DAH 7-P synthase.

Table 1. ^{1}H NMR Data for (3*S*)-[3-F]-DAH 7-P]

C no.	δ, ppm (multiplicity)	*J* values, Hz
3	4.8 (dd)	2.5 ($C3-H_{eq}-C4-H_{ax}$), 49.1 ($C3-H_{eq}-C3-F_{ax}$)
4	3.9 (ddd)	2.5 ($C4-H_{ax}-C3-H_{eq}$), 9.8 ($C4-H_{ax}-C5-H_{ax}$), 30.2 ($C4-H_{ax}-C3-F_{ax}$)
5	3.7 (dd)	9.8 ($C5-H_{ax}-C4-H_{ax}$), 9.8 ($C5-H_{ax}-C6-H_{ax}$)
6	3.8 (ddd)	9.8 ($C6-H_{ax}-C5-H_{ax}$)
7	4.1 (mult)	nd

[a] nd = not determined.

products is *R*.[52] The coupling constant values of $J = 2.5$ Hz between the C3 and C4 protons and $J = 9.8$ Hz between C4 and C5 protons observed here is consistent with an axial orientation of C4 proton and an equatorial orientation of C3 proton in the product. The three-bond coupling constant value of $J = 30.2$ Hz observed between C4 proton (axial) and the C3 fluorine indicates a trans-diaxial orientation of both the atoms in the product.[53,54] The major fluorine signal at $\delta = -130.2$ is thus assigned to the α-pyranose form of (3*S*)-[3-F]-DAH 7-P. The minor signal at $\delta = -140.4$ with similar coupling constant values of $J = 51.3$ Hz between the C3 fluorine and the C3 proton and $J = 29$ Hz between the C3 fluorine and the C4 proton (axial) indicates a similar axial orientation of the C3 fluorine and hence is assigned to the minor β-pyranose form of (3*S*)-[3-F]-DAH 7-P. Therefore, incubation of (*Z*)-[3-F]-PEP and E 4-P in the presence of DAH 7-P synthase forms (3*S*)-[3-F]-DAH 7-P. The other minor signal at $\delta = -123.4$ shows a coupling constant value of $J = 50$ Hz between the C3 fluorine and the C3 proton and $J = 12.9$ Hz between the C3 fluorine and the C4 proton (axial). The smaller three-bond coupling constant value of $J = 12.9$ Hz between the axial proton on C4 and the fluorine on C3 indicates a gauche arrangement of the two atoms, and thus the C3 fluorine in this product is equatorial. The minor signal at $\delta = -123.4$ is assigned to the minor product (3*R*)-[3-F]-DAH 7-P obtained from condensation of (*E*)-[3-F]-PEP (8% contaminant in (*Z*)-[3-F]-PEP) and E 4-P.

To determine if DAH 7-P synthase demonstrates a preference for one regioisomer over the other as a substrate as well as to further confirm the above stereochemical results, it was necessary to prepare (*E*)-[3-F]-PEP. A 60:40 mixture of (*Z*)-[3-F]-PEP and (*E*)-[3-F]-PEP was prepared by irradiating a 0.4 M solution of (*Z*)-[3-F]-PEP in a Rayonet photochemical reactor at 254 nm for 24 h.[3] The progress of the photoisomerization was monitored by ^{19}F NMR (Figure 2) over a period of 24 h. At equilibrium, the solution contained a 60:40 mixture of (*Z*:*E*)-[3-F]-PEP, as determined from the ^{19}F NMR as well as the ^{1}H NMR spectra. This mixture of regioisomers and A 5-P were incubated with *E. coli* KDO 8-P synthase, and the progress of the reaction was monitored by ^{19}F NMR. The results shown in Figure 3 confirm the earlier report by Kohen et al.[13] that KDO 8-P synthase shows a preference for the *E*-isomer over the *Z*-isomer. However, when an identical mixture of (*Z*)-[3-F]-PEP and (*E*)-[3-F]-PEP (60:40) was incubated with E 4-P in the presence of DAH 7-P synthase, both the isomers were consumed at equal rates as evidenced by ^{19}F NMR (Figure 4). The ^{19}F NMR spectrum of the DAH 7-P synthase reaction mixture shows four sets of signals (two major and two minor signals, each one as a doublet of a doublet) in the product region. The major signal at $\delta = -130.2$ and the minor signal at $\delta = -140.4$ are identical to those observed for the product obtained from the condensation of (*Z*)-[3-F]-PEP and E 4-P (shown in Figure 1) and are hence assigned to the α- and β-pyranose forms of (3*S*)-[3-F]-DAH 7-P, respectively. The other major signal at $\delta = -123.4$ is identical to that of the minor signal at $\delta = -123.4$ in Figure 1 and is assigned to the α-pyranose form of (3*R*)-[3-F]-DAH 7-P obtained from the condensation of (*E*)-[3-F]-PEP with E 4-P. The minor signal at $\delta = -125.1$ with a coupling constant values of $J = 47.8$ Hz between the fluorine on C3 and the C3 proton and $J = 15$ Hz between the C3 fluorine and the C4 proton (indicating an equatorial orientation for the C3 fluorine) is assigned to the β-pyranose form of the product (3*R*)-[3-F]-DAH 7-P. Therefore, in the case of DAH 7-P synthase-catalyzed condensation of [3-F]-PEP with E 4-P, the *Z*-isomer forms (3*S*)-[3-F]-DAH 7-P while the *E*-isomer forms (3*R*)-[3-F]-DAH 7-P. These results clearly indicate that the *si* face of PEP attacks the *re* face of E 4-P during the DAH 7-P synthase-catalyzed condensation reaction. Unlike KDO 8-P synthase, DAH 7-P synthase does NOT show any prefernce for one regioisomer over the other, at least in the case of [3-F]-PEP.

(52) Sheflyan, G. Y.; Howe, D. L.; Wilson, T. L.; Woodard, R. W. *J. Am. Chem. Soc.* **1998**, *120*, 11027−11032.
(53) Withers, S. G.; MacLennan, D. J.; Street, I. P. *Carbohydr. Res.* **1986**, *154*, 127−144.
(54) Withers, S. G.; Percival, M. D.; Street, I. P. *Carbohydr. Res.* **1989**, *187*, 43−66.

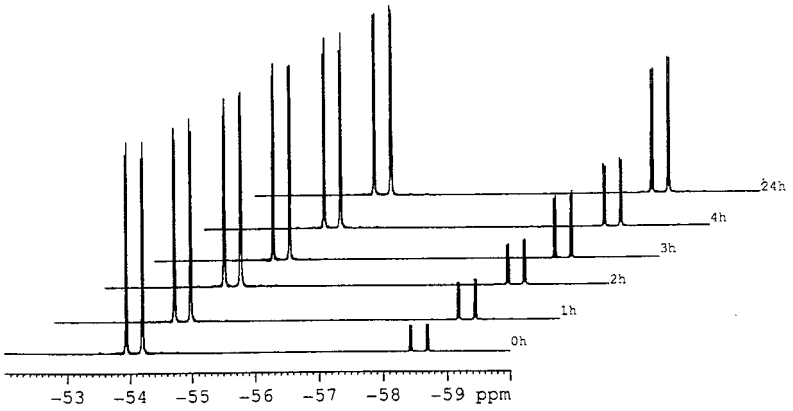


Figure 2. The ^{19}F NMR spectra of 0.4M (*Z*)-[3-F]-PEP solution at various time intervals during UV irradiation at 254 nm to form a mixture of (*Z*)- and (*E*)-[3-F]-PEP.

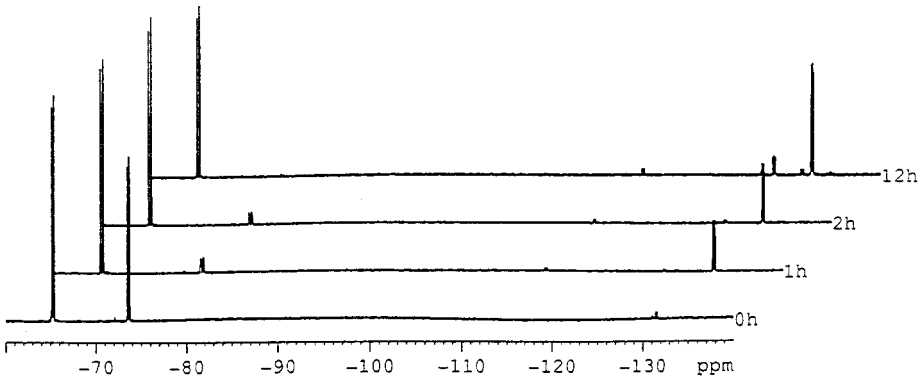


Figure 3. The ^{19}F NMR spectra, at various time intervals, of the reaction mixture containing A 5-P, a 60:40 mixture of (*Z*)-and (*E*)-[3-F]-PEP (−65 ppm and −73 ppm, respectively) and KDO 8-P synthase in 0.2 M Tris-acetate buffer (pH 7.5) and 10% D_2O at 37 °C. Chemical shifts of (*Z*)- and (*E*)-[3-F]-PEP are different from those in Figure 2 due to the prsence of the buffers and the difference in the pH.

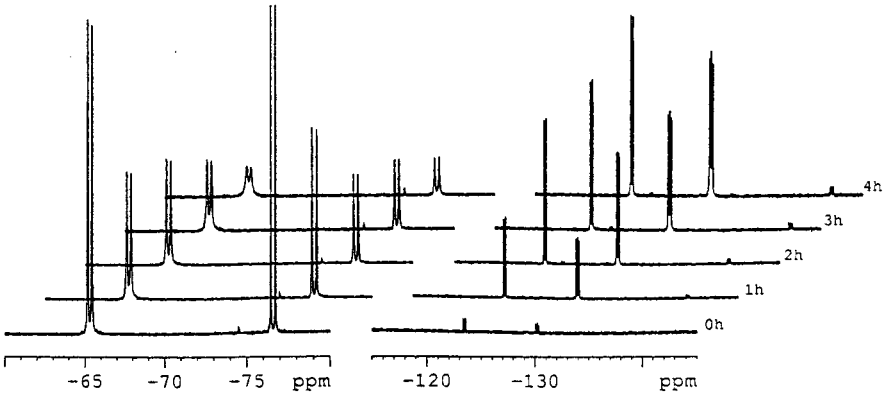
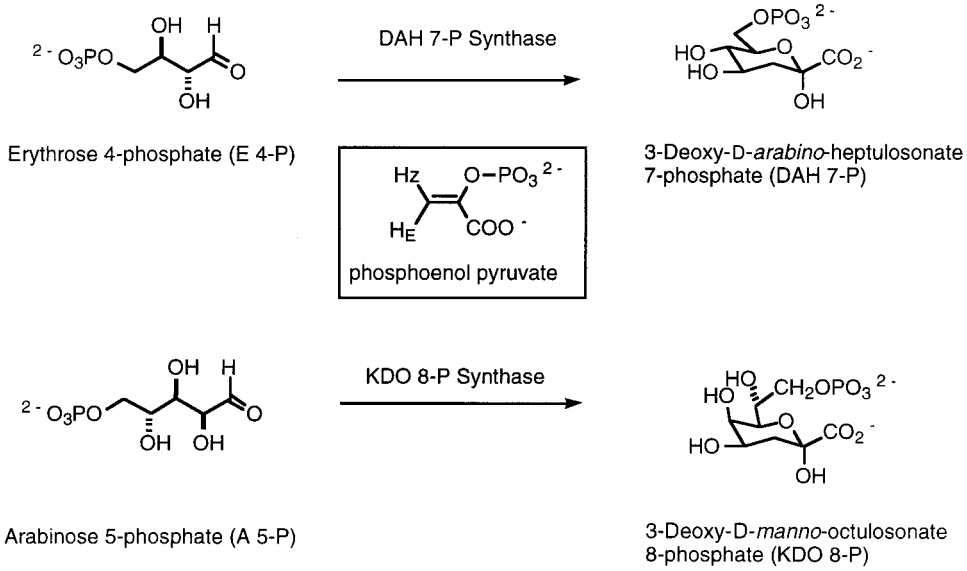


Figure 4. The ^{19}F NMR spectra of the reaction mixture containing E 4-P, a 60:40 mixture of (*Z*)- and (*E*)-[3-F]-PEP and manganese(II) chloride in BTP buffer (pH 6.8) and 10% D_2O at various time intervals after addition of DAH 7-P synthase at room temperature. Chemical shifts of (*Z*)-and (*E*)-[3-F]-PEP are different from those in Figure 2 due to the prsence of the buffers and the difference in the pH.

Since there is an electronic perturbation in [3-F]-PEP, caused by the substitution of F for H, we wanted to utilize a PEP analogue that is electronically more similar to PEP. Therefore, to substantiate the results obtained from the studies of [3-F]-PEP with DAH 7-P synthase as well as to probe the stereochemistry of the enzyme-catalyzed reaction when the five-carbon alternate substrates are incubated with DAH 7-P synthase, reactions employing (*Z*)- and (*E*)-[3-^{2}H]-PEP as PEP analogues were performed. Condensation products obtained from incubation of either (*Z*)- or (*E*)-[3-^{2}H]-PEP with E 4-P (Scheme 1), A 5-P, R 5-P, and dR 5-P in separate reactions were purified and then characterized by ^{1}H NMR spectroscopy. Incubation of (*Z*)-[3-^{2}H]-PEP with E 4-P, A 5-P, and dR 5-P forms (3*S*)-[3-^{2}H]-DAH 7-P, (3*S*)-[3-^{2}H]-KDO 8-P and (3*S*)-[3-^{2}H]-3,5-dideoxy-D-*altro(manno)*-octulosonate 8-phosphate, respectively, whereas incubation of (*E*)-[3-^{2}H]-PEP with E 4-P, A 5-P, and dR 5-P forms (3*R*)-[3-^{2}H]-DAH 7-P, (3*R*)-[3-^{2}H]-KDO 8-P and (3*R*)-[3-^{2}H]-3,5-dideoxy-D-*altro*(*manno*)-octulosonate 8-phosphate, respectively. The NMR analysis of the products obtained from the incubation of (*Z*)- and (*E*)-[3-^{2}H]-PEP with R 5-P in the presence

Scheme 1



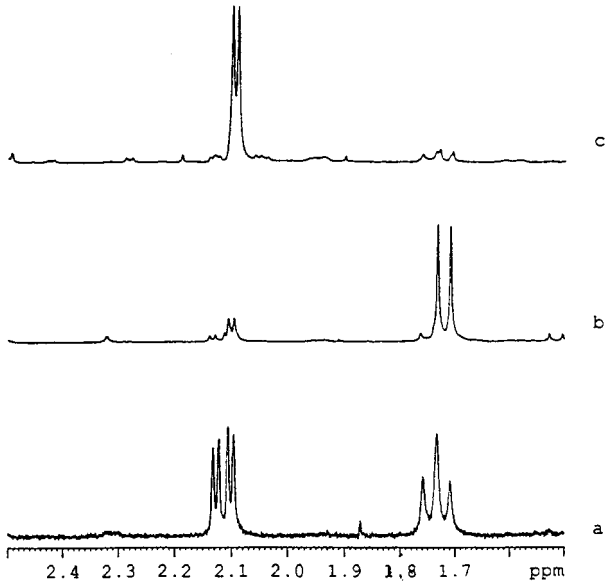


Figure 5. The partial ^{1}H NMR spectra (signals for the C3 protons) of the products obtained from DAH 7-P synthase-catalyzed condensation reactions between R 5-P and (a) PEP, (b) (*E*)-[3-^{2}H]-PEP, and (c) (*Z*)-[3-^{2}H]-PEP.

of DAH7P synthase is presented below as a typical example of product characterization.

The regions of the ^{1}H NMR spectra containing the signals for C3 protons of the C3 deuterated monosaccharides obtained from incubation of [3-^{2}H]-PEPs with R 5-P are shown in Figure 5. The partial ^{1}H NMR spectrum of the protio-compound (D-*altro*-octulosonate 8-phosphate) is also shown in Figure 5. In the case of D-*altro*-octulosonate 8-phosphate (Figure 5a), the C3 axial proton signal appears as a triplet at $\delta = 1.72$, while the C3 equatorial proton signal appears as a doublet of a doublet at $\delta = 2.1$.[52] The ^{1}H NMR spectra recorded for the product obtained from incubation of (*E*)-[3-^{2}H]-PEP with R 5-P (Figure 5b) shows the disappearance of the signal for the C3 equatorial proton and the appearance of a simple doublet (with a coupling constant values of $J = 11.5$ Hz between C3 and C4 protons) that would correspond to the signal for C3 axial proton. A coupling constant value of $J = 11.5$ Hz between the C3 proton and C4 axial proton suggests a trans-diaxial relationship between the two protons, consistent with incorporation of deuterium into the C3 equatorial position, indicating an *R* configuration at C3 for the product. The ^{1}H NMR spectrum recorded for the product obtained from incubation of (*Z*)-[3-^{2}H]-PEP with R 5-P (Figure 5c) shows the disappearance of the signal for the C3 axial proton and the presence of a simple doublet (with a coupling constant value of $J = 5$ Hz between C3 and C4 protons) which would correspond to the signal for C3 equatorial proton. A coupling constant value of 5 Hz between the C4-axial proton and the proton on C3, indicative of a gauche orientation between the two protons, suggests that the C3 proton is in an equatorial orientation, consistent with incorporation of deuterium into the C3 axial position in the product, indicating an *S* configuration at C3 of the product. Therefore, the condensation of (*Z*)-[3-^{2}H]-PEP with R 5-P forms (3*S*)-[3-^{2}H]-D-*altro*-octulosonate 8-phosphate while the condensation of (*E*)-[3-^{2}H]-PEP with R 5-P forms (3*R*)-[3-^{2}H]-D-*altro*-octulosonate 8-phosphate.

In this paper, we report the isolation, purification, and characterization of the products formed from the DAH 7-P synthase (phe)-catalyzed condensation of various stereospecifically labeled PEP analogues and four different phosphorylated monosaccharides. Characterization of these products confirms that the DAH 7-P synthase-catalyzed condensation of PEP analogues with E 4-P analogues proceeds via the same facial selectivity and hence follows the same stereochemical course, irrespective of the substrate. These results further confirm that the relative orientations of both the PEP and E 4-P analogues in the enzyme active site and that the reaction mechanism for alternate substrates is the same as for the normal substrates, E 4-P and PEP. These results provide direct evidence for the *si* face addition of PEP to the *re* face of the aldehyde group of the respective monosaccharide substrate during the DAH 7-P synthase-catalyzed condensation reaction, which is the same facial selectivity observed for KDO 8-P synthase. This adds to the list of PEP-utilizing enzymes that have been shown to be *si*-face specific, suggesting that there may be a common PEP binding motif for all these enzymes. Our observations indicate that both KDO 8-P synthase and

DAH 7-P synthase may have similar binding sites for both the substrates and some mechanistic commonalties. The results suggest that if a "carbanion-like species" is formed at C3 of PEP that attacks the electrophilic C1 aldehyde carbon of the phosphorylated monosaccharide, as hypothesized earlier by Floss and co-workers for DAH 7-P synthase and by our group for KDO 8-P synthase, that the lifetime of this anionic species must be shorter than the time required for the rotation of a methyl group ($<10^{-10}$ s). Although it is possible that the free rotation of the carbanion is significantly restricted in the enzyme active site, it seems rather unlikely suggesting that disappearance of the double bond between C2 and C3 of PEP and formation of a bond between C3 of PEP and C1 of the phosphorylated monosaccharide occur in concert. Finally, DAH 7-P synthase utilizes both the regioisomers of [3-F]-PEP at equal rates, unlike KDO 8-P synthase which exhibits a preference for (*E*)-[3-F]-PEP over (*Z*)-[3-F]-PEP.

Experimental Section

General. ^{1}H NMR spectra were acquired on a Bruker Avance DRX 500 (operating at 500.132 MHz for ^{1}H) with a 5 mm multinuclear inverse gradient probe using the water suppression program, WATERGATE Gradient Suppression.[57] ^{19}F NMR spectra were recorded on a Bruker Avance 300 (operating at 282.36 MHz for ^{19}F) and referenced externally to trifluoroacetic acid ($\delta = 0$). E 4-P, A 5-P, R 5-P, dR 5-P, manganese (II) chloride, and trifluoroacetic anhydride were obtained from Sigma Chemical Co. Bromine and *N*-bromosuccinimde were purchased from Fisher Scientific. Cyclohexylamine, diethyl oxalate, ethyl bromopyruvate, ethyl fluoroacetate, trimethyl phosphite, and tetrakis(triphenylphosphine)palladium(0) were purchased from Aldrich Chemical Co. Recombinant DAH 7-P synthase (phenylalanine-sensitive) and KDO 8-P synthase were isolated and purified as previously reported.[52]

(*Z*)-[3-F]-PEP Cyclohexylammonium Salt. The title compound was prepared by the procedure originally reported by Bergmann and Shahak[58] and modified by Stubbe and Kenyon.[1] In brief, diethyl oxalate was treated with sodium ethyl fluoroacetate to give the sodium enolate of diethyl fluorooxaloacetate which was directly brominated to give diethyl bromofluorooxaloacetate. After purification by distillation, the diethyl bromofluorooxaloacetate (86 °C at 0.7 mmHg) was heated at 80 °C for 8 h in concentrated HCl. Removal of the excess acid in vacuo followed by distillation gave pure bromofluoropyruvic acid which solidified upon cooling. The bromofluoropyruvic acid was converted into (*Z*)-3-F-2-[(dimethoxyphosphinyl)oxy]propenoic acid under standard Perkow-type reaction conditions (reaction with trimethyl phosphite at 0 °C followed by slow warming to room temperature over a 5 h period). This dimethyl ester was hydrolyzed by dissolving it in water and stirring at room temperature for 8 h. One equivalent of cyclohexylamine was then added, and the solution was immediately lyophilized to give the monocyclohexylammonium (CHA) salt of (*Z*)-[3-F]-PEP (92% *Z*-isomer and 8% *E*-isomer as determined by ^{1}H and ^{19}F NMR). NOTE: the addition of cyclohexylamine to the aqueous solution of the diester followed by stirring at room temperature, as in the standard procedure for the preparation of unlabeled PEP,[59] leads to total decomposition.

UV Isomerization of (*Z*)-[3-F]-PEP and (*E*)-[3-F]-PEP CHA Salts. A 60:40 mixture of (*Z*)-[3-F]-PEP and (*E*)-[3-F]-PEP was obtained by irradiating a 0.4 M solution of (*Z*)-[3-F]-PEP in D_2O (0.5 mL). The solution in a 5 mm quartz NMR tube (WILMAD) was irradiated at 254 nm at room temperature, in a Rayonet photochemical reactor for 24 h.[3] The conversion of (*Z*)-[3-F]-PEP to (*E*)-[3-F]-PEP was monitored by ^{19}F NMR. At equilibrium, the solution contained a 60:40 mixture of (*Z*)-[3-F]-PEP and (*E*)-[3-F]-PEP, as evidenced by ^{19}F NMR and ^{1}H NMR.

(*Z*)-[3-^{2}H]-PEP and (*E*)-[3-^{2}H]-PEP CHA salts. (*Z*)-[3-^{2}H]-PEP was prepared as previously reported.[49]

(*E*)-[3-^{2}H]-PEP was synthesized by the method reported by Dotson et al.[50] Ethyl bromopyruvate was brominated using *N*-bromosuccinimide to give ethyl 3,3-dibromopyruvate, which upon treatment with trimethyl phosphite under standard Perkow-type reaction conditions gave a 72:28 mixture of ethyl (*Z*)-3-bromo-2-[(dimethoxyphosphinyl)oxy]propenoate and ethyl (*E*)-3-bromo-2-[(dimethoxy-phosphinyl)oxy]propenoate. This mixture of *Z*- and *E*-isomers was treated with tetrakis-(triphenylphosphine)palladium(0) (1 equiv with respect to the *E*-isomer) to give an 80:20 mixture of (*E*)- and (*Z*)-[2-((dimethoxyphosphinyl)oxy)-3-ethoxy-3-oxo-1-propenyl]bromo-bis(triphenylphosphine)palladium. The *E*-vinyl palladium complex was separated from the *Z*-vinyl palladium complex by flash chromatography on silica gel 60 (230−400 mesh, E. Merck) using a stepwise gradient of 0, 1, 2, and 3% methanol in dichloromethane.[50]

The purified *E*-vinyl palladium complex was treated with a mixture of trifluoroacetic acid-D (99.5 atom % D, Aldrich) and trifluoroacetic anhydride, under anhydrous conditions to give ethyl (*E*)-3-deuterio-2-[(dimethoxyphosphinyl)oxy]propenoate, which was hydrolyzed using 1 N KOH and immediately loaded onto a Biorex-70 (BioRad) cation exchange column (1 × 5 cm, in the proton form). Cyclohexylamine (1 equiv with respect to the *E*-vinyl palladium complex) was added to the eluent from the column, and the mixture was lyophilized to give the CHA salt of (*E*)-[3-^{2}H]-PEP.

General Enzymatic Synthesis of [3-^{2}H]-DAH 7-Ps and [3-^{2}H]-KDO 8-P Analogues. To a Chemtube (BioRad, 12 × 75 mm), containing a solution of 1,3-bis[tris(hydroxymethyl)-methylamino]propane (BTP) (42.35 mg, 0.15 mmol) and manganese(II) chloride (0.3 mg, 1.5 μmol) in water, was added 13 mg of one of the following monosaccharides, E 4-P (0.044 mmol), A 5-P (0.056 mmol), R 5-P (0.056 mmol), or dR 5-P (0.06 mmol) and 12 mg of either (*Z*)- or (*E*)-[3-^{2}H]-PEP CHA salt (0.044 mmol), and the pH was adjusted to 6.8 using 1 N NaOH. DAH 7-P synthase (3 mg, 79 nmol) was then added to initiate the reaction, and the final volume of the reaction mixtures were made up to 2 mL. Reaction mixtures were incubated at 37 °C for 2 h. The enzymatic reactions were quenched by adding 0.5 mL of 10% trichloroacetic acid (TCA), vortexed for 30 s, and finally centrifuged for 30 min (1500*g*) to remove the precipitated protein. The supernatants were loaded onto 5 mL Econo-Pac HighQ (BioRad) anion-exchange columns (chloride form), preequilibrated with water. The columns were washed with 30 mL of water at a flow rate of 1 mL/min. The phosphorylated monosaccharides were eluted from the column using a linear gradient of 0 to 0.5 M LiCl solution over a period of 1 h. Fractions containing 3-deuterated DAH 7-P and KDO 8-P analogues, as identified by the thiobarbituric acid assay,[60,61] were pooled and lyophilized.

Enzymatic Synthesis of (3*S*)-[3-F]-DAH 7-P and (3*R*)-[3-F]-DAH 7-P. (*Z*)-[3-F]-PEP or a mixture of (*Z*)- and (*E*)-[3-F]-PEP (60:40) (15 mg, 0.052 mmol) was dissolved in 100 μL of 0.5 M BTP (pH = 11.0) in a 1.5 mL microcentrifuge tube. To these solutions was added 15 mg of E 4-P (0.056 mmol), and the pH was adjusted to 6.8 using 0.5 M BTP solution (pH = 11.0). After the addition of 30 μL of 50 mM manganese(II) chloride (1.5 μmol) solution, 50 μL of D_2O was added for deuterium lock. The reaction mixtures were transferred to

(55) Hansen, P. E. In *Annual Reports on NMR Spectroscopy*; Webb, G. A., Ed.; Academic Press: London, 1983; Vol. 15, pp 118−119.
(56) Hansen, P. E. In *Progress in NMR Spectroscopy*; Pergamon−Elsevier Science Ltd.: Oxford, U.K., 1988; Vol. 20, pp 207−255.
(57) Piotto, M.; Saudek, V.; Sklenar, V. *J. Biomol. NMR* **1992**, *2*, 661−665.
(58) Bergmann, E. D.; Shahak, I. *J. Chem. Soc.* **1960**, 462−463.
(59) Clark, V. M.; Kirby, A. J. *Biochim. Biophys. Acta* **1963**, *78*, 732.
(60) Aminoff, D. *Biochem. J.* **1961**, *81*, 384−392.
(61) Ray, P. H. *J. Bacteriol.* **1980**, *141*, 635−44.

NMR tubes. DAH 7-P synthase (3 mg, 79 nmol) was added to the NMR tubes to initiate the reactions, and the final volumes were adjusted to 500 μL. The reaction mixtures were incubated at room temperature, and the reaction progress was followed by ^{19}F NMR. After 4 h, the reactions were quenched by the addition of 500 μL of 10% TCA. [3-F]-DAH 7-P was purified as described above for [3-^{2}H]-DAH 7-P. Fractions containing [3-F]-DAH 7-P, as identified by ^{19}F NMR, were pooled and lyophilized.

Acknowledgment. This investigation was supported by U. S. Public Health Service Grant GM 53069. We thank M. Birck, D. Howe, and Drs. H. Duewel and W. Taylor for their critical reading and discussion of the manuscript. The contents of this paper are solely the responsibility of the authors and do not necessarily reflect the official views of NIGMS.

Supporting Information Available: The region of ^{1}H NMR spectra containing the signals for C3 protons of (3*R*)-[3-^{2}H]-DAH 7-P, (3*S*)-[3-^{2}H]- DAH 7-P, (3*R*)-[3-^{2}H]-3,5-dideoxy-D-*altro*(*manno*)-octulosonate 8-phosphate, and (3*S*)-[3-^{2}H]-3,5-dideoxy-D-*altro*(*manno*)-octulosonate 8-phosphate. This material is available free of charge via the Internet at http://pubs.acs.org.

JO991529L