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3-Deoxy-3-fluoropyridoxamine 5′-Phosphate: Synthesis and Chemical and Biological Properties of a Coenzyme B_6 Analog

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Abstract: The C-3 deoxygenation of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose is the key step in the biosynthesis of ascarylose which is a 3,6-dideoxy sugar found in the lipopolysaccharide of *Yersinia pseudotuberculosis*. This transformation, achieved by the catalysis of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (**E$_1$**) and CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase reductase (**E$_3$**), is initiated by a reversible dehydration followed by a stepwise electron transfer from NADH to reduce the resulting glucoseen−PMP adduct. An organic radical intermediate has been detected by EPR during **E$_1$**−**E$_3$** catalysis, and its characteristics are consistent with a phenoxyl radical. Its formation has been hypothesized to involve a tautomerization of the glucoseen−PMP intermediate to a PMP−quinone methide species, which then serves as the electron acceptor during the reduction. In order to gain further experimental evidence supporting this proposed mechanism, the title compound (F-PMP) was synthesized and tested for its competence as a cofactor for the **E$_1$**−**E$_3$** reaction. Upon incubation with F-PMP, no C-3 deoxysugar product could be detected in the mixture. This result initially appeared to support the prediction that the 3-F substituent would prevent the tautomerization and thus inhibit the subsequent reduction. However, further analysis showed that the catalysis was actually arrested at the dehydration step since no ^{18}O was incorporated at C-3 of the recovered substrate when the reaction was conducted in [^{18}O]H_2O, and no tritium was released when [4-^{3}H]F-PMP replaced PMP in the incubation. Interestingly, the pK_a of the ring nitrogen (N-1) of F-PMP was found to be 2.91, a value drastically altered from the 8.74 of PMP itself. Since the catalytic role of B_6 coenzymes is to act as an electron sink, storing the electrons that are later used for the cleavage and/or formation of covalent bonds, protonation at N-1 is clearly essential, as it allows the formation of a salt bridge/hydrogen bond with an active site aspartate residue and also enhances the electron-withdrawing capability of the pyridine ring. Because F-PMP is not expected to exist in the pyridinium form at neutral pH, its ability to promote 4′-H abstraction is hampered since the resulting anion cannot be delocalized by the cofactor. Although incubation of **E$_1$**−**E$_3$** with this new coenzyme B_6 analog fails to provide additional support for the mechanism of this unique deoxygenation process, the results reported herein, along with those deduced from studies of other 3-substituted PLP derivatives, illustrate the importance of having an intact 3-OH group of the coenzyme in PLP/PMP dependent catalysis.

Ascarylose (**1**, its CDP derivative), a 3,6-dideoxyhexose found in the lipopolysaccharide of *Yersinia pseudotuberculosis*,[1] is biosynthesized via a series of enzymatic steps beginning with CDP-D-glucose.[2] The deoxygenation at C-3 of CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose (**2**) is an intriguing step in this biosynthetic pathway and has been shown to be a novel C−O bond cleavage event.[3] This transformation is catalyzed by two enzymes: CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase (**E$_1$**),[4] a PMP/[2Fe-2S]-containing homodimeric enzyme,[5] and CDP-6-deoxy-L-*threo*-D-*glycero*-4-hexulose-3-dehydrase re-

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Scheme 1

ductase ($\mathbf{E_3}$),[6] a NADH dependent [2Fe-2S]-containing flavoenzyme.[7] As shown in Scheme 1, the overall reaction consists of two phases. The PMP coenzyme of $\mathbf{E_1}$ is responsible for the reversible dehydration of **2**.[3−5] The subsequent reduction, initiated by hydride transfer from NADH to the $\mathbf{E_3}$ FAD followed by sequential electron transfer from the reduced flavin through the iron−sulfur centers of $\mathbf{E_3}$ and $\mathbf{E_1}$, converts **4** to 3,6-dideoxy-D-*glycero*-D-*glycero*-4-hexulose (**7**).[7,8] Since these [2Fe-2S] centers are one-electron carriers, the reduction of the PMP-glucoseen intermediate (**4**) must occur via a radical process.[3d]

The above catalytic model was supported by the detection of an organic radical intermediate in an EPR study.[9] Interestingly, this radical signal is of Lorentzian-type and shows no hyperfine splitting. Of the few conceivable routes to form such an organic intermediate, that in which the unpaired spin develops primarily on the C-3 oxygen of PMP is considered most reasonable. It should be noted that formation of the proposed phenoxyl radical **6** is possible only if the reaction is initiated by a tautomerization to generate a PMP−quinone methide intermediate (**5**), which is a prominent tautomer of the coenzyme B_6 Schiff base. Thus, PMP in this catalysis appears to have a dual function of being responsible for the anion-induced dehydration and also for being an integral part of the subsequent redox process. While the kinetic properties of the organic radical intermediate have recently been characterized by stop-flow spectrophotometry and rapid freeze quench EPR spectroscopy,[10] the mechanistic details of its formation remain unresolved.

In an effort to assess whether tautomerization of **4** to generate the proposed PMP-quinone methide intermediate **5** is a prerequisite for the reductive half-reaction, a new coenzyme B_6 analog, 3-deoxy-3-fluoropyridoxamine 5′-phosphate (**8**, F-PMP) was prepared. The rationale of our design of this mechanistic probe is the following: (1) since the bond length and electronegativity of a C-F group resemble those of a C−OH group,[11] this fluorinated PMP analog is expected to be nearly isosteric to normal PMP and should have little problem fitting into the $\mathbf{E_1}$ active site; (2) the replacement of the hydroxyl group with a fluorine will not interrupt the hydrogen bonding between the C-3 substituent and the imine moiety which is crucial for PLP/

(1) Gorshkova, R. P.; Korchahina, N. I.; Ovodov, Yu. S. *Eur. J. Biochem.* **1983**, *131*, 345.

(2) (a) Glaser, L.; Zarkowsky, R. In *The Enzymes*; Boyer, P., Ed.; Academic Press: New York, 1971; Vol. 5, p 465. (b) Gabriel, O. In *Carbohydrates in Solution*; Gould, R., Ed.; Advances in Chemistry Series; American Chemical Society: Washington, DC, 1973; No. 117, p 387. (c) Grisebach, H. *Adv. Carbohydr. Chem. Biochem.* **1978**, *35*, 81. (d) Gabriel, O.; VanLenten, L. In *Biochemistry of Carbohydrates II*; University Park Press: Baltimore, 1978; Vol. 16, p 1. (d) Frey, P. A. In *Pyridine Nucleotide Coenzymes*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley Interscience: New York, 1987; Part B, p 461.

(3) (a) Gonzalez-Porque, P.; Strominger, J. L. *J. Biol. Chem.* **1972**, *247,* 6748. (b) Gonzalez- Porqué, P. In *Vitamin B6 Pyridoxal Phosphate, Chemical, Biochemical, and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1986; Part B, p 392. (c) Thorson, J. S.; Lo, S. F.; Ploux, O.; He, X.; Liu, H.-w. *J. Bacteriol.* **1994**, *176*, 5483. (d) Liu, H.-w.; Thorson, J. S. *Annu. Rev. Microbiol.* **1994**, *48*, 223.

(4) (a) Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3776. (b) Weigel, T. M.; Liu, L.-d.; Liu, H.-w. *Biochemistry* **1992**, *31*, 2129. (c) Weigel, T. M.; Miller, V. P.; Liu, H.-w. *Biochemistry* **1992**, *31*, 2140.

(5) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 7539.

(6) (a) Rubenstein, P. A.; Strominger, J. L. *J. Biol. Chem.* **1974**, *249*, 3782. (b) Han, O.; Liu, H.-w. *J. Am. Chem. Soc.* **1988**, *110*, 7893. (c) Han, O.; Miller, V. P.; Liu, H.-w. *J. Biol. Chem.* **1990**, *265*, 8033.

(7) Miller, V. P.; Liu, H.-w. *J. Am. Chem. Soc.* **1992**, *114*, 1880.

(8) Gassner, G. T.; Johnson, D. A.; Liu, H.-w.; Ballou, D. P. *Biochemistry* **1996**, *35*, 7752.

(9) Thorson, J. S.; Liu, H.-w. *J. Am. Chem. Soc.* **1993**, *115*, 12177.

(10) Johnson, D. A.; Gassner G. T.; Bandarian, V.; Ruzicka, F. J.; Ballou, D. P.; Reed, G. H.; Liu, H.-w. *Biochemistry*, in press.

(11) (a) Chambers, R. D. *Fluorine in Organic Chemistry*; Wiley: New York, 1973. (b) Smart, B. In *Chemistry of Functional Groups*; *Supplement D, The Chemistry of Halides, Pseudohalides and Azides*; Patai, S., Rappoport, Z., Eds.; Wiley: New York, 1983; p 603. (c) Leibman, J. F.; Greenburg, A.; Dolbier, W. R., Jr. *Fluorine-Containing Molecules*; VCH: Deerfield Beach, FL, 1988. (d) Baker, A. W.; Shulgin, A. T. *Nature* **1965**, *206*, 712.

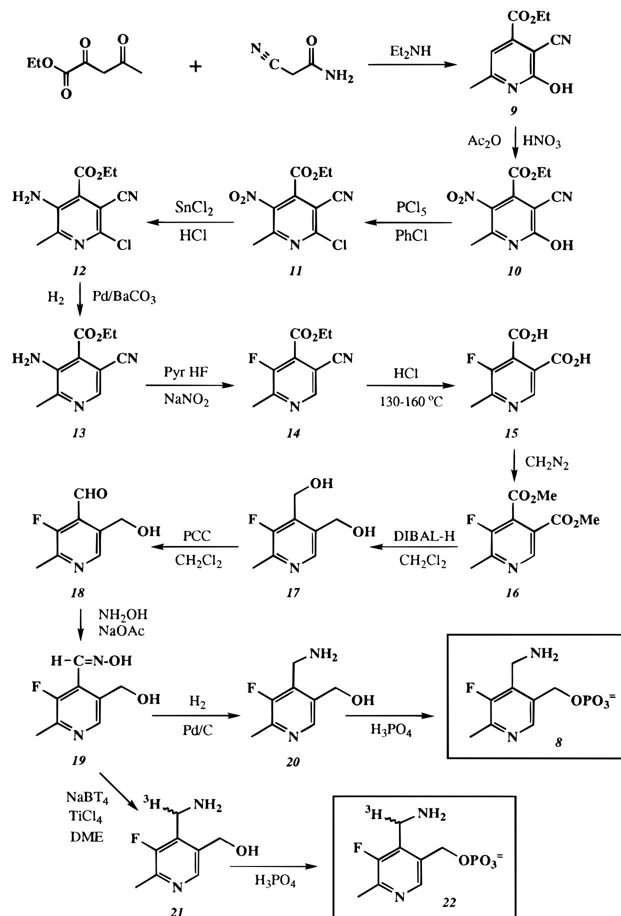
PMP catalysis;[12] (3) the electron-withdrawing capability of F-PMP should be augmented by the more electronegative 3-F substituent, and thus, F-PMP is expected to be a more effective "electron sink" able to better stabilize the C-4′ carbanion after the initial deprotonation; (4) the 3-F substituent would prevent the proposed tautomerization of the glucoseen intermediate **4** and stop the subsequent electron transfer if the $\mathbf{E_1}-\mathbf{E_3}$ catalyzed reduction follows the pathway shown in Scheme 1. The title compound, upon reconstitution with $\mathbf{E_1}$, was tested for its competence in $\mathbf{E_1}-\mathbf{E_3}$-catalyzed C-3 deoxygenation and $\mathbf{E_1}$-mediated reversible dehydration. The [4′-^{3}H]F-PMP was also prepared to determine whether the 4′-H is labile during the catalysis. Presented herein are the synthesis, characterization, and biological properties of this interesting coenzyme B_6 analog.

Results

Synthesis of F-PMP (8). To synthesize this PMP analog, it was necessary to construct a pyridoxine intermediate whose C-3 substituent could be readily manipulated. Such a requisite barred the most common approach based on Diels−Alder condensation of 4-methyloxazole with derivatives of maleic acid,[13] because the nascent product has a hydroxyl group at C-3 which cannot be easily converted to a fluoro substituent. Our initial plan to prepare F-PMP relied on a patented method of Stevens[14] to make the precursor (6-methyl-1,3-dihydro-furo⟨3,4-C⟩pyridin-7-yl)amine by coupling α-(methylimino)propionitrile with 3-ketotetrahydrofuran. However, all attempts to repeat this process failed. Although an alternative route to make this compound based on a Co-catalyzed cyclization of acetonitrile with substituted di-2-propynyl ether is available,[15] this multistep process is much more cumbersome. Thus, a new strategy, delineated in Scheme 2, was developed. The first five steps leading to the formation of ethyl 2-methyl-3-amino-5-cyanopyridine-4-carboxylate (**13**) are known reactions of Ichiba and Emoto.[16] The incorporation of a fluorine atom into C-3 of **13** was accomplished by using pyridine−HF in a dediazotization step reported by Fukuhara et al.[17] The alternative Baltz−Schiemann fluorination,[18] in which fluoroboric acid is used, is a much more cumbersome and lower yielding method. Using this improved procedure of Fukuhara et al., a 60% yield of **14** was obtained. This intermediate was then subjected to acid hydrolysis to give **15** which, after esterification, was reduced to 3-deoxy-3-fluoropyridoxine (**17**).

Selective oxidation of the C-4 hydroxymethyl group of **17** to the corresponding aldehyde proved to be the most challenging step. The 4-hydroxymethyl group of the normal pyridoxine is known to be much more reactive toward oxidation than the 5-hydroxymethyl group.[19] The regioselectivity has been ascribed to the electron-withdrawing effect of the ring nitrogen and the electron-donating ability of the 3-OH group, both of which make the C-4 benzylic position more reactive. However,

Scheme 2



the 3-OH has been substituted by an electron-withdrawing fluoro group in **17**, and consequently, the conversion of **17** to **18**, accomplished by pyridinium chlorochromate in methylene chloride at room temperature, only gave a 2 to 1 mixture of the C-4 and C-5 oxidized products. Attempts to oxidize **17** with manganese dioxide in sulfuric acid,[20] a procedure commonly used to convert pyridoxine to pyridoxal, afforded more of the overoxidized lactone and only a trace amount of the desired aldehyde. The NMR signal of the 5′-CH_2 of **17** at δ 4.77 is a singlet, and those of 4′-CH_2 at δ 4.84 and 2-Me at δ 2.52 are both doublets with $J_{H-F} = 1.6$ and 3.2 Hz, respectively. Interestingly, the peak of the aldehydic hydrogen of **18** at δ 10.13 is a doublet with a coupling constant of 2.3 Hz, and that of the hydroxymethyl moiety at δ 4.88 is only a singlet. These data clearly indicated that the aldehyde group must be in close proximity to 3-F and the major product **18** is indeed the desired C-4′ oxidized 3-deoxy-3-fluoropyridoxal.

Upon treatment with hydroxylamine hydrochloride and sodium acetate in an aqueous solution, aldehyde **18** was transformed to its oxime **19**, which was then subjected to catalytic hydrogenation followed by phosphorylation.[21] The resulting 3-deoxy-3-fluoropyridoxamine 5′-phosphate (**8**) was purified by an Amberlite XE-64 column. The structure of this product was confirmed by NMR and high-resolution MS analysis. The two doublets at δ 2.59 ($J_{H-F} = 3.0$) and 4.47 ($J_{H-F} = 1.0$) in the ^{1}H NMR spectra can be assigned to the 2-Me and 4′-CH_2 peaks, respectively. The doublet at δ 5.10

(12) Ivanov, V. I.; Karpeisky, M. Ya. *Adv. Enzymol.* **1969**, *32*, 21. (b) Braunstein, A. E. In *The Enzymes*; 3rd ed.; Boyer, P. D., Ed.; Academic Press: New York, 1973; Vol. 9, p 379.

(13) (a) Harris, E. E.; Firestone, K.; Pfister, R. R.; Boettcher, F. I.; Cross, R. B.; Currie, R. B.; Monaco, M.; Peterson, E. .; Reuter, W. *J. Org. Chem.* **1962**, *27*, 2705. (b) Firestone, R. A.; Harris, E. E.; Reuter, W. *Tetrahedron* **1967**, *23*, 943.

(14) Stevens, P. G. Synthesis of Pyridoxine. United States Patent, 2680743, 1954.

(15) Geiger, R. E.; Lalonde, M.; Stoller, H.; Schleich, K. *Helv. Chim. Acta* **1984**, *67*, 1274.

(16) Ichiba, A.; Emoto, S. *Sci. Pap. Inst. Phys. Chem. Res. (Tokyo)* **1941**, *38*, 347.

(17) Fukuhara, T.; Yoneda, N.; Sawada, T.; Suzuki, A. *Synth. Commun.* **1987**, *17*, 685.

(18) Baltz, G.; Schiemann, G. *Ber. Dtsch. Chem. Ges.* **1927**, *60*, 1186.

(19) Korytnyk, W.; Ahrens, H. *J. Med. Chem.* **1971**, *14*, 947.

(20) Ahrens, H.; Korytnyk, W. *J. Heterocycl. Chem.* **1967**, *4*, 625.

(21) (a) Harris, S. A.; Heyl, D.; Folkers, K. *J. Am. Chem. Soc.* **1944**, *66*, 2088. (b) Peterson, E. A.; Sober, H. A.; Meister, A. *J. Am. Chem. Soc.* **1952**, *74*, 570. (c) Peterson, E. A.; Sober, H. A.; Meister, A. *Biochem. Prep.* **1953**, *3*, 29.

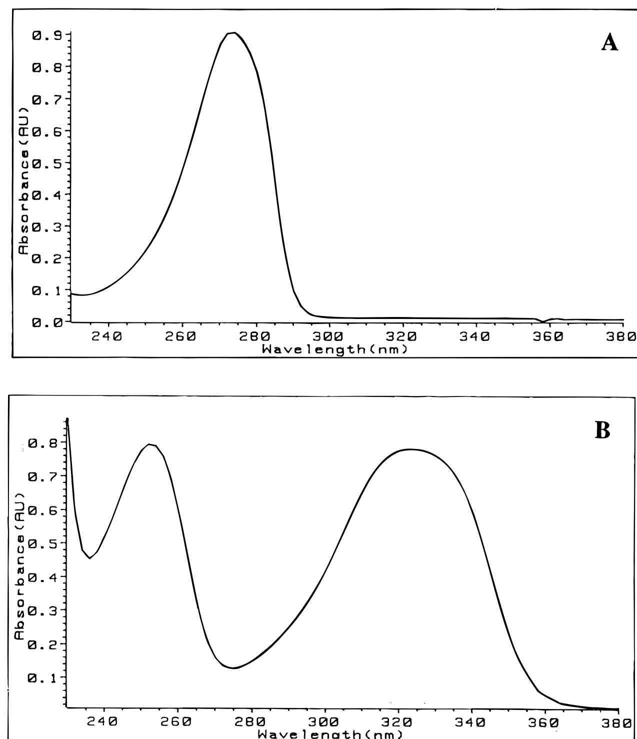


Figure 1. Ultraviolet−visible spectra of (A) F-PMP in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C and (B) PMP in 0.1 M potassium phosphate buffer (pH 7.0) at 25 °C.

with $J = 7.4$ Hz is the 5′-CH_2 signal coupled to the adjacent phosphorus nucleus. A resonance at −125.9 ppm was observed in ^{19}F NMR. While this signal is expected to be a multiplet due to the long-range coupling with the 2-Me and 4′-CH_2 hydrogens, it appears as a broad doublet with an apparent coupling constant of 2.7 Hz. The proton-coupled ^{31}P NMR spectrum of **8** reveals a triplet ($J = 7.4$) at 0.84 ppm, which is characteristic of a phosphorylated hydroxymethylene group.

UV-Vis Spectrum of F-PMP. As can be seen in Figure 1, the UV−vis spectrum of F-PMP is much different from that of PMP. The λ_{max} at 324 nm observed for PMP is clearly absent in the spectrum of F-PMP. It has been suggested that the absorbance at 324 nm may be ascribed to the electronic transition of PMP with an ionized 3-OH moiety.[22] An analogous structure is not available for F-PMP, thereby explaining this spectral difference.

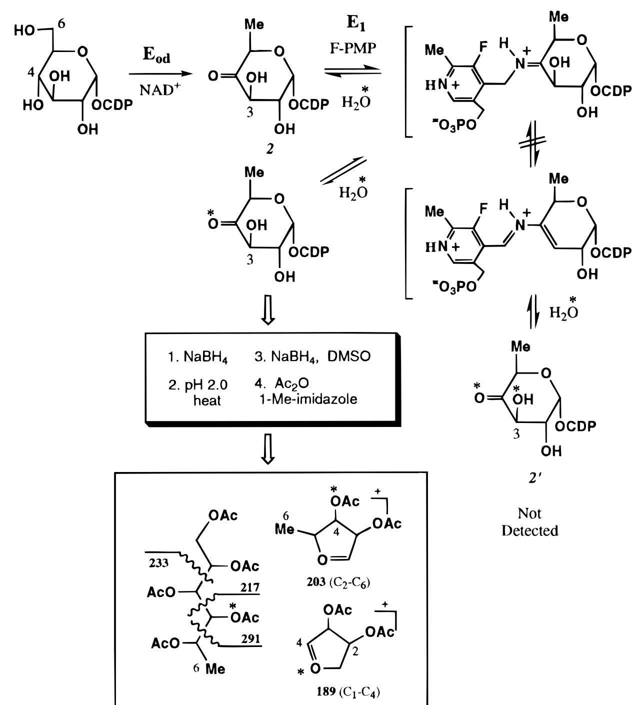
Enzyme Preparation. As shown in Scheme 1, the 3,6-dideoxygenations of CDP-D-glucose catalyzed by $\mathbf{E_{od}}$ and $\mathbf{E_1}$−$\mathbf{E_3}$ are two pivotal reactions in the biosynthesis of ascarylose. Purification of these enzymes was accomplished by previously reported procedures.[3c,23,24] The specific activities of the purified $\mathbf{E_{od}}$, $\mathbf{E_1}$, and $\mathbf{E_3}$ used in this work were 65, 119, and 69 U/mg, respectively. The purified $\mathbf{E_1}$ contained 0.84 equiv of iron per $\mathbf{E_1}$ monomer, and the iron content of $\mathbf{E_3}$ was 1.94 equivalents per monomer. Although most PMP was released from the $\mathbf{E_1}$ active site during the step of anion-exchange chromatography,[4] the isolated $\mathbf{E_1}$ still contained 0.2−0.3 equiv of PMP per monomer. Attempts to make $\mathbf{E_1}$(apo-PMP) by removing the residual PMP were complicated by the destruction of $\mathbf{E_1}$'s [2Fe-2S] center leading to irreversible denaturation of this enzyme. Hence, the purified $\mathbf{E_1}$ was used directly in subsequent studies without further treatment.

(22) Morozov, Yu. V. In *Vitamin B₆ Pyridoxal Phosphate, Chemical, Biochemical and Medical Aspects*; Dolphin, D., Poulson, R., Avramovic, O., Eds.; Wiley-Interscience: New York, 1986; Part A, p 132.

(23) Lei, Y.; Ploux, O.; Liu, H.-w. *Biochemistry* **1995**, *34*, 4643.

(24) Ploux, O.; Lei, Y.; Vatanen, K.; Liu, H.-w. *Biochemistry*, **1995** *34*, 4159.

Scheme 3



Effect of F-PMP on $\mathbf{E_1}$−$\mathbf{E_3}$ Product Formation. A GC-MS assay (see Scheme 3) was applied to determine whether the $\mathbf{E_1}$−$\mathbf{E_3}$ product was produced when F-PMP was used as the cofactor for $\mathbf{E_1}$. While the peracetylated alditols derived from the $\mathbf{E_1}$−$\mathbf{E_3}$ product could be easily detected in the positive control in which PMP was added to the assay mixture, no product formation was found in samples derived from incubations with F-PMP. These results clearly indicated that F-PMP is not a competent coenzyme for the $\mathbf{E_1}$−$\mathbf{E_3}$-catalyzed reaction.

Effect of F-PMP on $\mathbf{E_1}$-catalyzed C-O Bond Cleavage. The above results may be considered as preliminary evidence supporting the inability of the glucoseen−F-PMP adduct to undergo tautomerization, thereby inhibiting the reduction as predicted. However, it should be pointed out that C-3 deoxygenation catalyzed by $\mathbf{E_1}$ and $\mathbf{E_3}$ consists of two half-reactions: the reversible dehydration and the subsequent stepwise electron transfer reduction. Hence, one cannot exclude the possibility that it is the initial dehydration mediated by $\mathbf{E_1}$ being affected by the reconstitution with F-PMP. Thus, a series of incubations of $\mathbf{E_1}$, F-PMP, and $\mathbf{E_1}$ substrate **2** in buffer prepared with $[^{18}O]H_2O$ were conducted. The recovered substrate was subjected to borohydride reduction, acid hydrolysis, further reduction, and acetylation to yield glycitol pentaacetates whose MS fragmentation patterns are well characterized (Scheme 3).[25] As shown in Table 1, the MS fragments containing the C-4 oxygen linkage (*m/e* 289, 231, 201, 187) are all uniformly shifted by two mass units due to ^{18}O exchange with the 4-keto group. However, those containing the C-3 oxygen linkage were increased by two more mass units only when PMP was used in the incubation. No ^{18}O incorporation at C-3 was observed in samples derived from reconstitution with F-PMP. Apparently, F-PMP even fails to function as an effective coenzyme for glucoseen formation.

Effect of F-PMP on $\mathbf{E_1}$-Catalyzed C-4′ Deprotonation. It has been shown that $\mathbf{E_1}$ catalysis is initiated by a C-4′ deprotonation from the PMP-substrate complex **3** which triggers the subsequent C−O bond cleavage at C-3. The stereochemical

(25) Lönngren, J.; Svensson, S. *Adv. Carbohydr. Chem. Biochem.* **1974**, *29*, 41.

Table 1. Selected Mass Fragments of the Alditol Pentacetate Sample Derived from Recovered $\mathbf{E_1}$ Substrate **2**

incubation[a]	mass fragments (*m/z*)				
	C_1-C_4	C_3-C_6	C_1-C_3	C_2-C_6	C_1-C_4
standard	289	231	217	201	187
PMP + substrate + denatured $\mathbf{E_1}$	291	233	217	203	189
PMP + substrate + $\mathbf{E_1}$	293, 291	235, 233	219, 217	203, 205	189,191
F-PMP + substrate + denatured $\mathbf{E_1}$	291	233	217	203	189
F-PMP + substrate + $\mathbf{E_1}$	291	233	217	203	189

[a] The experimental details are described in the Experimental Section. All incubations were performed in $[^{18}O]H_2O$.

Table 2. Tritium Released by the $\mathbf{E_1}$-Catalyzed Deprotonation of the PMP−Substrate and the F-PMP−Substrate Complexes

incubation[a]	tritium released (dpm)
[4′-^{3}H]F-PMP + substrate	74
[4′-^{3}H]F-PMP + substrate + $\mathbf{E_1}$	75
[4′-^{3}H]PMP + substrate + $\mathbf{E_1}$	469

[a] Incubation conditions are described in the Experimental Section.

course of this deprotonation is *pro-S* specific,[4c] and the active site base is His-220.[23] In order to assess whether this C-4′ deprotonation step is affected by the reconstitution with F-PMP as the $\mathbf{E_1}$ cofactor, we have synthesized [4′-^{3}H]F-PMP (**22**). The key intermediate, 3-deoxy-3-fluoropyridoxal (**18**), was prepared as described earlier. Attempts to convert **18** to amine **20** in a model reaction using ammonia and sodium borohydride failed. Reduction of 3-deoxy-3-fluoropyridoxal oxime (**19**) by sodium borohydride or sodium cyanoborohydride was also unsuccessful. Eventually, oxime **19** was converted to **21** using [^{3}H]sodium borohydride and titanium(IV) chloride in 1,2-dimethoxyethane.[26] This labeled compound was then phosphorylated to give **22** (Scheme 2). When [4′-^{3}H]F-PMP was used in the incubation which was later treated with activated charcoal followed by centrifugation to sequester the PMP coenzyme, the radioactivity found in the supernatant was identical to that of the control (Table 2). Clearly, C-4′ abstraction did not occur when [4′-^{3}H]F-PMP was used as the coenzyme.

Competition between F-PMP and PMP for Binding with $\mathbf{E_1}$. The $\mathbf{E_1-E_3}$ coupled assay[23] which correlates the rate of $\mathbf{E_1-E_3}$ product formation to the rate of NADH consumption is a newly developed method to determine the activities of $\mathbf{E_1}$ and $\mathbf{E_3}$. When F-PMP was used as the $\mathbf{E_1}$ cofactor in this assay, no NADH consumption above background was observed. This result corroborates well with the conclusion derived earlier from the GC−MS assay. Interestingly, preincubation of $\mathbf{E_1}$ with 50 equiv of F-PMP followed by the addition of PMP, as in the normal assay procedure, decreased the reaction rate by nearly 20%. When 100 equiv of F-PMP was used in the preincubation, the rate reduction was more than 50%. From these results one may conclude that F-PMP can compete with PMP for binding in the $\mathbf{E_1}$ active site. Unfortunately, F-PMP is not fluorescent and coenzyme binding to $\mathbf{E_1}$ causes no discernible change of the fluorescence of $\mathbf{E_1}$. The lack of a convenient probe that is sensitive to the physical changes associated with the binding of F-PMP with $\mathbf{E_1}$ has hampered quantitative analysis of the binding affinity.

pK_a of the Ring Nitrogen (N-1) of F-PMP. To further characterize F-PMP and better understand why it fails to act as a competent coenzyme for the $\mathbf{E_1}$-$\mathbf{E_3}$ reaction, the pK_a of the ring nitrogen (pyridinium NH^+) of F-PMP was also determined. Since the chemical shift of the carbon adjacent to a nitrogen atom is sensitive to the ionization state of the nitrogen functional

(26) Kano, S.; Tanaka, Y.; Sugino, E.; Hibino, S. *Synthesis* **1980**, 695.

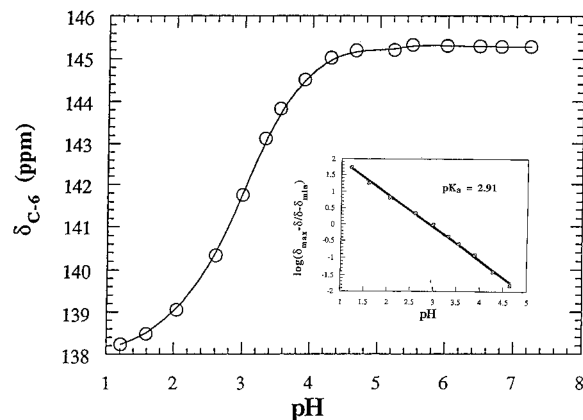


Figure 2. pH dependence of the C-6 chemical shift of (A) PMP in 50 mM potassium phosphate buffer and (B) F-PMP in 50 mM potassium phosphate buffer. The pH of the sample solution was adjusted by either a concentrated phosphoric acid or potassium hydroxide solution. Dioxane was used as the internal standard. The inset shows the semilogarithmic plot of $(\delta_{max} - \delta)/(\delta - \delta_{max})$ versus pH according to the Henderson−Hasselbach equation.

group, the pK_a of the ring nitrogen (N-1) of F-PMP can be estimated from the point of inflection of the ^{13}C shift versus pH plot for C-6. A pK_a value of 2.91 was derived from the semilogarithmic plot according to the Henderson−Hasselbach equation (eq 1),

$$\mathrm{pH} = \mathrm{p}K + \log[(\delta_{max} - \delta)/(\delta - \delta_{min})] \qquad (1)$$

where δ_{max} and δ_{min} are the maximum and and minimum shifts of C-6 in the pH invariant segment of the δ versus pH curve (Figure 2 inset).[27] The pK_a of N-1 of PMP was also determined by the same method. The value of 8.74 obtained compares very well to that of 8.61 determined by titration.[28] This difference in the protonation state of PMP and its F-PMP analog at neutral pH may account for the fact that F-PMP is not a competent cofactor for the $\mathbf{E_1-E_3}$ reaction.

Discussion

Since the most likely mechanism for the reductive half-reaction of $\mathbf{E_1-E_3}$ catalysis, shown in Scheme 1, begins with a tautomerization, leading to a PMP−quinone methide intermediate (**5**), the title compound **8**, owing to its inability to undergo such a tautomerization, was prepared to test the proposed mechanism. The fact that no product formation could be detected when F-PMP was substituted for PMP in $\mathbf{E_1-E_3}$ catalysis initially appeared to be consistent with the predicted outcome. However, the absence of ^{18}O incorporation at C-3 when F-PMP, $\mathbf{E_1}$, and its substrate **2** were incubated in [^{18}O]-buffer indicated that the reaction was arrested at the dehydration stage, not at the tautomerization as expected (Table 1). This conclusion was further supported by the lack of tritium release when [4′-^{3}H]F-PMP was used in the incubation (Table 2). Interestingly, while F-PMP fails to assume the normal catalytic role as a coenzyme B_6 analog, it can still compete, albeit weakly, with PMP for the $\mathbf{E_1}$ coenzyme binding site. Since a great excess of F-PMP (>1000-fold) was used in the assays, most of the residual PMP retained in the active site of the purified $\mathbf{E_1}$ was expected to be exchanged with F-PMP. Hence, no product formation was detected under the assay conditions. Clearly,

(27) Breitmaier, E.; Voelter, W. In *Carbon-13 NMR Spectroscopy: High Resolution Methods and Applications in Organic Chemistry and Biochemistry*; VCH Publishers: New York, 1987; p 121.

(28) Williams V. R.; Neilands, J. B. *Arch. Biochem. Biophys.* **1954**, *53*, 56.

the impotence of F-PMP to catalyze C−O bond cleavage of **3** is not due to binding problems alone but, more likely, to the alteration in the intrinsic properties of F-PMP.

Despite the fact that replacement of a fluorine for the OH group was considered a conservative substitution, drastic changes of the properties of F-PMP compared to PMP were observed. For example, the electronic absorption of PMP has two maxima at 324 and 252 nm in 0.1 M potassium phosphate buffer, pH 7.5, while that of F-PMP has only one λ_{max} at 274 nm in the same buffer (Figure 1); while PMP emits fluorescence at 393 nm when excited at 325 nm,[29] F-PMP is not fluorescent at all; 3-deoxy-3-fluoropyridoxine (**17**) is quite soluble in methylene chloride, but pyridoxine itself is soluble only in more polar solvents. These distinctions are at least partially due to the dipolar ionic nature of PMP whose 3-OH (pK_a of 3.69) is deprotonated and ring nitrogen (N-1, pK_a = 8.61) is protonated at neutral pH.[30] The substitution of a nonionizable fluorine at C-3 clearly has made F-PMP more hydrophobic than PMP and thus alters its physical as well as chemical characteristics.

Interestingly, the C-3 fluoro substitution has also affected the pK_a of the ring nitrogen (N-1). The pK_a of N-1 (pyridinium NH^+) of PMP had been measured to be 8.61 by Williams and Neilands[28] and 8.46 by Metzler et al.[31] On the basis of the pH dependence of the chemical shift of C-6, the pK_a of the ring nitrogen of PMP was determined to be 8.74, which compares quite well with literature values. For F-PMP, the pK_a of N-1 was estimated to be 2.91, a shift of almost 6 pH units (Figure 2). The large disparity between the pK_a of F-PMP and that of PMP reflects a major change in the electron density of the pyridine ring. It is obvious that the F-PMP ring nitrogen is not protonated at physiological pH due to the electron-withdrawing effects of the 3-fluoro substituent. A similar effect was also observed when H-6 was substituted with fluorine on the pyridoxal ring. The pK_a values of the 3-OH and N-1 of 6-fluoropyridoxal were reported to be 7.8 and 0.1, respectively.[32]

Although the $\mathbf{E_1}$ catalyzed reaction clearly represents a unique PMP-dependent catalysis, early mechanistic and stereochemical studies have demonstrated that $\mathbf{E_1}$ still behaves like a normal coenzyme B_6 dependent catalyst.[4c,33] This conclusion is consistent with the results of sequence alignment in which several invariant residues characteristic for PMP/PLP dependent enzymes are also conserved in the $\mathbf{E_1}$ sequence.[23] Thus, while the X-ray structure of $\mathbf{E_1}$ remains to be determined, information gathered from the known crystal structures of PLP-dependent enzymes, such as the chicken mitochondrial[34] as well as pig and chicken cytosolic aspartate aminotransferase,[35] can be used to predict and analyze the possible interactions between F-PMP and the $\mathbf{E_1}$ active site residues, from which reasons as to why F-PMP is not a competent coenzyme for $\mathbf{E_1}$ may be speculated.

First, it has been shown that the 3-hydroxyl group of PLP forms a hydrogen bond with Tyr-225 in mitochondrial aspartate aminotransferase from chicken heart.[36] It is also known that a salt bridge/ion pair interaction exists between the deprotonated 3-hydroxyl group and the protonated imine hydrogen of the PLP−substrate complex of these aminotransferases. The intermolecular hydrogen bonding with an active site residue may be important for proper anchoring of the coenzyme, and the intramolecular salt bridge/ion pair interaction between two oppositely charged groups within the PLP−substrate adduct has been suggested to be important in assisting the stabilization of the intermediates generated during the catalysis.[36] With the replacement of the 3-hydroxyl group by a fluorine in F-PMP, such an intramolecular salt bridge formation is no longer possible, which may partially account for the lack of catalytic competency of F-PMP with $\mathbf{E_1}$.

Another salt bridge/hydrogen bond interaction imperative for the catalysis of mitochondrial aspartate aminotransferase exists between Asp-222 and the protonated N-1 of PLP.[36] This ion pair contact stabilizes the positioning of the coenzyme in the active site and also sustains the electron-withdrawing capability of the coenzyme by raising the pK_a of N-1 so that the ring nitrogen remains protonated throughout catalysis. More importantly, the ion pair formation may help lower the pK_a of the 4′-H of the coenzyme−substrate by delocalizing the resulting carbanion into the pyridinium ring and thus facilitating the deprotonation step. Indeed, when Asp-222 of aspartate aminotransferase from *E. coli* was replaced by a nonionizable amino acid residue, such as Ala and/or Asn, the catalytic efficiency of these mutant proteins decreased drastically.[37] The significance of the salt bridge/hydrogen bond interaction for catalysis was further substantiated by the 20-fold increase of the transamination activity of the Ala-222 mutant (D222A) when reconstituted with *N*-methylpyridoxal 5′-phosphate instead of PLP itself.[38] Since alignment of the translated amino acid sequence of $\mathbf{E_1}$ with several representative aminotransferases had led to the identification of Asp-199 as the invariant Asp-220 equivalent in the $\mathbf{E_1}$ sequence,[23] a similar salt bridge/hydrogen bond formation should also occur when $\mathbf{E_1}$ binds with its substrate **2**. While the ring nitrogen of PMP in the active site of $\mathbf{E_1}$ is protonated at neutral pH, that of the F-PMP is not, due to the electronegative effect of the fluorine. Thus, F-PMP is not expected to promote 4′-H abstraction since the resulting anion cannot be readily delocalized by the cofactor. This notion corroborates well with the lack of tritium release when $\mathbf{E_1}$ was reconstituted with [4′-^{3}H]F-PMP (Table 2). Thus, the lack of N-1 protonation may be the main factor contributing to the catalytic incompetency of F-PMP with $\mathbf{E_1}$.

In summary, since the C−O bond cleavage of the first half-reaction of C-3 deoxygenation did not occur, using F-PMP as a coenzyme substitute for $\mathbf{E_1}$ failed to provide further insight directly related to the proposed mechanism for the second half-reaction (Scheme 1). Clearly, substituting an electron-withdrawing fluoro group at the C-3 position of PMP has a substantially negative impact on its capabilities as an effective coenzyme with the $\mathbf{E_1}$−$\mathbf{E_3}$ system. Although a large number of coenzyme B_6 analogs have been synthesized, only a few with modifications at the C-3 position have been made. These include 3-methoxy-,[39] 3-amino-,[40] 3-deoxy-,[40,41] 3-chloro-,[42] and

(29) Churchich, J. E. *Biochem. Biophys. Acta* **1969**, *178*, 480.
(30) Kallen, R. G.; Korpela, T.; Martell, A. E.; Matsushima, Y.; Metzler, C. M.; Metzler, D. E.; Morozov, Y. V.; Ralston, I. M.; Savin, F. A.; Torchinsky, Y. M.; Ueno, H. In *Transaminases*; Christen, P., Metzler, D. E., Eds.; Wiley-Interscience: New York, 1985; p 37.
(31) Metzler, D. E.; Harris, C. M.; Johnson, R. J.; Siano, D. B.; Thomson, J. S. *Biochemistry* **1973**, *12*, 5377.
(32) Korytnyk, W.; Srivastava, S. C. *J. Med. Chem.* **1973**, *16*, 638.
(33) Pieper, P. A.; Guo, Z.; Liu, H.-w. *J. Am. Chem. Soc.* **1995**, *117*, 5158.
(34) Ford, G. C.; Eichele, G.; Jansonius, J. N. *Proc. Natl. Acad. Sci. U.S.A.* **1980**, *77*, 2559.
(35) (a) Borisov, V. V.; Borisova, S. N.; Sostenov, N. I.; Vainshtein, B. K. *Nature* **1980**, *284*, 189. (b) Harutyunyan, E. G.; Malashkevich, V. N.; Tersyan, S. S.; Kochkina, V. M.; Torchinsky, Yu. M.; Braunstein, A. E. *FEBS Lett.* **1982**, *138*, 113. (c) Arnone, A; Briley, P. D.; Rogers, P. H.; Hyde, C. C.; Metzler, C. M.; Metzler, D. E. In *Molecular Structure and Biological Activity*; Griffen, J. F., Duax, W. L., Eds.; Elsevier, North-Holland: New York, 1987; p 57.
(36) Kirsch, J. F.; Eichele, G.; Ford, G. C.; Vincent, M. G.; Jansonius, J. N. *J. Mol. Biol.* **1984**, *174*, 497.
(37) Yano, T.; Kuramitsu, S.; Tanase, S.; Yoshimasa, M.; Kagamiyama, H. *Biochemistry* **1992**, *31*, 5878.
(38) Onuffer, J. J.; Kirsch, J. F. *Protein Eng.* **1994**, *7*, 413.
(39) (a) Pocker, A.; Fischer, E. H. *Biochemistry* **1969**, *8*, 5181. (b) Korytnyk, W.; Angelino, N. J. *J. Med. Chem.* **1977**, *20*, 745.

3-mercapto-[43] pyridoxine/PLP derivatives. Among those, 3-OMe- and 3-deoxy-PLPs are the most extensively studied. Although it had been shown that the 3-OMe-PLP can still bind to apoaspartate aminotransferase, its catalytic activity is, however, lower than the native enzyme by a factor of 10^6.[44] The 3-deoxy-PLP also fails to act as an effective coenzyme with aminotransferase,[45] and the 3-chloropyridoxine is only a weak coenzyme B_6 antagonist in rats.[42] These results, in conjunction with those of F-PMP, illustrate the importance of having the 3-OH group of the coenzyme intact in PLP/PMP-dependent catalysis. Changes in the electronic properties of this system which cause this enzyme inactivity reflect the delicacy of the process of natural selection of the PLP/PMP structures as coenzymes and the importance of the protonation states of the 3-substituent and ring nitrogen in the catalytic process.

Experimental Section

General Procedures. Melting points were determined with a Mel-Temp apparatus and are uncorrected. Mass spectra were obtained with either a VG 7070E-HF spectrometer (for FAB) or a Finnigan MAT 90 (for EI and CI). Ultraviolet−visible spectroscopy was performed on a Shimadzu UV-160 or a Hewlett-Packard 8452A spectrophotometer. ^{1}H and ^{13}C NMR spectra were recorded on an IBM NR/200, NR/300, Varian Unity 300, or Unity 500 spectrometer. ^{19}F NMR and ^{31}P NMR were recorded on a Varian Unity 300 spectrometer. Chemical shifts are reported in δ scale relative to an internal standard or appropriate solvent peak, with coupling constants given in hertz. NMR assignments labeled with an asterisk may be interchangeable. The pH measurements were performed with a Corning pH meter 240, and a MI-410 combination pH electrode from Microelectrodes, Inc. (Londonderry, NH), was used for microsamples. Flash chromatography was performed in columns of various diameters with J. T. Baker (230−400 mesh) silica gel by elution with the solvents specified. Analytical thin layer chromatography (TLC) was carried out on Merck silica gel 60 G-254 plates (25 mm) and developed with the appropriate solvents. The TLC spots were visualized with either UV light or by dipping the plates into a solution of phosphomolybdic acid (7% ethanolic solution) and then heating them. [^{18}O]H_2O was from Isotec (Miamisburg, OH) and [^{3}H]$NaBH_4$ was from New England Nuclear (Boston, MA). The racemic [4′-^{3}H]PMP was prepared according to published procedures[4a,46] with minor modifications. Large-scale preparation of the $\mathbf{E_1}$ substrate **2** was effected by the method of Lei et al.[23] Radioactivity was measured by liquid scintillation counting with a Beckman LS-3801 counter using Ecoscint A biodegradable scintillation solution from National Diagnostics (Manville, NJ). All chemicals and biochemicals were products of Sigma (St. Louis, MO) or Aldrich (Milwaukee, WI). Solvents were of analytical reagent grade or of the highest quality commercially available. Although not specified in each experimental procedure, the scale which was reported (small or large) varied depending on which gave the best yield and most of the synthetic steps were repeated a number of times.

Enzyme Preparation. The $\mathbf{E_3}$ was purified from a recombinant *E. coli* JM105-pOPI strain according to the method of Ploux et al.[24] Its activity was determined as previously described.[47] The activity unit (U) of $\mathbf{E_3}$ is defined as micromoles of DCPIP consumed per minute. Purification of $\mathbf{E_1}$ followed the procedure of Lei et al.[23] using the recombinant *E. coli* JM105-pJT18 overproducing strain.[3c] The $\mathbf{E_1}$ activity was determined by an $\mathbf{E_1}$−$\mathbf{E_3}$ coupled assay reported by Lei et al.[23] The activity unit (U) of $\mathbf{E_1}$ is defined as nanomoles of NADH consumed per minute. Protein concentrations were estimated by the Bradford method[48] using bovine serum albumin as the standard. The Bradford reagent was obtained from BioRad (Richmond, CA). The iron content of the purified enzymes was quantitated by the method of Fish,[49] and the PMP content was estimated by a fluorescence method.[23] The $\mathbf{E_{od}}$ was purified from *E. coli* HB101-pJT8 by the method of Thorson et al.,[3c] and the activity was assayed according to Yu et al.[50] The activity unit (U) of $\mathbf{E_{od}}$ is defined as micromoles of product formation per hour.

Synthesis of 3-Deoxy-3-fluoropyridoxamine 5′-Phosphate (8). Compounds **9**−**13** were prepared according to Ichiba and Emoto.[16]

Ethyl 2-Methyl-3-fluoro-5-cyanopyridine-4-carboxylate (14). Compound **13** (500 mg, 2.44 mmol) was dissolved in a hydrogen fluoride−pyridine solution (5 mL) in a 25 mL plastic container at 0 °C. This yellow mixture was stirred at room temperature for 15 min to dissolve the compound. After being cooled to 0 °C again, sodium nitrite (250 mg, 3.62 mmol) was slowly added to this solution. The mixture was maintained at 0 °C for 20 min and then warmed to 30 °C for 15 min. This was followed by heating at 60−70 °C for 30 min until the dediazotization was judged complete by TLC. During this period, evolution of nitrogen gas was noted. After cooling back to room temperature, the reaction mixture was quenched with ice−water (40 mL), and the aqueous solution was extracted with chloroform five times without neutralization. The organic extracts were dried with magnesium sulfate and concentrated under reduced pressure. The desired product was purified by flash chromotography (8% EtOAc/hexanes) to give 300 mg of **14** as a colorless liquid. The yield was 60%. TLC (50% EtOAC/hexanes): $R_f = 0.74$. ^{1}H NMR ($CDCl_3$): δ 8.65 (1H; s; 6-H), 4.54 (2H; q, $J = 7.1$; OCH_2), 2.66 (3H; d, $J = 3.4$; 2-Me), 1.46 (3H; t, $J = 7.1$; OCH_2CH_3). ^{13}C NMR ($CDCl_3$): δ 160.5 (C═O), 154.1 (C-3, d, $J = 268.7$), 154.3 (C-2, d, $J = 18.2$), 148.5 (C-6, d, $J = 7.2$), 128.4 (C-4, d, $J = 14.3$), 114.2 (C-5), 107.0 (CN), 63.5 (OCH_2), 18.9 (2-Me), 13.9 (OCH_2CH_3). High-resolution FAB-MS: calcd for $C_{10}H_{10}FN_2O_2$ $(M + 1)^+$ 209.0744, found 209.0726.

2-Methyl-3-fluoropyridine-4,5-dicarboxylic Acid (15). A solution of compound **14** (1.50 g, 7.21 mmol) in 8 mL of concentrated hydrochloric acid was heated in an oil bath at 120 °C for 3 h and then at 150 °C for 30 min. The solution was evaporated to dryness under reduced pressure. The resulting brown solid was redissolved in water and concentrated again to remove any residual acid. This procedure was repeated twice. The product was then recrystallized from a small amount of hot water to give 1.35 g of **15** as an off-white solid. The yield was 86%. Mp: 216−217 °C dec. ^{1}H NMR (CD_3OD): δ 8.77 (1H; s; 6-H), 2.52 (3H; d, $J = 2.9$; 2-Me). ^{13}C-NMR (CD_3OD): δ 167.5, 167.2 (C═O's), 155.8 (C-3, d, $J = 256.7$), 154.4 (C-2, d, $J = 17.4$), 148.2 (C-6, d, $J = 6.0$), 134.0 (C-4, d, $J = 18.3$), 125.6 (C-5), 19.4 (2-Me). High resolution FAB-MS: calcd for $C_8H_7FNO_4$ $(M + 1)^+$ 200.0359, found 200.0370.

Dimethyl 2-Methyl-3-fluoropyridine-4,5-dicarboxylate (16). A suspension of compound **15** (2 g, 10 mmol) in methanol (100 mL) was treated with a solution of diazomethane (ca. 12 mmol) in ether (30 mL) made from Diazald (Aldrich) according to the manufacturer's instructions. The reaction mixture was allowed to stand at room temperature and swirled occasionally until the evolution of nitrogen gas subsided. This procedure was repeated four more times until all the starting material was converted to product as indicated by TLC. The solution was then concentrated in vacuo to give **16** as a yellow liquid in 96% yield. TLC (50% EtOAc/hexanes): $R_f = 0.61$. ^{1}H NMR ($CDCl_3$): δ 8.87 (1H; s; 6-H), 3.96, 3.91 (3H each; s; OMe's), 2.59 (3H; d, $J = 3.2$; 2-Me). ^{13}C NMR ($CDCl_3$): δ 163.9, 163.7 (C═O's), 153.3 (C-3, d, $J = 260.4$), 152.6 (C-2, d, $J = 17.0$), 146.0 (C-6, d, $J = 6.0$), 129.6 (C-4, d, $J = 18.1$), 122.0 (C-5), 53.3, 52.9 (OMe's),

(40) Jones, R. G.; Kornfeld, E. C. *J. Am. Chem. Soc.* **1951**, *73*, 107.
(41) (a) Melius, P.; Marshall, D. L. *J. Med. Chem.* **1967**, *10*, 1157. (b) Harris, S. A.; Harris, E. E.; Peterson, E. R.; Rogers, E. F. *J. Med. Chem.* **1967**, *10*, 261.
(42) Gadekar, S. M.; Federick, J. L.; DeRenzo, E. C. *J. Med. Pharm. Chem.* **1962**, *5*, 531.
(43) Greene, J. L., Jr.; Montgomery, J. A. *J. Med. Chem.* **1964**, *7*, 17.
(44) Chen, V. J. Ph.D. Dissertation, Iowa State University, 1981.
(45) (a) Mora, S.; Bocharov, A. L.; Ivanov, V. I.; Karpeisky, M. Ya.; Mamaeva, O. K.; Stambolieva, N. A. *Mol. Biol. (USSR)* **1972**, *6*, 119. (b) Mora, S.; Nikolova, Z. V.;| Bocharov, A. L.; Ivanov. V. I.; Mamaeva, O. K.; Stambolieva, N.; Florentiev, V. L.; Karpiesky, M. Ya. *Mol. Biol. (USSR)* **1972**, *6*, 559. (c) Vasil'ev, V. Yu.; Mekhanik, M. L.; Severin, E. S.; Torchinsky, Yu. M. *Biokimiya* **1975**, *40*, 207.
(46) Voet, J.; Indenlang, P. M.; Blank, T.; Ulevitch, R.; Kallen, R.; Dunathan, H. *J. Biol. Chem.* **1973**, *248*, 841.
(47) Lo, S. F.; Miller, V. P.; Lei, Y.; Thorson, J. S.; Liu, H.-w.; Schottel, J. L. *J. Bacteriol.* **1994**, *176*, 460.
(48) Bradford, M. M. *Anal. Biochem.* **1976**, *72*, 248.
(49) Fish, W. W. *Methods Enzymol.* **1983**, *91*, 392.
(50) Yu, Y.; Russell, R. N.; Thorson, J. S.; Liu, L.-d.; Liu, H.-w. *J. Biol. Chem.* **1992**, *267*, 5868.

18.6 (2-Me). High-resolution FAB-MS: calcd for $C_{10}H_{11}FNO_4$ (M + 1)$^+$ 228.0672, found 228.0681.

3-Deoxy-3-fluoropyridoxine (17). Compound **16** (0.75 g, 3.30 mmol) was dissolved in anhydrous ether (8 mL) under nitrogen. To this solution, kept in an ice bath, was added dropwise 13.5 mL of 1.0 M diisobutylaluminum hydride (4.1 equiv). The reaction was stirred at 0 °C and quenched with an ammonium chloride solution after it was judged to be complete by TLC (ca. 1 h). The quenched solution was stirred vigorously, and some salts precipitated out. The pH of the water layer was neutralized with 1 N HCl, and the resulting mixture was thoroughly extracted with ethyl acetate. The combined organic extracts were dried, concentrated, and purified by flash chromatography (80% EtOAc/hexanes) to give a white solid: TLC (EtOAc): $R_f = 0.2$. The yield was 46%. Mp: 127−128 °C. ^{1}H NMR ($CDCl_3$): δ 8.32 (1H; s; 6-H), 4.84 (2H; d, J = 1.6; 4′-CH$_2$), 4.77 (2H; s; 5′-CH$_2$), 2.52 (3H; d, J = 3.2; 2-Me). ^{13}C NMR (CD_3OD): δ 158.8 (C-3, d, J = 256.6), 148.4 (C-2, d, J = 19.6), 146.0 (C-6, d, J = 5.9), 138.5 (C-5), 137.0 (C-4, d, J = 13.4), 61.5 (C-5′), 55.9 (C-4′, d, J = 7.6), 18.8 (2-Me). High-resolution FAB-MS: calcd for $C_8H_{11}FNO_2$ (M + 1)$^+$ 172.0773, found 172.0760. A minor compound was also isolated (eluted with 50% EtOAc/hexanes) which was identified as 2,5-dimethyl-3-fluoro-4-(hydroxymethyl)pyridine in ca. 15% yield. TLC (EtOAc): $R_f = 0.44$. ^{1}H NMR ($CDCl_3$): δ 8.07 (1H; s; 6-H), 4.73 (2H; d, J = 1.3; 4′-CH$_2$), 2.44 (3H; d; J = 3.0; 2-Me) 2.36 (3H; s; 5-Me). ^{13}C NMR ($CDCl_3$): δ 156.3 (C-3, d, J = 252.7), 145.4 (C-6, d, J = 5.8), 144.2 (C-2, d, J = 19.5),* 133.1 (C-4, d, J = 12.2),* 132.0 (C-5), 55.1 (C-4′, d, J = 5.9), 17.6 (2-Me), 15.3 (5-Me). High-resolution FAB-MS: calcd for $C_8H_{11}FNO$ (M + 1)$^+$ 156.0825, found 156.0815.

3-Deoxy-3-fluoropyridoxal (18). To a mixture of dry pyridinium chlorochromate (PCC; 25.3 mg, 0.12 mmol), sodium acetate (19.2 mg, 0.23 mmol), and powdered 3 Å molecular sieves (40 mg) in dry methylene chloride (8 mL) was added a suspension of compound **17** (20 mg, 0.12 mmol) in 2 mL of methylene chloride. The reaction was stirred at room temperature for 1 h and spiked with a small amount of PCC to ensure complete oxidation. After being stirred for another 30 min, an equal volume of anhydrous ether was added, and the resulting mixture was stirred vigorously for an additional 30 min. The solution was then filtered through silica gel and washed extensively with ether. The combined filtrates were evaporated to dryness to give the desired product **18**, along with a smaller amount of the 5′-oxidized product, as a white solid. The overall yield was 85%. Product **18** exists as a mixture of the aldehyde and the corresponding hemiacetal form in solution, whereas the 5′-oxidized byproduct is mainly in the hemiacetal form. Since the components of this mixture have similiar R_f values, it was difficult to fully purify the desired product **18**. Compound **18** in its aldehyde form are as follows. ^{1}H NMR ($CDCl_3$): δ 10.13 (1H; d, J = 2.3; 4′-H), 8.75 (1H; s; 6-H), 4.88 (2H; s; 5′-CH$_2$), 2.63 (3H; d, J = 3.3; 2-Me). ^{13}C NMR ($CDCl_3$): δ 193.1 (C-4′), 160−130 (Ar C's), 54.2 (C-5′, d, J = 7.2), 19.3 (2-Me). ^{1}H NMR ($CDCl_3$) of the hemiacetal form of compound **18**: δ 8.26 (1H; s; 6-H), 6.65 (1H; d, J = 1.9; 4′-H), 5.30, 5.05 (1H each; ABq, J = 13.3; 5′-CH$_2$),* 2.56 (3H; d, J = 3.0; 2-Me). ^{1}H NMR ($CDCl_3$) of the hemiacetal form of the 5′-oxidized product: δ 8.38 (1H; s; 6-H), 6.57 (1H; s; 5′-H), 5.27, 5.09 (1H each; ABq, J = 13.3; 4′-CH$_2$),* 2.56 (3H; d, J = 3.0; 2-Me). ^{13}C NMR ($CDCl_3$) of the mixture of both hemiacetals: δ 160−130 (Ar C's), 100.4, 99.8 (C-4′), 70.4, 68.8 (C-5′), 17.4 (2-Me's). High-resolution FAB-MS of **18**; calcd for $C_8H_9FNO_2$ (M + 1)$^+$ 170.0617, found 170.0609.

3-Deoxy-3-fluoropyridoxal Oxime (19). To a suspension of **18** (20 mg, 0.12 mmol) in water (3 mL) were added sodium acetate (12.9 mg, 0.16 mmol) and hydroxylamine hydrochloride (12.3 mg, 0.18 mmol). This mixture was heated to 100 °C for a few minutes until the solids dissolved and then cooled to room temperature, during which the oxime products precipitated. The precipitates were collected, washed with water, and dried. Two oxime products were formed, and they were separable by flash chromatography (60% EtOAc/hexane). The desired 4′-oxime product **19** was isolated in 78% yield. Mp: 208−210 °C dec. TLC (EtOAc): $R_f = 0.35$. ^{1}H NMR (CD_3OD): δ 8.46 (1H; s; 4′-CH), 8.45 (1H; s; 6-H), 4.83 (2H; s; 5′-CH$_2$), 2.55 (3H; d, J = 3.2; 2-Me). ^{13}C NMR (CD_3OD): δ 158.3 (C-3, d, J = 258.6), 148.3 (C-2, d, J = 18.5),* 145.9 (C-4, d, J = 6.7),* 144.0 (C-6, d, J = 5.7), 137.2 (C-5), 128.7 (C-4′, d, J = 9.8), 63.0 (C-5′), 18.8 (2-Me). High-resolution FAB-MS: calcd for $C_8H_{10}FN_2O_2$ (M + 1)$^+$ 185.0726, found 185.0717.

3-Deoxy-3-fluoropyridoxamine (20). To a solution of oxime **19** (100 mg, 0.54 mmol) in methanol (10 mL) was added 10% palladium on activated carbon (50 mg). The resulting mixture was stirred under hydrogen at room temperature for 5 h. The catalyst was removed by filtration and the filtrate concentrated to dryness. The desired product was isolated in 88% yield. TLC (40% MeOH/CHCl$_3$): $R_f = 0.41$. ^{1}H NMR (CD_3OD): δ 8.26 (1H; s; 6-H), 4.75 (2H; s; 5′-CH$_2$), 3.99 (2H; d; J = 1.4; 4′-CH$_2$), 2.54 (3H; d, J = 3.1; 2-Me). ^{13}C NMR (CD_3OD): δ 159.3 (C-3, d, J = 253.0), 148.7 (C-2, d, J = 19.7), 146.6 (C-6, d, J = 6.0), 139.6 (C-4, d, J = 13.4), 138.0 (C-5), 62.0 (C-5′), 37.6 (C-4′, d, J = 5.0), 18.9 (2-Me). High resolution FAB-MS: calcd for $C_8H_{12}FN_2O$ (M + 1)$^+$ 171.0855, found 171.0935.

3-Deoxy-3-fluoropyridoxamine 5′-Phosphate (8). To compound **20** (30 mg, 176 μmol) was added 10 times its weight of anhydrous phosphoric acid under nitrogen, and the mixture was heated to 100 °C for 24 h. Nine volumes of absolute ethanol was added slowly with stirring to the cooled reaction mixture to yield a white precipitate, which, after being collected and washed successively with absolute ethanol and ether, was dissolved in a minimal amount of water and brought to about pH 6 with concentrated ammonium hydroxide. This mixture was applied to the top of an Amberlite XE-64 column (a fine mesh, weak cation exchange resin) in the washed free acid form. The effluent fractions from the column, on elution with water, were examined by TLC. Fractions of the desired product were combined and lyophilized to give a white solid in 43% yield. TLC (1-propanol:NH$_4$OH:H$_2$O = 6:3:1): $R_f = 0.45$. Mp: 205−206 °C. ^{1}H NMR (D_2O): δ 8.40 (1H; s; 6-H), 5.10 (2H; d, J = 7.4; 5′-CH$_2$), 4.47 (2H; d, J = 1.0; 4′-CH$_2$), 2.59 (3H; d, J = 3.0; 2-Me). ^{13}C NMR (D_2O): δ 159.6 (C-3, d, J = 256.6), 150.9 (C-2, d, J = 19.1), 147.5 (C-6, d, J = 5.7), 134.8 (C-5, d, J = 7.3), 131.1 (C-4, d, J = 13.4), 65.4 (C-5′, d, J_{C-P} = 4.9), 36.2 (C-4′, d, J_{C-F} = 5.1), 19.5 (2-Me). ^{19}F NMR (D_2O as solvent, CFCl$_3$ as external standard): δ −125.9 (d, J = 2.7). ^{31}P NMR (D_2O as solvent, 85% H$_3$PO$_4$ as the external standard): δ 0.84 (t, J = 7.4). High-resolution FAB-MS: calcd for $C_8H_{13}FN_2O_4P$ (M + 1)$^+$ 251.0596, found 251.0586.

[4′-^{3}H]-3-Deoxy-3-fluoropyridoxamine (21). To a mixture of sodium borohydride (19 mg, 0.5 mmol) and titanium(IV) chloride (54.9 μL, 0.5 mmol) in dry 1,2-dimethoxyethane (DME, 6 mL) at 0 °C was added [^{3}H]NaBH$_4$ (1 mmol, 100 mCi), followed by compound **19** (100 mg, 0.538 mmol) in DME (20 mL). After stirring for 4 h, additional titanium(IV) chloride (295 μL, 2.69 mmol) and sodium borohydride (204 mg, 5.38 mmol) in DME (10 mL) were added to drive the reaction to completion. The reaction was quenched 17 h later with water, and the solution was neutralized with an ammonium hydroxide solution. The product was extracted with ether, and the combined organic extracts were dried, concentrated, and purified by flash chromatography (40% MeOH/CHCl$_3$) to give **21** as a white solid in 52% yield. The specific activity of tritium of this sample was 1.98 mCi/mmol.

[4′-^{3}H]-3-Deoxy-3-fluoropyridoxamine 5′-Phosphate (22). The labeled compound **21** (38 mg, 0.22 mmol) was mixed with the unlabeled **20** (48 mg, 0.28 mmol) and then reacted with anhydrous phosphoric acid (1.5 g) under nitrogen. After heating at 100 °C for 36 h, the mixture was cooled and treated with absolute ethanol (1.5 mL). The resulting precipitate was collected by filtration, washed with ether and absolute ethanol, and redissolved in a minimal amount of water. This solution was brought to pH 6 with concentrated ammonium hydroxide and purified by an Amberlite XE-64 column. Fractions containing the desired product were combined and lyophilized to give **22** as a white solid (39 mg) in 31% yield. The specific activity (SA) of tritium of this sample was 0.89 mCi/mmol.

GC-MS Assay. The **E$_1$**−**E$_3$** product formation was detected by a previously reported GC−MS procedure.[4b] A few minor modifications included (1) the enzymes were removed by placing the solution is a microcon 10 microconcentrator (Amicon, Beverly, MA) and spinning at 13000g for 30 min; (2) the crude products were not purified by HPLC, and (3) the amounts of reagents used in the derivatization were cut in half. The same procedure was also repeated using F-PMP (0.5 mg, 2.0 μmol) in place of PMP. Incubations with denatured **E$_1$** and/or **E$_3$** under identical conditions served as the controls.

Incubation of E_1 with CDP-D-glucose and PMP or F-PMP in [^{18}O]H_2O. The GC−MS assay described above was applied to detect the extent of ^{18}O incorporation into samples derived from incubation of substrate with E_1 in the presence of PMP or F-PMP (**8**). Except for the use of buffer prepared with [^{18}O]H_2O and the omission of E_3 in the incubation, the experiments followed the aforementioned procedure (Scheme 3). Additionally, after the incubation, $NaBH_4$ reduction, and enzyme removal were complete, another lyophilization step was added to remove the [^{18}O]H_2O. The resulting solids were redissolved in [^{16}O]H_2O and then subjected to acid hydrolysis to remove the CDP group. The control was prepared with denatured E_1.

Tritium Release Assay. Whether the C-4′ proton of the PMP coenzyme was labile during the incubation with E_1 and substrate was examined by a previously reported procedure using tritium-labeled F-PMP (**22**; 2.0 μg, 8 nmol) and E_1 (256 μg) with minor modifications.[4b] The control experiment was carried out with [4′-^{3}H]PMP (0.04 μg, 0.15 nmol, SA = 44.9 mCi/mmol) and a smaller amount of E_1 (10 μg) under identical conditions.

E_1−E_3 Coupled Assay. The competence of F-PMP as an effective coenzyme for E_1 was also assessed by an E_1−E_3 coupled assay previously reported by Lei et al.[23] Briefly, the E_1 substrate (0.1 μmol, 100 μM) was mixed with NADH (200 μM), F-PMP (10−100 molar equiv versus E_1 monomer), and E_3 (15.8 μg) in 20 mM Tris·HCl buffer, pH 7.5 (total volume 990 μL). The reaction was initiated by the addition of E_1 (80 μg, in 10 μL of the same buffer), and the overall catalysis was followed by monitoring the rate of reduction of the absorbance at 340 nm within the first 1 min after E_1 addition. An identical experiment in which PMP (25 μM) was used as the coenzyme was run in parallel for comparison. A sample without added coenzyme served as the control.

Competition between F-PMP and PMP for Binding with E_1. The E_1 (149 μg, 10 μL) was incubated with F-PMP (50 equiv per E_1 monomer) in 20 mM Tris·HCl buffer (pH 7.5) at room temperature for 3 h. To this solution was added PMP (10 equiv per E_1 monomer). The resulting mixture was incubated for 10 min, followed by the addition of a premixed assay solution (total volume 810 μL) containing NADH (200 μM), CDP-D-glucose (100 μM) and an appropriate amount of E_3 in 20 mM Tris·HCl, pH 7.5. This solution was mixed well, and the rate of reduction of the absorbance at 340 nm was measured. This experiment was repeated in which 100 equiv of F-PMP was used in the preincubation with E_1. The full activity was determined by the same procedure, except omitting the step of preincubating with F-PMP. The control for this assay included everything except coenzymes (both F-PMP and PMP). This background reading reflected the residual activity of the E_1 sample which was subtracted from the activity values determined above.

Determination of the pK_a of N-1 of PMP and F-PMP. The PMP in its HCl salt form (400 mg, 1.4 mmol) was dissolved in 10 mL of 50 mM potassium phosphate buffer, pH 5.4. Dioxane (2.6 mg, 25 μL of a 103.4 mg/mL solution) was added as a ^{13}C NMR internal reference. An aliquot of 500 μL of this solution was transferred to a NMR tube and diluted with two drops of D_2O. The pH of the remaining solution was adjusted to 6.2 with a concentrated KOH solution, and again a 500 μL aliquot of the resulting solution was transferred to a NMR tube along with two drops of D_2O. The same procedure was repeated in which the pH was increased by 0.3−0.5 unit each time, and aliquots of 500 μL were taken at each pH for ^{13}C NMR measurement. A total of 19 samples with pH varied from 5.4 to 13.0 were prepared. To determine the pK_a of the ring nitrogen of F-PMP, F-PMP (18.8 mg, 75 μmol) was dissolved in 500 μL of 50 mM potassium phosphate. The pH of this solution, measured with a microelectrode, was adjusted to 1.2 with concentrated phosphoric acid. Again, dioxane was added as the internal reference. The sample was placed in a NMR tube and a ^{13}C-NMR spectrum recorded. After the first spectrum was taken, the sample was retrieved from the NMR tube and the pH adjusted up 0.3−0.5 unit with a concentrated KOH solution. The sample was transferred back to a NMR tube to record the next ^{13}C NMR spectrum. This procedure was repeated until 12 spectra were taken at different pH values ranging from 1.2 to 5.2. The changes of the chemical shift of C-6 of PMP and/or F-PMP were followed during these experiments.

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Supporting Information Available: Spectral data of compounds **9**−**13** and the detailed assay conditions (4 pages). See any current masthead page for ordering and Internet access instructions.

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