Synthesis of a Deoxysugar Dinucleotide Containing an *exo*-Difluoromethylene Moiety As a Mechanistic Probe for Studying Enzymes Involved in Unusual Sugar Biosynthesis

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Introduction

Fluorine-containing compounds, while few are naturally occurring,[1] have attracted much attention due to their potential as enzyme inhibitors/chemotherapeutic agents.[2] It has been well documented that when fluorine(s) are introduced at appropriate loci, the chemical properties as well as the biological activities of the resulting compounds could be significantly altered.[3] In view of their potential applications, a diverse array of fluorine-containing molecules have been prepared and their activities evaluated. Particularly notable are compounds carrying a difluoromethylene moiety which can act as a bioisostere for aldehydes and ketones.[4] This functional group is chemically reactive under both ionic[5] and radical reaction conditions[6] and has been incorporated in the structure of a few mechanism-based inhibitors[7] of enzymes including squalene epoxidase,[7a] oxidosqualene cyclase,[7b] and monoamine oxidase.[7c] In addition, a number of difluoromethylene-containing compounds have been shown to possess antiviral[8] and/or antifungal activities.[9]

In an effort to explore nature's strategy for making unusual sugars,[10] we have recently isolated and characterized an important enzyme involved in the biosynthesis of yersiniose A,[11] which is an immunodominant branched-chain sugar found in the *O*-antigen of *Yersinia pseudotuberculosis* VI. This enzyme, YerE, is a thiamine pyrophosphate (TPP) dependent flavoenzyme that catalyzes the attachment of the branched-chain to a 3,6-dideoxy-4-hexulose precursor **3** to give **6** using pyruvate **2** as the side chain donor. As illustrated in Scheme 1, the reaction is initiated by the addition of TPP to pyruvate **2**, followed by the decarboxylation of the initial adduct to generate a nucleophilic carbanionic species **4**. This two-carbon acyl anion equivalent will then be used to attack the 4-keto group of **3** to give **5**. The subsequent breakdown of the coupling adduct **5** produces **6** and regenerates the TPP coenzyme. The last step of yersiniose A biosynthesis is the reduction of **6** by an NADPH-dependent reductase, YerF, to yield CDP-yersiniose A (**1**). Since the activity of YerE was not affected by the presence of the reducing agent dithionite, the enzyme bound FAD is unlikely to play a redox role in this transformation.[11]

In an attempt to develop methods of controlling this enzymatic transformation, which is essential to the biosynthesis of yersiniose, we prepared substrate analogues that, upon incubation with YerE, would lead to either inhibition or turnover, depending on the mode of catalysis. In view of the unique chemical, as well as steric, properties of the difluoromethylene group and the mechanistic insights gained from our early studies on the catalysis of YerE, we chose to pursue the synthesis of an *exo*-difluoromethylene containing analogue **7** as a potential inhibitor. If this compound is recognized by YerE, its difluoromethylene moiety may react with the nucleophilic acyl anion equivalent **4** at the active site to generate **8**. As illustrated in Scheme 2, the breakdown of this adduct may occur via two distinct routes. Pathway A is the same as that observed in normal catalysis. Carbon−carbon bond scission via this route resulted in a doubly branched-chain sugar **9** as the new product, together with the regeneration of TPP coenzyme after each catalytic cycle. In contrast, cleavage by route B gives a new product **10**, accompanied by the release of a fluoride anion. Since the TPP coenzyme is acetylated after each turnover, the enzyme is expected to be inactivated. This modified TPP coenzyme **11** may also function as an acylating agent to label an active-site nucleophile. Therefore, even the TPP coenzyme may be regenerated at the end, the covalently modified enzyme **12** may still be impaired.

It should be noted that nucleophilic addition to 1,1-difluoroolefins usually occurs at the terminal difluoromethylene carbon.[12] Therefore, if the acceptor **7** binds in the active-site in a manner that it is accessible to the

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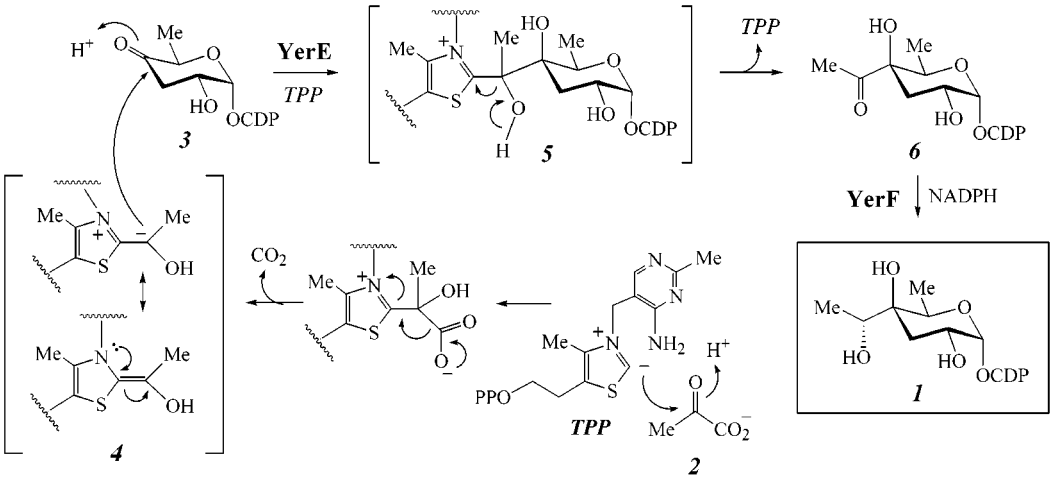
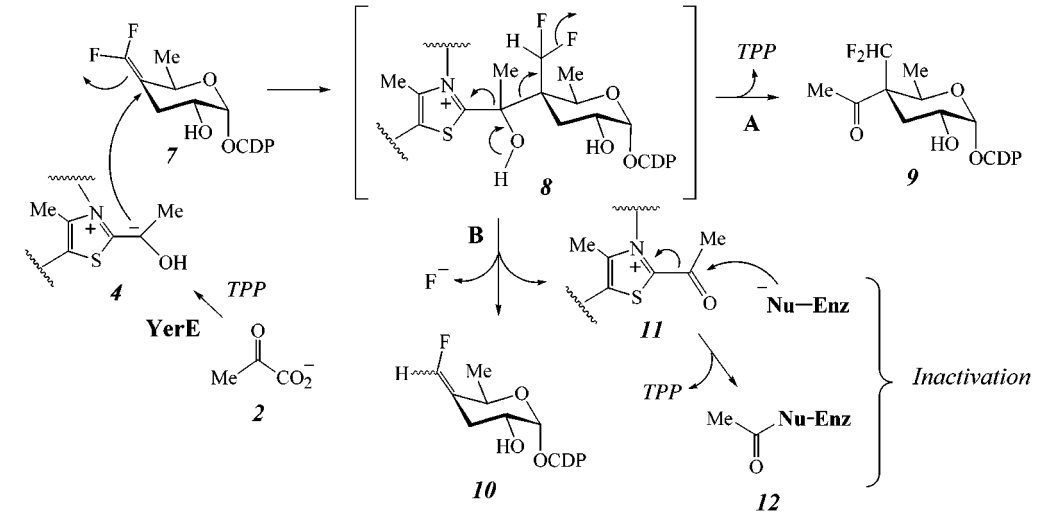
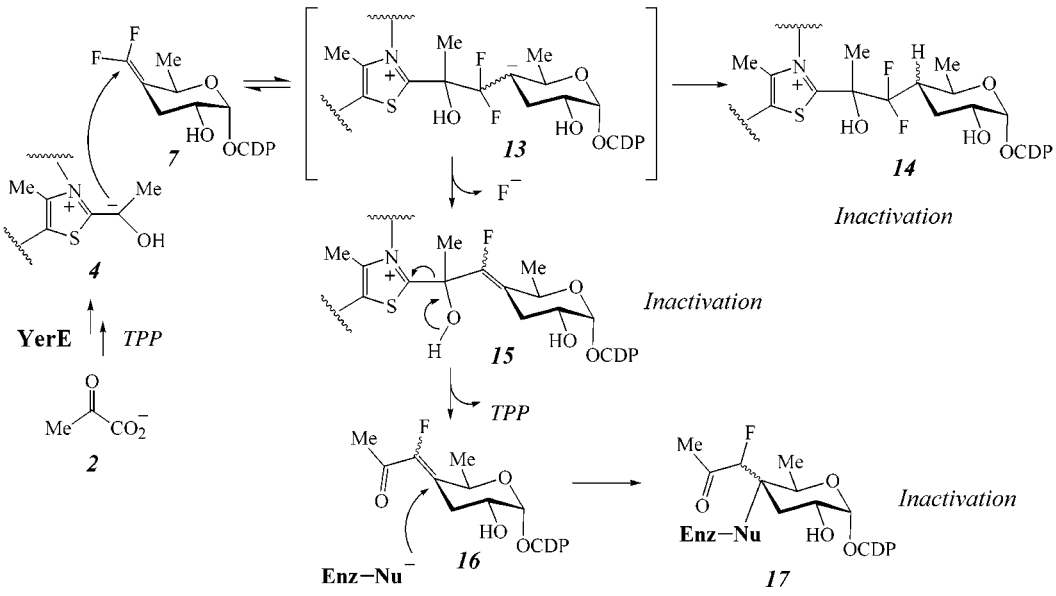
Scheme 1



Scheme 2

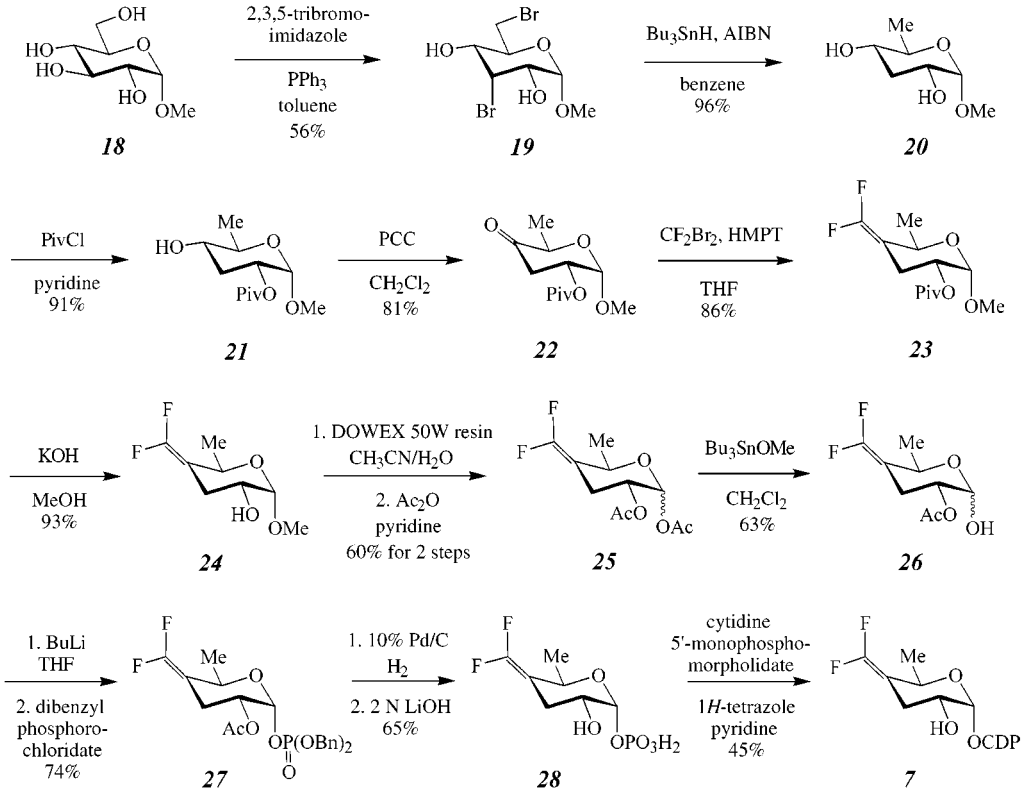


Scheme 3



nucleophile **4**, the chemical reactivity of the difluoromethylene moiety may direct the acyl anion equivalent **4** to attack its terminal carbon as shown in Scheme 3. The anion intermediate **13** may be protonated to give **14**, or it may undergo elimination to yield **15**. In either case, the enzyme is expected to be inactivated. Even if **15** can be further converted to **16** to generate TPP coenzyme, the resulting product **16** is a potential Michael acceptor that may trap an active site nucleophile to give **17**, leading to enzyme inactivation. Clearly, compound **7**

Scheme 4



holds promise as a mechanism-based inhibitor. In this paper, we report an efficient synthesis of this nucleotide sugar and the incubation results with the target enzyme YerE.

Results and Discussion

To test our idea, we have developed a convenient strategy to synthesize the desired molecule. The first phase of our synthesis called for the construction of 4-keto-3,6-dideoxyhexose **22**. As depicted in Scheme 4, a two-step procedure that was originally reported by Classon et al.[13] was used to make the 3,6-dideoxysugar precursor **20** from methyl α-D-glucopyranoside **18**. Fresh 2,3,5-tribromoimidazole[14] is essential to obtain the dibromide intermediate **19** in good yield. The C-2 hydroxyl group of **20** was then selectively acylated with pivaloyl chloride.[15] The C-4 hydroxy group of **21** was oxidized by PCC to give the 4-keto-sugar **22**. The incorporation of an *exo*-difluoromethylene moiety into **22** was accomplished using a Wittig-type reagent generated in situ with hexamethylphosphorus triamide (HMPT) and dibromodifluoromethane.[16] In this experiment, HMPT was added dropwise to the solution of **22** and CF_2Br_2 in dry THF at 0 °C, followed by warming the reaction mixture to room temperature. This modified procedure not only gave a very clean reaction with high yield but also alleviated the requirement of zinc metal in the reaction.[17]

Methyl pyranosides have been frequently used as starting material for carbohydrate synthesis; however, difficulties are often encountered when trying to remove the anomeric methoxy group after chemically sensitive functionalities are introduced in the target molecules. Indeed, complication was observed in our attempt to eliminate the C-1 methoxy moiety of **23** while keeping both the ester at C-2 and the difluoroolefin group at C-4 intact. Due to these complications, a three-step sequence was developed to convert **23** to its C-1 acetate derivative. As shown in Scheme 4, the pivaloyl ester of **23** was hydrolyzed first and the resulting product **24** was treated with an acidic resin in a mixture of acetonitrile and water. Then, acylation under basic condition produced **25** as a mixture of two anomeric isomers. Direct acetolysis occurred by treating **24** with acetic anhydride in the presence of catalytic amount of either sulfuric acid or triflic acid; however, this led to a defluorinated mixture as shown by ^{19}F NMR. It is worth mentioning that when a benzyl ether was used instead of pivaloyl ester to protect the C-2 hydroxy group in **20**, it could not be removed without destroying the difluoromethylene functionality.[18a]

Problems were also encountered during the deprotection of C-1 hydroxyl in **25**. Commonly used reagents such as hydrazine, hydrazine acetate, and benzylamine produced complicated results. Although the products were not well characterized, in most cases, the double bond seemed to have migrated into the pyranose ring upon treatment with these organic bases. Fortunately, this problem could be overcome by heating **25** with tributyltin methoxide in methylene dichloride.[19] The subsequent

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conversion of **26** to **27** was initially attempted by treatment of **26** with dibenzyl *N*,*N*-diethylphosphoramidite, followed by an oxidation step.[20] However, this two-step procedure via a phosphite intermediate gave very poor yield along with troubles in column purification. The transformation, however, could be readily achieved using dibenzyl phosphochloridate as the phosphatylation reagent.[21] This procedure has proven to be very dependable in our hands for the synthesis of diphosphonucleotidyl sugars in this laboratory.[18b] Judging from the coupling constants of 3.3 Hz for $^3J_{H-H}$ and 6.3 Hz for $^3J_{H-P}$ associated with the 1-H signal, product **27** clearly has the desired a configuration at C-1.

Compound **27** was then hydrogenolyzed and treated with 2 N lithium hydroxide to give **28**. The reaction sequence and conditions for the deprotection of **27** were very critical to produce **28** in good yield with high purity. Hydrogenolysis of the *O*-benzyl group of **27** should be performed for less than 10 min to prevent over reduction of the difluoromethylene double bond. Furthermore, alkaline hydrolysis to remove the acetyl group should be carried out after hydrogenation; this avoided deletion of the dibenzyl phosphate functionality. The final step of this synthesis was the 1*H*-tetrazole-promoted coupling of **28** with cytidine 5′-monophosphomorpholidate.[22] The desired product **7** was isolated by Bio-Gel P2 column eluted with 50 mM ammonium bicarbonate and characterized by NMR and MS.

Unfortunately, incubation of YerE with compound **7** showed no apparent effect on the enzyme activity. ^{19}F NMR analysis also failed to detect the release of fluoride anion during incubation. In addition, no new product was discernible in the incubation mixture by HPLC analysis. It is thus clear that **7** is neither an inhibitor nor a substrate for YerE. This result is disappointing; however, it only indicates that the active site of YerE is less flexible to accommodate this specific compound. Since many enzymes are known to be promiscuous with respect to substrate recognition, the strategy discussed in this paper may have general applicability to the design of probes to further the study of the mechanisms of related enzymatic catalyses. The chemistry described here should also be useful for the synthesis of compounds, especially modified sugar dinucleotides, containing an *exo*-difluoromethylene functionality.

Experimental Section

All chemicals were products of Aldrich Co. (Milwaukee, WI), unless otherwise specified. NMR spectra (^{1}H at 300 MHz, ^{13}C at 75 MHz, ^{19}F at 282 MHz, and ^{31}P at 121 MHz) were recorded with a Varian U-300 Spectrometer in $CDCl_3$, unless otherwise specified in the text. Chemical shifts (δ in ppm) are given relative to those for Me_4Si (for ^{1}H), $CDCl_3$ (for ^{13}C), external $CFCl_3$ (for ^{19}F), and external aqueous 85% H_3PO_4 (for ^{31}P). High-resolution mass spectroscopy was performed on a Finnigan Mat 95 (for HR-CI and HR-EI) or a VG Analytical 7070E-HF (for FAB) instrument. Flash chromatography was performed on Lagand Chemical silica gel (230−400 mesh) by elution with the specified solvents. Analytical thin-layer chromatography (TLC) was carried out on Polygram Sil G/UV_{254} plates (0.25 mm). Bio-Gel P2 resin (extra fine) for size-exclusion chromatography was purchased from Bio-Rad Laboratories (Hercules, CA). TLC spots were visualized by heating the plate previously stained with a solution of phosphomolybdic acid (3% in EtOH). Dry THF was distilled over sodium and benzophenone.

Preparation of 2,3,5-Tribromoimidazole. To a stirred solution of imidazole (68 g, 1.0 mol) in chloroform (500 mL) was added dropwise Br_2 (51.3 mL, 1.0 mmol) at room temperature. The resulting mixture was stirred for 1 h. The yellowish solid was collected by filtration, washed thoroughly with water, and then recrystallized from 95% ethanol. The desired tribromide[14] was isolated as a yellow solid (56 g, 55%).

Methyl 3,6-Dibromo-3,6-dideoxy-α-D-*allo*-hexopyranoside (19). A mixture of methyl α-D-glucoside **18** (22 g, 0.11 mol), PPh_3 (130 g, 0.49 mol), and tribromoimidazole (75 g, 0.24 mol) in toluene (1 L) was heated at 85 °C for 1 h and at 120 °C for 5 h. The mixture was cooled to room temperature and mixed with saturated sodium bicarbonate solution (600 mL). After the mixture was stirred for 10 min, I_2 was introduced in small increments with vigorous stirring until the solution turned purple. Excess I_2 was quenched with aqueous sodium thiosulfate solution. The organic layer was separated, and the aqueous layer was extracted with ether. The combined organic extracts were washed with brine, dried over sodium sulfate, and concentrated in vacuo. The solid residue was redissolved in a minimum amount of hot ethyl acetate. After cooling of the mixture to room temperature, the precipitate was filtered off and the filtrate was concentrated. The crude product was purified by silica gel chromatography (hexanes:ethyl acetate, 3:1) to give the 3,6-dibromosugar **19**[13] (20.5 g, 56%) as a white solid.

Methyl 3,6-Dideoxy-α-D-*ribo*-hexopyranoside (20). A suspension of the 3,6-dibromosugar **19** (20.4 g, 63 mmol), tributyltin hydride (42 g, 144 mmol), and AIBN (0.5 g, 3 mmol) in benzene (700 mL) was refluxed for 9 h under a nitrogen atmosphere. The solvent was removed in vacuo, and the residue was chromatographed on silica gel (hexanes:ethyl acetate, 2:1 to 1:2) to afford the 3,6-dideoxysugar **20**[13] (10.0 g, 96%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 4.58 (1H, d, J = 3.6 Hz), 3.70 (1H, m), 3.46 (1H, m), 3.43 (3H, s), 3.26 (1H, m), 2.18 (1H, m), 2.10 (1H, br s), 1.80 (1H, br s), 1.63 (1H, m), 1.25 (3H, d, J = 6.9 Hz). ^{13}C NMR ($CDCl_3$): δ 98.3, 70.8, 68.7, 67.6, 55.1, 37.0, 17.4.

Methyl 3,6-Dideoxy-2-*O*-pivaloyl-α-D-*ribo*-hexopyranoside (21). To a solution of **20** (4.8 g, 30 mmol) in pyridine (50 mL) was added portionwise an excess of pivaloyl chloride (12 mL) at 0 °C. The mixture was stirred for 2 h at the same temperature and then diluted with ethyl acetate. The resulting solution was washed successively with 5% HCl, brine, saturated sodium bicarbonate solution, and brine and dried over sodium sulfate. The solvents were removed in vacuo, and the residue was purified by silica gel chromatography (hexanes:ethyl acetate, 5:1) to give the pivalate **21** (6.7 g, 91%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 4.73 (1H, m), 4.66 (1H, m), 3.52 (1H, m), 3.34 (3H, s), 3.30 (1H, m), 2.50 (1H, br s), 2.05 (1H, m), 1.82 (1H, m), 1.21 (3H, d, J = 6.0 Hz), 1.14 (9H, s). ^{13}C NMR ($CDCl_3$): δ 178.1, 96.1, 70.7, 68.9, 68.5, 55.1, 38.7, 32.5, 27.0, 17.4. HRMS (CI): calcd for $C_{12}H_{23}O_5$ $[M + H]^+$, 247.1545; found, 247.1538.

Methyl 3,6-Dideoxy-2-*O*-pivaloyl-α-D-*erythro*-hexopyranosid-4-ulose (22). A mixture of pivalate **21** (0.58 g, 2.3 mmol), sodium acetate (0.39 g, 4.7 mmol), and PCC (2.06 g, 9.6 mmol) in dry CH_2Cl_2 (25 mL) was vigorously stirred at room temperature for 12 h. The resulting dark-brown mixture was filtered through a short silica gel column and washed with ether. The filtrate was collected and concentrated in vacuo. The residue was chromatographed on silica gel (hexanes:ethyl acetate, 5:1) to afford the keto sugar **22** (0.47 g, 81%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 5.11 (1H, ddd, J = 9.3, 6.9, 3.6 Hz), 4.93 (1H, d, J = 3.6 Hz), 4.16 (1H, q, J = 6.6 Hz), 3.46 (3H, s), 2.72 (1H, d, J = 6.9 Hz), 2.71 (1H, d, J = 9.3 Hz), 1.28 (3H, d, J = 6.6 Hz), 1.18 (9H, s). ^{13}C NMR ($CDCl_3$): δ 205.8, 177.5, 96.3, 70.4, 68.3, 55.9, 39.8, 38.7, 27.0, 14.8. HRMS (CI): calcd for $C_{12}H_{21}O_5$ $[M + H]^+$, 245.1389; found, 245.1347.

Methyl 3,4,6-Trideoxy-4-*C*-difluoromethylene-2-*O*-pivaloyl-α-D-*erythro*-hexopyranoside (23). To a solution of keto sugar **22** (2.56 g, 10.4 mmol) and dibromodifluoromethane (2.9 mL, 31 mmol) in dry THF (70 mL) was added dropwise HMPT (7.8 mL, 41 mmol) at 0 °C. After being vigorously stirred at room temperature for 2 h, the mixture was diluted with water and extracted with ethyl acetate. The organic extracts were dried over sodium sulfate and concentrated in vacuo. The residual oil was chromatographed on silica gel with hexanes to afford the

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difluoro sugar **23** (2.51 g, 86%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 4.78 (1H, m), 4.75 (1H, m), 4.41 (1H, m), 3.40 (3H, s), 2.51 (1H, m), 2.38 (1H, m), 1.40 (3H, dd, J = 6.6, 5.4 Hz), 1.19 (9H, s). ^{13}C NMR ($CDCl_3$): δ 177.7, 151.6 (dd, J = 289.2, 284.4 Hz), 96.8, 86.5 (dd, J = 18.5, 15.4 Hz), 68.7, 62.1 (t, J = 4.2 Hz), 55.4, 38.6, 26.9, 23.3 (d, J = 2.6 Hz), 18.2 (d, J = 7.4 Hz). ^{19}F NMR ($CDCl_3$): δ −99.1 (1F, d, J = 54.2 Hz), −101.7 (1F, dd, J = 54.2, 2.8 Hz). HRMS (CI): calcd for $C_{13}H_{21}F_2O_5$ $[M + H]^+$, 279.1408; found, 279.1412.

Methyl 4-*C*-Difluoromethylene-3,4,6-trideoxy-α-D-*erythro*-hexopyranoside (24). A solution of compound **23** (2.48 g, 8.9 mmol) in methanol (60 mL) containing potassium hydroxide (1.75 g, 31 mmol) was stirred at room temperature for 2 h, then filtered through a short silica gel column, and washed with THF. The filtrates were concentrated, and the residue was chromatographed on silica gel (hexanes:ethyl acetate, 5:1 to 2:1) to afford **24** (1.8 g, 93%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 4.69 (1H, d, J = 3.3 Hz), 4.39 (1H, m), 3.74 (1H, m), 3.47 (3H, s), 2.57 (1H, dd, J = 13.5, 5.4 Hz), 2.21 (1H, m), 2.06 (1H, br s), 1.41 (3H, dd, J = 6.9, 5.1 Hz). ^{13}C NMR ($CDCl_3$): δ 151.5 (dd, J = 289.7, 284.4 Hz), 98.7, 86.4 (dd, J = 19.1, 14.2 Hz), 67.3, 62.6 (dd, J = 4.7, 3.7 Hz), 55.4, 27.2, 18.0 (d, J = 7.4 Hz). ^{19}F NMR ($CDCl_3$): δ −89.7 (1F, d, J = 54.1 Hz), −92.4 (1F, dd, J = 54.1, 6.2 Hz). HRMS (CI): calcd for $C_8H_{16}NF_2O_3$ $[M + NH_4]^+$, 212.1098; found, 212.1090.

2-*O*-Acetyl-3,4,6-trideoxy-4-*C*-difluoromethylene-α/β-D-*erythro*-hexopyranosyl Acetate (25). A suspension of compound **24** (0.45 g, 2.3 mmol) and Dowex 50WX4-200 strongly acidic cation resin (H^+ type, ∼1 g) in a mixture of acetonitrile (4 mL) and water (16 mL) was stirred at 50 °C for 12 h. The resin was filtered off, and the filtrate was extracted with ethyl acetate. The combined organic extracts were dried over sodium sulfate and concentrated in vacuo. The residue was treated with a mixture of pyridine (3.0 mL) and acetyl anhydride (3.5 mL) for 2.5 h at 0 °C. The mixture was diluted with ethyl acetate and washed with 5% HCl, saturated sodium bicarbonate, and brine successively. After being dried over sodium sulfate, the solvents were evaporated under reduced pressure. The residue was chromatographed on silica gel (hexanes:ethyl acetate, 5:1 to 3:1) to give diacetyl sugar **25** (0.37 g, 60%) as a colorless oil, which was a mixture of two anomeric isomers. ^{1}H NMR ($CDCl_3$): δ 6.17 (0.4H, d, J = 3.3 Hz), 5.87 (0.6H, d, J = 3.3 Hz), 4.95 (0.4H, ddd, J = 8.4, 5.1, 3.3 Hz), 4.67 (0.6H, m), 4.63 (0.6H, m), 4.54 (0.4H, m), 2.66 (1H, m), 2.42 (1H, m), 2.11 (1.2H, s), 2.09 (1.8H, s), 2.07 (1.8H, s), 2.03 (1.2H, s), 1.41 (3H, m). ^{13}C NMR ($CDCl_3$): δ 169.9, 169.3, 169.0, 155.7, 155.3, 151.9, 151.5, 147.7, 91.4, 89.9, 89.3, 67.2, 65.2, 23.4, 21.2, 21.0, 20.9, 20.8, 18.2, 18.1. ^{19}F NMR ($CDCl_3$): δ −98.5 (0.43F, dd, J = 50.2, 2.8 Hz), −99.5 (0.57F, d, J = 51.4 Hz), −100.9 (0.43F, dd, J = 50.2, 2.8 Hz), −105.3 (0.57F, d, J = 51.4 Hz). HRMS (CI): calcd for $C_{11}H_{15}F_2O_5$ $[M + H]^+$, 265.0888; found, 265.0900.

Dibenzyl 2-*O*-Acetyl-3,4,6-trideoxy-4-*C*-difluoromethylene-α-D-*erythro*-hexopyranosyl Phosphate (27). A solution of peracetylated sugar **25** (0.36 g, 1.36 mmol) and tributyltin methoxide (0.5 mL) in dry CH_2Cl_2 (10 mL) was refluxed for 37 h under a nitrogen atmosphere. The mixture was concentrated, and the residue was chromatographed on silica gel (hexanes: ethyl acetate, 2:1) to yield the C1-deprotected intermediate **26** (0.17 g, 63%) as a colorless oil.

The intermediate **26** (0.168 g, 0.75 mmol) was dissolved in dry THF (10 mL) and cooled to −78 °C. *n*-BuLi solution (1.6 M in hexanes, 0.5 mL) was then added dropwise to this solution. After the mixture was stirred for 10 min at the same temperature, a solution of dibenzyl phosphorochloridate (prepared from 0.27 g of dibenzyl phosphite and 0.13 g of NCS in 4 mL of benzene)[21] in dry THF (4 mL) was added. The resulting solution was stirred at −78 °C for 1 h and then at 0 °C for 1 h. The mixture was concentrated at room temperature, diluted with ether, washed with water, and dried over sodium sulfate. The solvents were removed in vacuo, and the residue was purified by flash chromatography on silica gel (hexanes:ethyl acetate, 5:1 to 1:1) to give the desired product **27** (0.27 g, 74%) as a colorless oil. ^{1}H NMR ($CDCl_3$): δ 7.34 (10H, m), 5.79 (1H, dd, J = 6.3, 3.3 Hz), 5.08 (4H, m), 4.84 (1H, m), 4.50 (1H, m), 2.60 (1H, dd, J = 13.5, 5.4 Hz), 2.35 (1H, m), 1.91 (3H, s), 1.32 (3H, dd, J = 6.3, 6.0 Hz). ^{13}C NMR ($CDCl_3$): δ 189.8, 151.8 (dd, J = 291.3, 285.0 Hz), 135.6, 135.5, 128.6, 128.4, 127.8, 127.7, 94.5 (d, J = 5.3 Hz), 85.4 (dd, J = 19.5, 14.2 Hz), 69.3 (d, J = 4.8 Hz), 69.2 (d, J = 4.2 Hz), 68.2 (m), 64.5 (t, J = 4.8 Hz), 22.8, 20.5, 18.0 (d, J = 7.9 Hz). ^{19}F NMR ($CDCl_3$): δ −97.6 (1F, d, J = 49.7 Hz), −99.9 (1F, dd, J = 49.7, 5.4 Hz). ^{31}P NMR ($CDCl_3$): δ −1.7. HRMS (FAB) calcd for $C_{23}H_{26}F_2PO_7$ $[M + H]^+$, 483.1378; found, 483.1381.

3,4,6-Trideoxy-4-*C*-difluoromethylene-α-D-*erythro*-hexopyranosyl Phosphate (28). A suspension of compound **27** (131 mg, 0.27 mmol) and 10% Pd/C catalyst (45 mg) in a mixture of methanol and ethyl acetate solution (1:1, 10 mL) was hydrogenated with a hydrogen balloon at room temperature for 8 min. The mixture was filtered through Celite. The filtrate was mixed with 3 drops of triethylamine and concentrated under reduced pressure to give a colorless oil. The oil was dissolved in 2 N LiOH solution (2 mL) and stirred for 11 h at room temperature. The mixture was then diluted with water (4 mL) and stirred with Dowex 50WX-200, a strongly acidic cation resin (H^+ type, ∼ .5 g), for 30 min. The resin was filtered off, and the aqueous solution was neutralized with triethylamine. Lyophilization of the solution gave the coupling precursor **28** (64 mg, 62%) as a white powder. ^{19}F NMR (D_2O): δ −100.4 (1F, d, J = 55.0 Hz), −104.5 (1F, d, J = 55.0 Hz). ^{31}P NMR (D_2O): δ 0.8. HRMS (FAB): calcd for $C_7H_{10}F_2PO_6$ $[M - H]^-$, 259.0182; found, 259.0175.

Cytidine 5′-(3,4,6-Trideoxy-4-*C*-difluoromethylene-α-D-*erythro*-hexopyranosyl diphosphate) (7). A suspension of precursor **28** (64 mg, ∼0.17 mmol) and cytidine 5′-monophosphomorpholidate (200 mg, 0.29 mmol) in dry pyridine (1.5 mL) was evaporated over an oil pump to form a white powder. To ensure the removal of any residual moisture, this procedure was repeated three times. Dry pyridine (2.0 mL) and 1*H*-tetrazole (33 mg, 0.47 mmol) were then added to the above solid. After the mixture was stirred at room temperature for 60 h, the solvents were removed under vacuum. The residue was purified by Bio-Gel P2 column (2 cm × 1.1 m), eluted with 50 mM ammonium bicarbonate. The desired fractions, as indicated by their UV absorption at 267 nm, were collected and lyophilized to give the desired product **7** (45 mg, 45%) as a white powder. ^{1}H NMR (D_2O): δ 7.81 (1H, d, J = 7.2 Hz), 5.95 (1H, d, J = 7.2 Hz), 5.79 (1H, d, J = 3.6 Hz), 5.35 (1H, dd, J = 7.5, 2.7 Hz), 4.54 (1H, m), 4.0−4.2 (5H, m), 3.66 (1H, m), 2.39 (1H, m), 2.19 (1H, m), 1.22 (3H, dd, J = 6.9, 3.9 Hz). ^{13}C NMR (D_2O): δ 164.2, 155.2, 151.5, 142.0, 96.1, 94.2, 94.1, 89.4, 85.1 (dd, J = 19.8, 15.8 Hz), 82.6 (d, J = 9.0 Hz), 74.3, 68.9, 66.5, 65.3, 64.3 (d, J = 5.3 Hz), 25.0, 17.0 (d, J = 6.6 Hz). ^{19}F NMR (D_2O): δ −99.7 (1F, d, J = 54.2 Hz), −103.3 (1F, d, J = 52.8 Hz). ^{31}P NMR (D_2O): δ −10.7 (1P, d, J = 21.4 Hz), −12.4 (1P, d, J = 20.8 Hz). HRMS (FAB): calcd for $C_{16}H_{22}F_2N_3O_{13}P_2$ $[M - H]^-$, 564.0604; found, 564.0585.

Purification of YerE.[23] The *yerE* gene was amplified from *Yersinia pseudotuberculosis* VI by polymerase chain reaction (PCR) and cloned into a pET24(+) vector. After transformation into *Escherichia coli* DH5α, the positive clone was isolated and the corresponding plasmid DNA, pHC32, was transformed into *E. coli* BL21(DE-3). The BL21(DE-3)-pHC32 cells were grown in Terrific Broth medium supplemented with kanamycin (30 μg/mL) at 37 °C until the OD_{600} reached 0.6. The culture was then induced with isopropyl-thio-β-galactoside (IPTG, 0.1 mM), and grew at 24 °C for an additional 15 h. The cells were harvested by centrifugation at 6500*g* for 5 min at 4 °C, resuspended in lysis buffer (50 mM sodium phosphate, 150 mM NaCl, 10 mM imidazole, pH 8.0), and purified by Ni−NTA affinity chromatography. The YerE protein was further purified by FPLC−MonoQ chromatography with a linear gradient of 100−500 mM NaCl in 20 mM Tris-HCl buffer, pH 7.5. The collected fractions were concentrated by an Amicon concentrator using a YM-10 membrane.

Incubation Results. Compound **7** (2.3 mg, 4.0 μmol) was incubated with thiamine pyrophosphate (2 μmol), pyruvate (83 μmol), $MgCl_2$ (1 μmol), and YerE (0.25 nmol) in 100 mM potassium phosphate buffer prepared with D_2O (pD 8.0, 450 μL) at 24 °C. Aliquots were taken at appropriate time intervals to check enzyme activity under standard assay conditions.[11,23] It

(23) Chen, H. Ph.D. Thesis, University of Minnesota, Minneapolis, MN, 1999.

was found that preincubation of YerE with **7** led to no loss of activity as compared to the control. The incubation was also monitored by ^{19}F NMR to detect the possible release of fluoride ion. Again, no new signal was discernible after incubation for 15 h. The reaction mixture was finally analyzed by HPLC equipped with a SAX anion exchange column. No new peaks were found.

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Supporting Information Available: Reproductions of ^{1}H NMR spectra of compounds **7** and **19**−**28**. This material is available free of charge via the Internet at http://pubs.acs.org.

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