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Synthesis of α-Deoxymono and Difluorohexopyranosyl 1-Phosphates and Kinetic Evaluation with Thymidylyl- and Guanidylyltransferases

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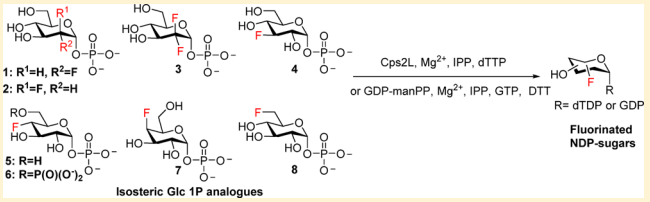
Supporting Information

ABSTRACT: Eight fluorinated isosteric α-D-glucopyranosyl 1-phosphate (**Glc 1P**) analogues have been synthesized. A promiscuity investigation of the thymidylyltransferase Cps2L and the guanidylyltansferase GDP-ManPP with these analogues showed that all were accepted by either enzyme, with the exception of 1,6-diphosphate **6**. Kinetic parameters were determined for these analogues using a continuous coupled assay. These data demonstrated the broad substrate promiscuity of Cps2L, with k_{cat}/K_m changes for monofluoro substitution at C-2, C-4, and C-6 and difluoro substitution at C-2 within two orders of magnitude. In contrast, the kinetic analysis of GDP-ManPP was only possible with three out of eight analogues. The pKa_2 values of analogues (**1**−**3**) were determined by proton decoupled ^{31}P and ^{19}F NMR titration experiments. Counterintuitively, the axial fluoro substituent in **3** did not change chemical shift upon titration, and there was no significant increase in acidity for the difluoro analogue over the monofluoro analogues. No strong Brønsted linear free-energy correlations were observed among all five substrates (**1**−**3**, **Glc 1P**, and **Man 1P**) for either enzyme-catalyzed reactions. However, Brønsted correlations were observed among selected substrates, indicating that the acidity of the nucleophilic phosphate and the configuration of the hexose each plays a significant role in determining the substrate specificity.



INTRODUCTION

Sugar nucleotides are essential to many biological processes, including acting as sugar donors in the biosynthesis of oligo- and polysaccharides by glycosyltransferases,[1] mediating cell−cell communication,[2] or as precursors of many deoxy-, amino-, or branched-chain sugars existing in many secondary metabolites produced by microorganisms or plants.[3] They are often biosynthesized from sugar 1-phosphates by nucleotidylyltransferases, also known as pyrophosphorylases (Scheme 1).[4] The mechanism of nucleotidylyltransferases has been described as ordered Bi−Bi where the nucleoside triphosphate binds first, followed by the sugar phosphate. Upon completion of the nucleophilic attack of the sugar phosphate upon the nucleoside triphosphate, pyrophosphate is released, followed by the sugar nucleotide.[5]

Cps2L[6] and guanosine diphosphate-mannopyranose pyrophosphatase (GDP-ManPP),[7] cloned from *Streptococcus pneumoniae* and *Salmonella enterica*, respectively, are responsible for catalyzing the synthesis of deoxythymidine diphosphate glucopyranose (dTDP-Glc) and guanosine diphosphate-mannopyranose (GDP-Man). Exploring the substrate specificity of these enzymes is integral to providing access to a variety of unnatural sugar nucleotides.[1a,4a,8] Unnatural sugar nucleotides prepared via chemical or enzymatic synthesis have potential applications as enzymes inhibitors[9] for drug discovery and as tools for providing insight into the structure and mechanism of enzymes. Fluorinated sugar nucleotides have received increasing attention since the C−F bond exhibits interesting biological properties,[9a,10] which is due to some unique features of the fluorine atom, including its high electronegativity, small size, ability to form hydrogen bonds, and resistance to metabolic transformation.[11] Furthermore, the preparation of unnatural sugar nucleotides will complement one-pot multienzyme (OPME) chemo-enzymatic approaches toward the post-translational modification of proteins to help examine the roles of glycans in biology.[12]

To expand our knowledge on the substrate specificity of these nucleotidylyltransferases and in particular the effects of acidity upon substrate reactivity, a small family of isosteric α-D-hexopyranosyl 1-phosphates with fluorine substituents on the glucopyranose ring (Figure 1) were designed and synthesized. Kinetic parameters were assessed using a continuous coupled enzyme assay involving inorganic pyrophosphatase (IPP), purine nucleoside phosphorylase (PNP), and xanthine oxidase (XO).[13] The use of the continuous assay for both enzymes permitted the collection of sufficient data for the construction of Brønsted linear free-energy relationships to analyze reaction rate data with measured pKa_2 values.

RESULTS AND DISCUSSION

Chemical Synthesis. The general strategy (Figure 2) used to synthesize these fluorinated α-D-hexopyranosyl 1-phosphates (**1**−**8**) included: (i) fluorine atom addition to monosaccharides

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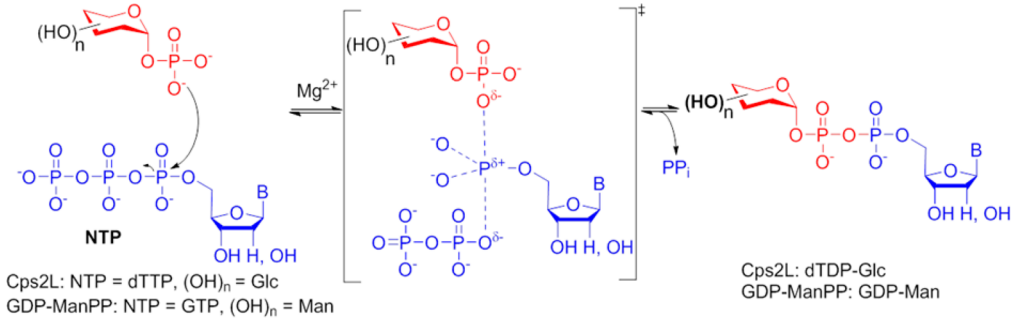
Scheme 1. General Mechanism of a Nucleotidylyltransferase-Catalyzed Reaction



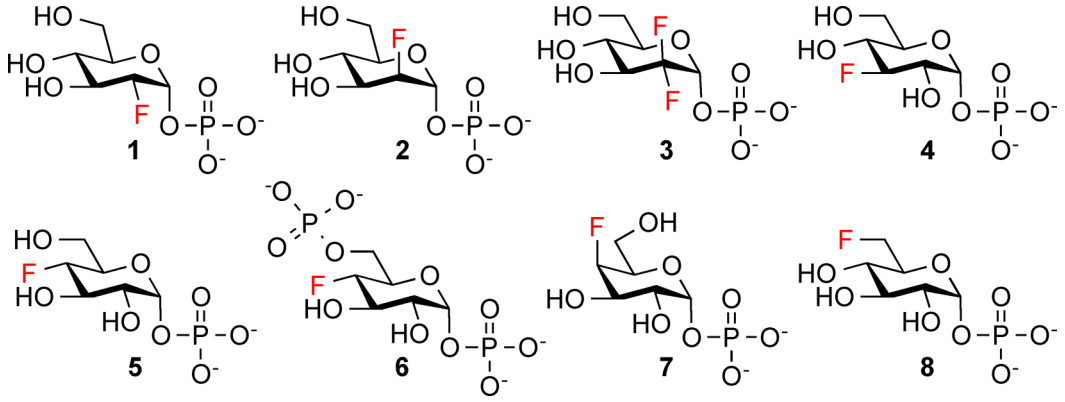


Figure 1. Isosteric α-D-glucopyranosyl 1-phosphate analogues prepared and evaluated.

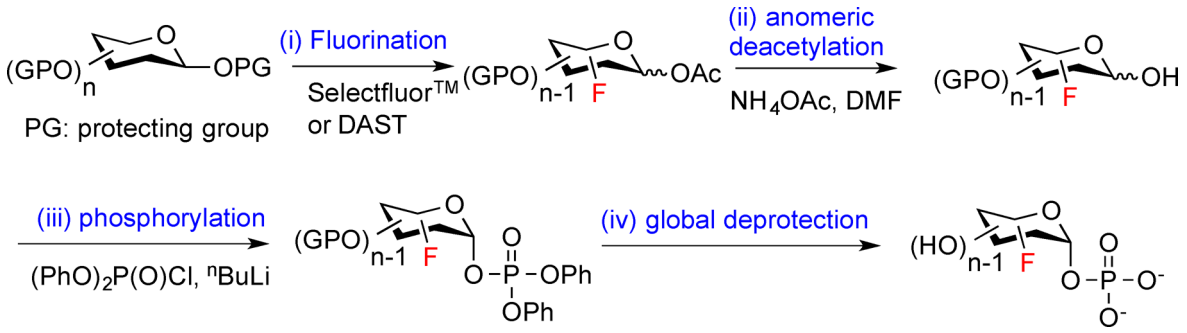
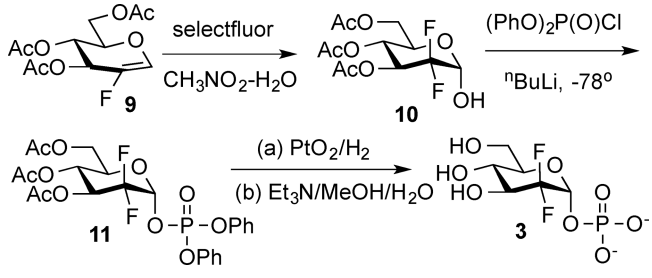


Figure 2. Strategy for synthesis of fluorinated α-hexopyranosyl 1-phosphates.

using either electrophilic (Selectfluor) or nucleophilic (diethylaminosulfur trifluoride, DAST) fluorinating reagents, (ii) selective hydrolysis of the anomeric acetate with NH_4OAc in DMF,[14] followed by (iii) phosphorylation of the anomeric hydroxyl group using diphenylphosphoryl chloride and *n*-BuLi at low temperature, and finally (iv) global deprotection.[15]

The synthesis of difluorinated sugar 1-phosphate **3** (Scheme 2) commenced with preparation of fluoroglycal **9** from commercially available tri-*O*-acetyl-D-glucal following published procedures.[16,17] Attempts to insert the second fluorine atom at the C-2 position of **9** using Selectfluor in acetone-H_2O failed with the recovery of starting material. However, the expected electrophilic fluorination of **9** with Selectfluor in nitromethane-H_2O produced **10** in 45% yield, furnishing exclusively the α anomer.[17] The coupling of **10** with diphenyl phosphoryl chloride and *n*-BuLi at −78 °C provided diphenyl phosphate **11** as the α anomer predominant in 57% yield. The anomeric configuration of **11** was unambiguously determined by the magnitude of the heteronuclear coupling constant $^1J_{C\text{-}1,H\text{-}1}$ of

Scheme 2. Synthesis of Compound 3



180 Hz, which is consistent for α-anomers.[18] The resulting diphenyl phosphate **11** was deprotected by hydrogenolysis using Adam's catalyst (PtO_2) to remove phenyl substituents, followed by hydrolysis of acetate protecting groups using Et_3N-H_2O-MeOH (1:3:7) to give rise to compound **3** in a quantitative yield. The identity of the two fluorine atoms in **3** was determined by a 2D $^1H-^{19}F$ HOESY experiment (Figure 3). The peak at −121.1 ppm corresponded to the fluorine in

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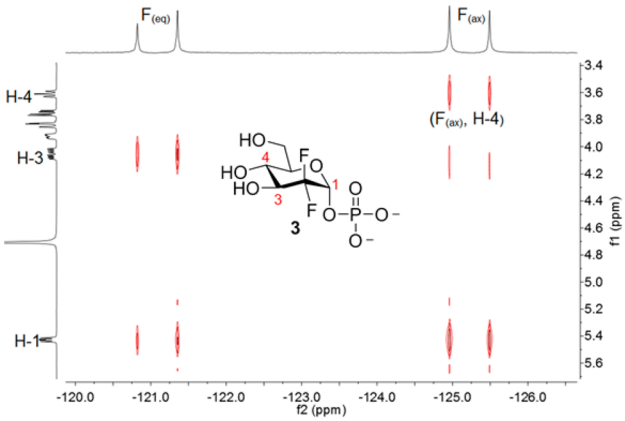

Figure 3. 2D $^{1}H-^{19}F$ HOESY spectrum of compound **3** (500 MHz, D_2O).

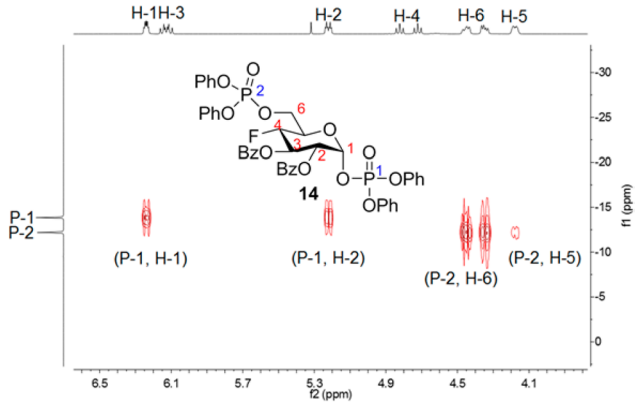

Figure 4. $^{1}H-^{31}P$ HMBC spectrum of compound **14** (500 MHz, $CDCl_3$).

the equatorial position, while the peak at −125.2 ppm belonged to the axial fluorine, as determined by the observation of a cross peak between the axial fluorine and H-4 in the 2D $^{1}H-^{19}F$ HOESY spectrum.

Phosphorylation of monosaccharides using diphenyl chlorophosphate to prepare sugar 1-phosphates **1**−**8** resulted in the isolation of only α-phosphorylated anomers after flash column chromatography (see the Supporting Information). This observation was consistent with ^{1}H NMR analysis of crude product mixtures which were absent of characteristic β-anomer resonance signals between 5.5−6.5 ppm. It was also interesting to find that, in the course of phosphorylation of **12**, in addition to the desired product **13**, a 1,6-diphosphorylated side product **14** was also isolated in 7% yield (Scheme 3), whereas a similar diphosphate product was not observed in the other phosphorylation reactions. Both **13** and **14** were treated with Adam's catalyst followed by NaOMe in MeOH to afford **5** and **6** both in 90% yield, respectively.

The structure of **14** was confirmed by analysis of the ^{1}H NMR, COSY, and 2D $^{1}H-^{31}P$ HMBC spectroscopy. Specifically, correlations between P-2 and H-5, P-2 and H-6 were observed (Figure 4), in addition to correlations between P-1 and H-1, P-1 and H-2 in the 2D $^{1}H-^{31}P$ HMBC spectrum.

Substrate Evaluation with Cps2L and GDP-manPP using HPLC. All of the synthetic α-D-deoxyfluorohexopyranosyl 1-phosphates (**1**−**8**) as well as α-D-glucopyranosyl 1-phosphate (**Glc 1P**) and α-D-mannopyranosyl 1-phosphate (**Man 1P**) were subject to the enzymatic reaction screening conditions as described previously.[6] Product formation was confirmed by HPLC and ESI-MS/MS analysis (see the Supporting Information). Enzymatic assay results are summarized in Table 1. It was found that Cps2L converted all substrates almost as effectively as it did for the physiological substrate **Glc 1P**, to provide corresponding dTDP-deoxyfluorosugars within 20−30 min. This result demonstrated the broad substrate promiscuity of Cps2L. 2-Deoxy-α-D-glucopyranosyl 1-phosphate (**2-deoxyGlc 1P**) has been demonstrated to be a poor substrate for a homologous nucleotidylyltransferase.[19] The reason for this inability to transform **2-deoxyGlc 1P** was ascribed to the absence of interactions between the C-2 hydroxyl group and the nucleotidylyltransferase active site. If the fluorine atom is an isostere of hydrogen, the turnover of **1**−**3** might be considered surprising. However, since all three compounds were accepted by Cps2L and effectively turned over to the corresponding nucleotides, it is more consistent to view the fluorine atom as isoelectronic to the hydroxyl group. Thus, these C-2 fluorinated analogues (**1**−**3**) either structurally resemble native **Glc 1P**, or sufficient binding interactions with Cps2L at C-2 position are restored through the C-2 fluorine atom serving as a hydrogen bonding acceptor.

Previously, GDP-ManPP has been shown to be capable of tolerating a variety of substrates structurally related to **Man 1P** including monoazidodeoxy, monomethoxy, and deoxy analogues of **Man 1P**.[7,20] In order to further probe its promiscuity, all of the above synthetic fluorinated α-D-hexopyrannosyl 1-phosphates, including both manno-configured and nonmanno-configured substrates, were incubated with GDP-ManPP under previously described assay conditions.[7] As shown in Table 1, when C-2 fluorinated substrates (**1**−**3**) were incubated with GDP-ManPP, the reaction proceeded well with the GTP conversion ranging from 93% to 98% within 2.5 h, which is consistent with native **Man 1P**, demonstrating the tolerance of GDP-ManPP for these structural changes. However, it was found that other fluorinated analogues, including **4**, **5**, **7**, and **8**, were poor substrates, as they required much longer reaction times (Figure 5) to achieve similar levels of conversions.

The poorest substrate of GDP-manPP was **7**, requiring 74 h to achieve about 78% GTP conversion. The observed slow conversion in the case of **4**, **5**, **7**, and **8** could be attributed to

Scheme 3. Formation of a 1,6-Diphosphorylated Product

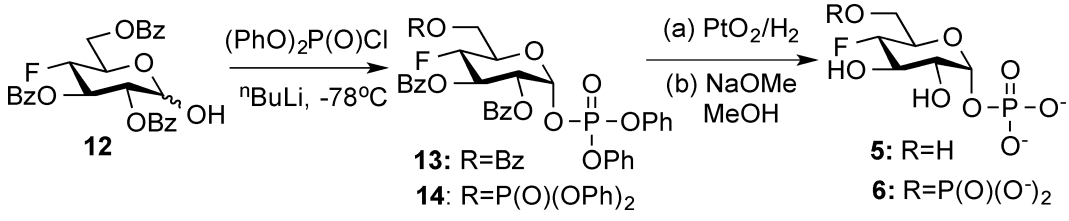

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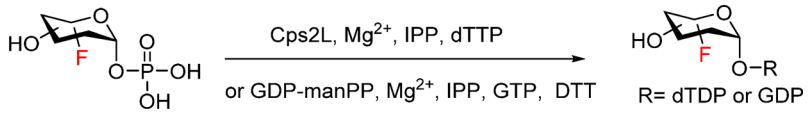
Table 1. HPLC Assay Results of Substrates with Cps2L and GDP-manPP



Glc 1P, Man 1P, 1-8

	conv. (%)[a]		incubation time (h)		Rt (min)[b]	
substrate	dTTP	GTP	dTTP	GTP	dTTP	GTP
1	94	95	0.33	2.5	5.61	6.01
2	93	98	0.33	2.5	5.62	6.00
3	95	93	0.33	2	5.85	6.14
4	93	87	0.5	48	5.84	6.11
5	94	79	0.5	33	5.90	5.81
6	—[c]	—	—	—	—	—
7	93	78	0.5	74	6.14	6.14
8	95	94	0.5	8.5	5.76	6.10
Glc 1P[d]	97	94	0.33	1	5.47	6.03
Man 1P[e]	92	98	0.5	0.5	5.49	5.92

[a]Percentage conversion = $[A_P/(A_P + A_T + A_D)] \times 100$, where A_P = the NDP-sugar product peak area, A_T = the remaining NTP peak area, A_D = the hydrolysis product NDP peak area. [b]The provided retention times are representative, as some variability in the retention times (±0.2 min) were observed due to instrument variability. Standard retention time: dTTP, 6.79 min; dTDP, 5.94 min; GTP, 6.82 min; GDP, 5.85 min. [c]No desired product formation under standard conditions. [d]Reported 90% dTTP conversion.[6] [e]Reported above 90% GTP conversion.[7]

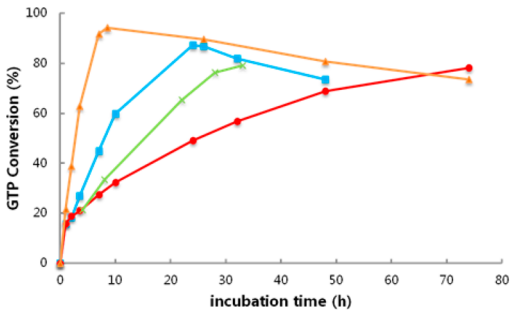


Figure 5. GDP-ManPP-catalyzed conversion of fluorohexopyranosyl 1-phosphates to GDP-Man derivatives: (blue solid square) **4**, (green "×") **5**, (red solid circle) **7**, (orange solid triangle) **8**.

GDP-ManPP preferring manno- over gluco-configured substrates. Nevertheless, the presence of products over extended incubation times demonstrates the potential for the chemoenzymatic synthesis of unnatural sugar nucleotides. As expected, both Cps2L and GDP-ManPP failed to turn over the 1,6-diphosphate **6**, as no product signals were detected by HPLC or ESI-MS, indicating the inability of these two enzymes to accept **6**.

Kinetic Analysis of Fluorinated Substrates (1−8) with Cps2L and GDP-ManPP. To better understand how these synthetic fluorinated isosteric analogues of **Glc 1P** interacted with the two enzymes Cps2L and GDP-ManPP, kinetic analyses were performed using a continuous coupled enzyme assay.[13] As shown in Table 2, the kinetic parameters of **8** with Cps2L were shown to be the closest to those of **Glc 1P**, indicating the C-6 hydroxyl group of **Glc 1P** makes the smallest contribution to binding and catalysis with Cps2L, which is consistent with the early reported results for this class of enzyme.[19] With regard to the three C-2 fluorinated analogues (**1**−**3**), they displayed a 10- to 77-fold decrease in k_{cat} and 2 orders of magnitude drop in k_{cat}/K_m in comparison to **Glc 1P**. The presence of an axial fluoro substituent in **2** is less detrimental to activity than the presence of an axial hydroxyl group (**Man 1P**). The difluoro substitution at C-2 in **3** results in tighter binding than either monofluoro analogue **1** or **2**. The difluoro analogue **3** was the least reactive, consistent with a reduction in the second ionization constant (pKa_2) of the

Table 2. Kinetic Data for Substrates with Enzyme Cps2L and GDP-ManPP

	Cps2L			GDP-ManPP		
substrates	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($mM^{-1}\ s^{-1}$)
1	267 ± 23	2.3 ± 0.1	8.6 ± 4.3	523 ± 73	$(3.2 \pm 0.2) \times 10^{-3}$	$(6.1 \pm 0.3) \times 10^{-2}$
2	243 ± 30	0.94 ± 0.07	3.9 ± 2.3	187 ± 20	0.16 ± 0.01	0.9 ± 0.3
3	97 ± 15	0.31 ± 0.02	3.3 ± 1.4	1110 ± 193	$(8.9 \pm 0.6) \times 10^{-3}$	$(8 \pm 3) \times 10^{-3}$
4	300 ± 113	$(1.1 \pm 0.2) \times 10^{-3}$	$(3.7 \pm 1.8) \times 10^{-3}$	nd[a]	nd	nd
5	130 ± 29	0.57 ± 0.05	4.4 ± 1.8	nd	nd	nd
7	297 ± 98	0.02 ± 0.002	$(6 \pm 2) \times 10^{-2}$	nd	nd	nd
8	52 ± 9	4.2 ± 0.2	80 ± 30	nd	nd	nd
Glc 1P	83 ± 10[b]	24[b]	280[b]	1304 ± 150	$(7 \pm 0.4) \times 10^{-2}$	$(5.7 \pm 2.5) \times 10^{-2}$
Man 1P	632 ± 108	0.49 ± 0.04	0.8 ± 0.4	165 ± 40	1.65 ± 0.18	10 ± 4.5
3NH$_2$Glc 1P[b]	1100 ± 100	0.08	7.2×10^{-2}	nd	nd	nd
3N$_3$Glc 1P[b]	1100 ± 330	3×10^{-4}	3×10^{-4}	nd	nd	nd

[a]Not determined. [b]Data previously reported.[13]

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anomeric phosphate and, therefore, rendering the phosphate group less nucleophilic. Compound **5** has similar kinetic parameters to those of the C-2 fluorinated counterparts, whereas its C-4 epimer (**7**) was a demonstrably worse substrate. It was found that **4** was the poorest Cps2L substrate among these fluorinated analogues, as revealed by its almost 10- and 20-fold decrease in k_{cat} and k_{cat}/K_m, respectively. This result is in parallel to those of 3-deoxy-3-amino-α-D-glucopyranosyl 1-phosphate (**3NH$_2$Glc 1P**) and 3-deoxy-3-azido-α-D-glucopyranosyl 1-phosphate (**3N$_3$Glc 1P**), which were two very poor substrates for Cps2L.[13] Indeed, substitutions of the C-3 hydroxyl group with fluoro-, amino-, or azido- functionalities all have a negative impact on Cps2L catalysis. The K_m for compound **4** was comparable to compound **1** and **2** and significantly better than for **3NH$_2$Glc 1P** and **3N$_3$Glc 1P**. This suggests that the fluorine replacement at C-3 position has a small effect on the formation of the Michaelis−Menten complex, but significantly affects the chemical transformation to product, which indicates that **4** may be a potential inhibitor of Cps2L.

Kinetic parameters of the five substrates (**Man 1P**, **Glc 1P**, and **1**−**3**) with GDP-ManPP were determined using the same continuous coupled assay method as previously described for Cps2L by measuring the release of pyrophosphate (Table 2).[13] For the physiological substrate **Man 1P**, our calculated K_m and k_{cat} data were not identical to those reported for the same enzyme using an end point assay, which showed its K_m and k_{cat} of 40 μM and 6 μM^{-1} s^{-1} respectively.[7] This discrepancy presumably arises as a result of the different assay method. Nevertheless, the comparison between different substrate analyses using the continuous coupled assay provides clear insight into substrate specificity trends. Substitution of fluorine for the C-2 hydroxyl group in **Man 1P** did not affect the K_m, but did result in a 10-fold decrease in both k_{cat} and k_{cat}/K_m. Introducing one additional fluorine to the C-2 position (i.e., **3**), however, led to approximately 6-, 200-, and 1300-fold decline in K_m, k_{cat}, and k_{cat}/K_m, respectively, with respect to **Man 1P**. Alteration of the C-2 hydroxyl group orientation from axial (Man) to equatorial (Glc) resulted in binding affinity (K_m), turnover (k_{cat}), and catalytic efficiency (k_{cat}/K_m) decreases of approximately 1, 2, and 3 orders of magnitude, respectively. A similar decrease was also observed in the k_{cat} and k_{cat}/K_m of **1**, but surprisingly, its binding affinity did not decrease as much as expected when compared with **Man 1P**. These results suggest the indispensable engagement of the C-2 axial hydroxyl group of **Man 1P** in binding with GDP-ManPP, which is consistent with previously reported results[7] and the observed sluggish reaction rate when incubated with gluco-configured fluorinated analogues including **4**, **5**, **7**, and **8** for which we were unable to characterize kinetically due to low turnover (Figure 4).

The comparison between Cps2L and GDP-ManPP with compounds (**1**−**3**) shows very different profiles. For Cps2L, there is no significant difference between the catalytic efficiency of **2** and **3**, whereas for GDP-ManPP, the difluoro compound (**3**) is an order of magnitude less reactive.

Acidity of Substrates and Determination of Linear Free-Energy Relationships. The pKa_2 values were readily determined from the ^{31}P and ^{19}F chemical shift data for all three C-2 fluorinated compounds, and the values were consistent between each nucleus (Table 3). The pKa_2 value measured for **Glc 1P** was consistent with that obtained from literature.[21] All three synthesized compounds were more acidic than **Glc 1P**, however, the magnitude of the changes varied substantially with each analogue. Compound **1**, with the equatorial fluoro-substituent, had only small increase in acidity (0.2 pH units) relative to **Glc 1P** (Table 3). By contrast, compound **3**, with both axial and equatorial fluoro-substituents, had a larger increase in acidity (0.4 pH units), relative to **Glc 1P**. Similar magnitude differences in acidity were not observed between **Man 1P**, **2**, and **3**. Compound **3**, was anticipated to be the most acidic, yet it was only marginally more acidic than compound **2**. Furthermore, only the equatorial fluorine resonance changed chemical shift (Figure 6) for compound **3**, while the axial fluorine resonance remained unchanged upon titration. By contrast, both the equatorial and axial fluorine resonances changed chemical shift for compounds **1** and **2** (see the Supporting Information). The reason behind this phenomenon is not clear. It does, however, demonstrate a limitation to the concept of providing additional inductively electron-withdrawing substituents to increase acidity.

Table 3. NMR Determined pKa_2 Values of 1−3, Man 1P, and Glc 1P

compds	reported pKa_2 value	pKa_2 determined by ^{19}F NMR	pKa_2 determined by ^{31}P NMR	average pKa_2[a]
1	5.90 ± 0.03[b]	6.12 ± 0.02	6.10 ± 0.02	6.11 ± 0.02
2		5.78 ± 0.01	5.69 ± 0.02	5.74 ± 0.02
3		5.73 ± 0.02	5.69 ± 0.02	5.71 ± 0.02
Man 1P	6.08[c]			6.08
Glc 1P	6.15[c]		6.25 ± 0.1	6.25 ± 0.1

[a]Average pK_{a2} refers to the average values determined by ^{19}F and ^{31}P NMR. [b]Values determined by ^{19}F NMR,[22] [c]pK_{a2} determined by potentiometric titration.[21,23]

With both k_{cat}/K_m and pKa_2 of compounds **Glc 1P**, **Man 1P**, and **1**−**3** in hand, linear free-energy Brønsted plots comparing turnover efficiency of Cps2L and GDP-ManPP with average pKa_2 values of these substrates were constructed (Figure 7). There were no strong Brønsted correlations with all five substrates and their pKa_2 values in both Cps2L or GDP-ManPP-catalyzed reactions, indicating that hexose configuration is prioritized over negative charge recognition by both enzymes. However, grouping the substrates into physiological and nonphysiologically configured substrates for each enzyme resulted in moderate to good correlations (Figure 7A,B). Enzyme Cps2L with physiologically configured substrates (**Glc 1P**, **1**, and **3**) demonstrated a positive dependence on nucleophile acidity (slope = 3.01, R^2 = 0.69). Similarly, for GDP-ManPP with physiologically configured substrates (**Man 1P**, **2**, and **3**) there was a positive dependence on nucleophile acidity (slope = 6.2, R^2 = 0.64). These trends are consistent with a nucleotidylyltransferase mechanism, whereby the sugar 1-phosphate directly attacks the P_α of the nucleoside triphosphate. The nonphysiologically configured substrates for Cps2L, (**Man 1P**, **2**, and **3**) resulted in a negative dependence on the nucleophile acidity (slope = −1.8, R^2 = 0.97), while for GDP-ManPP, the nonphysiologically configured substrates (**Glc 1P**, **1**, and **3**) resulted in a positive dependence on nucleophile acidity (slope = 2.3, R^2 = 0.45). Thus, the two enzymes affect catalysis with their nonphysiologically configured substrates in a different manner.

CONCLUSION

A small library of α-deoxyfluoro hexopyranosyl 1-phosphates (**1**−**8**) have been accessed by a general chemical synthesis

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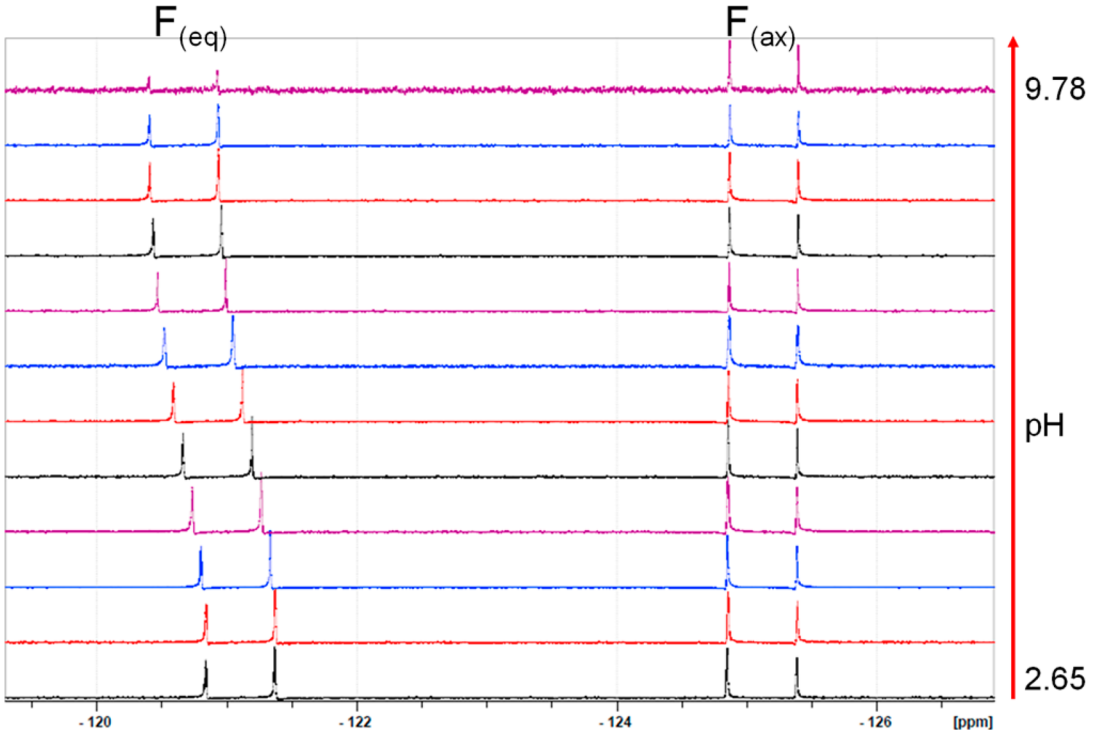

Figure 6. Sensitivity of two fluorine atoms in **3** to pH as measured by ^{19}F NMR.

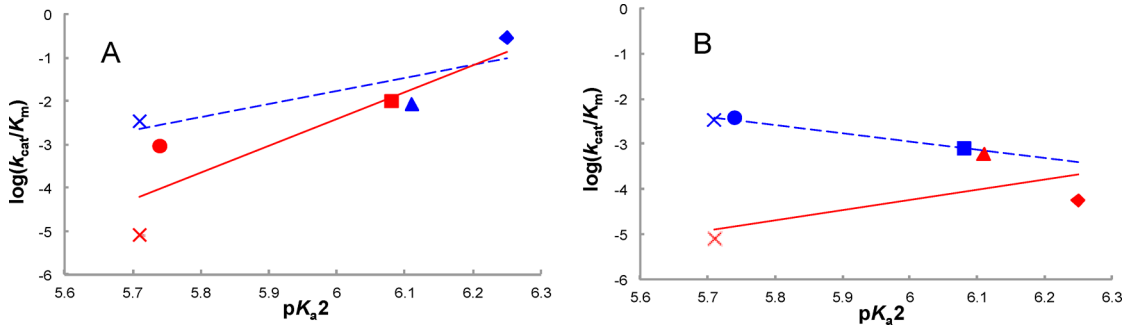

Figure 7. Brønsted linear free-energy plot of Cps2L and GDP-ManPP-catalyzed formation of sugar nucleotides as a function of phosphate nucleophile pKa_2. (A) Physiological substrate configuration for Cps2L (**Glc 1P** (blue solid diamond), **1** (blue solid triangle), **3**(blue "×"), dashed regression line) or GDP-ManPP (**Man 1P** (red solid square), **2** (red solid circle), **3** (red "×"), solid regression line); (B) Nonphysiological substrate configuration for Cps2L (**Man 1P** (blue solid square), **2** (blue solid circle), **3** (blue "×"), dashed regression line) or GDP-ManPP (**Glc 1P** (red solid diamond), **1** (red solid triangle), **3** (red "×") solid regression line).

featuring the fluorine introduction, anomeric deacetylation, phosphorylation, and global deprotection. These analogues were then evaluated as substrates for the thymidylyltransferase Cps2L and the guanidylyltansferase GDP-ManPP. Both enzymes turned over all of the isosteric hexopyranosyl 1-phosphates analogues provided sufficient incubation time, with the exception of the 1,6-diphosphate **6**. Kinetic analysis of these analogues was performed using a continuous coupled assay. Monofluoro substitutions at either C-2, C-4, or C-6 of **Glc 1P** were well tolerated by Cps2L, whereas monofluoro substitution at C-3 was significantly more adverse. Difluoro substitution at C-2 maintains binding affinity in comparison to **Glc 1P** but decreases turnover and catalytic efficiency approximately 10- and 100-fold with Cps2L. Both HPLC assay and kinetic analysis with GDP-ManPP demonstrated that the axial hydroxyl group at the C-2 position of **Man 1P** is critical for GDP-ManPP catalytic activity, and in contrast to Cps2L, replacement of axial C-2 hydroxyl group with either a mono- or difluoro substituent results in significantly lower turnover and efficiency of GDP-ManPP. The axial fluoro substituent in the difluoro analogue **3** did not change chemical shift upon titration. The pKa_2 values of three C-2 fluoro-substituted analogues are surprisingly in the order of **1** < **2** ≈ **3**, which were measured by both ^{31}P and ^{19}F NMR titrations. No strong Brønsted correlations among all five substrates (**1**−**3**, **Glc 1P**, and **Man 1P**) were observed for either Cps2L or GDP-ManPP-catalyzed reactions. However, moderate correlations were observed among physiological configured substrates for each enzyme, and a positive and a negative correlation were found among the nonphysiological configured substrates accepted by GDP-ManPP and Cps2L, respectively. These correlations demonstrate that the Brønsted catalysis law applies with selected substrates for each enzyme. The described broad substrate promiscuity of these catalysts will enable the preparation of building blocks for chemo-enzymatic synthesis of unnatural glycans to address the needs of glycobiology.

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EXPERIMENTAL SECTION

General Methods and Instrumentation: Chemical Synthesis. All chemicals and reagents were purchased from commercial sources and were used as received, unless otherwise noted. Syntheses that required anhydrous conditions were performed under an inert atmosphere of dried high-purity nitrogen. HPLC grade methanol was employed where stated. Glassware was dried overnight in an oven set at 120 °C and assembled under a stream of inert gas. Analytical thin-layer chromatography (TLC) was performed using silica gel 60 F254 precoated glass plates; compound spots were visualized by ultraviolet light at 254 and/or by charring after treatment with a vanillin stain. Flash chromatography was performed using silica gel 60 (230−400 mesh). NMR spectra were recorded on either 300 or 500 MHz spectrometers. All ^{1}H, ^{13}C, ^{19}F, and ^{31}P chemical shifts are reported in ppm using tetramethylsilane (0.00 ppm) or the solvent signal [$CDCl_3$ (^{1}H 7.26 ppm; ^{13}C 77.16 ppm); D_2O (^{1}H 4.79 ppm)] as the internal reference or MeOD (^{13}C 49.50 ppm in D_2O) or 85% aq. H_3PO_4 (^{31}P 0.00 ppm) or CF_3COOH (^{19}F, −76 ppm) as an external reference. Splitting patterns are indicated as follows: br, broad; s, singlet; d, doublet; t, triplet; at, apparent triplet; q, quartet; m, multiplet. All coupling constants (*J*) are reported in Hertz (Hz). The ratios of counterion (Et_3NH) in compounds **1**−**8** were determined by the integration peaks of Et_3N in their corresponding ^{1}H NMR spectra. High-resolution mass spectra were recorded using ion trap (ESI TOF) spectrometers. For specific schemes for the synthesis of all intermediates and final compounds, please see the Supporting Information.

General Procedure A: Selective Anomeric Deacetylation. NH_4OAc (12 mmol) was added to the solution of peracetated sugar (3 mmol) in anhydrous DMF (3 mL) and stirred for 2 days at rt. When the reaction was deemed complete by TLC, the remaining NH_4OAc was filtered off, and the filtrate was concentrated to dryness.

General Procedure B: Phosphorylation of Anomeric Hydroxyl Group. *n*-BuLi (1.2 mmol, 2.5 M in hexane) was added dropwise to the solution of sugars (1 mmol) in anhydrous THF (5 mL) at −78 °C under a N_2 atmosphere. After stirring for 15 min, diphenyl phosphoryl chloride (1.2 equiv) was added dropwise to the above solution, and the reaction mixture was stirred for another 0.5 h at the same temperature. TLC analysis indicated the complete consumption of the starting material. The reaction was gradually warmed to rt, quenched with aq sat. NH_4Cl solution (2 mL), and extracted with EtOAc (3 × 10 mL). The combined organic layer was washed with aq sat. NaCl solution (3 mL), dried over $MgSO_4$, filtered, and concentrated *in vacuo* to afford the crude product.

General Procedure C: Global Deprotection. PtO_2 (20 mol %) was added to a diphenyl ester phosphate solution in EtOH (3 mL). The resulting mixture was stirred overnight at rt under a H_2 atmosphere (1 atm). Reactions were monitored by TLC; completed reaction was quenched by filtering off the catalyst and concentrating the reaction mixture under reduced pressure. The resulting crude product was subject to ^{1}H NMR analysis to confirm the completion of the reaction before proceeding to the next step. Et_3N (1 mL) was added to the crude product residue in MeOH (2 mL), and the resulting reaction mixture was concentrated under reduced pressure. The resulting residue was dissolved in Et_3N-H_2O-MeOH (1:3:7 v/v/v, 5 mL) and stirred overnight at rt. Lastly, the reaction mixture was concentrated *in vacuo*.

*2-Deoxy-2-fluoro-3,4,6-tri-O-acetyl-α-D-glucopyranosyl 1-Diphenylphosphate (**16**).* 2-Deoxy-2-fluoro-3,4,6-tri-*O*-acetyl-α-D-glucopyranose **15** was prepared from 3,4,6-tri-*O*-acetyl-α-D-glucal over three steps according to a reported procedure.[16] Following general procedure B, **15** (500 mg, 1.62 mmol) was phosphorylated to yield **16**. After silica gel column chromatography eluting with CH_2Cl_2/MeOH (80:1), **16** was obtained (525 mg, 60% yield) as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.41−7.22 (m, 10H), 6.17−6.15 (m, 1H, H-1), 5.58 (q, *J* = 10.0 Hz, 1H), 5.09 (t, *J* = 10.0 Hz, 1H), 4.69−4.58 (m, 1H, H-2), 4.17 (dd, J = 10.0 and 5.0 Hz, 1H), 4.04−4.02 (m, 1H), 3.85−3.82 (m, 1H), 2.10−2.01 (m, 9H), ^{19}F{^{1}H} NMR (471 MHz, $CDCl_3$) δ −200.45, ^{31}P {^{1}H} NMR (202 MHz, $CDCl_3$) δ −13.51, {lit.[24] ^{19}F{^{1}H} NMR (377 MHz, $CDCl_3$) δ −200.9, ^{31}P NMR (162 MHz, $CDCl_3$) δ −12.9}.

*2-Deoxy-2-fluoro-α-D-glucopyranosyl 1-Phosphate (**1**).* Following general procedure C, (50 mg, 79% yield) of **1** was obtained as colorless solid after lyophilization from **16** (76 mg, 0.14 mmol). ^{1}H NMR (500 MHz, D_2O) δ5.56 (dd, *J* = 6.5 and 3.0 Hz, 1H), 4.31 (dd, JH-$_{2,F}$ = 49.5 and *J* = 9.0 Hz, 1H), 3.97−3.91 (m, 1H), 3.83−3.77 (m, 2H), 3.67 (dd, *J* = 12 and 4.5 Hz, 1H), 3.39 (t, *J* = 4.5 Hz, 1H), 3.11 (q, *J* = 7.5 Hz, 11 H), 1.20 (t, *J* = 7.5 Hz, 17 H). ^{19}F{H} NMR (471 MHz, D_2O) δ −199.59, ^{31}P{H} NMR (202 MHz, D_2O) δ 0.57. {lit.[22] ^{19}F{H} NMR (471 MHz, D_2O) δ −200.20}.

*2-Deoxy-2-fluoro-3,4,6-tri-O-acetyl-α-D-mannopyranosyl 1-Diphenylphosphate (**18**).* 2-Deoxy-2-fluoro-3,4,6-tri-*O*-acetyl-α-D-mannopyranose **17** was prepared from 3,4,6-tri-*O*-acetyl-α-D-glucal over three-steps according to a reported procedure.[16] Following general procedure B, **17** (308 mg, 1.0 mmol) was phosphorylated to yield **18**. After silica gel column chromatography by eluting with *n*-hexane/EtOAc (3:1), **18** was obtained (318.6 mg, 59% yield) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.32−7.12 (m,10H), 5.98 (dt, *J* = 6.9 and 2.1 Hz, 1H, H-1), 5.37−5.16 (m, 2H), 4.73 (dt, $J_{H\text{-}2,F}$ = 48.6 and *J* = 2.1 Hz, H-2), 4.13 (dd, *J* = 12.6 and 4.5 Hz, H-6a), 4.02−3.98 (m, 1H), 3.87 (dd, *J* = 12.6 and 2.1 Hz, H-6b), 2.01−1.88 (m, 9H), ^{19}F NMR (282 MHz, $CDCl_3$) δ −203.19, ^{31}P NMR (122 MHz, $CDCl_3$) δ −14.19, {lit.[25] ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.35−7.19 (m, 10H), 5.99 (ddd, *J* = 8.5, 7.1, and 2.2 Hz, 1H, H-1), 5.37 (ddd, *J* = 10.3, 10.2, and 1.2 Hz, 1H, H-4), 5.25 (ddd, $J_{H\text{-}3,F}$ = 27.2, *J* = 10.3 and 2.2 Hz, 1H, H-3), 4.74 (ddd, $J_{H\text{-}2,F}$ = 48.9, *J* = 2.2 and 0.5 Hz, 1H, H-2), 4.13 (dd, *J* = 12.5 and 4.2 Hz, 1H, H-6), 4.04−3.98 (m, 1H, H-5), 3.88 (dd, *J* = 12.5 and 1.4 Hz, 1H, H-6), 4.13 (dd, J = 12.5 and 4.2 Hz, 1H, H-6), 2.09−1.95 (m, 9H)}.

*2-Deoxy-2-fluoro-α-D-mannopyranosyl 1-Phosphate (**2**).* Following general procedure C, **2** (34 mg, 81% yield) was obtained as a colorless solid after lyophilization from **18** (54 mg, 0.1 mmol). ^{1}H NMR (500 MHz, D_2O) δ 5.53 (t, *J* = 6.5 Hz, 1H, H-1), 4.80 (m, 1H), 3.96 (ddd, $J_{H\text{-}2,F}$ = 30.0, *J* = 10.0 and 2.5 Hz, 1H), 3.87−3.85 (m, 2H), 3.75 (dd, *J* = 12.5 and 5.5 Hz, 1H, H-6a), 3.70−3.66 (m, 1H), 3.19−3.14 (m, 5H), 1.26−1.23 (m, 6.6H), ^{19}F{^{1}H} NMR (471 MHz, D_2O) δ −204.10, ^{31}P{^{1}H} NMR (202 MHz, MeOD) δ −2.75, {lit.[25] tributylammonium salt, ^{1}H NMR (300 MHz, D_2O) δ 5.38 (ddd, *J* = 8.1 and 6.0 Hz, 1H, H-1), 4.59 (ddd, $J_{H\text{-}2,F}$ = 48.7, *J* = 2.5 and 2.4 Hz, 1H, H-2), 3.76 (ddd, $J_{H\text{-}3,F}$ = 30.8, *J* = 9.6 and 2.5 Hz, 1H, H-2), 3.48−3.69 (m, 4H), 2.98−2.85 (m, 12H), 1.51−1.10 (m, 24H), 0.72 (m, 18H)}.

*2-Deoxy-2,2-difluoro-3,4,6-tri-O-acetyl-α-D-arabino-hexopyranosyl 1-Diphenylphosphate (**11**).* 2-Deoxy-2,2-difluoro-3,4,6-tri-*O*-acetyl-α-D-arabinopyranose **10** was synthesized according to a reported method.[16,17] Following general procedure B, **10** (284 mg, 0.87 mmol) was phosphorylated to yield **11**. After silica gel column chromatography eluting with *n*-hexane/EtOAc (4:1) and a second column chromatography purification eluting with CH_2Cl_2/MeOH (400:1), **11** was obtained (278 mg, 57% yield) as colorless oil. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43−7.25 (m, 10H), 5.93 (dd, *J* = 10.0 and 5.0 Hz, 1H, H-1), 5.60 (ddd, *J* = 15.0, 10.0, and 5.0 Hz, 1H, H-3), 5.32−5.25 (m, 1H, H-4), 4.18 (dd, *J* = 10.0 and 5.0 Hz, 1H, H-6), 4.10 (ddd, *J* = 10.5, 4.0, and 2.0 Hz, 1H, H-5), 3.92 (dd, *J* = 12.5 and 2.5 Hz, 1H, H-6), 2.16 (s, 3H), 2.06 (s, 3H), 2.01 (s, 3H). ^{19}F{^{1}H} NMR (471 MHz, $CDCl_3$) δ −120.21 (d, $J_{F,F}$ = 259.1 Hz), −121.11 (d, $J_{F,F}$ = 259.1 Hz). ^{31}P{^{1}H} NMR (202 MHz, $CDCl_3$) δ −14.16. ^{13}C{^{1}H} NMR (126 MHz, $CDCl_3$) δ 170.6, 169.7, 169.2, 150.3, 150.2, 150.17, 150.1, 130.2, 130.16, 126.19, 126.16, 120.42, 120.38, 120.23, 120.2, 114.3 (dt, $J_{C\text{-}2,F}$ = 970.3, 47.1 Hz), 94.8, 94.6, 94.3, 70.4, 68.4 (t, $J_{C,F}$ = 75.4 Hz), 66.8 (d, $J_{C,F}$ = 23.6 Hz), 61.0, 20.7, 20.6, 20.5. HRMS (ESI^{+}): found $[M + Na]^+$ 581.1003. $C_{24}H_{25}F_2NaO_{11}P$ requires $[M + Na]^+$ 581.0995.

*2-Deoxy-2,2-difluoro-α-D-arabino-hexopyranosyl 1-Phosphate (**3**).* Following general procedure C, **3** (45 mg, 94% yield) was obtained as colorless solid after lyophilization from **11** (60 mg, 0.11 mmol). ^{1}H NMR (500 MHz, D_2O) δ 5.41 (dd, *J* = 8.5 and 5.5 Hz, 1H, H-1), 4.06 (ddd, $J_{H,F}$ = 32.0, *J* = 9.5 and 4.5 Hz, 1H), 3.93 (dd, *J* = 10.0 and 3.0 Hz, 1H), 3.84 (dd, *J* = 12.5 and 2.0 Hz, 1H), 3.74 (dd, *J* = 15.0 and 5.0 Hz, 1H), 3.59 (t, *J* = 9.5 Hz, 1H), 3.15 (q, *J* = 5.0 Hz,

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9H), 1.23 (t, J = 5.0 Hz, 14H). $^{19}F\{^1H\}$ NMR (471 MHz, D_2O) δ −121.01 (d, $J_{F,F}$ = 249.6 Hz), −125.22 (d, $J_{F,F}$ = 249.6 Hz). $^{31}P\{^1H\}$ NMR (202 MHz, D_2O) δ −0.26, $^{13}C\{^1H\}$ NMR (126 MHz, D_2O) δ 92.3, 91.9, 91.7 (m, C-1), 72.6, 70.3 (t, $J_{C\text{-}2,F}$ = 187.5 Hz, C-2), 68.3 (d, J = 7.5 Hz), 60.2, 46.7, 8.3. HRMS (ESI$^-$): found $[M - H]^-$ 279.0084. $C_6H_{10}F2O_8P$ requires $[M - H]^-$ 279.0087.

*3-Deoxy-3-fluoro-2,4,6-tri-O-acetyl-α-D-glucopyranose (**20**).* 3-Deoxy-3-fluoro-1,2,4,6-tetra-*O*-acetyl-α-D-glucopyranose **19** was prepared from diacetone glucose according to a published method.[26] Following general procedure A, **19** (1.0 g, 2.86 mmol) was selectively deacetylated to yield **20**. After silica gel column chromatography eluting with *n*-hexane/EtOAc (1.5:1), **20** was obtained (513 mg, 58% yield) as light yellow oil. ^1H NMR (500 MHz, $CDCl_3$) (anomeric mixture) δ 5.35 (m, 1H), 5.12−5.07 (m, 2H), 5.01 (m, 1H), 4.96−4.90 (m, 0.4H), 4.86−4.85 (m, 1H), 4.84−4.82 (m, 0.5H), 4.77−4.73 (m, 0.5H), 4.16−4.01 (m, 4H), 2.05−2.00 (m, 12H), {lit.[27] ^1H NMR (300 MHz, $CDCl_3$) (α-anomer) δ 5.45 (br, 1H), 5.10 (m, 2H), 4.64 (m, 1H), 4.16 (m, 3H), 2.13−2.08 (m, 9H)}.

*3-Deoxy-3-fluoro-2,4,6-tri-O-acetyl-α-D-glucopyranosyl 1-diphenylphosphate (**21**).* Following the general procedure B, **20** (403 mg, 1.31 mmol) was phosphorylated to yield **21**. After silica gel column chromatography eluting with *n*-hexane/EtOAc (2:1), **21** was obtained (450 mg, 64% yield) as colorless oil. ^1H NMR (500 MHz, $CDCl_3$) δ 7.37−7.19 (m, 10H), 6.10 (dt, J = 7.0 and 3.5 Hz, 1H, H-1), 5.32−5.25 (m, 1H), 5.12−5.07 (m, 1H), 4.84 (dt, $J_{H\text{-}3,F}$ = 50.0 and J = 5.0 Hz, 1H, H-3), 4.18 (dd, J = 15.0 and 5.0 Hz, 1H), 4.04−4.02 (m, 1H), 3.94−3.92 (m, 1H), 2.15 (s, 3H), 2.05 (s, 3H), 1.98 (s, 3H). $^{19}F\{^1H\}$ NMR (471 MHz, $CDCl_3$) δ −200.81. $^{31}P\{^1H\}$ NMR (202 MHz, $CDCl_3$) δ −13.98. ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.4, 169.7, 169.1, 150.3, 150.2, 130.0, 129.9, 125.8, 120.2, 120.1, 120.0, 119.96, 95.2 (dd, J = 10.0 and 6.3 Hz, C-1), 88.6 (d, $J_{C\text{-}3,F}$ = 188.8 Hz, C-3), 71.1 (dd, J = 18.0 and 7.3 Hz), 69.6 (d, $J_{C,F}$ = 67.5 Hz), 67.4 (d, $J_{C,F}$ = 18.6 Hz), 60.9, 20.5, 20.3. HRMS (ESI$^+$): found $[M + Na]^+$ 563.1064. $C_{24}H_{26}FNaO_{11}P$ requires $[M + Na]^+$ 563.1089.

*3-Deoxy-3-fluoro-α-D-glucopyranosyl 1-Phosphate (**4**).* Following general procedure C, **4** (67 mg, 100% yield) was obtained as a colorless solid after lyophilization from **21** (80 mg, 0.15 mmol). ^1H NMR (300 MHz, D_2O) δ 5.44 (dt, J = 6.0 and 3.0 Hz, 1H, H-1), 4.55 (dt, $J_{H\text{-}3,F}$ = 57.0 and J = 9.0 Hz, 1H, H-3), 3.85−3.62 (m, 5H), 3.12 (q, J = 6.0 Hz, 11H), 1.20 (t, J = 6.0 Hz, 17H). $^{19}F\{H\}$ NMR (282 MHz, D_2O) δ −198.86, $^{31}P\{H\}$ NMR (122 MHz, D_2O) δ −0.05, {lit.[26] $^{19}F\{H\}$ NMR (376 MHz, D_2O) δ −200.5, $^{31}P\{H\}$ NMR (162 MHz, D_2O) δ −0.08}.

*4-Deoxy-4-fluoro-2,3,6-tri-O-benzoyl-α-D-glucopyranose (**12**).* 4-Deoxy-4-fluoro-1-*O*-acetyl-2,3,6-tri-*O*-benzoyl-α-D-glucopyranose (1.76 g, 3.28 mmol) was prepared according to the reported method,[28] which was selectively deacetylated to yield **25**, following general procedure A. After silica gel column chromatography by eluting with *n*-hexane/EtOAc (3:1), **12** was obtained (1.26 mg, 78% yield) as light yellow solid. ^1H NMR (500 MHz, DMSO-d_6) anomeric mixture δ 8.09−7.86 (m, 9H), 7.71−7.46 (m, 14H), 6.01 (dt, J = 14.5 and 9.5 Hz, 1H), 5.96−5.93 (m, 0.27H), 5.51 (m, 1H), 5.25−5.20 (m, 1H), 5.06 (dt, $J_{H\text{-}4,F}$ = 51.0 and J = 9.5 Hz, 1H), 4.69−4.65 (m, 1H), 4.59−4.52 (m, 2H), 4.41−4.38 (m, 0.27H). $^{19}F\{^1H\}$ NMR (471 MHz, DMSO-d_6) anomeric mixture: δ −196.68, −199.12. {Lit.[28] $^{19}F\{^1H\}$ NMR (282 MHz, $CDCl_3$) δ −199.50}.

*4-Deoxy-4-fluoro-2,3,6-tri-O-benzoyl-α-D-glucopyranosyl 1-Diphenylphosphate (**13**) and 4-Deoxy-4-fluoro-2,3,6-tri-O-benzoyl-α-D-glucopyranosyl 1,6-Diphenylphosphate (**14**).* Following general procedure B, **12** (500 mg, 1.01 mmol) was phosphorylated to yield **13** and **14**. After silica gel column chromatography eluting with *n*-hexane/EtOAc (3.5:1) and a second column chromatography purification eluting with *n*-hexane/acetone (4:1), **13** (300 mg, 55% yield) and **14** (61 mg, 7% yield) were obtained as colorless oils. Data for **13**: ^1H NMR (500 MHz, $CDCl_3$) δ 8.11−8.06 (m, 4H), 7.93−7.91 (m, 2H), 7.61−7.13 (m, 21H), 6.41 (dt, J = 6.0 and 3.0 Hz, 1H, H-1), 6.27 (dt, J = 13.5 and 9.5 Hz, 1H, H-3), 5.48 (dt, J = 10.5 and 3.0 Hz, 1H, H-2), 4.94 (dt, $J_{H\text{-}4,F}$ = 50.5 and J = 9.5 Hz, 1H, H-4), 4.59−4.58 (m, 2H, H-6), 4.49 (ddd, J = 10.0, 7.0, and 3.0 Hz, 1H, H-5). $^{19}F\{^1H\}$ NMR (471 MHz, $CDCl_3$) δ −197.69. $^{31}P\{^1H\}$ NMR (202 MHz, $CDCl_3$) δ −13.57. $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$) δ 166.3, 165.8, 150.54, 150.48, 133.9, 133.8, 133.6, 130.3, 130.2, 130.13, 130.08, 129.8, 129.3, 128.8, 128.6, 126.0, 125.9, 120.3, 120.3, 120.3, 95.4 (d, J = 6.3 Hz, C-1), 86.8 (d, $J_{C\text{-}4,F}$ = 187.5 Hz, C-4), 70.6 (t, J = 7.5 Hz, C-3), 70.1 (d, J = 6.3 Hz, C-2), 69.9 (d, J = 10.0 Hz, C-5), 62.1 (C-6). HRMS (ESI$^+$): found $[M + Na]^+$ 749.1548. $C_{39}H_{32}FNaO_{11}P$ requires $[M + Na]^+$ 749.1558.

Data for **14**: ^1H NMR (500 MHz, $CDCl_3$) δ 8.05−8.03 (m, 2H), 7.90−7.88 (m, 2H), 7.60−7.13 (m, 27H), 6.24 (dt, J = 6.0 and 3.0 Hz, 1H, H-1), 6.12 (dt, J = 14.0 and 10.0 Hz, 1H, H-3), 5.22 (dt, J = 10.0 and 3.0 Hz, 1H, H-2), 4.77 (dt, $J_{H\text{-}4,F}$ = 50.0 and J = 10.0 Hz, 1H, H-4), 4.47−4.43 (m, 1H, H-6), 4.35 (dd, J = 10.0 and 5.0 Hz, 1H, H-6), 4.19−4.17 (m, 1H, H-5), $^{19}F\{^1H\}$ NMR (471 MHz, $CDCl_3$) δ −198.39. $^{31}P\{^1H\}$ NMR (202 MHz, $CDCl_3$) δ −12.20, −13.86. $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$) δ 165.6, 165.5, 150.6, 150.6, 150.4, 150.31, 150.25, 133.8, 133.7, 130.2, 130.1, 130.0, 129.1, 128.6, 128.6, 128.5, 128.4, 125.9, 125.8, 120.3, 120.2, 120.2, 120.14, 120.10, 95.1 (d, J = 5.0 Hz), 85.4 (d, $J_{C\text{-}4,F}$ = 187.5 Hz), 70.3 (t, J = 8.8 Hz), 70.0 (dd, $J_{C,F}$ = 25.0 and J = 7.5 Hz), 69.7 (d, $J_{C,F}$ = 20.0 Hz), 65.5 (d, J = 5.0 Hz). HRMS (ESI$^+$): found $[M + Na]^+$ 877.1617. $C_{44}H_{37}FNaO_{13}P_2$ requires $[M + Na]^+$ 877.1586.

*4-Deoxy-4-fluoro-α-D-glucopyranosyl 1-Phosphate (**5**).* PtO_2 (40 mg, 200 mol %) was added to the solution of **13** (66 mg, 0.091 mmol) in MeOH (3 mL). The resulting reaction mixture was stirred overnight at rt under H_2 atmosphere (balloon). When the reaction was complete as determined by TLC, it was quenched by filtering off the catalyst and concentrated under reduced pressure. The resulting crude product was subject to ^1H NMR analysis to further confirm the success of the hydrogenolysis reaction before proceeding to the next step. Freshly made NaOMe in MeOH (4 mL, 1.3 mmol/mL) was added to the above product residue in MeOH (2 mL) and stirred 5 h at rt. Dowex × 50 (H^+ form) resin was added to neutralize the reaction mixture (until pH 5) and then filtered off the resin. The filtrate was immediately neutralized with Et_3N (until pH about 8). The reaction mixture was then concentrated *in vacuo* and lyophilized to give **5** (37.8 mg, 90% yield) as colorless solid. ^1H NMR (300 MHz, D_2O) δ 5.44 (dt, J = 6.6 and 3.3 Hz, 1H, H-1), 4.33 (ddd, $J_{H\text{-}4,F}$ = 51.0, J = 9.9 and 9.0 Hz, 1H, H-4), 4.05−3.94 (m, 2H), 3.84−3.71 (m, 2H), 3.57−3.52 (m, 1H), 3.16 (q, J = 7.2 Hz, 22H), 1.24 (s, J = 7.2 Hz, 34H), $^{19}F\{^1H\}$ NMR (282 MHz, D_2O) δ −198.34, $^{31}P\{^1H\}$ NMR (122 MHz, D_2O) δ −0.70, {lit.[22] $^{19}F\{^1H\}$ NMR (254 MHz, D_2O) δ −199.07, **5** as a bis(cyclohexylammonium) salt}.

*4-Deoxy-4-fluoro-α-D-glucopyranosyl 1,6-Diphosphate (**6**).* Following the same procedure as described for **5**, **6** (31.4 mg, 90% yield) was obtained as a colorless solid from **14** (67.8 mg, 0.079 mmol) after purification using a LH20 column (eluted with H_2O) and lyophilized. ^1H NMR (300 MHz, D_2O) δ 5.43 (dt, J = 6.6 and 3.3 Hz, 1H), 4.40 (dt, $J_{H\text{-}4,F}$ = 50.7 and J = 9.6 Hz, 1H), 4.16−4.14 (m, 1H), 4.05−3.94 (m, 3H), 3.55 (dt, J = 7.2 and 2.7 Hz, 1H), 3.15 (q, J = 7.5 Hz, 6.7 H), 1.23 (t, 7.5 Hz, 10H), ^{19}F NMR (471 MHz, D_2O) δ −197.97, ^{31}P NMR (202 MHz, D_2O) δ 1.17, 0.02, ^{13}C NMR (125 MHz, D_2O) δ 94.1 (d, J = 5.4 Hz), 88.8 (d, $J_{C\text{-}4,F}$ = 179 Hz, C-4), 71.2 (d, J = 5.1 Hz), 71.1 (d, J = 3.9 Hz), 68.9 (dd, $J_{C,F}$ = 24.1 and J = 7.1 Hz), 63.0 (d, J = 4.1 Hz), 46.7, 8.3. HRMS (ESI$^+$): found $[M - H]^-$ 340.9833. $C_6H12FO_{11}P_2$ requires $[M - H]^-$ 340.9844.

*4-Deoxy-4-fluoro-1,2,3,6-tetra-O-acetyl-α-D-galactopyranose (**23**).* Methyl 4-deoxy-4-fluoro-6-trityl-α-D-galactopyranoside **22** was synthesized from methyl-α-D-glucopyranoside according to a reported method.[29] Following a known procedure,[30] to a solution of **22** (2.00 g, 4.57 mmol) in Ac_2O (60 mL) was added dropwise concentrated H_2SO_4 (0.6 mL) at rt. The resulting reaction mixture was stirred overnight at rt. $NaHCO_3$ (3 g) and aq. sat. $NaHCO_3$ solution (20 mL) were added to quench the reaction at 0 °C. The reaction mixture was then extracted with CH_2Cl_2 (3 × 60 mL). The combined organic layer was washed sequentially with water (10 mL), brine (10 mL), dried over $MgSO_4$, concentrated, and purified by silica gel column chromatography using 2:1 *n*-hexane/EtOAc as an eluent to provide **23** (1.59 g, 87% yield) as a colorless oil. ^1H NMR (500 MHz, $CDCl_3$) anomeric mixture:δ 6.40 (d, J = 5.0 Hz, 1H, H-1β), 5.72 (d, J = 10.0 Hz, 0.18H, H-1α), 5.41 (dd, J = 10.0 and 5.0 Hz, 1H, H-2β), 5.28

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(ddd, J = 26.5, 10.5, and 5.0 Hz, 1H, H-3β), 5.03 (ddd, $J_{H\text{-}3\alpha,F}$= 26.5, J = 10.5 and 5.0 Hz, 0.18H, H-3α), 4.98 (dd, $J_{H\text{-}4\beta,F}$= 50.0 and J = 2.5 Hz, H-4β), 4.90 (dd, $J_{H\text{-}4\alpha,F}$= 50.0 and J = 2.5 Hz, 0.18H, H-4α), 4.32−4.20 (m, 3.45H), 3.97 (dt, $J_{H\text{-}5\alpha,F}$ = 27.0 and J = 6.5 Hz, 0.18H, H-5α), 2.17−2.04 (m, 15H). $^{19}F\{^1H\}$ NMR (471 MHz, $CDCl_3$) anomeric mixture: δ −217.03, −219.20. $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$) δ 170.5, 169.8, 168.9, 92.1, 89.8, 87.3, 86.6, 85.8, 85.1, 72.1, 72.0, 71.6, 71.4, 69.3, 69.2, 68.1, 68.0, 67.9, 66.3, 61.5, 61.5, 61.4, 61.4, 21.0, 20.9, 20.8, 20.8, 20.6. HRMS (ESI$^+$): found $[M + Na]^+$ 373.0900. $C_{14}H_{19}FNaO_9$ requires $[M + Na]^+$ 373.0905.

*4-Deoxy-4-fluoro-2,3,6-tri-O-acetyl-α-D-galactopyranose (**24**).* Following general procedure A, **23** (1.47 g, 4.2 mmol) was deacetylated to yield **24**. After silica gel column chromatography eluting with *n*-hexane/EtOAc (1.3:1), **24** was obtained (779 mg, 52% yield) as a colorless oil. ^1H NMR (500 MHz, $CDCl_3$) δ 5.54 (d, J = 3.5 Hz, 1H), 5.41−5.33 (m, 1H), 5.23 (dd, J = 10.5 and 3.5 Hz, 1H), 5.16 (dd, J = 10.0 and 8.5 Hz, 0.41H), 5.02 (ddd, $J_{H,F}$ = 25.0, J = 10.5, and 2.5 Hz, 0.41H), 5.00 (dd, $J_{H\text{-}4,F}$ = 50.5 and J = 2.5 Hz, 1H), 4.89 (dd, $J_{H\text{-}4,F}$ = 50.0 and J = 2.5 Hz, 0.43H), 4.72−4.71 (m, 0.40H) 4.40−4.22 (m, 4H), 3.89 (dt, $J_{H,F}$ = 26.5 and J = 6.5 Hz, 0.54H), 2.15−2.04 (m, 14H). $^{19}F\{^1H\}$ NMR (471 MHz, $CDCl_3$) anomeric mixture: δ −216.84, −219.43, $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$) δ 171.1, 170.9, 170.6, 170.5, 95.9, 90.7, 87.2 (d, $J_{C\text{-}4,F}$ = 183.8 Hz), 86.2 (d, $J_{C\text{-}4,F}$ = 185.0 Hz), 77.5, 77.2, 77.0, 71.1, 71.0, 68.3, 68.1 (d, J = 17.5 Hz), 66.7 (d, $J_{C,F}$ = 18.8 Hz), 62.0 (d, J = 6.3 Hz), 61.8 (d, J = 5.0 Hz), 20.9, 20.8 HRMS (ESI$^+$): found $[M + Na]^+$ 331.0793. $C_{12}H_{17}FNaO_8$ requires $[M + Na]^+$ 331.0800.

*4-Deoxy-4-fluoro-2,3,6-tri-O-acetyl-α-D-galactopyranosyl 1-Diphenylphosphate (**25**).* Following general procedure B, **24** (779 mg, 2.53 mmol) was phosphorylated to yield **25**. After silica gel column chromatography by eluting with *n*-hexane/EtOAc (2.2:1) and further purification eluting with *n*-hexane/acetone (3:1), **25** was obtained (675 mg, 49% yield) as colorless oil. ^1H NMR (500 MHz, $CDCl_3$) δ 7.39−7.21 (m, 10H), 6.16 (dd, J = 6.0 and 2.5 Hz, 1H, H-1), 5.39−5.30 (m, 2H), 4.98 (dd, $J_{H\text{-}4,F}$ = 50.0 and 1.5 Hz, 1H, H-3), 4.30−4.16 (m, 3H), 2.15 (s, 3H), 1.98 (s, 3H), 1.89 (s, 3H). $^{19}F\{^1H\}$ NMR (471 MHz, $CDCl_3$) δ −218.68. $^{31}P\{^1H\}$ NMR (202 MHz, $CDCl_3$) δ −13.67, $^{13}C\{^1H\}$ NMR (126 MHz, $CDCl_3$) δ 170.3, 170.2, 169.9, 150.4, 150.4, 130.1, 123.0, 125.8, 120.4, 120.4, 120.2, 120.1, 95.7 (d, J = 5.0 Hz, C-1), 86.5 (d, $J_{C\text{-}4,F}$ = 185.0 Hz, C-3), 69.2 (d, $J_{C,F}$ = 18.8 Hz), 67.4 (d, J = 17.5 Hz), 66.9 (d, J = 6.3 Hz), 61.3 (d, J = 6.3 Hz), 20.8, 20.6, 20.4. HRMS (ESI$^+$): found $[M + Na]^+$ 563.1094. $C_{24}H_{26}FNaO_{11}P$ requires $[M + Na]^+$ 563.1089.

*4-Deoxy-4-fluoro-α-D-galactopyranosyl 1-Phosphate (**7**).* Following general procedure C, **7** (83 mg, 100% yield) was obtained as colorless solid after lyophilization from **25** (96 mg, 0.18 mmol). ^1H NMR (500 MHz, D_2O) δ 5.46 (dd, J = 7.0 and 3.5 Hz, 1H), 4.83 (dd, $J_{H\text{-}4,F}$ = 51.0 and J = 2.0 Hz, 1H), 4.15 (dt, $J_{H,F}$ = 31.5 and 6.0 Hz, 1H), 3.96 (dd, $J_{H,F}$ = 30.0 and 2.5 Hz, 1H), 3.76−3.74 (m, 1H), 3.71−3.70 (m, 2H), 3.11 (q, J = 7.5 Hz, 12H), 1.20 (t, J = 7.5 Hz, 19H). $^{19}F\{^1H\}$ NMR (471 MHz, D_2O) δ −219.69. $^{31}P\{^1H\}$ NMR (202 MHz, D_2O) δ 0.59, $^{13}C\{^1H\}$ NMR (125 MHz, D_2O) δ 94.8 (d, J = 5.0 Hz, C-1), 90.4 (d, $J_{C\text{-}4,F}$ = 176.3 Hz, C-3), 70.3 (d, J = 17.5 Hz), 68.4 (d, J = 7.5 Hz), 68.0 (d, J = 8.8 Hz), 60.0 (d, J = 5.0 Hz), 46.7, 8.3. {Lit.[31] $^{31}P\{^1H\}$ NMR (121 MHz, D_2O) δ −0.22}.

*6-Deoxy-6-fluoro-2,3,4-tri-O-acetyl-α-D-glucopyranose (**27**).* According to a reported method,[32,30] following general procedure A, 6-deoxy-6-fluoro-1,2,3,4-tetra-*O*-acetyl-α-D-glucopyranose **26** (1.47 g, 4.2 mmol) was selectively deacetylated to yield **27**. After silica gel column chromatography eluting with *n*-hexane/EtOAc (1.3:1), **27** was obtained (779 mg, 52% yield) as a colorless oil. ^1H NMR (500 MHz, $CDCl_3$) anomeric mixture: δ 5.54−5.51 (m, 1H), 5.45 (m,1H), 5.26−5.22 (m, 0.2H), 5.06−5.01 (m, 1H), 4.90−4.88 (m, 0.2H), 4.85 (dd, J = 10.0 and 3.5 Hz, 1H), 4.79 (t, J = 2.5 Hz, 0.2H), 4.54−4.33 (m, 4H), 4.28−4.20 (m, 1H), 3.80−3.73 (m, 0.3H), 2.06−2.00 (m, 11H). $^{19}F\{H\}$ NMR (471 MHz, $CDCl_3$) anomeric mixture: δ −232.15, −232.70. $^{13}C\{^1H\}$ NMR (125 MHz, $CDCl_3$) δ 170.6, 170.5, 167.0, 95.4, 90.0, 81.5 (d, $J_{C\text{-}6,F}$ = 173.8 Hz), 81.3 (d, $J_{C\text{-}6,F}$ = 175.0 Hz), 72.9 (d, $J_{C\text{-}5,F}$ = 62.5 Hz), 72.7 (d, $J_{C\text{-}4,F}$ = 20.0 Hz), 71.2, 70.0, 68.3 (d, J = 6.3 Hz), 68.2 (d, J = 7.5 Hz), 68.0 (d, $J_{C,F}$ = 18.8 Hz), 20.7, 20.7. HRMS (ESI$^+$): found $[M + Na]^+$ 331.0793. $C_{12}H_{17}FNaO_8$ requires $[M + Na]^+$ 331.0800.

*6-Deoxy-6-fluoro-2,3,4-tri-O-acetyl-α-D-glucopyranosyl 1-Diphenylphosphate (**28**).* Following general procedure B, **27** (343 mg, 1.11 mmol) was phosphorylated to yield **28**. After silica gel column chromatography eluting with *n*-Hexane/EtOAc (2.5:1), **28** was obtained (344 mg, 57% yield) as a colorless oil. ^1H NMR (500 MHz, $CDCl_3$) δ 7.41−7.23 (m, 10H), 6.11 (dd, J = 7.0 and 3.5 Hz, 1H, H-1), 5.57 (t, J = 10.0 Hz, 1H), 5.18 (t, J = 10.0 Hz, 1H), 5.02 (dt, J = 10.0 and 3.0 Hz, 1H), 4.38 (ddd, J = 14.5, 11.0, and 3.5 Hz, 1H), 4.29 (ddd, J = 14.5, 11.0, and 3.5 Hz, 1H), 4.10−4.02 (m, 1H), 2.07 (s, 3H), 2.05 (s, 3H), 1.85 (s, 3H). $^{19}F\{H\}$ NMR (471 MHz, $CDCl_3$) δ −233.93. $^{31}P\{H\}$ NMR (202 MHz, $CDCl_3$) δ −13.80. ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.0, 169.8, 169.3, 150.3, 150.2, 123.0, 129.9, 125.8, 120.4, 120.4, 120.1, 120.1, 95.0 (d, J = 5.0 Hz, C-1), 80.4 (d, $J_{C\text{-}6,F}$ = 175.0 Hz, C-6), 70.4 (d, $J_{C\text{-}5,F}$ = 18.8 Hz), 69. Seven (d, J = 17.5 Hz), 69.2, 67.2 (d, J = 2.5 Hz), 20.6, 20.5, 20.2. HRMS (ESI$^+$): found $[M + Na]^+$ 563.1097. $C_{24}H_{26}FNaO_{11}P$ requires $[M + Na]^+$ 563.1089.

*6-Deoxy-6-fluoro-α-D-glucopyranosyl 1-Phosphate (**8**).* Following general procedure C, **8** (53 mg, 83% yield) was obtained as a colorless solid after lyophilization from **28** (73 mg, 0.14 mmol). ^1H NMR (500 MHz, D_2O) δ 5.43 (dd, J = 7.0 and 3.5 Hz, 1H), 4.77−4.57 (m, 2H), 3.95 (dd, $J_{H\text{-}6,F}$ = 30.5 and 10 Hz, 1H), 3.74 (t, J = 9.5 Hz, 1H), 3.51−3.46 (m, 2H), 3.15 (q, J = 7.5 Hz, 12H), 1.23 (t, J = 7.5 Hz, 18H). $^{19}F\{H\}$ NMR (471 MHz, D_2O) δ −236.29. $^{31}P\{H\}$ NMR (202 MHz, D_2O) δ 0.34. {Lit.[22] $^{19}F\{H\}$ NMR (471 MHz, D_2O) δ −237.48}

General Methods and Instrumentation: Enzymatic Analysis. Cps2L, purine nucleoside phosphorylase (PNP), and GDP-ManPP were overexpressed, isolated, and quantified as previously described.[7] Microbial xanthine oxidase (XO) and recombinant inorganic pyrophosphatase (IPP) were obtained from a supplier. IPP stock solutions (0.1 EU/μL) were prepared in double distilled water; thawed aliquots were kept in a fridge and were used for up to 1 month after thawing. XO stock solutions (120 U/mL) were prepared in Tris·HCl (pH 7.5, 25 mM) and stored at −30 °C; aliquots were used immediately after thawing. Kinetic reactions were performed in 96-well plates and were monitored using a UV spectrophotometer with SoftMax Pro version 4.8. Nonlinear regression analysis was performed using GraFit 5.0.4 Erithacus Software.

Analytical HPLC Assays. HPLC analysis of enzymatic reactions was performed as previously described.[6] A linear gradient from 90/10 A/B to 40/60 A/B over 8.0 min followed by a plateau at 40/60 A/B over 2.0 min at 1.0 mL/min^{-1} was used, where A was an aqueous buffer containing 12 mM Bu_4NBr, 10 mM KH_2PO_4 (pH 4.0), and 5% HPLC grade CH_3CN and B is HPLC grade CH_3CN.

IPP-PNP-XO Coupled Kinetic Assays with Cps2L. Stock solutions containing Tris·HCl (pH 7.5, 25 mM), **Glc 1P**, or fluorinated analogues (25−250 μM), dTTP (1 mM), $MgCl_2$ (5.7 mM), inosine (1 mM), IPP (1.7 EU/mL), PNP (1 μM), and XO (1.5 EU/mL) were allowed to preincubate at room temperature for 5 min, in order to consume contaminating P_i present in the solution. An appropriate concentration of Cps2L was then added to initiate the reaction. The initial reaction velocity was monitored spectrophotometrically over 10 min at a wavelength of 290 nm. Rates were linear. Observed initial kinetic rates were halved to account for the 2 equiv of P_i derived from each PP_i unit produced in the Cps2L reaction. Rates were converted from absorbance units (mAu) to concentration units (μM) using a phosphate standard curve. The standard curve was generated using identical conditions to those described for kinetic assays, except with variable P_i (10−100 mM $NaH_2PO_4{\cdot}H_2O$) instead of sugar phosphate. λ_{290} values were taken once the reaction had reached completion (after approximately 7 min) and plotted against phosphate concentration producing data that were fit by linear regression, providing a slope that was used as the conversion factor between mAu and μM units.

IPP-PNP-XO Coupled Kinetic Assays with GDP-ManPP. The GDP-ManPP kinetic assay was performed exactly the same as described in Cps2L, with stock solutions containing Tris·HCl (pH 7.5, 25 mM), **Man 1P**, or fluorinated analogues (25−250 μM), GTP

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(1 mM), $MgCl_2$ (5.7 mM), inosine (1 mM), IPP (1.7 EU/mL), PNP (1 μM), and XO (1.5 EU/mL), and 1,4-dithiothretiol (DTT) (1 mM).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b01485.

^{1}H NMR, ^{31}P{^{1}H} NMR, ^{19}F{^{1}H} NMR and ^{13}C{^{1}H} NMR spectra for all new compounds. HPLC and continuous assay methods and pK_a data (PDF)

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The authors declare no competing financial interest.

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