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Chemoenzymatic Synthesis of 4-Fluoro-*N*-Acetylhexosamine Uridine Diphosphate Donors: Chain Terminators in Glycosaminoglycan Synthesis

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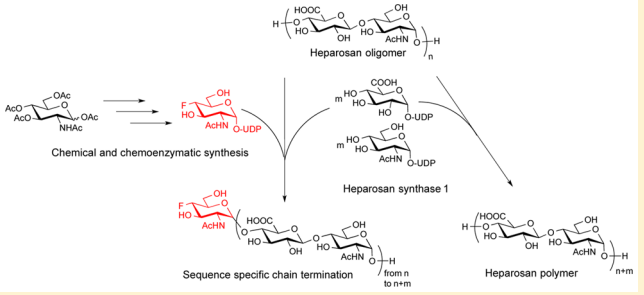
Supporting Information

ABSTRACT: Unnatural uridine diphosphate (UDP)-sugar donors, UDP-4-deoxy-4-fluoro-*N*-acetylglucosamine (4FGlcNAc) and UDP-4-deoxy-4-fluoro-*N*-acetylgalactosamine (4FGalNAc), were prepared using both chemical and chemoenzymatic syntheses relying on *N*-acetylglucosamine-1-phosphate uridylyltransferase (GlmU). The resulting unnatural UDP-sugar donors were then tested as substrates in glycosaminoglycan synthesis catalyzed by various synthases. UDP-4FGlcNAc was transferred onto an acceptor by *Pastuerella multocida* heparosan synthase 1 and subsequently served as a chain terminator.



Sugar nucleotides are the primary building blocks from which most complex carbohydrates are prepared and, thus, are crucial intermediates in carbohydrate metabolism.[1] Uridine diphosphate (UDP) monosaccharides are among the most common sugar nucleotide donors and are transferred to glycosyl acceptors by glycosyltransferases or synthases in glycan biosynthetic pathways. Both natural and unnatural UDP-sugars, can be chemically, enzymatically, or chemoenzymatically synthesized.[2] The synthesis of natural UDP-sugars is well established in literature,[3] however, the synthesis and application of unnatural UDP-sugars, which are otherwise not synthesized *in vivo* is not well explored. Such unnatural donors have great potential as enzymatic substrates in carbohydrate synthesis, as enzyme inhibitors in biochemical studies, as tools for assay development, and as reagents for the study of glycoconjugate biosynthesis.[4]

UDP-*N*-acetylglucosamine (GlcNAc), UDP-*N*-acetylgalactosamine (GalNAc), and UDP-glucuronic acid (GlcA) are widely studied natural donor substrates involved in glycosaminoglycan (GAG) synthesis.[2,5,6] GAGs are linear polysaccharides composed of disaccharide repeating units of hexosamine and uronic acid residues that are frequently sulfated.[2,5] Due to the presence of 1 → 4 linkages in GAGs, particularly those of the heparan sulfate/heparin family, the C4 position of the UDP-donor offers an interesting target for modification. By chemically modifying the monosaccharide prior to the addition of a UDP moiety, the structure of the final polysaccharide chain can be manipulated. Access to the C4 position can be complicated by its low relative reactivity compared to neighboring sites.[7] This and the presence of charged or polar functional groups requires multiple protection/deprotection steps for C4 modification. Purely chemical approaches for UDP-sugar synthesis generally are associated with low yields due to tedious protection/deprotection strategies, limiting scale-up and commercialization. A chemoenzymatic approach offers a synthetic route that circumvents many of these challenges. Chemoenzymatic synthesis of natural UDP-sugars has been accomplished using recombinant *Escherichia coli*-expressed *N*-acetylglucosamine-1-phosphate uridylyltransferase (GlmU).[8] We hypothesized that UDP-donors chemically modified at the C4 position could be prepared using GlmU and used to synthesize heparan sulfate/heparin oligosaccharides with novel structures and also potentially serve as chain terminators.[9]

The chemical synthesis of an unnatural UDP-GlcNAc donor generally relies on standard protection−deprotection chemistry for site-specific control. Previous work in our laboratory[8] reported the chemical synthesis of a series of GlcNR-1-phosphate analogs (R = H_2, N_2, COR′) and the recognition of these analogs by GlmU uridylyltransferase. In this study we concluded that C4 orientation of the hexosamine sugar had little impact on GlmU utilization of an UDP-HexNR (i.e., UDP-GlcNR or UDP-GalNR), yet the amide linkage to the C-2

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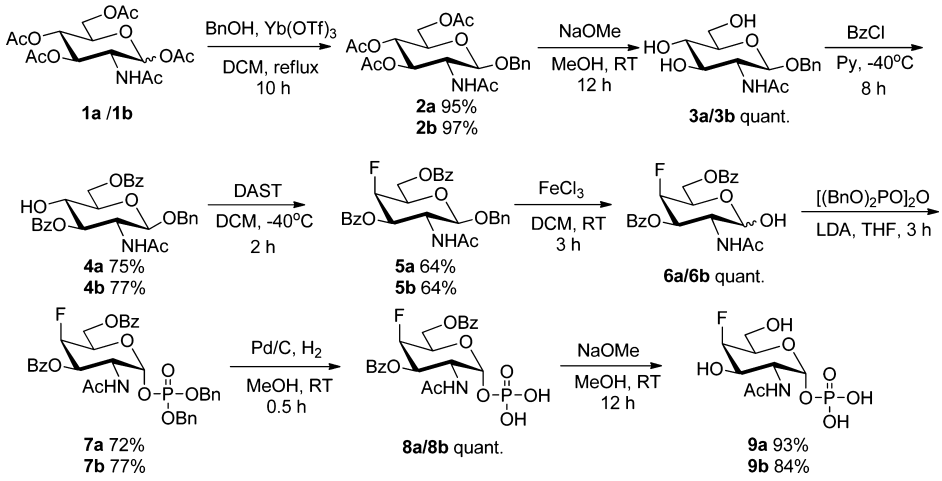
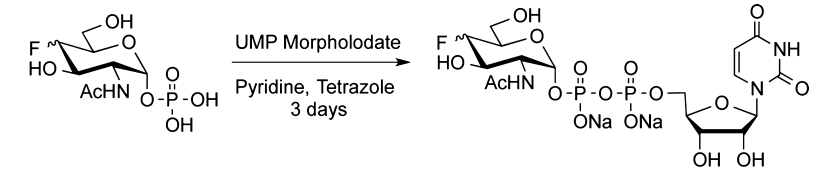
Scheme 1. Synthesis of 4FGalNAc-1-Phosphate 9a and 4FGlcNAc-1-Phosphate 9b[a]



[a]Although only one stereochemical series is shown (starting from D-glucosamine pentaacetate **1a** to 4FGalNAc-1-phosphate **9a**), both series were synthesized and the yields for both are provided.

Scheme 2. Chemical Synthesis of UDP-4FGalNAc 10a and UDP-4FGlcNAc 10b Donors



nitrogen of the phosphate analogs was crucial for GlmU recognition. While the promiscuity of GlmU has been notably utilized with 4-OH hexosamine phosphates[10,11] subsequent studies, by Wang et al.,[12] demonstrated that GlmU was intolerant of any functional group and the C4 position larger than a hydroxyl functionality. Previous syntheses of 4-fluoro-substituted sugar nucleotides have been reported, however these schemes were either purely chemical[13−15] or utilized a lengthy chemoenzymatic route with relatively low conversion rates by means of UDP-GalNAc pyrophosphorylase.[16] The use of fluorinated nucleotides as chain extension terminators has been well established with galactofuranose derivatives[17] while metabolic precursors of fluoro-substituted sugar nucleotides have been shown to disrupt nucleotide sugar synthesis at the point of incorporation.[18,19] Here we report the first chemoenzymatic synthesis of the 4-fluoro UDP-GlcNAc/-GalNAc analogs using GlmU in good yields (45%−50%) and the application of UDP-4FGlcNAc as a glycosylation terminator in heparan sulfate/heparin synthesis.

Our first objective in accessing the target UDP-4FGlcNAc/-4FGalNAc donors was to synthesize the corresponding 4FGlcNAc-/4FGalNAc-1-phosphate analogs. We began the synthesis with commercially available D-glucosamine/D-galactosamine pentaacetate **1a**/**1b** (Scheme 1).

Refluxing **1** with benzyl alcohol and $Yb(OTf)_3$ in dichloromethane at 60 °C provided the β-benzyl glycoside of D-glucosamine/galactosamine tetraacetate **2** in good yields (Glc 95% and Gal 97%) and with excellent selectivity. Zemplen deacetylation reaction with sodium methoxide in methanol afforded triol **3** in quantitative yield. The C-4 fluoride functionality was installed by a selective benzoylation of C-3 and C-6 followed by (diethylamino) sulfur trifluoride (DAST) reaction resulting in a C-4 configuration inverted intermediate **5a**. Using the same approach, the other corresponding compound **5b** was also successfully prepared. Deprotection of the anomeric hydroxyl followed by phosphorylation with tetrabenzyl pyrophosphate afforded the phosphorylated intermediate **7** with excellent α-selectivity. The targeted substrates **9a**/**9b** were obtained in high yields by using de-*O*-benzylation under hydrogenation followed by base catalyzed de-*O*-benzoylation.

With two monophosphate hexosamines in hand, we next focused our attention on the chemical approach to preparing the UDP-4FGalNAc **10a**/UDP-4FGlcNAc **10b** donors (Scheme 2). Compounds **9a**/**9b** were converted to their pyridinium salts and then stirred with uridine monophosphate (UMP)-morpholidate and tetrazole in pyridine for 3 days to afford the target unnatural UDP-sugars. However, at this stage, the purification became problematic. Neither size exclusion chromatography (SEC) nor strong anion exchange (SAX) chromatography could satisfactorily purify the target compounds as numerous organic phosphate salts, contained in the crude, showed very little difference in molecular weight and polarity. We were able to circumvent this problem by first converting all the phosphate salts to their sodium form using an ion-exchange resin before subjecting the product to SEC on a 1.2 m BioGel P2 column with elution by water. Lyophilization afforded the 4FGalNAc-/4FGlcNAc-UDP derivatives **10a**/**10b** in satisfactory isolated yields.

Next, we examined the ability of GlmU uridylyltransferase to use 4FGlcNAc-/4FGalNAc-1-phosphate analogs as substrates. Both 4FGlcNAc-1-phosphate **9b** and 4FGalNAc-1-phosphate

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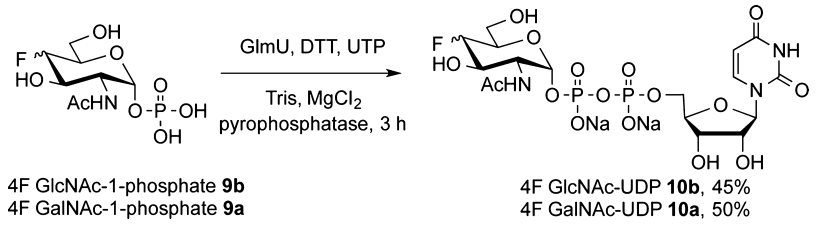
Scheme 3. 4FGlcNAc-1-Phosphate 9b and 4FGalNAc-1-Phosphate 9a as Substrates for GlmU



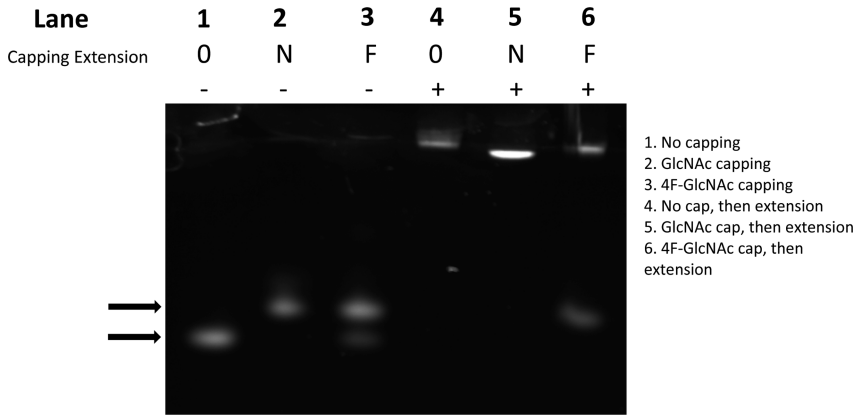


Figure 1. FACE analysis of dye-tagged heparosan oligosaccharide elongated with authentic or analog UDP-sugar donors by PmHS1.

9a were accepted by GlmU affording the corresponding UDP-sugar nucleotides in satisfactory yields of 45% and 50%, respectively (Scheme 3).

In our previous study,[8] we had found that the configuration of the 4-OH group appeared not to play a critical role in enzyme recognition. This study further confirmed our previous observation demonstrating that the 4-fluoro substituted substrates were both similarly good GlmU substrates.

With the chemically and enzymatically synthesized unnatual UDP-donors in hand, we next tested their utility as substrates in GAG synthesis. Two tests were conducted to determine 4F GlcNAc-/4FGalNAc-UDP were incorporated into a growing GAG chain or served as a chain extension terminator.[20] Copolymerization assays were conducted using radiolabeled sugar incorporation to monitor synthase-catalyzed copolymerization of UDP-[^{3}H]-GlcA with the UDP-4FHexNAc donor in the presence of a GAG acceptor. Controls with natural UDP-HexNAc (either UDP-GlcNAc or UDP-GalNAc) functioned as expected for the GAG synthases, *Pasturella multocida* (*Pm*) hyaluranan synthase HAS, *Pm* chondroitin synthase (CS), and *Pm* heparosan synthase HS 1 or 2.[21,22] However, the UDP-4FHexNAc donors showed no signal above background for the presence of incorporated UDP-4FHexNAc, leading to the conclusion that these unnatural sugars once incorporated are not substrates for further GAG chain elongation. Any inhibitory effect on chain polymerization was approximately the same or less than shown by UDP alone (~25−78% at 0.5 mM test range). Since an acceptor with UDP-4FHexNAc at its nonreducing terminus is missing a C4-hydroxyl required to form the glycosidic linkage, this was an expected result. It was, however, possible that such an acceptor could have been extended through the nearby C3-OH instead, as enzyme promiscuity is unpredictable, but this was not observed.

Termination studies were next performed using synthase-catalyzed polymerization assays that normally extends natural GAG chains to ~50−100 kDa. Parallel reactions were performed in which UDP-4FHexNAc sugar was added to test if the presence had any effect on GAG chain length, or if the sugar itself was a synthase inhibitor. The UDP-4FHexNAc substrate were tested with each of the *Pasteurella* GAG synthases that are capable of efficiently polymerizing HA (PmHAS), heparosan (PmHS1 or 2), or chondroitin (PmCS) chains. Interestingly, the two-step termination-type experiments, reactions with PmHS1 appeared to block chain extension when the acceptor was pretreated with the UDP-4FGlcNAc analog. Therefore, we did more direct experiments to verify that the 4FGlcNAc analog sugar from the UDP-4FGlcNAc donor was transferred onto the nonreducing end of the heparosan acceptor. As observed by fluorophore-assisted carbohydrate electrophoresis (FACE) gel (Figure 1) and by mass spectrometry (Table 1), new species were observed that had properties consistent with 4FGlcNAc incorporation. While the 4-fluoro group did block subsequent chain extension, it was not transferred efficiently as observed by the partial transformation of the starting material.

Table 1. Mass Spectrometric Analysis of Heparosan Anhydromannitol Tetrasaccharide or Hexasaccharide Acceptors and PmHS1-Catalyzed Extension Products[a]

reaction	heparosan tetrasaccharide	heparosan hexasaccharide
starting oligosaccharide	718.20	1097.34
+ GlcNAc	921.30	1300.42
+ 4FGlcNAc	923.30	1302.41

[a]The molecular weight was obtained in negative mode by matrix-assisted laser desorption (MALDI)-time of flight (ToF)-mass spectrometry (MS). The predicted masses were observed for the analog-terminated products.

In summary, the successful synthesis of UDP-4FGlcNAc/UDP-4FGalNAc analogs was completed using both an improved chemical method as well as a new chemoenzymatic

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approach. This constitutes the first synthesis of UDP-4FHexNAc analogs by the uridylyltransferase GlmU. These UDP-sugars were then tested for incorporation and termination capabilities using copolymerization assays. While neither analog showed signs of incorporation and extension of GAGs, reactions with PmHS1 pretreated with UDP-4FGlcNAc resulted in the incorporation of a single 4FGlcNAc at the nonreducing end of the acceptor acting as a chain terminator. These results suggest that the unnatural UDP-donor, 4FGlcNAc, can be used to synthesize heparan sulfate/heparin oligosaccharides having novel functionality and of defined sequence length.

EXPERIMENTAL SECTION

General Information. Unless noted otherwise, commercially available materials were used without further purification. All reagents were purchased from commercial corporations. Flash chromatography (FC) was performed using silica gel (200−300 mesh) according to the standard protocol. All reactions under standard conditions were monitored by thin-layer chromatography (TLC) on gel F254 plates. Mass data were acquired by MALDI-ToF-MS or electrospray ionization (ESI)-high-resolution (HR)-MS on an LTQ-Orbitrap XL FT-MS spectrometer. ^{1}H and ^{13}C NMR spectra were recorded on a 800 MHz (200 MHz for ^{13}C NMR) or 600 MHz (150 MHz for ^{13}C NMR) spectrometer. ^{19}F, ^{31}P NMR spectra were recorded on a 500 MHz NMR spectrometer.

*Synthesis of Benzyl 2-Acetamido-3,6-di-O-benzoyl-2-deoxy-β-D-gluco-/galacto-pyranoside (**4a/4b**).* Commercially available D-glucosamine/galactosamine pentaacetate **1a**/**1b** (1.0 g, 2.6 mmol) was refluxed with benzyl alcohol (0.85 mL, 8.2 mmol) and Yb(OTf)$_3$ (200 mg, 0.3 mmol) in dichloromethane (20 mL) at 60 °C for 3 h. The solution was allowed to cool to room temperature and then washed three times with deionized water. The organic layer was concentrated under reduced pressure and the product was recrystallized from methanol and hexanes to afford **2a**/**2b** as clear crystals in 95 and 97% yields, respectively.

To a solution of **2a**/**2b** (350 mg, 0.8 mmol) in anhydrous MeOH (10 mL) was added sodium methoxide solution (4.82 mL, 0.5 M in methanol) at room temperature and the resulting solution left to stir for 2 h. The solution was then neutralized with prewashed and acidified Amberlite IR-120 hydrogen ion-exchange resin and the reaction mixture filtered through cotton wool to remove the resin. The eluent was then concentrated under reduced pressure to give the compound **3a**/**3b** as white solids in quantitative yields. The resulting solids were used in the following step without further purification.

Compound **3a**/**3b** (240 mg, 0.77 mmol) was dissolved in pyridine (4 mL) and the solution cooled to −40 °C in a dry ice/ethyl acetate bath. Benzoyl chloride (196 μL, 1.69 mmol) was added dropwise and the solution was allowed to warm to room temperature overnight. The pyridine was removed under vacuum and the resulting crude mixture was purified by column chromatography (hexanes/EtOAc 3:1) to yield compound **4a**/**4b** as white solids (300 mg, 75% and 307 mg, 77% respectively).

*Benzyl 2-Acetamido-3,6-di-O-benzoyl-2-deoxy-β-D-glucopyranoside (**4a**).* ^{1}H NMR (800 MHz, CDCl$_3$) δ: 8.06 (d, *J* = 7.8, 2H, H−Ar), 7.96 (d, *J* = 7.8, 2H, H−Ar), 7.56 (t, *J* = 7.38, 1H, H−Ar), 7.53 (t, *J* = 7.38, 1H, H−Ar), 7.43 (t, *J* = 7.63, 2H, H−Ar), 7.37 (t, *J* = 7.63, 2H, H−Ar), 7.27−7.23 (m, 5H, H−Ar), 5.89 (d, *J* = 9.28, 1H, H-1), 5.36 (dd, *J* = 9.07, 10.45, 1H, H-6a), 4.88 (d, *J* = 12.14, 1H, H−OCH$_2$Bn), 4.70 (dd, *J* = 4.85, 12.14, 1H, H-3), 4.66−4.63 (m, 3H, H-2, H-5, H-6b), 4.62 (d, *J* = 12.14, 1H, H′-OCH$_2$Bn), 3.77−3.74 (m, 1H, H-4), 1.77 (s, 3H, H−Ac); ^{13}C NMR (200 MHz, CDCl$_3$) δ 170.4, 167.6, 167.1, 137.2, 133.6, 133.4, 130.0, 129.9, 129.6, 129.1, 128.5, 128.5, 128.4, 128.0, 127.9, 99.6, 76.1, 74.3, 70.3, 69.4, 63.7, 54.1, 23.3. ESI-HRMS calcd. for $C_{29}H_{29}NO_8$ ([M+Na]$^+$) 542.1785, found 542.1789.

*Benzyl 2-Acetamido-3,6-di-O-benzoyl-2-deoxy-β-D-galactopyranoside (**4b**).* White solid. ^{1}H NMR (600 MHz, CDCl$_3$) δ 8.14−8.03 (m, 4H, H−Ar), 7.66−7.57 (m, 2H, H−Ar), 7.54−7.43 (m, 4H, H−Ar), 7.39−7.29 (m, 5H, H−Ar), 5.51 (d, *J* = 8.96, 1H, H-1), 5.35 (dd, *J* = 2.81, 11.14, 1H, H-6a), 4.94 (d, *J* = 12.14, 1H, H−OCH$_2$OBn), 4.74−4.61 (m, 3H, H-3, H-5, H′-OCH$_2$OBn), 4.51−4.44 (m, 1H, H-2), 4.25 (d, *J* = 2.47, 1H, H-6b), 4.01 (t, *J* = 6.39, 1H, H-4), 1.87 (s, 3H, H−Ac); ^{13}C NMR (150 MHz, CDCl$_3$) δ 170.5, 166.5, 166.4, 137.1, 133.7, 133.5, 130.3, 130.1, 129.9, 129.8, 129.3, 128.7, 128.6, 128.3, 128.2, 99.8, 73.4, 72.5, 70.5, 67.1, 63.0, 51.2, 23.5. ESI-HRMS calcd. for $C_{29}H_{29}NO_8$ ([M+Na]$^+$) 542.1785, found 542.1760.

*Synthesis of Benzyl 2-Acetamido-4-fluoro-3,6-di-O-benzoyl-2,4-deoxy-β-D-galacto/gluco-pyranosides (**5a/5b**).* Diethylamino sulfur trifluoride (76 μL, 0.57 mmol) was added to a solution of **4a**/**4b** (100 mg, 0.19 mmol) in dry DCM (5 mL) at −40 °C. The mixture was then allowed to warm to room temperature over 2 h, and was quenched by the addition of MeOH (several drops) at 0 °C. The residue was diluted with DCM and the solution was washed with water, sat. NaHCO$_3$ aq., and brine and then was dried over anhydrous MgSO$_4$. The solvent was removed by evaporation and purified by silica gel chromatography (hexanes/EtOAc 3:1) to yield **5a**/**5b** as yellow oils (64 mg, 64% for both compounds).

*Benzyl 2-Acetamido-4-fluoro-3,6-di-O-benzoyl-2,4-deoxy-β-D-galactopyranoside (**5a**).* Yellow oil. ^{1}H NMR (600 MHz, CDCl$_3$) δ: 8.06 (d, *J* = 7.90, 4H), 7.59 (t, *J* = 7.50, 2H), 7.48−7.43 (m, 4H), 7.34−7.29 (m, 5H), 5.55 (ddd, *J* = 2.48, 11.24, 27.30, 1H), 5.45 (d, *J* = 8.83, 1H), 5.01 (dd, *J* = 2.48, 50.51, 1H), 4.94 (d, *J* = 12.09, 1H), 4.90 (d, *J* = 8.46, 1H), 4.70−4.64 (m, 2H), 4.56 (dd, *J* = 6.79, 11.24, 1H), 4.26−4.20 (m, 1H), 4.06 (dt, *J* = 6.74, 26.40, 1H), 1.85 (s, 3H); ^{13}C NMR (150 MHz, CDCl$_3$) δ 173.8, 169.5, 169.4, 140.3, 137.0, 136.8, 133.4, 133.2, 133.1, 132.8, 132.3, 131.9, 131.8, 131.6, 131.4, 102.5, 89.2 (d, *J* = 186.67), 74.5 (d, *J* = 19.57), 74.1, 74.0, 65.4, 55.2, 26.8. ESI-HRMS calcd. for $C_{29}H_{28}FNO_7$ ([M+H]$^+$) 522.1923, found 522.1910.

*Benzyl 2-Acetamido-4-fluoro-3,6-di-O-benzoyl-2,4-deoxy-β-D-glucopyranoside (**5b**).* Yellow oil. ^{1}H NMR (600 MHz, CDCl$_3$) δ: 8.10−8.02 (m, 4H), 7.64−7.55 (m, 2H), 7.50−7.43 (m, 4H), 7.35−7.28 (m, 5H), 5.80−5.75 (br, 1H), 5.42 (d, *J* = 9.03, 1H), 5.00−4.93 (m, 2H), 4.74−4.62 (m, 2H), 4.49−4.39 (m, 2H), 4.30−4.29 (m, 1H), 3.97−3.92 (m, 1H), 1.85 (s, 3H); ^{13}C NMR (150 MHz, CDCl$_3$) δ 173.7, 169.4, 169.2, 140.3, 137.0, 136.8, 133.4, 133.2, 132.9, 132.3, 131.9, 131.9, 131.6, 131.5, 102.95, 90.4 (d, *J* = 188.90), 75.0 (d, *J* = 22.21), 74.2, 74.0, 66.3, 57.7, 26.9. ESI-HRMS calcd. for $C_{29}H_{28}FNO_7$ ([M+H]$^+$) 522.1923, found 522.1902.

*Synthesis of 1-Phospho-2-acetamido-4-fluoro-α-D-galacto-/gluco-pyranosides (**7a/7b**).* To a solution of **5a**/**5b** (200 mg, 0.38 mmol) in dry DCM (5 mL) was added FeCl$_3$ (136 mg, 0.84 mmol) under N$_2$ protection. After stirring for 3 h, the reaction was quenched by the addition of aq. NH$_4$Cl. The aqueous layer was extracted with DCM (3 × 15 mL). The combined organic phase was washed with brine, dried over Na$_2$SO$_4$, and concentrated under vacuum. The resulting crude product **6a**/**6b** was used in the following step without further purification. Crude **6a**/**6b** was dissolved in dry THF (5 mL) and the solution cooled to −78 °C. To the cooled solution, lithium diisopropylamide solution (LDA, 2 M in THF) (0.42 mL, 0.84 mmol) was added dropwise (CAUTION: this is a pyrophoric reagent and care must be taken in undertaking this reaction). After 30 min, tetrabenzyl pyrophosphate (307 mg, 0.57 mmol) was added to the reaction mixture. The reaction was stirred at −78 °C for 30 min and slowly warmed to 0 °C over 3h. Then the reaction was quenched by the addition of aq. NH$_4$Cl. The aqueous layer was extracted with EtOAc (3 × 15 mL), and the combined organic phase was washed with brine, dried over Na$_2$SO$_4$, and concentrated under vacuum. The crude was purified by FC (silica gel, Hexanes/EtOAc 3:1) to give compounds **7a**/**7b** as yellow oils (191 mg, 72% and 204 mg, 77% respectively).

*Dibenzyl 2-Acetamido-4-fluoro-3,6-di-O-benzoyl-2,4-deoxy-α-D-galactopyranosyl Phosphate (**7a**).* Yellow oil. ^{1}H NMR (600 MHz, CDCl$_3$) δ: 8.08−7.97 (m, 4H), 7.62−7.51 (m, 2H), 7.49−7.42 (m, 2H), 7.41−7.23 (m, 12H), 5.82−5.76 (m, 1H), 5.66−5.60 (m, 1H), 5.35−5.24 (m, 1H), 5.12−4.96 (m, 5H), 4.93−4.86 (m, 1H), 4.52−4.41 (m, 2H), 4.38−4.28 (m, 1H), 1.67 (s, 3H); ^{13}C NMR (150 MHz, CDCl$_3$) δ 173.6, 169.8, 169.2, 138.7, 138.5, 137.1, 136.7, 133.4, 133.1, 132.7, 132.3, 132.1, 132.1, 132.0, 131.8, 131.5, 100.4 (d, *J* = 6.04), 89.4

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(d, J = 189.64), 73.4, 73.3, 72.7, 72.6, 71.9, 71.8, 65.2, 65.2, 50.8, 26.2. ESI-HRMS calcd. for $C_{36}H_{36}FNO_{10}P$ ($[M+H]^+$) 692.2055, found 692.2041.

*Dibenzyl 2-Acetamido-4-fluoro-3,6-di-O-benzoyl-2,4-deoxy-α-D-glucopyranosyl Phosphate (**7b**).* Yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.09−8.01 (m, 4H), 7.96−7.94 (m, 2H), 7.91−7.88 (m, 1H), 7.84−7.80 (m, 2H), 7.63−7.56 (m, 2H), 7.52−7.43 (m, 9H), 5.93−5.89 (m, 2H), 5.67−5.64 (m, 1H), 5.49 (dd, J = 3.16, 11.46, 1H), 5.15−5.03 (m, 4H), 4.97−4.91 (m, 1H), 4.54−4.49 (m, 1H), 4.46−4.41 (m, 1H), 4.34−4.29 (m, 1H), 1.66 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.7, 169.7, 169.5, 138.8, 138.7, 137.0, 136.9, 136.7, 136.6, 133.3, 133.2, 133.1, 132.7, 132.4, 132.2, 132.2, 132.1, 132.0, 131.9, 131.8, 131.7, 100.5 (d, J = 6.04), 89.5 (d, J = 181.56), 73.4, 73.3, 72.6, 71.6, 71.2, 65.3, 51.6, 26.2. ESI-HRMS calcd. for $C_{36}H_{36}FNO_{10}P$ ($[M+H]^+$) 692.2055, found 692.2041.

*Phosphate 2-Acetamido-4-fluoro-α-D-galacto-/gluco-pyranoside (**9a/9b**).* The protected phosphate (25 mg, 0.036 mmol) was dissolved in MeOH (2 mL). Pd/C (10 wt% on activated carbon) (3 mg) was added and the mixture was stirred under a hydrogen atmosphere for 2 h. The palladium was filtered off and the solvent removed to yield a crude **8a**/**8b**, which was used in the next step without further purification. To a solution of crude **8a**/**8b** in anhydrous MeOH (3 mL) was added sodium methoxide solution (20 μL, 5.5 M in methanol) at 0 °C and the resulting solution was left to stir for 2 h. The solution was then neutralized with prewashed and acidified Amberlyte IR-120 hydrogen ion-exchange resin and the reaction mixture filtered through cotton wool to remove the resin. The eluent was then concentrated under reduced pressure to give the corresponding 4-F phospho-sugar analog **9a**/**9b** as white solids (10 mg, 93% and 9 mg, 84% respectively).

*Phosphate 2-Acetamido-4-fluoro-α-D-galactopyranoside (**9a**).* White solid. ^{1}H NMR (800 MHz, D_2O) δ 5.25 (d, J = 3.8, 1H, H-1), 4.32 (dt, J = 9.12, 50.87, 1H, H-4), 4.01−3.93 (m, 2H, H-3, H-5), 3.89−3.84 (m, 1H, H-2), 3.77−3.73 (m, 1H, H-6a), 3.71−3.66 (m, 1H, H-6b), 1.93 (s, 3H, H−Ac); ^{13}C NMR (200 MHz, D_2O) δ 174.5, 92.8 (d, J = 5.0), 89.3 (d, J = 181.57), 69.7 (d, J = 23.82), 69.4 (d, J = 17.53), 59.8, 53.5, 22.0; ^{31}P NMR (202.5 MHz, D_2O) δ −0.5; ^{19}F NMR (470 MHz, D_2O) δ −198.0 (dd, J = 14.1, 51.7). (ESI-HRMS calcd. for $C_8H_{15}FNO_8P$ ($[M-H]^-$) 302.0447, found 302.0435.

*Phosphate 2-Acetamido-4-fluoro-α-D-glucopyranoside (**9b**).* White solid. ^{1}H NMR (800 MHz, D_2O) δ: 5.31 (s, 1H, H-1), 4.34 (dt, J = 9.48, 50.44, 1H, H-4), 4.00−3.93 (m, 2H, H-3, H-5), 3.93−3.86 (m, 1H, H-2), 3.76−3.68 (m, 2H, H-6a, H-6b), 1.93 (s, 3H, H−Ac); ^{13}C NMR (200 MHz, D_2O) δ 174.7, 93.2 (d, J = 5.3), 89.0 (d, J = 179.91), 70.2 (d, J = 24.67), 68.8 (d, J = 19.51), 59.7, 53.3, 21.9; ^{31}P NMR (202.5 MHz, D_2O) δ −1.7; ^{19}F NMR (470 MHz, D_2O) δ −198.2 (dd, J = 14.1, 51.7). ESI-HRMS calcd. for $C_8H_{15}FNO_8P$ ($[M-H]^-$) 302.0447, found 302.0440.

*Chemical Synthesis of Disodium Uridine 5′-(2-Acetamido-2,4-dideoxy-4-fluoro-α-D-galacto-/gluco-pyranosyl) Diphosphate (**10a/10b**).* A solution of monophosphate **9a**/**9b** (4 mg, 0.013 mmol) in MeOH (2 mL) was treated with Et_3N (9 μL, 0.07 mmol), and then concentrated under vacuum to yield crude bis(triethylammonium) phosphate. Without purification, this crude material was coevaporated with dry pyridine (3 × 3 mL). Uridine 5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt (14 mg, 0.02 mmol) was coevaporated with pyridine (3 × 3 mL), then transferred via a cannula into the reaction flask. The combined reagents were coevaporated with pyridine (2 × 2 mL) then pyridine (2 mL), 1H-tetrazole (4 mg, 0.05 mmol) were added and the reaction mixture stirred at room temperature for 3 d. The reaction mixture was then concentrated under vacuum and was converted into Na^+ form by passing through a Dowex (Na^+) column. The resulting fraction was concentrated under vacuum and the residue was loaded onto a Bio-Gel P2 column (1 × 120 cm) and eluted with H_2O. Fractions were collected, and those containing the product as determined by MS were combined and freeze-dried to afford 4F-GlcNAc/GalNAc-UDP as white powders (5 mg, 61%).

*Disodium Uridine 5′-(2-Acetamido-2,4-dideoxy-4-fluoro-α-D-galactopyranosyl) Diphosphate (**10a**).* White solid. ^{1}H NMR (800 MHz, D_2O) δ: 7.83 (d, J = 7.72, 1H, uridine-H″-6), 5.87−5.81 (m, 2H, uridine-H″-5, rib-H′-1), 5.39 (br, 1H, H-1), 4.35 (dt, J = 8.93, 50.75, 1H, H-4), 4.26−4.20 (m, 2H, rib-H′-2, rib-H′-5a), 4.18−4.10 (m, 2H, rib-H′-3, rib-H′-4), 4.09−4.03 (m, 1H, rib-H′-5b), 4.01−3.91 (m, 3H, H-2, H-3, H-5), 3.75 (d, J = 12.12, 1H, H-6a), 3.68 (d, J = 12.12, 1H, H-6b), 1.95 (s, 3H, H−Ac); ^{13}C NMR (200 MHz, D_2O) δ 174.7, 166.4, 151.7, 141.5, 102.6, 94.1, 88.7 (d, J = 179.60), 88.5, 83.1 (d, J = 9.50), 73.8, 70.4 (d, J = 25.0), 69.5, 69.2 (d, J = 18.56), 64.9 (d, J = 5.14), 59.6, 53.2, 22.0; ^{31}P NMR (202.5 MHz, D_2O) δ −11.5, −13.3; ^{19}F NMR (470 MHz, D_2O) δ −163.4 (dd, J = 14.1, 51.7). ESI-HRMS calcd. for $C_{17}H_{24}FN_3O_{16}P_2Na_2$ ($[M-2Na-H]^-$) 608.0700, found 608.0720.

*Disodium Uridine 5′-(2-Acetamido-2,4-dideoxy-4-fluoro-α-D-glucopyranosyl) Diphosphate (**10b**).* White solid. ^{1}H NMR (800 MHz, D_2O) δ: 7.87 (d, J = 8.15, 1H, uridine-H″-6), 5.90−5.85 (m, 2H, uridine-H″-5, rib-H′-1), 5.43−5.41 (m, 1H, H-1), 4.39 (dt, J = 9.49, 50.95, H-4), 4.29−4.25 (m, 2H, rib-H′-2, rib-H′-5a), 4.21−4.18 (m, 1H, rib-H′-3,), 4.18−4.14 (m, 1H, rib-H′-4), 4.12−4.08 (m, 1H, rib-H′-5b), 4.04−3.95 (m, 3H, H-2, H-3, H-5), 3.81−3.76 (m, 1H, H-6a), 3.74−3.70 (m, 1H, H-6b), 1.99 (s, 3H, H−Ac); ^{13}C NMR (200 MHz, D_2O) δ 174.8, 166.2, 151.8, 141.6, 102.6, 94.2 (d, J = 5.85), 88.8 (d, J = 182.54), 88.5, 83.1 (d, J = 9.25), 73.8, 70.4 (d, J = 24.58), 69.5, 69.2 (d, J = 18.56), 65.0, 59.6, 53.2, 22.0; ^{31}P NMR (202.5 MHz, D_2O) δ −11.5, −13.3; ^{19}F NMR (470 MHz, D_2O) δ −198.4. ESI-HRMS calcd. for $C_{17}H_{24}FN_3O_{16}P_2Na_2$ ($[M-2Na-H]^-$) 608.0700, found 608.0714.

General Procedure for the Enzymatic Preparation of the UDP-Sugar Nucleotides. A reaction mixture containing the monophosphate, glucosamine-1-phosphate acetyltransferase/*N*-acetyl glucosamine-1-phosphate uridylyltransferase (GlmU), Tris-HCl (46 mM, pH 7.0), $MgCl_2$ (5 mM), dithiothreitol (200 μM), UTP (2.5 mM), and inorganic pyrophosphatase (0.012 units/μL), from Sigma-Aldrich, was incubated at 30 °C for 3 h. For GalNAc substrates, a second portion of GlmU was added to the reaction mixture on the second day. The reaction was monitored through MS. Then three reaction volumes of ethanol were added to quench the reaction and stored overnight at −20 °C. Centrifugation at 3000 rcf for 30 min afforded the crude product in the supernatant which was then concentrated and further purified on a BioGel (Bio-Rad) P2 column (1 × 100 cm) and eluted with H_2O. Fractions were collected, and those containing the product as determined by UV absorbance at 262 nm were combined and freeze-dried to afford the pure product. Characterization data were consistent with chemically synthesized products.

Expression of GlmU Enzyme. The procedures for the expression and purification of GlmU were described previously. Briefly, GlmU was cloned into a PET 21b vector (Novagen) to form a C-terminal $(His)_6$-tagged fusion protein. The expression was carried out in BL21 star cells (Invitrogen). The protein was purified by a Ni-Sepharose 6 Fast Flow column (GE Health) following a standard procedure.

Polymerization Assays. To assess if the analogs would serve as inhibitors or terminators of the GAG synthases, various protocols were used to detect if the reagents could slow polymerization rates or block elongation of nascent chains. For the kinetic experiments, each of the fluorine-containing UDP-hexosamine analogs (0.5 mM) was co-incubated in a reaction containing radiolabeled UDP-[^{3}H]GlcA (0.05−0.5 mM; 0.1 μCi; PerkinElmer), with or without the addition of the authentic UDP-hexosamine. Reactions (25 μL) also contained 50 mM Tris, pH 7.2, and 1 mM $MnCl_2$ with 0.35−25 μg of purified recombinant enzyme (PmHS1 or PmHS2; PmHAS or PmCS) (as well as 1 M ethylene glycol for PmHAS and PmCS) at 30 °C for 10 min to 24 h. In other control reactions, UDP, the byproduct of GAG polymerization and a part of the analog structure, was employed to assess for any inhibitor-like effects. The reaction mixtures were quenched with sodium dodecyl sulfate detergent (2% final conc) and analyzed by descending paper chromatography (overnight in 65:35 ethanol/1 M ammonium acetate buffer, Whatmann 3MM paper). As a negative control for assay background, a reaction with no UDP-hexosamine was tested in parallel (GAG chain polymerization can only occur when UDP-GlcUA and a functional UDP-hexosamine are present simultaneously).

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To assess the analogs' termination functionality (i.e., stop GAG chain growth), a two-step assay was used with unlabeled UDP-sugars and enzyme (6−10 μg). First, a quasi-monodisperse hyaluronan (for PmHAS and PmCS) or heparosan (PmHS1) polysaccharide acceptor (each ∼12 kDa) was capped by reacting it with an excess (1.1 to 10 molar excess over acceptor) of the various UDP-hexosamine analogs (or no analog) for 2 h at 30 °C. Second, both the authentic UDP-sugars (8 mM each UDP-GlcA and the natural UDP-hexosamine) were added and the reactions were continued overnight. If the acceptor had been capped with an nonextendable 4-F group, then the polysaccharide acceptor's size would not increase. The polysaccharide products were analyzed on agarose gels with Stains-All staining.[23]

FACE Electrophoresis. To confirm that the analogs were indeed being added to the GAG acceptor, a heparosan trisaccharide was labeled with AMAC (2-aminoacridone) to facilitate fluorescent visualization of the extended oligosaccharides and polysaccharides.[24] First, the AMAC-labeled oligosaccharide was capped by reacting it with a 10-fold molar excess of the either the fluorine-containing analog or UDP-GlcNAc over acceptor or no analog in a buffer containing 50 mM Hepes, pH 7.2, 1 mM $MnCl_2$, and purified recombinant PmHS1 (5 μg) for 9 h at 30 °C. Second, both of the authentic UDP-sugars required for polymerization were added (final 1.6 mM each) to a portion of these reactions and the incubation was continued overnight. The products of each step were analyzed on a 21% 1X TAE polyacrylamide gel run in Tris-Tricine buffer and visualized by UV fluorescence.

Mass Spectroscopy. Heparosan oligosaccharides (mixture of 4 and 6 sugars long; 1 μg) were reacted with a 10-fold molar excess of either UDP-GlcNAc, the fluorine-containing analog, or no analog in a buffer containing 50 mM Tris, pH 7.2, 1 mM $MnCl_2$, and purified recombinant PmHS1 or PmHS2 (5 μg) for 5.5 h at 30 °C. The reactions were assessed by MALDI-ToF-MS, with the matrix 6-aza-2-thiothymine at a concentration of 5 mg/mL in 50% acetonitrile, 0.1% trifluoroacetic acid (TFA) (ref 3). HA oligosaccharides were employed as mass calibrants (Hyalose, LLC, Oklahoma City, OK).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.joc.6b02929.

^{1}H, ^{13}C, ^{19}F, and ^{31}P NMR spectra of the components (PDF)

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Notes

The authors declare no competing financial interest.

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