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Synthesis of 4-azido-*N*-acetylhexosamine uridine diphosphate donors: Clickable glycosaminoglycans

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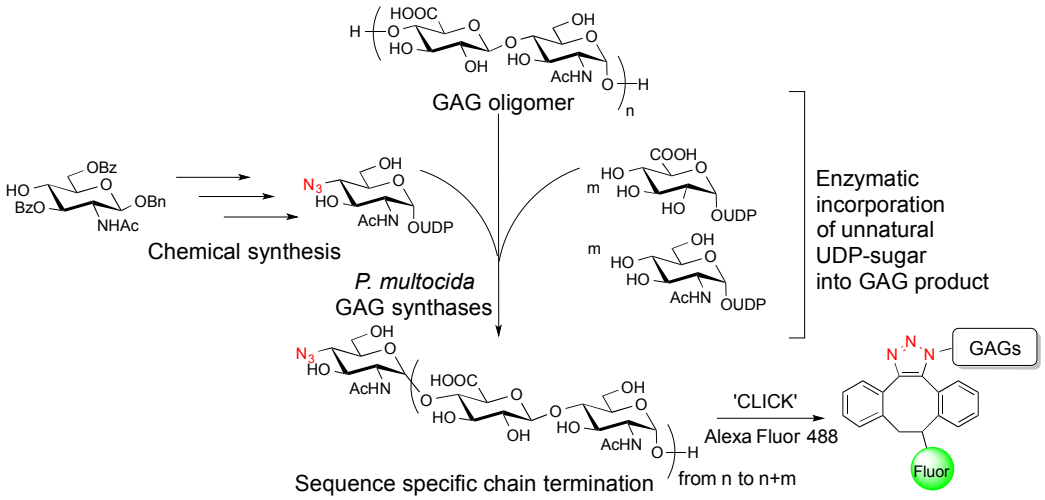
Abstract:

Unnatural chemically modified, nucleotide sugars, UDP-4-N_3-GlcNAc and UDP-4-N_3-GalNAc were chemically synthesized for the first time. These unnatural UDP-sugar products were then tested for incorporation into hyaluronan, heparosan or chondroitin using polysaccharide synthases. The UDP-4-N_3-GlcNAc served as a chain termination substrate for hyaluronan or heparosan synthases; the resulting 4-N_3-GlcNAc-terminated hyaluronan and heparosan were then successfully conjugated with Alexa Fluor 488 DIBO alkyne, demonstrating that this approach is generally applicable for labeling and detection of suitable GAGs.



Glycosaminoglycans (GAGs) are a family of structurally complex heteropolysaccharides composed of repeating disaccharide units containing hexosamine residue.[1] GAGs are prevalent as free glycans or in the core structures of glycans in glycoproteins and glycolipids, and control a wide range of physiological and pathological events including cell-cell interactions,[2] enzyme inhibition,[3] cell proliferation[4] and growth factor receptor[5] during various metabolic processes. Heparin/heparan sulfate, hyaluronan and chondroitin/dermatan sulfate are the three most studied and abundant classes of GAG polymers. Nucleotide sugars are key intermediates in carbohydrate metabolism and glycoconjugate biosynthesis.[6] Uridine diphosphate (UDP) monosaccharides are common sugar nucleotide donors being transferred to the non-reducing terminus of carbohydrate chains by glycosylation reactions with glycosyltransferases in the GAGs biosynthetic pathway.[6,7]

GlcNAc and GalNAc are naturally occurring saccharide residues prevalent in GAGs, *N*-acetylglucosamine (GlcNAc) found in heparin/ heparan sulfate/hyaluronan while *N*-acetylgalactosamine (GalNAc) found in chondroitin/dermatan sulfate, and playing essential roles in biological processes.[6,8] Thus, the corresponding naturally occurring UDP-GlcNAc/GalNAc as well as structural analogues, are of great interest for enzymatic reactions in carbohydrate synthesis and would be a good approach to understand the mechanism of GlcNAc/GalNAc-related pathways.[9] Due to its unique reactivity in biological system, azide-derivatized UDP-sugar becomes an exciting target and has been well established.[10]

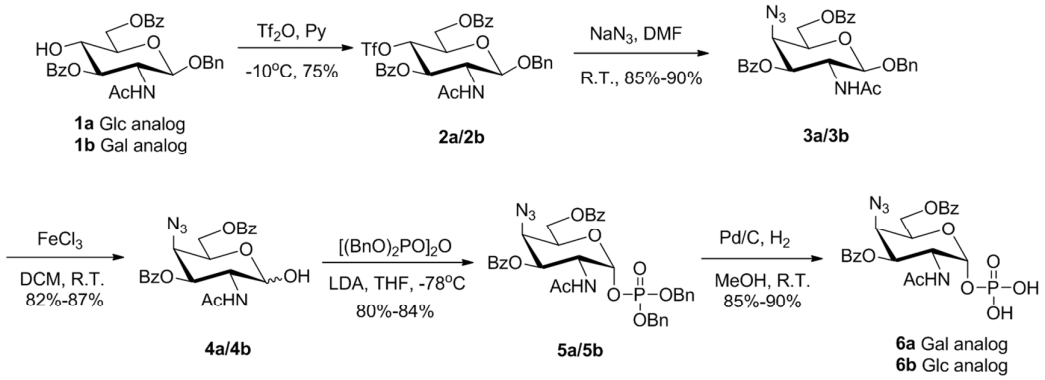
The azido moiety is inert to natural processing or reactivity within biological systems, but can be readily covalently tagged with imaging probes or epitope using an azide-specific reaction, such as Staudinger ligation[11] with phosphines and the [3+2] cycloaddition with alkynes,[12] which permits the detection of specific glycoconjugate types on cells or in living organisms or the selective capture of glycoproteins from cell or tissue lysates. At this time, various chemical or enzymatic approaches have been developed to synthesize azide-derivatized UDP-sugars. Chen *et al.*[10b] reported a one-pot three enzymes system to produce UDP-6N_3GlcNAc; Wang *et al.*[10a] developed a chemoenzymatic approach involved *Escherichia coli* GlcNAc-1-P

uridyltransferase (GlmU) catalysis to obtain UDP-6N_3GalNAc and UDP-GalNAz. However, due to the more challenging modification for C4 position either in chemical or enzymatic way, very few C4 modified azido-UDP sugars have been reported even though the 1→4 linkage is particular in GAGs.[13]

In our recent work, we reported the first chemoenzymatic synthesis of the UDP-4-fluoro (F) GlcNAc/-4-FGalNAc analogs using recombinantly–expressed *N*-acetylglucosamine-1-phosphate uridylyltransferase (GlmU) and the application of UDP-4-FGlcNAc as a glycosylation terminator in the chemoenzymatic synthesis of heparan sulfate/heparin.[14] Here we present the first synthesis of 4-N_3 derivatized GlcNAc and GalNAc UDP sugars, that were tested for their ability to mimic two natural donor substrates required in GAGs synthesis, resulting in metabolic conversion to azide-functionalized GAGs as potential substrates for Huisgen 1,3-dipolar cycloaddition.[12] The corresponding unnatural GlcNAc-1-phosphate substrate specificity of enzyme GlmU was also investigated.

Our first objective was the synthesis of 4-N_3-GlcNAc-/4-N_3-GalNAc-1-phosphate analogs. These precursors could then be used as substrates for either chemical or enzymatic reactions, to access the target UDP-4-N_3-GlcNAc or UDP-4-N_3-GalNAc donors. Based on our previous studies,[14] we started the synthesis from the known compounds benzyl 2-acetamido-2-deoxy-β-D-gluco-/galacto-pyranoside **1a/1b** (Scheme 1)[14,15]. The placement of the C-4 azide functionality was initiated through the reaction of Tf_2O in pyridine with **1a/1b** to afford the triflates **2a/2b**. Nucleophilic displacement of triflate groups in **2a/2b** with sodium azide in dimethylformamide afforded 4-N_3- GalNAc/4-N_3-GlcNAc derivatives **3a/3b** in 85-90% yields with the inversion of the C-4 configuration.[16] The anomeric benzyl group was oxidatively removed affording **4a**/**4b**, followed by phosphorylation[17] with tetrabenzyl pyrophosphate to obtain the phosphorylated intermediate **5a/5b** in good yield (80%-84%) and with excellent α-selectivity. Deprotection of benzyl groups was achieved under hydrogenation to obtain the target benzoate-protected substrates **6a/6b** in good yields (85%-90%). Hydrogenation needed to be strictly controlled, as longer reaction times would cause azido reduction.

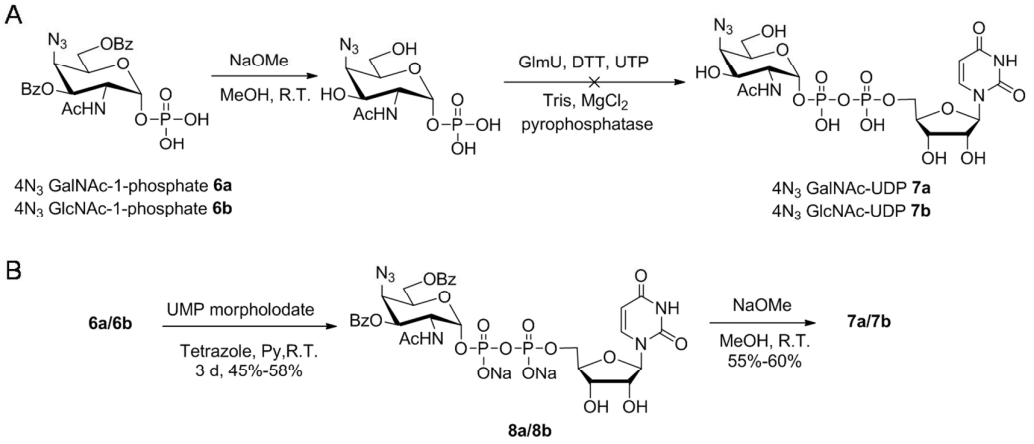
Scheme 1. Synthesis of 4-N_3-GalNAc-1-phosphate **6a** and 4-N_3-GlcNAc-1-phosphate **6b**[a]



[a]Although only one stereochemical series is shown (starting from *β*-D-glucopyranoside **1a** to **6a**), both series were synthesized and the yields for both are provided.

With both protected 4-N_3-*N*-acetylhexosamine-1-phosphates in hand, we next focused our attention on their deprotection and to determining whether they could be enzymatically converted with GlmU uridyltransferase to the two unnatural nucleotide sugar donors.[14] Treatment of **6a**/**6b** by sodium methoxide to afforded the deprotected 4-N_3-GalNAc-1-phosphate/4-N_3-GlcNAc-1-phosphate analogs. Unfortunately, GlmU recognized neither of the analogs and, thus, no corresponding UDP-sugars were observed by LC-MS (Scheme 2A). Wang *et al.*[10a] investigated the UDP-GlcNAc/UDP-GalNAc substrate specificity of GlmU and suggested that GlmU was intolerant of any functional group on the C4 position larger than a hydroxyl functionality. We also reported the successful GlmU recognition for 4-F-GlcNAc-1-phosphate /4-F-GalNAc-1-phosphate analogs.[14] Since the fluorine atom is considerably smaller and the azido group is considerably larger than a hydroxy group, our observations of the failure of GlmU to utilize the 4-N_3- and 4-F-*N*-actylhexosamine-1-phosphate analogs are consistent with Wang *et al.*[10a]

Scheme 2. GlmU attempt and chemical synthesis of UDP-4-N_3-GalNAc and UDP-4-N_3-GlcNAc donors



Next, we turned our attention to the chemical approach to preparing the unnatural UDP-sugar donors (Scheme 2B). The protected 4-N_3-*N*-acetylhexosamine-1-phosphates **6a**/**6b** were converted to their pyridinium salts and then stirred with UMP-morpholidate and tetrazole in pyridine for 3 days to afford the benzoate-protected UDP sugars.[18] By treating the reaction crude with (Na^+) resin, all the phosphate salts were converted to their sodium form and purified on a Biogel P2 column afforded the protected UDP derivatives **8a**/**8b** in modest yields (45%-58%). Deprotection of **8a**/**8b** was carried out under standard Zemplén conditions, affording the corresponding donors UDP-4-N_3-GalNAc **7a** and UDP-4-N_3-GlcNAc **7b**.

The utility of the two chemically synthesized unnatural UDP-donors as substrates in GAG synthesis was next examined. In the pilot trials, the incorporation of these new synthetic analogs was attempted using indirect radiochemical sugar assays that rely on co-incorporation of a labeled natural sugar when the analog is used by the *Pasteurella* GAG synthases[19], but the potential signals were very close to background levels. Therefore, a direct mass spectrometry-based assay was employed to monitor the incorporation of any analog directly. Basically, a short GAG acceptor terminating in GlcA was reacted with the analog and the reaction mixture is analyzed for the presence of elongated species by MALDI-ToF MS. A single azido-monosaccharide

derived from UDP-4-N_3-GlcNAc was incorporated into a growing GAG chain of heparosan (HEP) with *Pasteurella multocida* heparosan synthases (PmHS1 or 2) or hyaluronan (HA) with *Pasteurella multocida* hyaluronan synthase (PmHAS) based on the very close agreement between the predicted and the observed masses of the elongated species (Table 1).

In parallel tests with UDP-4-N_3-GalNAc and the *Pasteurella multocida* chondroitin synthase, PmCS, the analog was not incorporated into the chain of the chondroitin acceptor (*not shown*). This is somewhat surprising as PmHAS and PmCS are very similar at the amino acid sequence level (~87% identical) and the tested reaction conditions were equivalent, but currently there is no structural information for these two enzymes, thus some differences in their respective active sites seem a likely explanation for the observed usage of the 4-azido precursors.

Table 1. Mass spectrometric analysis of GAG synthase reaction products[a]

Acceptor	Donor	PmHAS	PmCS	PmHS1	PmHS2
HA4[b]	none	775.05	775.04		
	+ UDP-GlcNAc	978.09	978.08		
	+ UDP-4-N_3-GlcNAc	1003.12	ND[e]		
HEP6[c]	none			1097.10	
	+ UDP-GlcNAc			1300.10	
	+ UDP-4-N_3-GlcNAc			1325.08	
HEP3-Benz[d]	none			747.28	747.33
	+ UDP-GlcNAc			950.39	950.43
	+ UDP-4-N_3-GlcNAc			ND[e]	975.50

[a]The theoretical azide mass addition compared to native sugars is 25.02 Da. [b]Hyaluronan tetrasaccharide. [c]Heparosan hexasaccharide. [d]Heparosan trisaccharide-benzaldehyde (aldehyde at the reducing end). [e]No desired product.

We successfully prepared two azido-tagged GAGs (see the Experimental Section for details), either heparosan (HEP) or hyaluronan (HA), by employing the UDP-4-N_3-GlcNAc as a chain terminating donor. Thus, these end-labeled GAGs both possess a 4-N_3-GlcNAc residue at their non-reducing end. We next examined whether these modified chains were 'Click'-reactive. Reactions between the 4-N_3-GlcNAc-terminated HEP or HA (~10-100 kDa) and Alexa Fluor 488 DIBO

alkyne[20] were performed and the products were analyzed by polyacrylamide gel electrophoresis (PAGE) analysis and fluorescence detection (excitation 488 nm; emission 494 nm) (Figure 1, lanes 1 and 2). As negative controls, either the starting material 4-N_3-GlcNAc-terminated HA or HEP (~10-100 kDa) without added Alexa Fluor 488 DIBO alkyne were also loaded on the gel (lane 3 and 4 in Figure 1). The gel was subjected to electrophoresis for 4 h to run the unreacted, excess Alexa Fluor 488 DIBO alkyne off the gel to reduce the level of residual fluorescent reagent in the GAG area. All four lanes containing GAG samples were then visualized by Alcian blue staining; each lane displayed a blue-stained band corresponding to the expected molecular weight and polydispersity of the input GAG samples (Figure 1, left panel). As expected, only the GAG bands present in lanes 1 and 2 (Figure 1, right panel) displayed fluorescence, confirming that 4-N_3-GlcNAc-terminated HEP and HA had been successfully click-tagged with the alkyne dye.

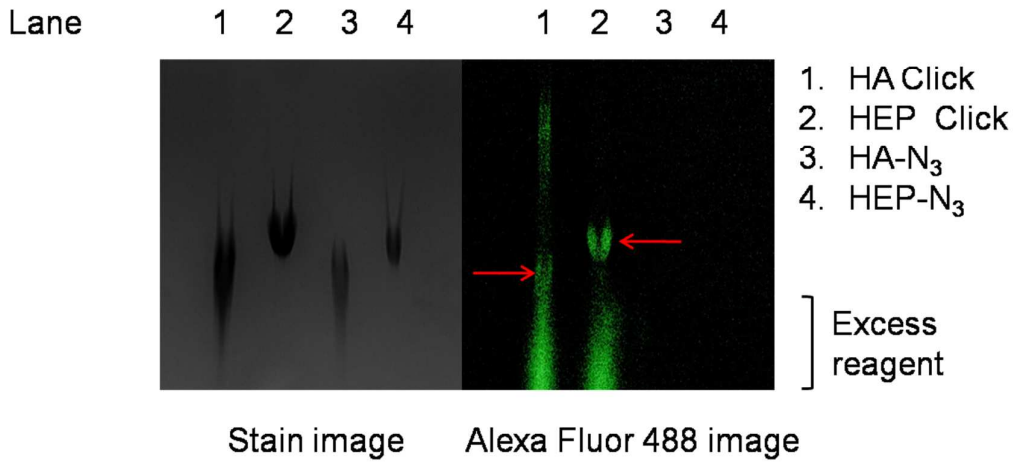


Figure 1. PAGE analysis of click reaction between 4-N_3-GlcNAc-terminated HEP and HA and Alexa Fluor 488 DIBO alkyne. Products are marked with red arrows. The excess reagent runs as a smear running faster than the GAGs (denoted with a bracket).

In summary, this is the first synthesis of UDP-4-N_3-GlcNAc/UDP-4-N_3-GalNAc analogs reported using an efficient all-chemical approach. The final unnatural UDP-sugars were then tested for incorporation into HA, HEP and chondroitin using polysaccharide synthases and UDP-4-N_3-GlcNAc was added to the non-reducing end of the sugar chain and served as a chain termination substrate in HA and HEP.

Compared to chemical method to introduce azido groups into polysaccharides, this enzymatic technique provided a "green" approach to access regioselectively azide-functionalized GAGs, which would bring better control of the final structure and composition of these macromolecules. The resulting 4-N_3-GlcNAc-terminated HEP and HA were then conjugated with Alexa Fluor 488 DIBO alkyne, demonstrating that this technology is generally applicable for labeling and detection of suitable GAGs.

EXPERIMENTAL SECTION

General information.

All reagents were purchased from commercial vendors, and unless noted otherwise, used without further purification. Flash chromatography (FC) was performed using silica gel (200−300 mesh) according to standard protocols. Reactions were monitored by thin-layer chromatography (TLC) on silica 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, ^{13}C, ^{1}H-^{1}H COSY and ^{1}H-^{13}C HSQC NMR spectra were recorded on a 800 MHz (200 MHz for ^{13}C NMR) spectrometer or 600 MHz (150 MHz for ^{13}C NMR).

Synthesis of benzyl 2-acetamido-4-azido-3,6-di-O-benzoyl-2,4-dideoxy-β-D-galacto/gluco-pyranosides **(3a/3b)**

The dibenzoylated compound **1a/1b** (520 mg, 1.0 mmol, 1.0 equiv) was dissolved in DCM (5 mL) and pyridine (0.2 mL) solvent system. The solution was cooled to 0 ^{o}C and trifluoromethane sulfonic anhydride (0.34 mL, 2.0 mmol, 2.0 equiv) was added dropwise and the resulting mixture was stirred at 0^{o}C for 3 h. The mixture was diluted with 20 mL EtOAc and washed with H_2O and dried over anhydrous Na_2SO_4. After filtering, the solvent was removed under vacuum to afford the triflate derivative **2a/2b** as yellow oils, which were used without further purification.

The triflate derivative **2a/2b** from above was dissolved in DMF (5 mL) and sodium

azide (195 mg, 3.0 mmol, 3.0 eq) was added and stirred at room temperature overnight. The solvent was removed under vacuum and the residue was dissolved in 20 mL EtOAc and washed with H_2O. After drying over anhydrous $MgSO_4$, the solvent was removed and the resulting crude mixture was purified by column chromatography (Hexanes/EtOAc 1:2) to yield **3a/3b** as white solids (348 mg, 64% and 364 mg, 67% respectively, two steps).

Benzyl 2-acetamido-4-azido-3,6-di-O-benzoyl-2,4-dideoxy-β-D-galactopyranosides **(3a)**

White solid. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.13-8.10 (m, 2H), 8.05-8.02 (m, 2H), 7.64-7.58 (m, 2H), 7.53-7.49 (m, 2H), 7.48-7.43 (m, 2H), 7.34-7.29 (m, 3H), 7.29-7.27 (m, 2H), 5.33 (m, 1H), 4.89 (d, J = 12.50, 1H), 4.72 (dd, J = 12.20, 2.27, 1H), 4.64-4.58 (m, 2H), 4.56 (d, J = 8.47, 1H), 4.30-4.25 (m, 1H), 3.88 (dd, J = 10.20, 1H), 3.65-3.62 (m, 1H), 1.84 (s, 3H); ^{13}C NMR (150 MHz, $CDCl_3$) δ 173.6, 170.1, 169.6, 140.2, 137.2, 136.8, 133.4, 133.3, 133.1, 133.0, 132.0, 131.9, 131.8, 131.7, 131.4, 103.0, 77.6, 75.9, 73.7, 66.8, 64.3, 57.6, 26.6. HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for $C_{29}H_{29}N_4O_7$ 545.2031; Found 545.2022.

Benzyl 2-acetamido-4-azido-3,6-di-O-benzoyl-2,4-dideoxy-β-D-glucopyranosides **(3b)**

White solid. The NMR and MS data was consistent with the literature.[16]

Synthesis of 1-phospho-2-acetamido-4-azido-3,6-di-O-benzoyl-α-D-galacto/gluco -pyranosides **(5a/5b)**

To a solution of **3a/3b** (207 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_4Cl. The aqueous layer was extracted with DCM (3 × 15 mL). The combined organic phase was washed with brine, dried over Na_2SO_4 and concentrated under vacuum. The resulting crude product **4a/4b** was used in the following step without further purification.

Crude **4a/4b** 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. After 30 minutes, tetrabenzyl pyrophosphate (307 mg, 0.57 mmol) was added to the reaction mixture. The reaction was stirred at -78°C for 30 minutes and slowly warmed to 0°C over 3 h. Then the reaction was quenched by the addition of aq. NH_4Cl. The aqueous layers were extracted with EtOAc (3 x 15 mL), and the combined organic phase was washed with brine, dried over Na_2SO_4 and concentrated under vacuum. The crude was purified by FC (silica gel, Hexanes/EtOAc 1:2) to give compounds **5a/5b** as yellow oils (187 mg, 69% and 198 mg, 73% respectively, two steps).

1-phospho-2-acetamido-4-azido-3,6-di-O-benzoyl-α-D-galactopyranosides **(5a)**

Yellow oil. ^{1}H NMR (800 MHz, $CDCl_3$) δ: 8.06-8.04 (m, 2H), 7.99-7.97 (m, 2H), 7.60-7.57 (m, 1H), 7.54-7.51 (m, 1H), 7.47-7.44 (m, 2H), 7.39-7.36 (m, 2H), 7.35-7.28 (m, 10H), 5.71 (dd, J = 5.82, 3.29, 1H), 5.60 (d, J = 9.36, 1H), 5.45 (dd, J = 3.29, 11.28, 1H), 5.07-4.97 (m, 4H), 4.89-4.84 (m, 1H), 4.45-4.41 (m, 1H), 4.40-4.36 (m, 1H), 4.35-4.32 (m, 1H), 4.20-4.18 (m, 1H), 1.65 (s, 3H); ^{13}C NMR (200 MHz, $CDCl_3$) δ 170.2, 166.4, 165.9, 135.4, 135.3, 135.2, 135.1, 134.0, 133.4, 130.2, 129.7, 129.3, 128.9, 128.8, 128.7, 128.7, 128.5, 128.3, 128.2, 128.1, 97.0 (d, J = 6.13), 70.2, 70.0 (d, J = 5.11), 69.9 (d, J = 4.86), 68.8, 62.9, 60.5, 47.9 (d, J = 6.90), 22.9. HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for $C_{36}H_{36}N_4O_{10}P$ 715.2164; Found 715.2153.

1-phospho-2-acetamido-4-azido-3,6-di-O-benzoyl-α-D-glucopyranosides **(5b)**

Yellow oil. ^{1}H NMR (600 MHz, $CDCl_3$) δ: 8.07-8.04 (m, 2H), 8.04-8.02 (m, 2H), 7.61-7.57 (m, 2H), 7.48-7.44 (m, 4H), 7.41-7.33 (m, 10H), 5.85 (d, J = 9.60, 1H), 5.73 (dd, J = 3.20, 5.98, 1H), 5.42 (dd, J = 9.28, 10.88, 1H), 5.15-5.03 (m, 4H), 4.53-4.46 (m, 2H), 4.41 (dd, J = 2.03, 12.27, 1H), 3.94-3.87 (m, 2H), 1.63 (s, 3H);^{13}C NMR (150 MHz, $CDCl_3$) δ 173.7, 170.0, 169.4, 138.7, 138.6, 138.5, 138.4, 137.2, 136.7, 133.3, 133.1, 132.8, 132.3, 132.2, 132.1, 132.0, 131.9, 131.8, 131.5, 99.8 (d, J

= 5.73), 74.8, 73.7, 73.4 (d, *J* = 4.46), 73.3 (d, *J* = 4.71), 65.9, 63.5, 55.4 (d, *J* = 7.50), 26.1.

HRMS (ESI-TOF) m/z: $[M + H]^+$ calcd for $C_{36}H_{36}N_4O_{10}P$ 715.2164; Found 715.2147.

Synthesis of disodium uridine 5′-(2-acetamido-2,4-dideoxy-4-azido-3,6-di-O-benzoyl-α-D-galacto/gluco-pyranosyl) diphosphate **(8a/8b)**

The protected phosphate **5a/5b** (10 mg, 0.013 mmol) was dissolved in MeOH (2 mL). Pd/C (10 wt% on activated carbon) (6 mg) was added and the mixture was stirred under a hydrogen atmosphere for 2 h. The palladium was then filtered off and the solvent removed *in vacuo* to produce crude **6a/6b** in 90% and 85% yield, respectively, which was used in the next step without further purification.

A solution of mono-phosphate **6a/6b** (8 mg, 0.013 mmol) in MeOH (2 mL) was treated with Et_3N (9 µL, 0.07 mmol), and then concentrated to yield crude bis(triethylammonium) phosphate. Without purification, this crude material was repeatedly co-evaporated with dry pyridine (3 × 3 mL). Uridine 5′-monophosphomorpholidate 4-morpholine-*N*,*N*′-dicyclohexylcarboxamidine salt (14 mg, 0.02 mmol) was co-evaporated with pyridine (3 × 3 mL) in a separate vessel, then transferred in 2 mL pyridine via a cannula into the reaction flask. The combined reagents were co-evaporated with pyridine (2 × 2 mL), then 1H-tetrazole (4 mg, 0.05 mmol) in pyridine (2 mL),was added, and the reaction mixture stirred at rt for 3 d. The reaction mixture was then concentrated *in vacuo* and was converted into Na^+ form by passing through a Dowex (Na^+) column. The resulting fraction was concentrated in vacuum and the residue was loaded onto a Bio Gel P2 column (1 × 120 cm) eluted with H_2O. Fractions were collected, and those containing the product as determined by MS were combined and freeze-dried to afford **8a/8b** as white powders (5 mg, 45% and 7 mg, 58% respectively).

Disodium uridine 5′-(2-acetamido-2,4-dideoxy-4-azido-3,6-di-O-benzoyl

-α-D-galactopyranosyl) diphosphate **(8a)**

White solid. ^{1}H NMR (600 MHz, D_2O) δ: 7.98-7.90 (m, 3H), 7.86 (d, J = 8.15, 1H), 7.81 (d, J = 8.15, 1H), 7.63-7.54 (m, 3H), 7.48-7.41 (m, 3H), 5.87-5.81 (m, 1H), 5.76-5.74 (m, 1H), 5.65 (d, J = 8.22, 1H), 5.61-5.58 (m, 1H), 5.45-5.41 (m, 1H), 4.53-4.43 (m, 2H), 4.30-4.19 (m, 3H), 4.18-4.12 (m, 2H), 4.00-3.89 (m, 2H), 1.85 (s, 3H); ^{13}C NMR (150 MHz, D_2O) δ 178.2, 170.7, 169.7, 169.4, 155.1, 145.2, 144.9, 144.6, 137.7, 137.3, 133.0, 132.9, 132.2, 132.1, 106.1, 105.2, 97.8, 91.6, 87.1, 86.5, 77.6, 77.2, 75.1, 73.3, 73.1, 72.3, 71.4, 67.0, 63.8, 25.4. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{31}H_{33}N_6O_{18}P_2$ 839.1332; Found 839.1340.

Disodium uridine 5'-(2-acetamido-2,4-dideoxy-4-azido-3,6-di-O-benzoyl -α-D-glucopyranosyl) diphosphate **(8b)**

White solid. ^{1}H NMR (600 MHz, D_2O) δ: 7.99-7.91 (m, 4H), 7.84-7.80 (m, 2H), 7.64-7.60 (m, 2H), 7.48-7.44 (m, 3H), 5.85-5.81 (m, 1H), 5.78 (d, J = 3.05, 1H), 5.73 (d, J = 8.08, 1H), 5.59 (dd, J = 3.12, 6.74, 1H), 5.44 (m, 1H), 4.63-4.53 (m, 2H), 4.45 (dt, J = 3.05, 10.49, 1H), 4.31-4.27 (m, 1H), 4.24-4.18 (m, 3H), 4.17-4.11 (m, 2H), 1.78 (s, 3H); ^{13}C NMR (150 MHz, D_2O) δ 177.8, 171.4, 171.0, 169.2, 154.8, 144.9, 144.7, 137.7, 137.5, 133.0, 132.8, 132.2, 132.1, 132.0, 106.0, 105.5, 97.7, 92.4, 86.1, 77.4, 77.1, 76.1, 72.9, 72.8, 72.6, 68.4, 66.8, 63.1, 55.2, 25.0. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{31}H_{33}N_6O_{18}P_2$ 839.1332; Found 839.1335.

Synthesis of disodium uridine 5'-(2-acetamido-2,4-dideoxy-4-azido-α-D-galacto/gluco-pyranosyl) diphosphate **(7a/7b)**

To a solution of **8a/8b** (6 mg, 0.007 mmol) in anhydrous MeOH (1 mL) was added Sodium methoxide solution (20 µL, 5.5 M in methanol) and the resulting solution left to stir overnight. The solution was then neutralized with pre-washed and acidified Amberlyte IR-120 Hydrogen ion exchange resin and the reaction mixture filtered

through cotton wool to remove the resin. The reaction mixture was concentrated under reduced pressure and the residue was loaded onto a Bio Gel P2 column (1 × 75 cm) and eluted with H_2O to give **7a**/**7b** as white solids. (2 mg, 55% and 3 mg, 60% respectively).

Disodium uridine 5′-(2-acetamido-2,4-dideoxy-4-azido-α-D-galactopyranosyl) diphosphate **(7a)**

White solid. ^{1}H NMR (600 MHz, D_2O) δ: 7.82 (d, J = 8.06, 1H, uridine-H''-6), 5.86-5.82 (m, 2H, uridine-H''-5, rib-H'-1), 5.38-5.35 (m, 1H, H-1), 4.26-4.20 (m, 2H, rib-H'-2, rib-H'-5a), 4.17-4.08 (m, 4H, H-2, H-5, rib-H'-3, rib-H'-4), 4.07-4.00 (m, 2H, H-3, rib-H'-5b), 3.67-3.63(m, 1H, H-6a), 3.62-3.56 (m, 2H, H-4, H-6b), 1.95 (s, 3H); ^{13}C NMR (150 MHz, D_2O) δ 185.8, 172.9, 171.7, 151.9, 141.7, 102.6, 94.6, 88.5, 83.1, 73.6, 70.5, 69.5, 68.0, 65.1, 62.8, 60.7, 21.9.

HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{17}H_{25}N_6O_{16}P_2$ 631.0808; Found 631.0808.

Disodium uridine 5′-(2-acetamido-2,4-dideoxy-4-azido-α-D-glucopyranosyl) diphosphate **(7b)**

White solid. ^{1}H NMR (600 MHz, D_2O) δ: 7.86 (d, J = 7.95, 1H, uridine-H''-6), 5.88-5.84 (m, 2H, uridine-H''-5, rib-H'-1), 5.42 (dd, J = 3.18, 7.32, 1H, H-1), 4.27-4.22 (m, 2H, rib-H'-2, rib-H'-5a), 4.19-4.13 (m, 2H, rib-H'-3, rib-H'-4), 4.10-4.06 (m, 1H, rib-H'-5b), 3.95(dt, J = 2.84, 10.70, 1H, H-2), 3.79-3.66 (m, 4H, H-3, H-5, H-6a, H-6b), 3.48 (m, 1H, H-4), 1.97 (s, 3H, H-NAc); ^{13}C NMR (150 MHz, D_2O) δ 178.5, 169.9, 145.1, 106.0, 98.0, 92.3, 86.5, 77.2, 74.9, 73.7, 72.7, 68.2, 64.9, 63.8, 56.9, 25.6. HRMS (ESI-TOF) m/z: $[M - H]^-$ calcd for $C_{17}H_{25}N_6O_{16}P_2$ 631.0808; Found 631.0809.

GAG synthase assays

Either azido-containing UDP-hexosamine analog or authentic UDP-hexosamine (0.5 mM) was incubated in a reaction (25 µl) containing 50 mM Tris, pH 7.2, 1 mM

$MnCl_2$, radiolabeled UDP-[^{3}H]GlcA (0.05 mM; 0.1 μCi; Perkin Elmer), and 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 5.25 h. The reaction mixtures were quenched with sodium dodecyl sulfate (2% final conc) and analyzed by descending paper chromatography (overnight in 65:35 ethanol/1 M ammonium acetate buffer, Whatman 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-GlcA and a functional UDP-hexosamine are present simultaneously).

Mass Spectroscopy

HA[21a] or Heparosan oligosaccharides[21b] (0.1-1 μg) were reacted with a 2-10-fold molar excess of either UDP-GlcNAc, UDP-GalNAc, the azido-containing analog, or no analog in a buffer containing 50 mM Tris, pH 7.2, 1 mM $MnCl_2$, and purified recombinant synthase (5-10 μg) for 16 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). HA oligosaccharides were employed as mass calibrants.

Azido tagged GAG preparations

GAGs (HA, 68 kDa or HEP, 40.1 kDa) were azido end-labeled at the non-reducing terminus using the donor sugar UDP-4-N_3-GlcNAc in a reaction buffer containing 50 mM HEPES, pH 7.2, 1 mM $MnCl_2$, and 1 μg/μl purified enzyme (PmHAS or PmHS1).

Click chemistry

The 4-N_3-GlcNAc-terminated HEP or HA polymers (30 μg) were mixed with a 10-fold excess Alexa Fluor 488 DIBO alkyne in 15 μL deionized water and incubated at room temperature in the dark for 5 h. The crude reaction was then directly subjected to PAGE analysis.

Polyacrylamide gel electrophoresis (PAGE)

Phenol red dye was added to the sample for visualization of the ion front during electrophoresis. A 10-µg aliquot of each sample was analyzed on a 4-15% Mini-Protean® TGX™ Precast Gel ran in Tris-Glycine buffer (at constant 30 mA for 4 h). The gel was first visualized by UV fluorescence and followed by Alcian Blue staining (0.5% w/v Alcian blue dye and 2% v/v aqueous acetic acid) and destained in water.[22]

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS publications website at DOI:

HRMS, ^{1}H, ^{13}C, COSY and HSQC NMR spectra of the components (PDF)

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Notes

The authors declare no completing financial interest.

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