A Combined Chemical and Enzymatic Strategy for the Construction of Carbohydrate-Containing Antigen Core Units[1]

Gary C. Look,† Yoshitaka Ichikawa,† Gwo-Jenn Shen,† Pi-Wan Cheng,‡ and Chi-Huey Wong*,†

Department of Chemistry, The Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, California 92037, and Department of Pediatrics, The University of North Carolina, Chapel Hill, North Carolina 27599

Received March 5, 1993

Glycosidase-mediated coupling of glycal acceptors with galactose donors affords β1,3-linked disaccharides that are versatile intermediates for further chemical and enzymatic manipulation. The utility of these disaccharides is demonstrated by the elaboration of these compounds into the type I and type IV core disaccharides of carbohydrate-containing antigens. Further conversion of the type IV disaccharide to a mucin-type trisaccharide (Galβ1,3(GlcNAcβ1,6)GalNAc) was accomplished with the use of a β1,6-*N*-acetylglucosaminyltransferase. This combined use of enzymatic and chemical methodologies allows for the rapid assembly, with high regio- and stereoselectivity, of core oligosaccharides of biologically important glycoconjugates.

Introduction

The galactosyl β1,3-linkage is an important structural motif of many complex carbohydrates with significant biological functions (Figure 1).[2] As such, there exists a clear interest in the development of strategies to assemble these molecules and analogs in a rapid and efficient manner.[3] As part of our continuing studies in the use of enzymes in organic synthesis, we have developed strategies for the formation of glycosidic linkages with a minimal use of protecting groups through the employment of glycosidases and glycosyltransferases.[4-6] There is, however, no β1,3-galactosyltransferase available for the enzymatic synthesis of these molecules. We turned our attention to the use of β-galactosidase and glycal acceptors.

Glycal disaccharides have been shown to be valuable intermediates in the synthesis of complex natural and unnatural oligosaccharides.[3c,e,f,7] While conventional synthesis of oligosaccharides involves a measure of flexibility in terms of the variety of monosaccharide synthons that may be employed, this strategy often involves multiple protection and functional group manipulation steps in order to form regio- and stereospecific linkages. We have recently reported the facile glycosidase-mediated synthesis of β1,3-linked disaccharides using glycals as acceptors (Figure 2).[5] The appeal of this methodology is that versatile β1,3-linked compounds can be formed in good yields with few protecting groups from commercially available material. We now report our further studies of enzymatic glycosylation and the elaboration of the products into the core disaccharides of carbohydrate antigens and the further use of a β1,6-*N*-acetylglucosaminyltransferase to prepare a core trisaccharide of a mucin-type antigen.

Results and Discussion

Subtilisin-Mediated Acetylation. Our preliminary studies indicated that 6-*O*-acetylated glycals were good acceptors for galactosidase-mediated glycosylation reactions.[5] Previously, Holla had employed lipases in the preparation of 6-*O*-acetylated glucal **2a** and galactal **2b**.[8,9] Subtilisin BPN′ and some of its engineered variants with improved stability in dimethylformamide have also been used in the selective acetylation of carbohydrates.[10] We have determined that Subtilisin can be employed in the acetylation of glycals as well as 2-acetamido-2-deoxy sugars (Scheme I).

Galactosidase-Mediated Coupling. Of the large variety of enzymes studied for their ability to use glycals as acceptors, β-galactosidase from *E. coli* has proven the most amenable to this methodology. As reported earlier,[5] 6-*O*-acetyl glucal **2a** was accepted by the enzyme in coupling to *p*-NO_2-Ph-β-galactopyranoside, affording exclusively the β1,3-linked disaccharide **5a** (Scheme II).

† The Scripps Research Institute.
‡ University of North Carolina.

(1) Supported by the NIH and an American Cancer Society Postdoctoral Fellowship (PF-3525 to G.C.L.).

(2) (a) Hakomori, S. *Adv. Cancer Res.* **1989**, *52*, 257. (b) Fenzi, T. *Nature* **1985**, *314*, 53. (c) Berg, E. L.; Robinson, M. K.; Mansson, O.; Butcher, E. C.; Magnani, J. L. *J. Biol. Chem.* **1991**, *266*, 14869. (d) Hodgson, J. *Biotechnology* **1991**, *9*, 609.

(3) (a) Wong, T. C.; Haque, W.; Abbas, S. Z.; Noujam, A. A. *J. Carbohydr. Chem.* **1990**, *9*, 745. (b) Sabesan, S.; Lemieux, R. U. *Can. J. Chem.* **1984**, *62*, 644. (c) Jacquinet, J. C.; Paulsen, H. *Tetrahedron Lett.* **1981**, *22*, 1387. (d) Paulsen, H.; Paal, M. *Carbohydr. Res.* **1984**, *135*, 71. (e) Wang, L.-X.; Sakairi, N.; Kuzuhara, H. *Carbohydr. Res.* **1991**, *219*, 133. (f) Schaubach, R.; Hemberger, J.; Kinzy, W. *Liebigs Ann. Chem.* **1991**, 607.

(4) For a review, see: Ichikawa, Y.; Look, G. C.; Wong, C.-H. *Anal. Biochem.* **1992**, *202*, 215.

(5) Look, G. C.; Wong, C.-H. *Tetrahedron Lett.* **1992**, *33*, 4253.

(6) For other examples on glycosidase-mediated β1,3-linked disaccharide synthesis, see: (a) Brockhaus, M.; Lehmann, J. *Carbohydr. Res.* **1977**, *53*, 21. (b) Lehmann, J.; Schroter, E. *Carbohydr. Res.* **1977**, *58*, 65. (c) Lehmann, J.; Zieger, B. *Carbohydr. Res.* **1977**, *58*, 72. (d) Crout, D. H. G.; Howarth, O. W.; Singh, S.; Swoboda, B. E. P.; Critchley, P.; Gibson, W. T. *J. Chem. Soc., Chem. Commun.* **1991**, 1550. (e) Hedbys, L.; Johansson, E.; Mosbach, K.; Larsson, P.-O.; Gunnarsson, A.; Svensson, S.; Lönn, H. *Glycoconjugate J.* **1989**, *6*, 161. (f) Bay, S.; Cantacuzene, D. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 423. Petit, J.-M.; Paquet, F.; Beau, J.-M. *Tetrahedron Lett.* **1991**, *32*, 6125.

(7) For examples, see: (a) Gervay, J.; Danishefsky, S. J. *J. Org. Chem.* **1991**, *56*, 5448. (b) Fu, H.; Kondo, H.; Ichikawa, Y.; Look, G. C.; Wong, C.-H. *J. Org. Chem.* **1992**, *57*, 7265. (c) Bencomo, V. V.; Jacquinet, J.-C.; Sinaÿ, P. *Carbohydr. Res.* **1982**, *110*, C9. (d) Wong, C.-H.; Dumas, D. P.; Ichikawa, Y.; Koseki, K.; Danishefsky, S. J.; Weston, B. W.; Lowe, J. B. *J. Am. Chem. Soc.* **1992**, *114*, 7321. (e) Danishefsky, S. J.; Gervay, J.; Peterson, J. M.; McDonald, F. E.; Koseki, K.; Oriyama, T.; Griffith, D. A.; Wong, C.-H.; Dumas, D. P. *J. Am. Chem. Soc.* **1992**, *114*, 8329. (f) Danishefsky, S. J.; Koseki, K.; Griffith, D. A.; Gervay, J.; Peterson, J. M.; McDonald, F. E.; Oriyama, T. *J. Am. Chem. Soc.* **1992**, *114*, 8331.

(8) For another example of lipase-catalyzed acylation of glycals, see Holla, E. W. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 220.

(9) Wang, Y.-F.; Lalonde, J. J.; Momongan, M.; Bergbreiter, D. E.; Wong, C.-H. *J. Am. Chem. Soc.* **1988**, *110*, 7200.

(10) Zhong, Z.; Liu, J. L.-C.; Dinterman, L. M.; Finkelman, M. A. J.; Mueller, W. T.; Rollence, M. L.; Whitlow, M.; Wong, C.-H. *J. Am. Chem. Soc.* **1991**, *113*, 683.

0022-3263/93/1958-4326$04.00/0

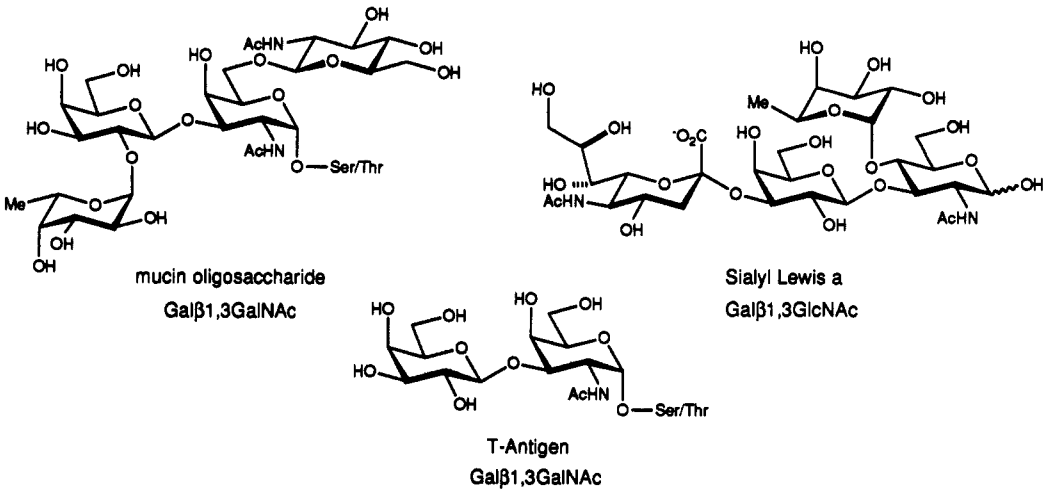


Figure 1. Examples of glycoconjugates possessing a galactosyl β1,3-disaccharide motif.

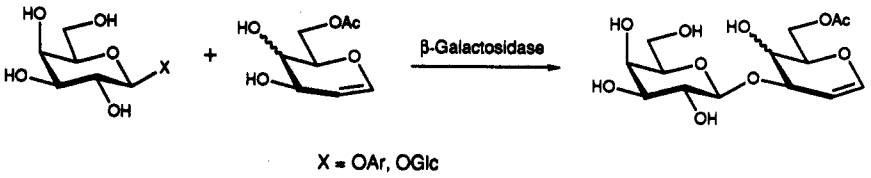
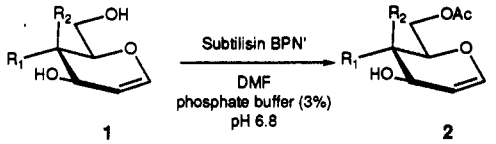
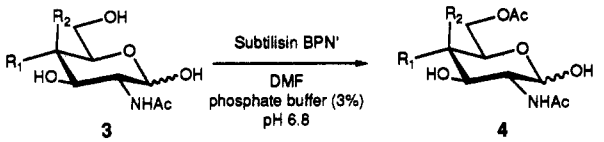


Figure 2. Enzymatic strategy to form galactosyl β1,3-linked disaccharides.

Scheme I. Subtilisin-Catalyzed Acetylation of Monosaccharides with Vinyl Acetate



1a, 2a: R_1 = OH, R_2 = H, yield = 77%
1b, 2b: R_1 = H, R_2 = OH, yield = 83%



3a, 4a: R_1 = OH, R_2 = H, yield = 75%
3b, 4b: R_1 = H, R_2 = OH, yield = 64%

While galactal was an inhibitor of β-galactosidase,[11,12] the enzyme-catalyzed reaction of *p*-NO_2-Ph-β-galactopyranoside with 6-*O*-acetyl galactal **2b** produced the β1,3-linked disaccharide **5b** in 42% yield (Scheme II). As observed earlier for the glucal case,[5] the product disaccharide displays product inhibition, thus attenuating the yield of **5b**. Disaccharide **5b** may be isolated, however the peracetylated derivative **6b** proved easier to purify and characterize. The β1,3-linkage of the disaccharide (mono- or peracetylated) was determined by 2D NMR experiments as described in the Experimental Section. Disaccharide **6b** is an intermediate used by the Sinaÿ group in the synthesis of the T-antigen.[7c]

The acetylated monosaccharides **4a** and **4b** proved to be poor acceptors for the β-galactosidase, giving mixtures of compounds with hydrolysis of the donor *p*-NO_2-Ph-β-galactopyranoside being the major reaction pathway. While lactose proved to be a galactose donor, the reaction was complicated by additional monosaccharide products, thus rendering impractical the use of this disaccharide as a substrate.

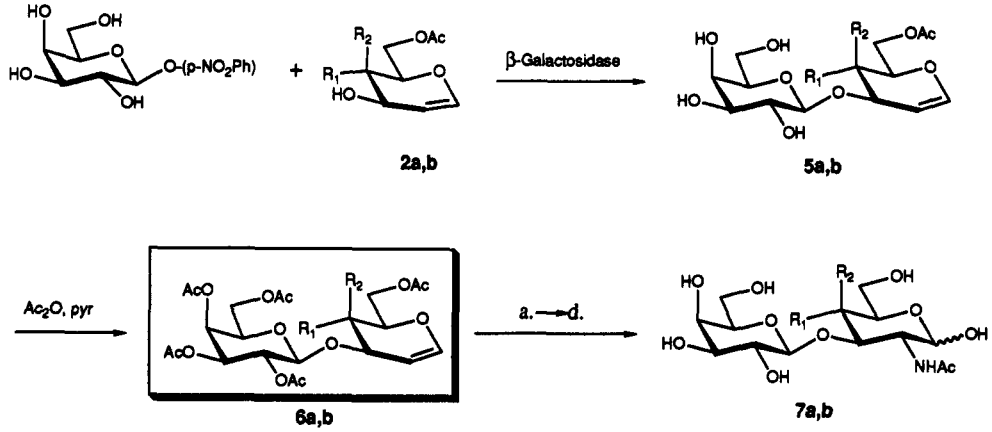
As an example of the utility of the glycal disaccharides, the peracetylated compounds **6a** and **6b** were converted by the known route of azidonitration,[13] reduction, and *N*-acetylation to the protected type I and type IV disaccharides, respectively. Whereas azidonitration of **6a** afforded an approximately 1:1 ratio of manno- to gluco-products, this reaction process performed on **6b** afforded exclusively the galacto-product. The manno- and gluco-products were separated after the reduction/acetylation steps. The unoptimized azidonitration–reduction sequence is somewhat poor, giving low yields of desired disaccharides **7a** and **7b**. Deprotection afforded the free sugars which are substrates for further enzymatic manipulation.[14]

***N*-Acetylglucosaminyltransferase-Mediated Coupling.** To further demonstrate the utility of enzymes in carbohydrate synthesis, we explored the use of the recently isolated β1,6-*N*-acetylglucosaminyltransferase.[15] This enzyme is responsible for the transfer of *N*-acetylglucosamine to the 6-hydroxy position of either the *N*-acetylgalactosamine or galactose-reducing terminus of mucin-type oligosaccharides.[15,16] During our studies of glycosyltransferase-mediated oligosaccharide syntheses with *in situ* cofactor formation, we explored UDP-GlcNac regenera-

(11) Lee, Y. C. *Biochem. Biophys. Res. Commun.* **1969**, *35*, 161.
(12) Bay and Catacuzene report that galactal acts as a donor and an acceptor for galactosidase at higher concentrations, see: ref 6f.

(13) (a) Lemieux, R. U.; Radcliffe, R. M. *Can. J. Chem.* **1979**, *57*, 1244. (b) Lin, C.-H.; Sugai, T.; Halcomb, R. L.; Ichikawa, Y.; Wong, C.-H. *J. Am. Chem. Soc.* **1992**, *114*, 10138.
(14) For example, see: Palcic, M. M.; Venot, A. P.; Ratcliffe, R. M.; Hindsgaul, O. *Carbohydr. Res.* **1989**, *190*, 1.
(15) (a) Ropp, P. A.; Little, M. R.; Cheng, P.-W. *J. Biol. Chem.* **1991**, *266*, 23863. (b) van Halbeek, H.; Vliegenthart, J. F. G.; Kochetkov, N. K.; Arbatsky, N. P.; Derevitskaya, V. A. *Eur. J. Biochem.* **1982**, *127*, 21.
(16) (a) Dutti, A.; Orlacchio, A.; Siminovitch, K. A.; Dennis, J. W. *Anal. Biochem.* **1992**, *206*, 262. (b) Cheng, P.-W.; Wingert, W. E.; Little, M. R.; Wei, R. *Biochem. J.* **1985**, *227*, 405.

Scheme II.[a] Chemical and Enzymatic Synthesis of the Type I and Type IV Disaccharides



2a, 5a, 7a: R_1 = OH, R_2 = H
2b, 5b, 7b: R_1 = H, R_2 = OH

6a: R_1 = OAc, R_2 = H
6b: R_1 = H, R_2 = OAc

[a] (a) $Ce(NH_4)_2(NO_3)_6$, NaN_3; (b) NaOAc, AcOH, 1.4:1 gluco/manno products; (c) Pd–C, H_2, Ac_2O; (d) NH_3, MeOH.

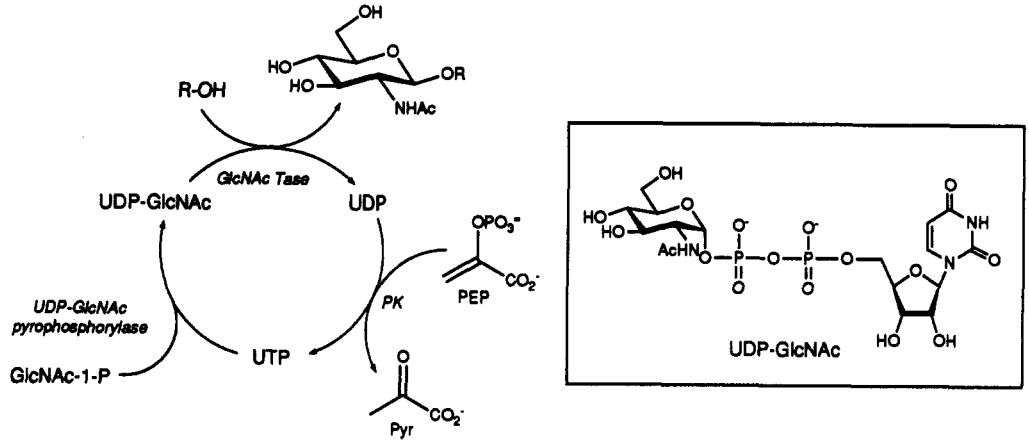


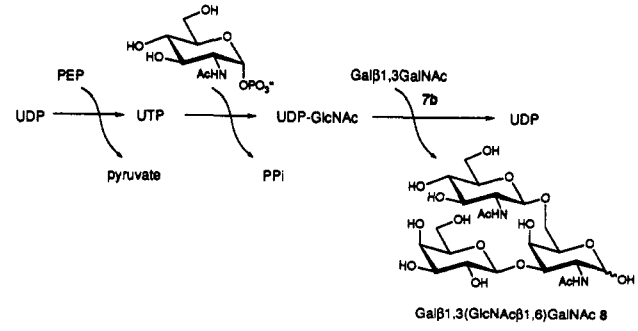
Figure 3. System for the in situ regeneration of UDP-GlcNAc.

tion. In such a regeneration system, GlcNAc-1-P and UTP are converted to UDP-GlcNAc and inorganic pyrophosphate in the presence of UDP-GlcNAc pyrophosphorylase. The formed UDP-GlcNAc is consumed by GlcNAc transferase and the released UDP is converted to UTP by pyruvate kinase in the presence of phospho(enol)pyruvate.

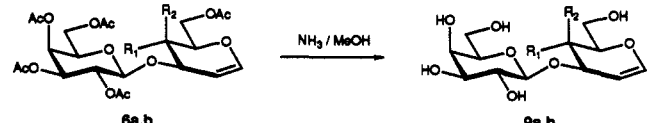
In preliminary experiments, crude yeast cell extracts were used as the source of UDP-GlcNAc pyrophosphorylase in a "one pot" system (Figure 3).[4] However, this enzyme system proved problematic due to the presence of phosphatases that gradually degraded the phosphate-containing species (UDP-GlcNAc, GlcNAc-1-P, UDP, UTP) in the reaction mixture and led to a low-yield product.

The difficulties encountered with the cell extract system were circumvented with the utilization of whole yeast cells in a stepwise synthetic sequence (Scheme III). Under these reaction conditions, the formed UDP-GlcNAc was found to be stable. After the formation of UDP-GlcNAc, as judged by TLC (10 h), the acceptor **7b** and β1,6-*N*-acetylglucosaminyltransferase were added to the reaction mixture, and the desired trisaccharide **8** was subsequently isolated. We are currently examining conditions to obtain pure UDP-GlcNac pyrophosphorylase from the yeast lysate in order to effectively utilize the *in situ* regeneration system for UDP-GlcNAc.

Scheme III. GlcNAc Transferase Reaction with in Situ Generation of UDP-GlcNAc



Scheme IV. Deacetylation of Glycal Disaccharides



6a: R_1 = OAc, R_2 = H
6b: R_1 = H, R_2 = OAc
9a: R_1 = OH, R_2 = H, yield = 97%
9b: R_1 = H, R_2 = OH, yield = 83%

The free glycal disaccharides can be readily prepared from the peracetylated species, **6a** and **6b** (Scheme IV). Compound **9a** has been used for enzymatic halohydration

using chloroperoxidase,[7b] and compound **9b** is being studied as a substrate for β1,6-*N*-acetylglucosaminyltransferase.

Conclusions

We have demonstrated the utility of galactosidase in the efficient formation of useful glycal disaccharides with complete regio- and stereofidelity. These glycal disaccharides have been converted in a series of straightforward transformations to the important type I and type IV core disaccharides. In addition, a mucin-type trisaccharide has been formed using β1,6-*N*-acetylglucosaminyltransferase on the chemoenzymatically prepared disaccharide **7b**. The core trisaccharide 8 can be further fucosylated utilizing an α1,2-fucosyltransferase[17] to form a complex mucin-type tetrasaccharide structure.

Experimental Section

General Experimental Details. Reagents were used as purchased without further purification. *Saccharomyces cerevisiae* yeast cells, pyruvate kinase, and *Escherichia coli* β-galactosidase were purchased from Sigma. Bovine β-1,6-*N*-acetylglucosaminyltransferase was isolated as described previously and used as a crude cell extract.[15] The following chemical and enzyme abbreviations are used in the experimental section: PIPES = piperazine-*N*,*N*′-bis(2-ethanesulfonic acid), HEPES = *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid), EDTA = ethylenediaminetetraacetic acid, PEP = phospho(enol)pyruvate; PK = pyruvate kinase (EC 2.7.1.40), DTT = DL-dithiothreitol, PMSF = phenylmethylsulfonyl fluoride, ^{1}H NMR spectra run in D_2O were measured using HOD as the internal standard with a chemical shift of 4.68 ppm. ^{13}C NMR spectra run in D_2O were measured using CH_3CN as an internal standard with the chemical shift of the methyl group at 1.3 ppm.

6-*O*-Acetyl-D-*arabino*-hex-1-enitol (2a).[8] In a typical procedure, a mixture of glucal (2.68 g, 18.3 mmol), DMF (90 mL), vinyl acetate (60 mL), and PIPES/EDTA/NaOAc buffer (3 mL, of a 0.07, 0.1, 0.2 M solution, pH 6.5) were treated with Subtilisin BPN′ (1.00 g, 8100 U). After 24 h, the mixture was filtered and concentrated. The residue was purified by silica gel column chromatography ($CHCl_3$/MeOH 10:1) to afford **2a** (2.67 g, 77%) as a pale yellow oil: ^{1}H NMR (500 MHz, $CDCl_3$) β 6.34 (dd, *J* = 1.7, 6.0 Hz, 1H, H-1), 4.76 (dd, *J* = 2.1, 6.1 Hz, 1H, H-2), 4.56 (dd, *J* = 4.2, 12.4 Hz, 1H, H-6a), 4.35 (dd, *J* = 2.3, 12.4, Hz, 1H, H-6b), 4.30 (dt, *J* = 1.9, 7.3 Hz, 1H, H-3), 3.92 (ddd, *J* = 2.3, 4.2, 10.2 Hz, 1H, H-5), 3.57 (dd, *J* = 7.4, 10.2 Hz, 1H, H-4), 2.15 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) 172.1, 144.0, 102.8, 76.0, 69.5, 69.4, 62.7, 20.7; IR (film) 3389, 2917, 1738, 1658, 1443, 1372, 1230 cm^{-1}; HRMS (LSIMS, NaI) *m/e* 211.0582 (211.0582 calcd for $C_8H_{12}O_5Na$, M + Na$^+$), $[\alpha]_D$ = −27° (*c* = 0.33, H_2O).

6-*O*-Acetyl-D-*lyxo*-hex-1-enitol (2b).[8] This compound was prepared following the procedure for the preparation of **2a**: yield = 91%; ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.40 (dd, *J* = 1.3, 6.2 Hz, 1H, H-1), 4.75–4.73 (m, 1H, H-2), 4.43–4.40 (m, 2H, H-3 and H-6a), 4.33 (dd, *J* = 7.3, 11.7 Hz, 1H, H-6b), 4.10 (app t, *J* = 6.3 Hz, 1H, H-5), 3.92 (br s, 1H, H-4), 2.77 (br s, 1H, OH), 2.65 (br s, 1H, OH), 2.12 (s, 3H, CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 171.1, 144.0, 103.1, 74.3, 65.1, 63.8, 20.7; IR (film) 3417, 2905, 1738, 1650, 1372, 1228, 1154, 1073, 1035 cm^{-1}; HRMS (LSIMS, NaI) *m/e* 211.0582 (211.0582 calcd for $C_8H_{12}O_5Na$, M + Na$^+$); $[\alpha]_D$ = −3° (*c* = 3.1, $CHCl_3$).

2-Acetamido-6-*O*-acetyl-2-deoxy-D-glucopyranose (4a). This compound was prepared following the procedure for the preparation of **2a**: recystallized from MeOH/$CHCl_3$; yield = 83% as a 6.5:1 mixture of α and β anomers. Data for α anomer: ^{1}H NMR (500 MHz, D_2O) δ 5.03 (d, *J* = 3.5 Hz, 1H, H-1), 4.19 (2H, H-6), 3.91–3.88 (m, 1H, H-5), 3.74 (dd, *J* = 3.6, 10.8 Hz, 1H, H-2), 3.61 (t, *J* = 9.3 Hz, 1H, H-3), 3.39 (t, *J* = 9.5 Hz, 1H, H-4), 1.97 and 1.89 (s, 3H each, CH_3); ^{13}C NMR (125 MHz, D_2O) δ 155.6, 155.3, 72.0, 54.7, 51.1, 50.4, 44.6, 35.1, 3.0; HRMS (LSIMS, CsI) *m/e* 396.0059 (396.0059 calcd for $C_{10}H_{17}NO_7Cs$, M + Cs$^+$); mp 167.5–169° dec; $[\alpha]_D$ = +62.5° (*c* = 0.16, H_2O).

2-Acetamido-6-*O*-acetyl-2-deoxy-D-galactopyranose (4b). This compound was prepared following the procedure for the preparation of **2a**: recrystallized from MeOH/$CHCl_3$, yield = 86% as a 2:1 mixture of α and β anomers. Data for mixture of anomers: ^{1}H NMR (500 MHz, D_2O) δ 5.08 (d, *J* = 3.3 Hz, H-1 α-anomer), 4.50 (dd, *J* = 2.7, 8.3 Hz, H-1 β-anomer), 4.16–4.06 (m contains H-6a,b), 4.00–3.94 (m, contains H-2 as dd, *J* = 3.3, 11.1 Hz), 3.89–3.71 9m, contains H-3), 3.64–3.55 (m, contains H-5), 2.05 and 1.98 (s each, CH_3); ^{13}C NMR (125 MHz, D_2O) δ 160.3, 155.1, 76.4, 72.1, 56.2, 53.5, 52.2, 51.9, 51.6, 49.6, 49.1, 48.9, 48.4, 48.2, 45.3, 45.0, 42.3, 42.0, 34.7, 34.6, 31.3, 31.2, 3.1, 3.0, 1.3; HRMS (LSIMS, NaI) *m/e* 286.0903 (286.0903 calcd for $C_{10}H_{17}NO_7Na$, M + Na$^+$), $[\alpha]_D$ = +91.7° (*c* = 0.12, H_2O).

D-Galactopyranosyl-β-(1,3)-6-*O*-acetyl-D-*lyxo*-hex-1-enitol (5b). In the manner described previously,[5] a solution of 6-*O*-acetyl galactal **2b** (72.3 mg, 0.38 mmol) and β-4-nitrophenyl galactopyranoside (79.8 mg, 0.26 mmol) was prepared in 0.07 M PIPES–0.1 M NaOAc–0.2 M EDTA solution (3 mL) and acetone (100 μL) at 23 °C. β-Galactosidase (*E. coli*, 75 U) was added and the reaction mixture was maintained for 26 h. The mixture was purified (silica gel, 5:2:2 $CHCl_3$–MeOH–EtOAc) to afford **5b** (38 mg, 42%) and recovered **2b** (39 mg, 54%): ^{1}H NMR (500 MHz, D_2O) δ 6.31 (dd, *J* = 1.8, 6.3 Hz, 1H, H-1 galactal), 4.74 (dt, *J* = 1.9, 6.3 Hz, 1H, H-2 galactal), 4.49–4.48 (m, 1H, H-3 galactal), 4.42 (d, *J* = 7.8 Hz, 1H, H-1 Gal), 4.25–4.17 (m, 2H, H-6a galactal), 4.15 (dd, *J* = 4.1, 8.1 Hz, 1H, H-5 galactal), 4.11–4.10 (m, 1H, H-4 galactal), 3.79 (br d, *J* = 3.1 Hz, 1H, H-4 Gal), 3.66–3.59 (m, 2H, H-6b Gal), 3.38–3.55 (m, 1H, H-5 Gal), 3.52 (dd, *J* = 3.4, 9.9 Hz, 1H, H-3 Gal), 3.51–3.45 (m, 1H, H-2 Gal), 1.98 (s, 3H, C(O)CH_3); ^{13}C NMR (125 MHz, D_2O) δ 164.4, 144.7, 102.9, 102.9, 75.6, 74.6, 73.1, 73.1, 71.1, 69.1, 64.6, 61.4, 49.3, 20.7; HRMS (LSIMS, NaI) *m/e* 373.1122 (373.1111 calcd for $C_{14}H_{22}O_{10}Na$, M + Na$^+$).

The assignments for H-3 and H-4 of the galactal portion of the molecule were determined through DQF COSY ^{1}H NMR experiments as evidenced by long-range coupling of H-1 (at δ 6.49) with H-3 (at δ 4.67–4.66) but not H-4 (at δ 4.29–4.28). The linkage was assigned based on the strong downfield shift of H-4 galactal to δ 5.3 versus the slight shift of H-3 to δ 4.5 upon per-*O*-acetylation of the disaccharide (These spectra were run in $CDCl_3$).

Compound **5b** was per-*O*-acetylated to **6b** with pyridine and acetic anhydride. **6b**: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.41 (d, *J* = 6.4 Hz, 1H, H-1 galactal), 5.37 (d, *J* = 3.4 Hz, 1H, H-4 Gal), 5.34 (m, 1H, H-4 galactal), 5.18 (dd, *J* = 8.0, 10.4 Hz, 1H, H-2 Gal), 5.01 (dd, *J* = 3.4, 10.5 Hz, 1H, H-3 Gal), 4.81 (dd, *J* = 2.9, 6.3 Hz, 1H, H-2 galactal), 4.58 (d, *J* = 7.9 Hz, 1H, H-1 Gal), 4.55 (m, 1H, H-3 galactal), 4.28–4.20 (m, 3H, H-5, H-6a galactal), 4.15–4.07 (m, 3H, H-6b Gal), 3.88 (t, *J* = 6.7 Hz, 1H, H-5 Gal); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6, 170.3, 170.2, 170.2, 169.3, 144.9, 99.3, 98.8, 73.3, 70.8, 70.8, 68.5, 67.9, 66.9, 65.4, 62.3, 61.2, 29.7, 20.8, 20.8, 20.7, 20.6; IR (film) 1747, 1645, 1371 cm^{-1}; HRMS (LSIMS, NaI) *m/e* 583.1668 (583.1639 calcd for $C_{24}H_{32}O_{16}Na$, M + Na$^+$); $[\alpha]_D$ = −13° (*c* = 1.28, $CHCl_3$) (lit.[7f] $[\alpha]_D$ = −3°).

D-Galactopyranosyl-β-(1,3)-2-acetamido-2-deoxy-D-galactopyranose (7b). Following the procedure of Lemieux,[13] a solution of **6b** (90 mg, 0.16 mmol) in CH_3CN (5 mL) was added dropwise to a mixture of NaN_3 (16 mg, 0.2 mmol) and $Ce(NH_4)_2(NO_3)_6$ (320 mg, 0.6 mmol) at −23 °C under argon. The reaction mixture was allowed to warm to room temperature and maintained for 14 h whereupon the starting material had been consumed as evidenced by TLC (1:1 EtOAc–hexanes). The solution was poured into ice–water and extracted with EtOAc, washed with water and brine, dried ($MgSO_4$), and concentrated. The residue was used directly for the next reaction without purification. The residue was dissolved in acetic acid (5 mL) and treated wtih anhydrous NaOAc (65 mg, 1.2 mmol). The reaction mixture was warmed to 100 °C and maintained overnight. The mixture was cooled, diluted with EtOAc, rinsed with water and saturated $NaHCO_3$/brine, dried ($MgSO_4$), and concentrated. The crude material was used for the next step without further purification: IR (film) 2114, 1747, 1369, 1222, 1059 cm^{-1}; HRMS (LSIMS, CsI) *m/e* 794.0997 (794.1021 calcd for $C_{26}H_{35}N_3O_{17}Cs$, M + Cs$^+$).

(17) Cheng, P.-W.; DeVries, A. *Carbohydr. Res.* **1986**, *149*, 253.

A solution of the crude azido compound (100 mg) in Ac_2O (5 mL) was hydrogenated over Pd–C (25 mg) under a H_2 atmosphere (1 atm). After 14 h, the solution was filtered and concentrated to afford the peracetylated acetamido derivative of **7b** (56 mg, 52% from **6b**) which was a mixture of anomers: IR (film) 2924, 2854, 1747, 1371, 1225, 1053 cm^{-1}; HRMS (LSIMS, CsI) *m/e* 810.1229 (810.1221 calcd for $C_{28}H_{39}O_{18}NCs$, M + Cs^+).

The above compound (10 mg, 0.015 mmol) was dissolved in a 1 M solution of ammonia in MeOH (2 mL). The reaction mixture was maintained at room temperature for 12 h, whereupon the starting material has been consumed as evidenced by TLC. The reaction mixture was concentrated and purified by gel filtration (Bio Gel P-2, H_2O) to afford **9b** as a white amorphous solid (4.8 mg, 83%) which was a 1:1 mixture of α and β isomers: ^1H NMR (500 MHz, D_2O) δ 5.07 (d, J = 3.7 Hz, H-1 GalNAc α-anomer), 4.55 (d, J = 8.5 Hz, H-1 GalNAc β-anomer), 4.35 and 4.29 (d each, J = 7.8 Hz, H-1 Gal), 4.16 (d, J = 3.7, 11.1 Hz, H-2 GalNAc), 4.11 (m), 4.04 (m), 4.0 (t, J = 5.7 Hz), 3.89 (dd, J = 3.1, 11.2 Hz, H-3 GalNAc), 3.77–3.76 (m, contains H-4 Gal), 3.72 (dd, J = 3.1, 11.0 Hz), 3.64–3.58 (m), 3.57–3.53 (m, H-5 Gal), 3.52–3.46 (m, H-3 Gal), 3.40–3.36 (m, H-2 Gal), 1.89 and 1.88 (s each, $C(O)CH_3$); ^{13}C NMR (125 MHz, D_2O) δ 175.4, 175.1, 105.3. 105.1, 95.6, 91.6, 80.5, 77.5, 75.4, 75.3, 73.0, 71.1, 71.0, 70.6, 69.2, 69.0, 68.5, 61.6, 61.4, 52.9, 49.4, 22.7, 22.5; HRMS (LSIMS, NaI) *m/e* 406.1325 (406.1325 (calcd for $C_{14}H_{25}O_{11}NNa$, M + Na^+); $[\alpha]_D$ = +45° (*c* = 0.22, H_2O). This data corresponds to that of an authentic sample purchased from Sigma Chemical Co..

2,3,4,6-Tetra-*O*-acetyl-D-galactopyranosyl-β-(1,3)-4,6-di-*O*-acetyl-D-*arabino*-hex-1-enitol (6a). This compound was prepared in 50% yield as described previously:[5] ^1H NMR (500 MHz, $CDCl_3$) δ 6.48 (d, J = 6.37 Hz, 1H, H-1 glucal), 5.39 (d, J = 3.4 Hz, 1H, H-4 Gal), 5.23 (app t, J = 3.7 Hz, 1H, H-4 glucal), 5.17 (dd, J = 7.9, 10.4 Hz, 1H, H-2 Gal), 5.02 (dd, J = 3.4, 10.4 Hz, 1H, H-3 Gal), 4.87 (app t, J = 5.5 Hz, 1H, H-2 glucal), 4.66 (d, J = 7.9 Hz, 1H, H-1 Gal), 4.41 (dd, J = 8.1, 12.1 Hz, 1H, H-6a glucal), 4.36–4.34 (m, 1H, H-3 glucal), 4.19–4.08 (m, 4H, Gal H-6a,b, H-5,6b glucal), 3.93 (app t, J = 6.7 Hz, 1H, H-5 Gal), 2.17, 2.09, 2.09, 2.06, 2.05, 1.98 (s, 3H each, 6 × $C(O)CH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.5, 170.4, 170.3, 170.1, 169.5, 169.3, 145.2, 99.1, 97.1, 73.8, 70.9, 70.8, 69.3, 68.7, 67.9, 66.9, 61.5, 61.2, 29.7, 20.9, 20.8, 20.7, 20.7, 20.5; IR (film) 1747, 1650, 1370 cm^{-1}; HRMS (LSIMS, NaI) 583.1639 (583.1639 calcd for $C_{24}H_{32}O_{15}Na$, M + Na^+); $[\alpha]_D$ = −22° (*c* = 1.08, $CHCl_3$).

D-Galactopyranosyl-β-(1,3)-2-acetamido-2-deoxy-D-glucopyranose 7a. This compound was prepared from **6a** (300 mg, 0.54 mmol) in the same manner as for **7b**. A 1.4:1 ratio of gluco- to manno-products was formed at the azidonitration stage. The gluco-product was separated after acetate displacement of the nitrated azido species (96 mg, 26%): IR (film) 2113, 1747, 1369, 1222, 1056 cm^{-1}; HRMS (LSIMS, CsI) *m/e* 794.1000 (794.1021 calcd for $C_{26}H_{35}N_3O_{17}Cs$ M + Cs^+). The azido product was reduced as described for **7b** to afford the acetylated derivative of **7a** (42 mg, 43%). Peracylated **7a**: ^1H NMR (500 MHz, $CDCl_3$) δ 6.07 (d, J = 3.6 Hz, H-1 GlcNAc α-anomer), 5.86 (d, J = 7.8 Hz) and 5.76 (d, J = 8.6 Hz, NH), 5.48 (d, J = 9.7 Hz, H-1 GlcNAc β-anomer), 5.37–5.36 (m), 5.09–5.03 (m), 5.02–4.96 (m), 4.61 (d, J = 7.7 Hz, H-1 Gal), 4.55 (dt, J = 3.1, 9.9 Hz, H-2 GlcNAc), 4.26 (dd), 4.22 (dd, J = 4.4, 12.6 Hz), 4.18–4.14 (m), 4.10–4.06 (m), 4.04–4.00 (m), 3.96–3.89 (m), 2.22, 2.22, 2.16, 2.16, 2.09, 2.09, 2.08, 2.08, 2.08, 2.06, 2.06, 2.03, 2.03, 1.99 (s each, $C(O)CH_3$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.8, 170.8, 170.1, 169.6, 169.5, 169.0, 100.9, 91.7, 91.2, 75.7, 72.7, 70.9, 70.8, 70.6, 70.2, 69.8, 69.1, 69.0, 68.2, 66.8, 66.6, 62.2, 61.7, 61.0, 60.8, 51.1, 29.7, 23.3, 21.0, 20.7, 20.6, 20.5; HRMS (LSIMS, CsI) *m/e* 810.1229 (810.1221 calcd for $C_{28}H_{39}O_{18}NCs$, M + Cs^+).

Compound **7a** was isolated as a 2.8:1 mixture of α and β anomers (21 mg, 93%): ^1H NMR (500 MHz, D_2O) δ 5.02 (d, J = 3.5 Hz, H-1 GlcNAc α anomer), 4.59 (d, J = 7.7 Hz, H-1 GlcNAc β anomer), 4.31 and 4.27 (d each, J = 7.8 Hz, 1H, H-1 Gal), 3.91 (dd, J = 3.5, 10.6 Hz, H-2 GlcNAc), 3.79–3.70 (m, includes H-3 GlcNAc), 3.68-3.56 (m), 3.48 (dt, J = 3.3, 10.0 Hz, H-4 GlcNAc), 3.43 (t, J = 9.3 Hz), 3.41–3.35 (m, H-2 Gal), 1.88 (s, 3H, C(O)-CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 175.2, 175.0, 104.0, 103.9, 95.2, 91.5, 83.1, 80.6, 75.9, 75.7, 75.7, 73.0, 73.0, 71.7, 71.2, 71.1, 69.2, 69.1, 69.9, 61.5, 61.2, 61.0, 56.1, 53.3, 22.7, 22.4; HRMS (LSIMS, NaI) *m/e* 406.1325 (406.1325 calcd for $C_{14}H_{25}O_{11}NNa$, M + Na^+); $[\alpha]_D$ = +8.3° (*c* = 0.36, H_2O). This data is consistent with that previously reported.[18]

Formation of Trisaccharide 8. A solution of GlcNAc-1-P (25 mg, 73 μmol), UDP (35 mg, 73 μmol), PEP Na_3 salt (20 mg, 85 μmmol), EDTA (3 mM), and $MgCl_2$ (5 mM) in HEPES (100 mM, 5 mL) was adjusted to pH 7.5 with 1 N NaOH. To this solution were added freeze-dried yeast cells (50 mg) and PK (200 U). The mixture was gently stirred for 10 h at 23 °C under an argon atmosphere. After the formation of UDP-GlcNAc was observed as evidenced by TLC ($iPrOH/H_2O/NH_4OH$ 7:3:1), **7b** (10 mg, 26 mmol), leupeptin (0.5 mg/mL), pepstatin (0.7 mg/mL), Tween 20 (a detergent) (50 μL), DTT (3.8 mg), PMSF (0.2 mM), and GlcNAcT′ase-containing crude cell extract[15] (100 mg) were added to the mixture. The reaction was maintained for 2 days under an argon atmosphere, concentrated, and chromatographed on silica gel (EtOAc/AcOH/water 3:2:1) followed by Bio Gel P-2 filtration with water to afford **7** (2.7 mg, 6.3%), after lyophilization, as a 5:4 mixture of α and β anomers: ^1H NMR (500 MHz, D_2O) (partial) δ 5.14 (d, J = 3.7 Hz, H-1α of GalNAc), 4.61 (d, J = 8.5 Hz, H-1β of GalNAc), 4.52 (d, J = 8.5 Hz, H-1 of GlcNAc), 4.51 (d, J = 8.5 Hz, H-1 of GlcNAc), 4.43 (d, J = 7.8 Hz, H-1 of Gal), 4.38 (d, J = 7.8 Hz, H-1 of Gal), 1.99, 1.99, 1.98, 1.97 (s, 3H each, NAc); HRMS (LSIMS, NaI) *m/e* 609.2119 (609.2119 calcd for $C_{22}H_{38}N_2O_{16}Na$, M + Na^+). This data is in agreement with that previously reported.[15]

D-Galactopyranosyl-β-(1,3)-D-*lyxo*-hex-1-enitol (9b). Compound **6b** (50 mg, 0.09 mmol) was dissolved in a 1 M solution of ammonia in MeOH (10 mL) at room temperature. The reaction was maintained for 24 h whereupon no starting material remained as evidenced by TLC (2:3 EtOAc–hexanes). The reaction mixture was concentrated and purified by gel filtration chromatography (Bio-Gel P-2) with H_2O as the eluant to afford **9b** (23 mg, 83%) as a white amorphous solid: ^1H NMR (500 MHz, D_2O) δ 6.30 (dd, J = 1.6, 6.2 Hz, 1H, H-1 galactal), 4.46 (m, 1H, H-2 galactal), 4.39 (d, J = 7.8 Hz, 1H, H-1 Gal), 4.03–4.02 (m, 1H, H-3 galactal), 3.91 (dd, J = 4.5, 8.2 Hz, 1H, H-4 galactal), 3.76 (m, 1H, H-4 Gal), 3.69 (dd, J = 8.2, 11.2 Hz, 1H, H-5 Galactal), 3.66–3.58 (m, 4H, H-6 galactal and Gal), 3.54–3.52 (m, 1H, H-5 Gal), 3.49 (dd, J = 3.3, 10.0 Hz, 1H, H-3 Gal), 3.39 (dd, J = 7.8, 9.9 Hz, 1H, H-2 Gal); ^{13}C NMR (125 MHz, D_2O) δ 145.2, 102.9, 100.6, 77.3, 75.6, 73.5, 73.2, 71.2, 69.1, 64.7, 61.8, 61.5; HRMS (LSIMS, NaI) *m/e* 331.1005 (331.1005 calcd for $C_{12}H_{20}O_9Na$, M + Na^+); $[\alpha]_D$ = −10°, (*c* = 0.2, H_2O).

D-Galactopyranosyl-β-(1,3)-D-*arabino*-hex-1-enitol (9a). Compound **9a** was prepared from **6a** (570 mg, 1.0 mmol) following the procedure used for the preparation of **9b**. The reaction mixture was concentrated *in vacuo* to afford **9a** (300 mg, 97%) as a white amorphous solid: ^1H NMR (500 MHz, D_2O) δ 6.35 (d, J = 6.2 Hz, 1H, H-1 glucal), 4.81 (dd, J = 2.7, 6.2 Hz, 1H, H-2 glucal), 4.42 (d, J = 7.9 Hz, 1H, H-1 Gal), 4.27–4.26 m 1H, H-3 glucal), 3.84–3.81 (m, 1H), 3.79–3.77 (m, 2H, H-4 glucal and H-4 Gal), 3.76–3.75 (m, 2H), 3.68–3.63 (m, 2H), 3.61–3.57 (m, 1H), 3.53 (dd, J = 3.5, 9.6 Hz, 1H, H-3 Gal), 3.39 (dd, J = 7.9, 9.9 Hz, 1H, H-2 Gal); ^{13}C NMR (125 MHz, D_2O) δ 145.2, 101.8, 100.1, 78.4, 77.1, 75.8, 73.2, 71.1, 69.0, 67.4, 61.5, 60.4; HRMS (LSIMS, NaI) *m/e* 331.1005 (331.1005 calcd for $C_{12}H_{20}O_9Na$, M + Na^+); $[\alpha]_D$ = −21° (*c* = 0.28, H_2O).

Supplementary Material Available: Copies of NMR spectra (21 pages). This material is contained in libraries on microfiche, immediately follows this article in the microfilm version of the journal, and can be ordered from the ACS; see any current masthead page for ordering information.

(18) Lemieux, R. U.; Driguez, H. *J. Am. Chem. Soc.* **1975**, *97*, 4063.