Glycoconjugate Journal 16, 767–772, 1999

Synthesis of selectively radiolabeled hexasaccharides for the determination of enzymatic regioselectivity

J. C. McAuliffe[1], M. Ujita[2], M. Fukuda[1] and O. Hindsgaul[1]*

[1]*The Burnham Institute, 10901 North Torrey Pines Rd., San Diego, CA, 92122, USA,* [2]*Present Address; National Institute for Basic Biology, Okazaki, 444-8585, Japan*

Poly-*N*-acetyllactosamines provide backbone structures for functional modifications such as sialyl Lewis X. To understand how the biosynthesis of poly-*N*-acetyllactosamines is regulated, two branched oligosaccharides of the structure Galβ1,4GlcNAcβ1, 6(Galβ1,4GlcNAcβ1,2)-Manα1,6Manβ-octyl 1 and 2 were synthesized in which one of the terminal galactose units was selectively radiolabeled. Hexasaccharides 1 and 2 were assembled from the chemically synthesized pentasaccharide precursors GlcNAcβ1,6(Galβ1,4GlcNAcβ1,2)-Manα1,6Manβ-octyl3 and Galβ1,4GlcNAcβ1, 6(GlcNAcβ1, 2) - Manα1,6 Manβ-octyl 4 respectively, through treatment with UDP-1-[^{3}H]-Gal and β1,4 galactosyltransferase. Compounds 1 and 2 were subsequently incubated with UDP-GlcNAc and the UDP-GlcNAc: Galβ1-4Glc(NAc) β1,3-*N*-acetylglucosaminyltransferase (*i*-GlcNAc transferase) resulting in a partial conversion to a mixture of heptasaccharides which were purified by HPLC. The branch selectivity of the addition of *N*-acetylglucosamine to compounds 1 and 2 was then characterized by endo-β-galactosidase digestion of the heptasaccharides, followed by isolation of the resultant pentasaccharides on C18 reverse-phase silica cartridges. Comparison of the amount of radiolabel to a control reaction lacking endo-β-galactosidase indicated the favored site of GlcNAc addition to be the lower β1,2-branch over the β1,6-branch by a 3 : 1 ratio.

Keywords: **Glycosyltransferase, *N*-acetyllactosamine, biosynthesis, radiolabel**

Introduction

N-Acetyllactosamine-containing oligosaccharides are found in a large proportion of both *O*- and *N*-linked glycans, often further substituted by *N*-acetylglucosamine, galactose, fucose, sialic acids and sulfate [1]. These molecules fulfill a range of functions including blood type determination and immune response, and have also been implicated in cancer metastasis [2–5]. For instance, Ohyama et al. demonstrated recently that the ability of injected B16 melanoma cells to colonize mouse lung tissue correlates to the amount of sialyl Le^x bearing poly-*N*-acetyllactosamine structures on the cell surface [6,7]. The glycosyltransferases responsible for the assembly and further modification of *N*-acetyllactosamines often exhibit branch selectivity toward structures with multiple acceptor sites, thereby influencing the structure and composition of the final products [8]. Ultimately, the acceptor preferences of these glycosyltransferases have direct functional effects *in vivo*, however, characterization of this enzymatic regioselectivity has traditionally required several chemical or enzymatic steps combined with repetitive chromatography [9].

In this paper we describe the chemoenzymatic synthesis of the selectively branch-labeled hexasaccharides **1** and **2** and their application to the determination of enzymatic regioselectivity of a soluble form of the UDP-GlcNAc:Galβ1-4Glc(NAc) β1-3-*N*-acetylglucosaminyltransferase (*i*-GlcNAcT) [10]. Our synthetic approach involved chemical syntheses of the pentasaccharide acceptors **3** and **4** which, upon treatment with UDP-1-[^{3}H]-Gal by β1,4galactosyltransansferase, afforded the target hexasaccharides **1** and **2**.

Materials and Methods

General

Chemicals and solvents were obtained from Aldrich Chemical Co. (Milwaukee, WI) and were used as received. Molecular sieves (Aldrich) were held at 160°C for 48 h and cooled under

*To whom correspondence should be addressed.

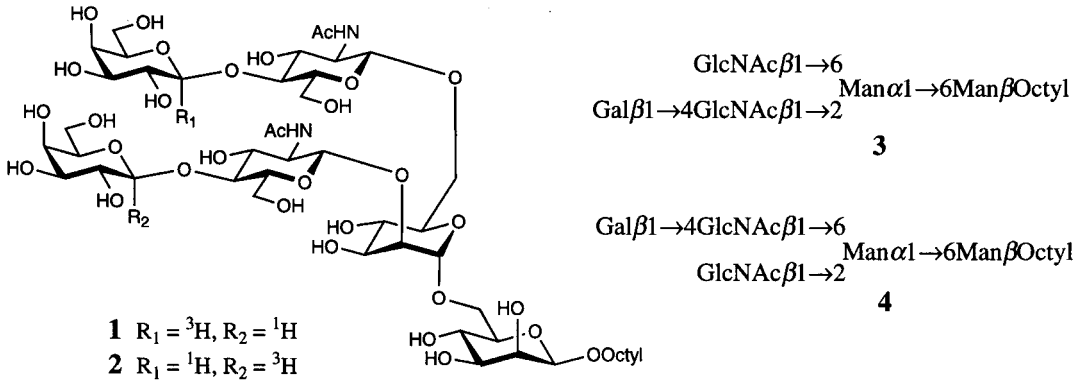


Figure 1. Synthesis of radiolabeled hexosaccharides I and 2 from pentasaccharides 3 and 4.

vacuum immediately before use. Thin-layer chromatography was performed on silica gel 60 glass-backed plates obtained from Merck (Germany). Plates were developed using the specified solvents and developed by heating after treatment with 10% H_2SO_4 in ethanol. Flash chromatography was performed with silica gel (230–400 mesh) obtained from Fluka (Switzerland). ^{1}H-NMR spectra were acquired on a Varian 300 MHz INOVA NMR spectrometer. MALDI-TOF mass spectra were obtained using a PE Biosystems Voyager DE-RP.

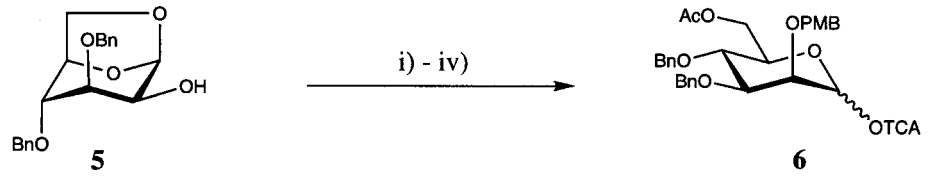
Plasmid cDNA, encoding the catalytic domain of human *i*-GlcNAcT (amino acid residues 53-415), a signal peptide sequence and *Staphylococcus aureus* protein A, was expressed in COS-1 cells as described by Sasaki et al. [10]. The culture medium was concentrated 100-fold in a Centricon 10 concentrator (Amicon) and used directly as an enzyme source. Enzyme activity was determined using 0.5 mM Galβ1, 4GlcNAcβ-p-nitrophenol (Toronto Research Chemicals) as an acceptor.

Chemical Synthesis of Pentasaccharides **3** and **4**

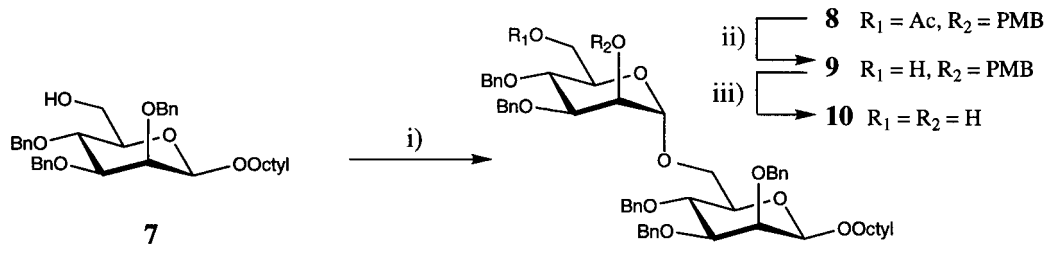
Conversion of 3,4-di-*O*-benzyl-1,6-anhydro-D-mannose **5** [11] into the trichloroacetimidate 6 was achieved in four steps and 66% overall yield as outlined in Scheme 1.

Glycosylations

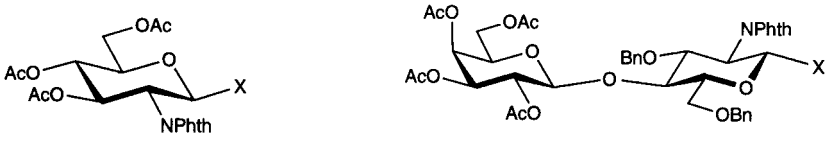
Glycosylation of octyl 2, 3, 4-tri-O-benzyl-β-D-mannopyranoside **7** [12,13] with 1.2 equivalents of the imidate **6** and a catalytic amount (10–20 µL) of triethylsilyl trifluoromethanesulfonate (TESOTf) was performed in ether at −45° in the presence of powdered 4Å molecular sieves over 30 min and gave the α-glycoside **8** in 66% yield following purification on silica gel (ethyl acetate/toluene, 1 : 4), along with minor amounts (10–15%) of the β-anomer (Scheme 2). Treatment of compound **8** with 50 mM NaOMe in MeOH over 2 h gave the OH-6′ compound **9** in excellent yield (>90%). Subsequent treatment of compound **9** with 80% AcOH at 50°C for 1 h gave the diol **10** (75%). Partial ^{1}H NMR (300 MHz, $CDCl_3$).



Scheme 1. i) *p*-MeOBnCl, NaH, DMF ii) Ac_2O/TFA, 10 : 1, 2h iii) hydrazine acetate, DMF iv) Cl_3CCN, DBU, CH_2Cl_2



Scheme 2. i) compound **6**, TESOTf, Et_2O, −45° ii) NaOMe, MeOH iii) AcOH, H_2O, 50°



11a X = Cl
11b X = OTCA

12a X = Cl
12b X = OTCA

Figure 2. Glycosyl donors used in the synthesis pentasaccharides 3 and 4.

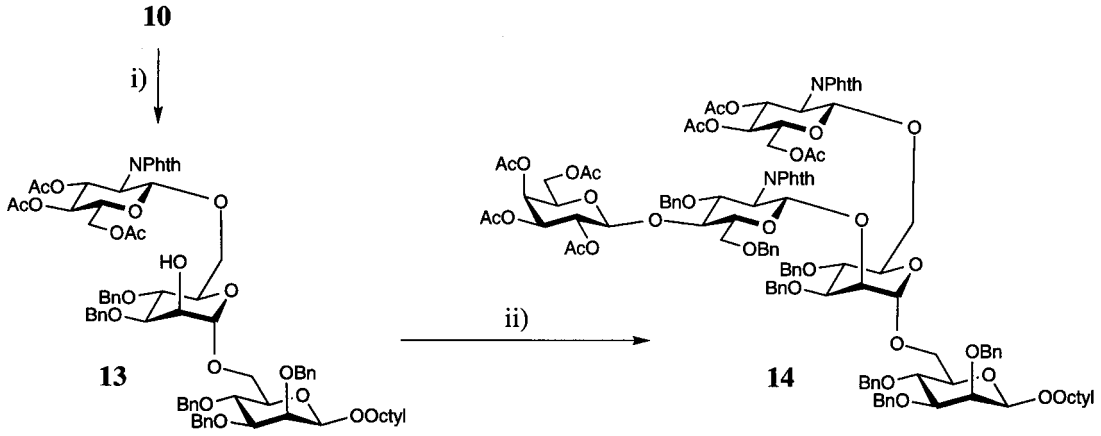
For compound **8**: δ7.18-7.48 (m, 27 H, Ar), 6.76-6.86 (m, 2 H, *Ar*O*Me*), 5.11 (d, $J = 1.5$ Hz, H-1′), 4.34 (s, H-1), 3.77 (s, 3H, ArO*Me*), 2.00 (s, 3H, Ac), 0.86 (t, 3 H, octyl *Me*). For compound **10**: δ 7.20-7.52 (m, 25H, Ar), 5.02 (d, $J = 1.8$ Hz, H-1′), 4.34 (s, H-1), 4.13 (t, $J = 2$ Hz, H-2′), 0.88 (t, 3 H, octyl *Me*).

Glycosylation of **10** with the chloride **11a** [14] (1.5 eq), silver trifluoromethanesulfonate (AgOTf) (2 eq) and collidine (2 eq) was performed in CH_2Cl_2 at $-30°$ over 1 h and gave initially the trisaccharide **13** in 62% yield following chromatography (Scheme 3). Subsequent treatment of **13** with the trichloroacetimidate **12b** [15] and catalytic TESOTf in CH_2Cl_2 at $-40°$ gave the pentasaccharide **14** in 63% yield after purification on a silica gel column (acetone/toluene 1 : 2). Partial ^{1}H NMR (300 MHz, $CDCl_3$). For compound **13**: δ 5.82 (dd, $J = 9.1$, 10.7 Hz, H-3″), 5.38 (d, $J = 8.5$ Hz, H-1″), 5.15 (dd, $J = 9.2$, 10.0 Hz, H-4′), 4.82 (d, $J = 1.7$ Hz, H-1′), 4.30 (s, H-1), 1.92, 2.02, 2.08 (3s, 9 H, Ac), 0.85 (t, 3 H, octyl *Me*). For compound **14**: δ 5.82 (dd, $J = 9.1$, 10.8 Hz, H-3″), 5.46 (dd, $J = 9.2$, 10.8 Hz, H-4″), 5.38 (d, $J = 8.5$ Hz, H-1′), 5.26 (bd, $J = 3.6$ Hz, H-4^{Gal}), 1.82-2.06 (7s, 21 H, Ac), 0.88 (t, 3 H, octyl *Me*).

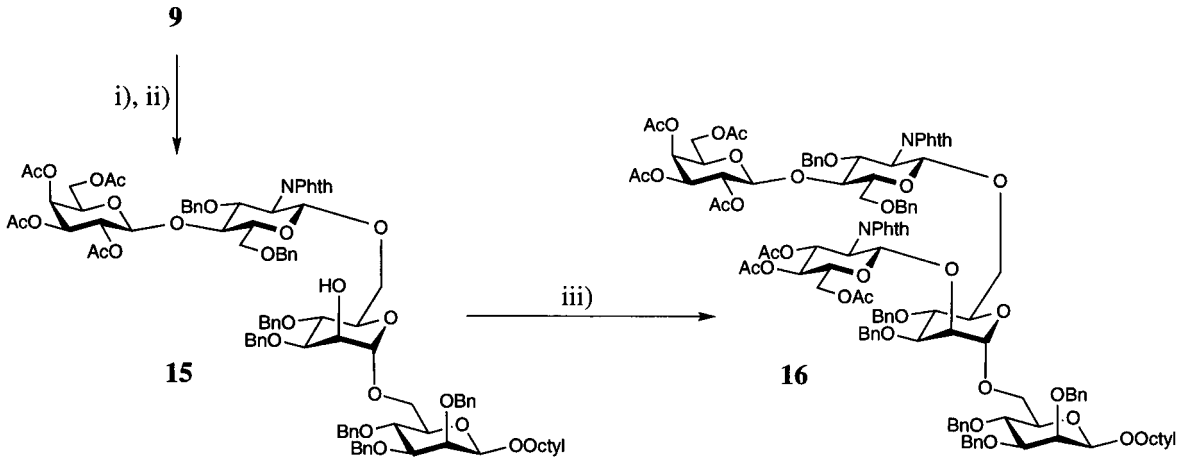
Glycosylation of **9** with the imidate **12b** (1.5 eq) and catalytic TESOTf in CH_2Cl_2 at $-45°$C over 30 min gave a major product (TLC) which was isolated and treated with 80% AcOH at 50°C for 1 h to give the tetrasaccharide **15** in 54% yield following chromatography (Scheme 4). Subsequent glycosylation of **15** with the trichloroacetimidate **11b** [16] (2.5 eq) and catalytic TESOTf in CH_2Cl_2 at $-20°$C over 2 h gave the pentasaccharide **16** in 31% yield after purification on a silica gel column (acetone/toluene 1 : 2). Partial ^{1}H NMR (300 MHz, $CDCl_3$). For compound **15**: δ 5.22 (d, $J = 3.4$ Hz, H-4^{Gal}), 4.26 (s, H-1), 1.96, 2.00, 2.03 (3s, 12 H, Ac), 0.84 (t, 3 H, octyl *Me*). For compound **16**: δ 5.77 (dd, $J = 8.7$, 10.3 Hz, H3″), 5.45 (d, $J = 8.2$ Hz, H1″), 5.22 (bd, $J = 3.6$ Hz, H-1^{Gal}), 5.10 (dd, $J = 9.2$, 10.3 Hz, H-4″), 1.85-2.04 (6s, 21H, Ac), 0.90 (m, 3H, octyl *Me*).

Deprotection of the Pentasaccharides

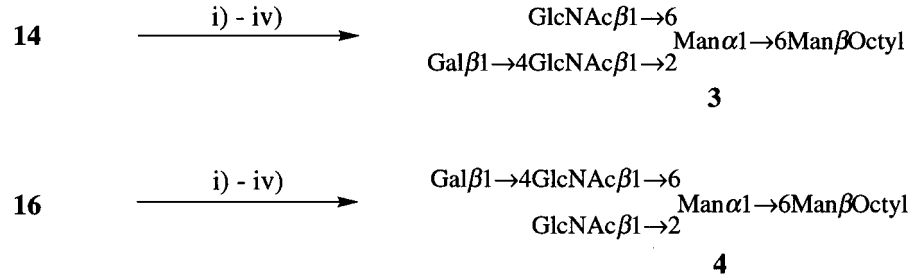
Deprotection of the pentasaccharides **14** and **16** was achieved through standard conditions and gave the deprotected pentasaccharides **3** and **4** in yields of 44% and 58%, respectively (Scheme 5). The crude products were isolated on C18 reverse-phase Sep-Pak cartridges (Waters) and subsequently purified on LH-20 Sephadex eluted with water. Partial ^{1}H NMR (300 MHz, D_2O). For compound **3**: δ 4.90 (s, H-1′), 4.68 (s, H-1), 4.63 (d, $J = 7.8$ Hz, 1 H), 4.56 (d, $J = 7.7$ Hz, 1 H), 4.48 (d, $J = 8.3$ Hz, 1 H), 2.09, 2.05 (2s, 6 H, NHAc). *m/z* (found) 1045.16. (calc.) 1045.04 $[M + Na]^+$-1. For compound **4**: δ 4.88 (s, H-1′), 4.66 (s, H-1), 4.60-4.55 (m, 2 H), 4.47 (d, $J = 8.3$ Hz, 1H), 2.06, 2.03 (2s, 6H, NHAc). *m/z* (found) 1045.33. (calc.) 1045.04 $[M + Na]^+$-1.



Scheme 3. i) **11a**, AgOTf, collidine, CH_2Cl_2, $-30°$ ii) **12b**, TESOTf, CH_2Cl_2, $-45°$



Scheme 4. i) **12b**, TESOTf, CH_2Cl_2, −45° ii) AcOH/H_2O (4 : 1), 50° iii) **11b**, TESOTf, CH_2Cl_2, −20°



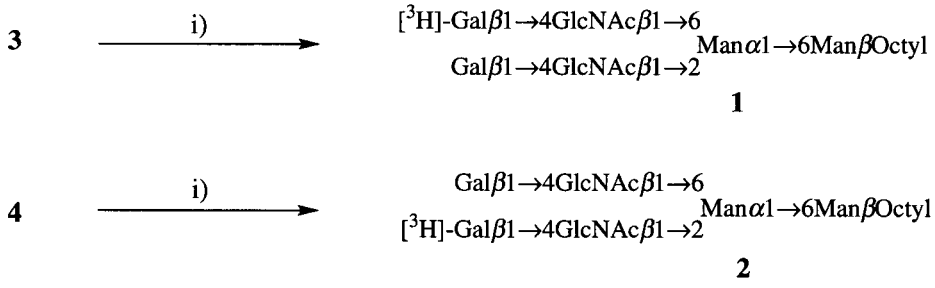
Scheme 5. i) H_2NNH_2.AcOH, MeOH, reflux, 48 h ii) Ac_2O, pyridine, o/n iii) NaOMe, MeOMe, MeOH, 2 h iv) H_2, $Pd(OH)_2/C$, AcOH, MeOH, 3 days

Enzymatic synthesis of radiolabeled hexasaccharides

The enzymatic additions of 1-[^{3}H]-Gal to **3** and **4** were performed on a 1–2 μmol scale utilizing bovine milk β1-4 galactosyltransferase (50 mU) and UDP-1-[^{3}H]-Gal (10 μCi/μmol acceptor) in 50 mM HEPES buffer (pH 7.4) containing 15 mM $MnCl_2$ at 37°C. UDP-1-[^{3}H]-Gal (10 μCi/μmol acceptor) was added to the reaction medium containing either **3** or **4**, along with unlabeled UDP-Gal (0.5 μmol). After 12 h another 2.5 μmol of UDP-Gal was added and incubation continued for another 12 h. Thin-layer chromatography indicated absence of the starting material. The products were isolated on Sep-Pak cartridges resulting in excellent yields (>90%) of labeled hexasaccharides **1** and **2** with a specific activity of approximately 8.5 Ci/mol, indicating the incorporation of 85% of the label.

Determination of the branch selectivity of the i-GlcNAc transferase

An aliquot of **1** (100 μL of 1 mM) was incubated with UDP-GlcNAc and the soluble *i*-GlcNAcT at 37°C for 1 h, resulting in a partial conversion to a mixture of heptasaccharides **17a,b,** which were subsequently purified by HPLC on an amido-column (Varian MicroPak AX-5) (Scheme 7). The mixture of heptasaccharides **17a,b** was then digested with endo-β-



Scheme 6. i) [^{3}H]-Gal-UDP, β1, 4Gal T, 15 mM $MnCl_2$, 50 mM HEPES, pH 7.4, 37°

galactosidase, effectively removing the galactose units functionalized by *N*-acetylglucosamine. The resulting pentasaccharides were isolated by solid-phase extraction and the amount of radioactivity recovered compared to a control that did not contain endo-β-galactosidase.

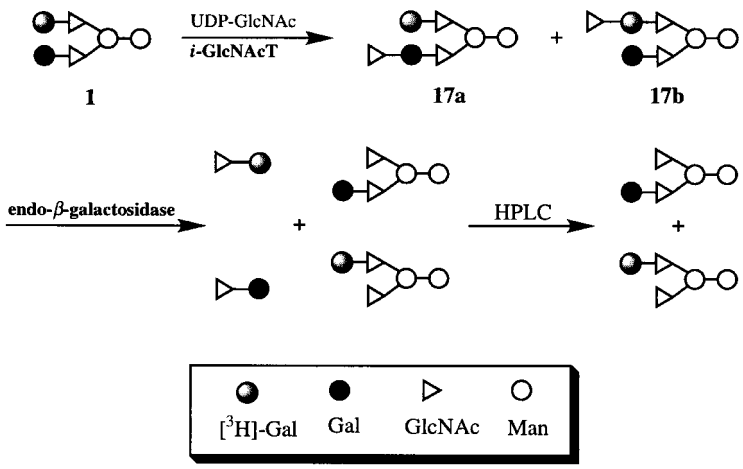
Results and Discussion

We sought to assemble the desired pentasaccharides **3** and **4** through a common strategy involving regioselective glycosylation at OH-6′ of the diol **10** with a moderately reactive glycosyl chloride, followed by subsequent glycosylation at OH-2′ with a highly reactive trichloroacetimidate donor. Accordingly, sequential glycosylation of **10** with the chloride **11a** and the trichloroacetimidate **12b** gave initially the trisaccharide **13** in 62% yield, followed by the pentasaccharide **14** in 63% yield (Scheme 3). Having synthesized the pentasaccharide **14**, we then attempted the inverse procedure. Unfortunately, attempts to glycosylate the diol **10** with the lactosaminyl chloride **12a** were unsuccessful, resulting in unreacted acceptor and decomposition products derived from the donor.

We then pursued a modified approach involving glycosylation of the alcohol **9** with the trichloroacetimidate **12b**, followed by acid hydrolysis of the 2′-*O*-PMBn ether to give the tetrasaccharide **15** in 54% overall yield (Scheme 4). Glycosylation of **15** was achieved with the imidate **11b**, to give the pentasaccharide **16**, albeit in low yield (31%). This was not unexpected given the reported unreactivity at 2′-OH of a similar compound [17]. Compounds **14** and **16** were subsequently deprotected (Scheme 5) to give the pentasaccharides **3** and **4** in satisfactory yields.

The enzymatic incorporation of 1-[^{3}H]-Gal into compounds **3** and **4** was performed in such a manner as to maximize the specific activity of the products. This was achieved through the initial addition of the radiolabeled donor substrate UDP-1-[^{3}H]-Gal, along with UDP-Gal (0.5 eq). After a 2 h incubation, a further aliquot of UDP-Gal (1.2 eq) was added so as to drive the reaction to completion. The resulting hexasaccharides **1** and **2** were isolated on C18 Sep-pak cartridges in nearly quantitative yields. It was determined that in this manner approximately 85% of the radiolabel had been incorporated into the products.

Having obtained the desired radiolabeled hexasaccharides **1** and **2**, we next examined their utility in the determination of enzymatic regioselectivity, specifically that of the addition of *N*-acetylglucosamine by a soluble form of the *i*-GlcNAc transferase [10]. Incubation of the hexasaccharide **1** with the *i*-GlcNAc transferase was performed as detailed in the methods section and resulted in the conversion of approximately 10% of the starting material into a mixture of heptasaccharides **17a,b** (Scheme 7). Parallel experiments with the unlabeled analog of hexasaccharide **1** demonstrated that the octasaccharide resulting from the addition of two equivalents of [^{3}H]-GlcNAc only accounted for 5.7% of the total products even after incubation times of 10 h. Heptasaccharides **17a,b** were then isolated by HPLC and subjected to an endo-β-galactosidase digest resulting in the removal of only those galactosyl residues functionalized with GlcNAc. The resultant pentasaccharides were isolated and it was determined that 75% of the radioactivity, attributed to [^{3}H]-Galβ1,4-GlcNAcβ1, 6(GlcNAcβ1,2)-Manα1,6Manβ-octyl, was recovered, as compared to a control that did not contain endo-β-galactosidase. Repetition of these experiments with hexasaccharide **2,** resulted in the recovery of 26% of the radioactivity, relative to control, as GlcNAcβ1, 6([^{3}H]-Galβ1,4GlcNAcβ1,2)-Manα1, 6Manβ-octyl. On the basis of these two experiments it was determined that the preference of the *i*-GlcNAcT towards compounds **1** and **2** to be for the β1,2 branch over the β1,6 branch by a ratio of approximately 3 : 1. These results, in



Scheme 7. Determination of the regioselectivity of GlcNAc addition by the *i*-GlcNAc transferase. Addition of GlcNAc to hexasaccharide **1** gave a mixture of heptasaccharide products **17a,b**. Subsequent treatment of **17a,b** with endo-β-galactosidase removed only the functionalized galactose residues and allowed the branch specificity of the reaction to be characterized.

conjunction with recent insights regarding the β1, 4galactosyltransferse family of enzymes [18,19], help explain the distribution of complex *N*-type glycans observed in nature. Specifically, the preference of the *i*-GlcNAcT for the β1,2 branch of complex *N*-type glycans is offset by the preference of β1,4Gal-T1 for GlcNAc present on the upper β1, 6 branch of GlcNAcβ1,6(GlcNAcβ1,2)Manα1, 6Manβ-octyl [20]. As a result, polylactosamine extension occurs on both the β1,2 and β1,6 branches of complex *N*-type glycans.

In summary, we have successfully demonstrated the synthesis of two selectively radiolabeled hexasaccharides **1** and **2** and their subsequent application to the elucidation of enzymatic regioselectivity through a convenient protocol. We expect to apply this procedure to other glycosyltransferases in the near future.

Acknowledgment

This work was supported by National Institutes of Health Grant PO1 CA71932.

References

1 Fukuda M, *Cancer Res* **56**, 2237–44 (1996).
2 Yamashita K, Ohkura T, Tachibana Y, Takasaki S, Kobata A, *J Biol Chem* **259**, 10834–40 (1984).
3 Pierce M, Arango J, *J Biol Chem* **261**, 10772–7 (1986).
4 Dennis, JW, Laferte S, Waghorne C, Breitman ML, Kerbel RS, *Science* **236**, 582–5 (1987).
5 Saitoh O, Wang W-C, Lotan R, Fukuda M, *J Biol Chem* **267**, 5700–11 (1992).
6 Ohyama C, Tsuboi S, Fukuda M, *EMBOJ* **18**, 1516–25 (1999).
7 Fukuda MN, Ohyama C, Lowitz K, Matsuo O, Pasqualini R, Ruoslahti E, Fukuda M, *Cancer Res* **60**, 450–6.
8 Ujita M, McAuliffe JC, Suzuki M, Hindsgaul O, Clausen H, Fukuda MN, Fukuda M, *J Biol Chem* **274**, 9296–304 (1999).
9 Van den Eijnden DH, Koenderman AHL, Schiphorst WECM, *J Biol Chem* **263**, 12461–71 (1988).
10 Sasaki K, Kurata-Miura K, Ujita M, Angata K, Nakgawa S, Sekine S, Nishi T, Fukuda M, *Proc Natl Acad Sci* USA **94**, 14294–9 (1997).
11 Hori H, Nishida Y, Ohrui H, Meguro H, *J Org Chem* **54**, 1346–53 (1989).
12 Synthesized from 6-*O*-acetyl-2,3,4-tri-*O*-benzyl-α-D-mannosyl bromide[13], 1-octanol and silver zeolite in CH_2Cl_2 at -30°C.
13 Paulsen H, Lockhoff O *Chem Ber* **114**, 3102–14 (1981).
14 Jansson K, Noori G, Magnussen G, *J Org Chem* **55**, 3181–5 (1990).
15 McAuliffe JC, Fukuda M, Hindsgaul O, *Bioorg Med Chem Lett* **9**, 2855–8 (1999).
16 Grundler G, Schmidt RR, *Carbohydr Res* **135**, 203–16 (1985).
17 Zhu X-X, Cai M-S, Zhou R-L, *Carbohydr Res* **303**, 261–6 (1997).
18 Schwientek T, Almeida R, Levery S, Holmes E, Bennett E, Clausen H, *J Biol Chem* **273**, 29331–40 (1998).
19 Ujita M, McAuliffe JC, Schwientek T, Almeida R, Hindsgaul O, Clausen H, Fukuda M, *J Biol Chem* **273**, 34843–9 (1998).
20 Ujita M, McAuliffe JC, Hindsgaul O, Sasakai K, Fukuda, MN, Fukuda M, *J Biol Chem* **274**, 16717–26 (1999).

Received 2 February 2000, revised 15 April 2000, accepted 30 April 2000