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Use of *N*-Acetylglucosaminyltransferases I and II in the Synthesis of a Dideoxypentasaccharide

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Abstract—Partially purified forms of N-acetylglucosaminyltransferases I and II were used, in sequence, to preparatively convert the readily available synthetic 6-deoxy-α-D-Man(1→3)[6-deoxy-α-D-Man(1→6)]β-D-Man-O$(CH_2)_8$COOMe to a dideoxy-analog of the pentasaccharide core common to all complex N-linked oligosaccharides.

Introduction

The biosynthesis of asparagine-linked oligosaccharide chains is under the control of a series of enzymes termed *N*-acetylglucosaminyltransferases I-VI (GlcNAcT-I-VI).[1-3] The sequence of action and substrate specificities of these enzymes have been extensively studied. The specific branches initiated by addition of GlcNAc residues by each of these enzymes is summarized in Figure 1.[1-3]

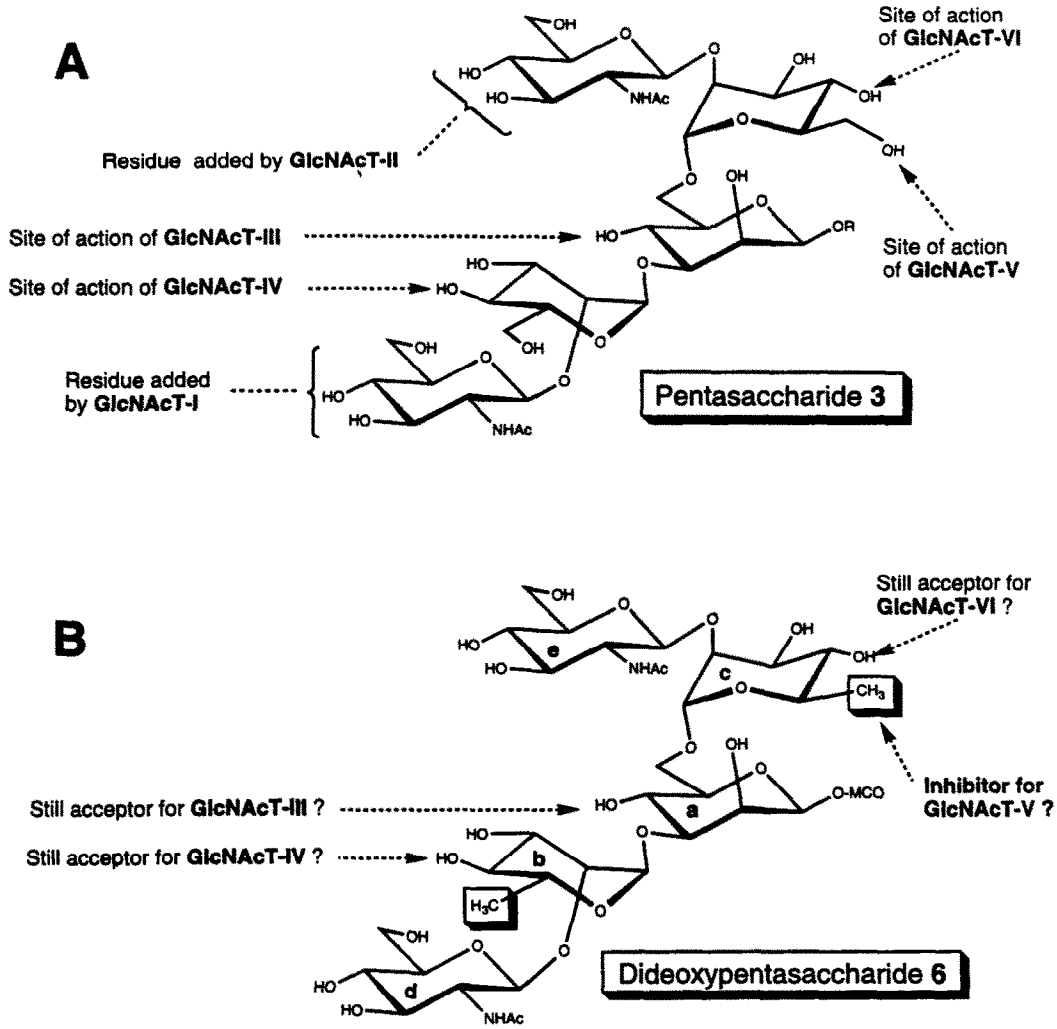


Figure 1. A: Summary of the substrate specificities of GlcNAcT-I through VI. In the natural acceptors, R is chitobiose linked to asparagine. Synthetic acceptors with R = $(CH_2)_7CH_3$ and R = $(CH_2)_8COOCH_3$ (MCO) are also active. B: Structure and possible activities of the dideoxypentasaccharide **6** prepared in this work.

Tremendous effort[4–21] has been expended in the synthesis of both partial structures of the naturally occurring sequences and chemically modified analogs thereof. The special interest in preparing deoxy-analogs of acceptor structures follows observations that removal of certain OH groups often leads to derivatives which are specific acceptors for a given glycosyltransferase. Sometimes, these derivatives can also act as competitive inhibitors for a GlcNAcT which normally glycosylates the OH group that was removed.[22,23] Deoxyoligosaccharide analogs have also been extensively used to study the recognition of acceptors by the GlcNAc-transferases. A major collaborative effort between the groups of Paulsen, Schachter and Brockhausen should be noted in this regard.[4,7,10,11, 14,15,18–20]

In a continuation of such studies, we decided to prepare the new di-6-deoxy pentasaccharide derivative **6** (Figure 1) for several reasons. Compound **6** could in principle exhibit many useful properties, some of which are summarized in Figure 1. For example, the 6"-OH group normally acted on by GlcNAcT-V has been removed in this structure which might then exhibit inhibitory activity towards that enzyme. Both hydroxyl groups that have been deoxygenated in **6** are also in proximity to positions acted on by GlcNAcT-III, IV and VI. Pentasaccharide **6** should therefore be useful in probing the recognition of acceptors by these latter enzymes.

Several deoxy- and otherwise modified pentasaccharides have been previously prepared for similar specificity studies.[8,10,12,13–15,18–20] Compound **6**, however, would have an advantage of symmetry, and therefore ease of synthesis, over most other derivatives since both 'branches' are identical. In planning the synthesis of **6** we decided to examine the feasibility of a combined chemical–enzymatic approach using GlcNAcT-I and II to add the final GlcNAc residues. That way we might also learn more about the substrate specificity of these two important enzymes which have previously been shown to be efficient synthetic adjuncts when the unmodified trimannoside **1** was used as the acceptor to produce the 'native' pentasaccharide **3**. The 6-OH groups of the mannose residues in **1** have been reported to not be 'critical' recognition elements for these two enzymes when deoxygenated one at a time. It was not known, however, whether the kinetic parameters for the transfer to the di-6-deoxygenated tri- and tetrasaccharides would remain sufficiently favorable to permit their use in a preparative synthesis.

Discussion

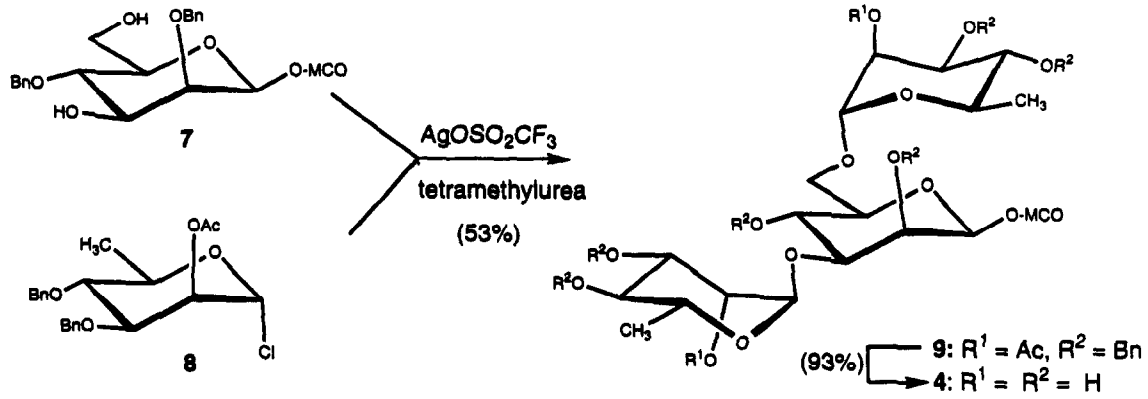
The target oligosaccharides were synthesized as their 8-methoxycarbonyloctyl[24] (MCO) glycosides. This hydrophobic aglycone was included to facilitate the measurement of enzyme kinetics using a reported "Sep-Pak Assay".[25] The isolation of product from preparative scale reactions by bulk reverse-phase extraction was thus also simplified. The synthesis of dideoxytrisaccharide **4** was straightforward using reagents previously prepared in our laboratory. Glycosylation of the known diol **7**[9] with an excess of donor **8**[22] yielded the protected trisaccharide **9** in 53 % yield. In principle, 6-deoxyacetochloro- or acetobromomannose could equally well have been used for this purpose. The 3,4-di-O-benzyl derivative was used instead, however, in the event that the enzymes could not be used to complete the synthesis. The two GlcNAc residues would then have had to be introduced chemically. This turned out not to be required *(vide infra)*. Deprotection of **9** produced the required trisaccharide **4** (93 %).

The reactivity of **4** with GlcNAcT-I from two different sources was examined. Partially purified soluble enzyme from human milk[8] and detergent solubilized enzyme from rabbit liver[9] were used. Kinetic parameters for the transfer reactions were determined by quantitating the transfer of ^{3}H-tracer-labeled GlcNAc from UDP-GlcNAc to **4**. The results are presented in Table 1 where they are compared with parameters similarly obtained for the 'native' dihydroxytrisaccharide **1**.

As seen in Table 1, the enzyme from both sources had very similar kinetic parameters for trisaccharide **1** with K_m values of 0.70 and 0.88 mM. For the dideoxytrisaccharide **4**, the K_m values increased 3-fold to 2.22 and 2.75 mM while the V_{max} decreased 6-fold. Dideoxygenation therefore had a serious deleterious effect on the efficiency of the reaction. Nevertheless, extended incubation (4 days) with the liver enzyme resulted in a 60 % conversion of **4** to **5** which could be separated from unreacted **4** by size exclusion chromatography on BioGel P-2. Key NMR data for compounds **1–6** are collected in Table 2.

Only GlcNAcT-II from rabbit liver[9] was examined for its ability to use dideoxytetrasaccharide **5** as a substrate. The deoxygenation was found to have very little effect on the kinetics of the transfer which turned out to be slightly faster with the dideoxytetrasaccharide acceptor **5** (Table 1). For the preparative synthesis of **6**, TLC indicated that glycosylation of **5** was complete after 3 days. The NMR data for purified **6** are collected in Table 2.

This work expands the utility of GlcNAcT-I and II to the preparation of analogs of the naturally occurring N-linked oligosaccharide sequences. As these enzymes become cloned [23] and successfully overexpressed they should become routine reagents in the combined chemical–enzymatic synthesis of oligosaccharides. Evaluation of the enzymatic activity of the deoxyoligosaccharides **5** and **6** produced in the present work is in progress and will be reported elsewhere.


7
8
AgOSO2CF3
tetramethylurea
(53%)
(93%)
9: R1 = Ac, R2 = Bn
4: R1 = R2 = H

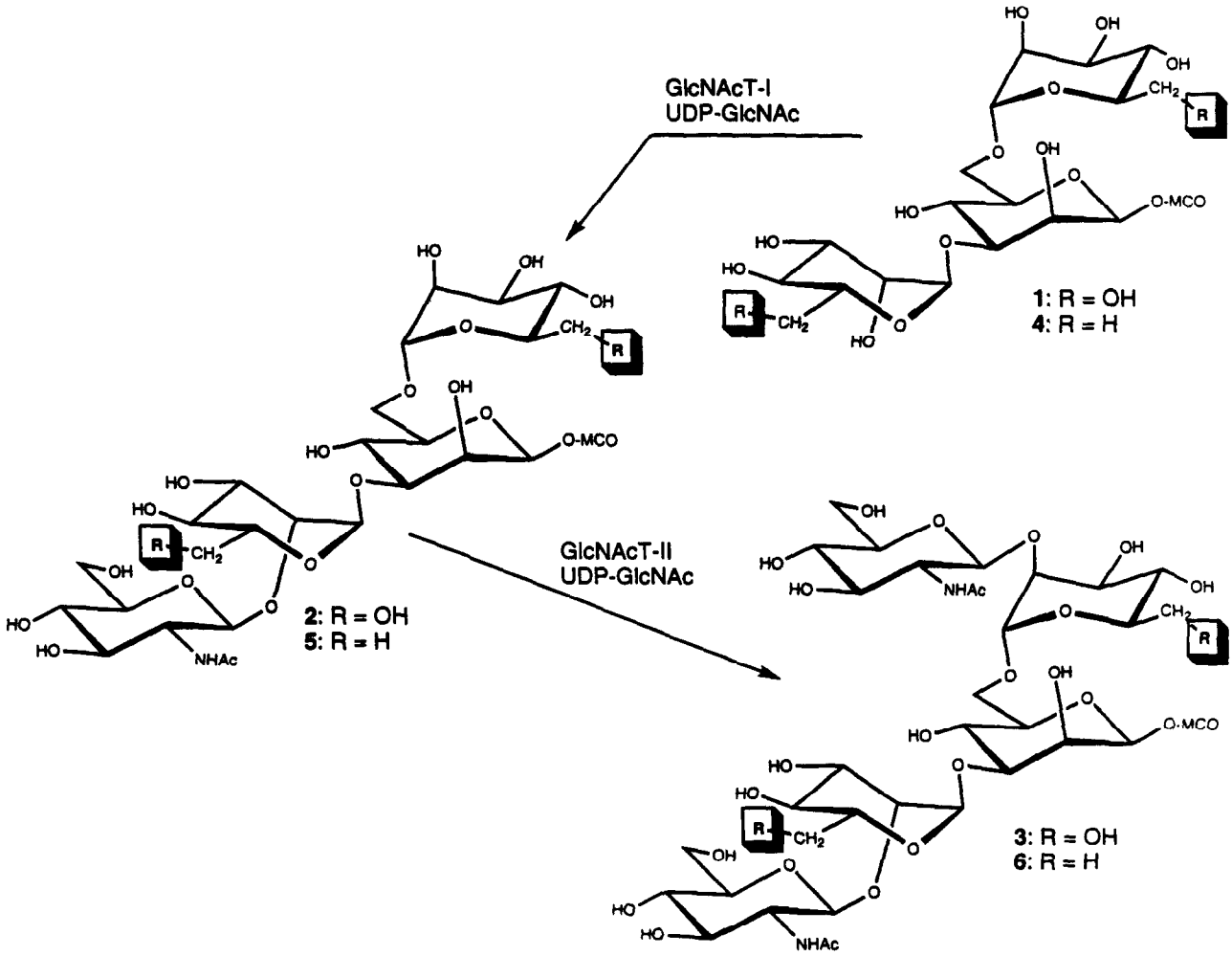

GlcNAcT-I
UDP-GlcNAc
1: R = OH
4: R = H
2: R = OH
5: R = H
GlcNAcT-II
UDP-GlcNAc
3: R = OH
6: R = H

Table 1. Kinetics data for compounds **1**, **2**, **4**, and **5** with GlcNAcT-I and II[‡]

ACCEPTOR	PRODUCT	ENZYME	K_m (mM)	V_{max} (pmol/min)
1	2	GlcNAcT-I (Human milk)	0.70	48.3
1	2	GlcNAcT-I (Rabbit liver)	0.88	48.1
4	5	GlcNAcT-I (Human milk)	2.22	6.0
4	5	GlcNAcT-I (Rabbit liver)	2.75	6.4
2	3	GlcNAcT-II (Rabbit liver)	0.14	35.1
5	6	GlcNAcT-II (Rabbit liver)	0.13	50.4

[‡]All kinetic constants have standard errors of less than 10 %.

Table 2. ^1H and ^{13}C-NMR chemical shifts and mass spectral data for compounds **1–6**[‡]

COMPOUND	M+Na$^+$	H1a ($J_{1,2}$)	H1b ($J_{1,2}$)	H1c ($J_{1,2}$)	H1d ($J_{1,2}$)	H1e ($J_{1,2}$)	C1a	C1b	C1c	C1d	C1e
1	697	4.675 (<1)	5.104 (1.6)	4.908 (1.7)			103.2	100.6	100.2		
4	665	4.687 (<1)	5.018 (1)	4.839 (1.5)			103.3	100.6	100.3		
2	900	4.672 (<1)	5.127 (1.7)	4.910 (1.7)	4.550 (8.3)		100.6	100.5	100.2	100.1	
5	868	4.689 (<1)	5.059 (1)	4.845 (1.4)	4.545 (8.3)		100.6	100.3	100.3	100.3	
3	1103	4.571 (<1)	5.128 (1)	4.918 (1.5)	4.552 (8.3)	4.582 (8.3)	100.6	100.2	97.6	100.4	100.3
6	1071	4.673 (<1)	5.057 (<1)	4.868 (1)	4.547 (8.0)	4.560 (8.3)	100.6	100.1	97.7	100.3	100.2

[‡]Recorded in 99.96 % D_2O. Compounds **2–5** recorded at 360 MHz and 90 MHz (^{13}C). Compounds **1** and **6** recorded at 300 MHz and 75.5 MHz (^{13}C); coupling constants reported in Hz; chemical shifts relative to internal acetone (δ = 2.225, ^1H) and external dioxane (δ = 67.4, ^{13}C); monosaccharide labelling a–e as in Figure 1.

Experimental

General methods

Optical rotations were measured with a Perkin–Elmer 241 polarimeter at 22 ° ± 2 °. TLC was performed on silica gel 60-F_{254} (Merck) with detection by quenching of fluorescence and/or by charring with sulfuric acid. Iatrobeads refers to a beaded silica gel (product number 6RS-8060, Iatron Laboratories, Tokyo). For gel filtration, Bio-Gel P-2 (200–400 mesh) (Bio-Rad Laboratories) was used. C_{18} Sep–Pak sample-preparation cartridges were obtained from Waters Associates. UDP-6-[^{3}H]-GlcNAc (specific activity 18.9 Ci/mmol) was obtained from New England Nuclear and liquid scintillation counting was performed with a Beckman LS-5000 instrument using quench correction as described by the manufacturer. ^1H NMR spectra were recorded at 300 or 360 MHz (Bruker spectrometers) on solutions in $CDCl_3$ (internal Me_4Si) or D_2O (internal acetone, δ 2.225). ^{13}C NMR spectra were recorded at 75.5 MHz on solutions in $CDCl_3$ (internal Me_4Si) or D_2O (external 1 % 1,4-dioxane in D_2O, δ 67.4). Only partial NMR data are reported; the other data were in accord with the proposed structures. The chemical shifts and coupling constants (as observed splittings) for ^1H resonances are reported as though they were first order.

The assignments of ^{13}C resonances are tentative. Fast atom bombardment mass spectra (FAB-MS) were obtained using an AEI MS-9 instrument with Xe as the bombarding gas with 1,4-dithiothreitol: 1,4-dithioerythritol (5:1) as the matrix. The microanalyses were carried out by the Analytical Services Laboratory of this Department.

Chemical synthesis

8-Methoxycarbonyloctyl 3,6-di-O-(2-O-acetyl-6-deoxy-3,4-di-O-benzyl-α-D-mannopyranosyl)-2,4-di-O-benzyl-β-D-mannopyranoside (9). A mixture of **7** (102.5 mg, 0.19 mmol) and silver trifluoromethanesulfonate (198.5 mg, 0.77 mmol) was dried *in vacuo* in the presence of P_2O_5 for 5 h at 20 °C and dissolved in 1,2-dichloroethane (2 mL) under nitrogen. To this stirred mixture was added 1,1,3,3-tetramethylurea (46.2 μL, 0.39 mmol) and chloride **8** (156.4 mg, 0.39 mmol) in 1,2-dichloroethane (2 mL). After 5 h further addition of 1,1,3,3-tetramethylurea (46.2 μL, 0.39 mmol) and chloride (0.39 mmol) were made. The mixture was then stirred for an additional 15 h and diluted with 1,2-dichloroethane (25 mL). Solids were removed by filtration and washed with dichloromethane (50 mL). The filtrate was then washed twice with saturated aqueous $NaHCO_3$ and twice with water before evaporation to a syrup which was purified by chromatography on Iatrobeads using (hexane:ethyl acetate, 3:1) as eluant to provide the trisaccharide **9** (129 mg, 53 %), $[\alpha]_D = +1.40$ ° (*c* 0.5, chloroform), R_f 0.76 (hexane:ethyl acetate, 1:1); ^1H NMR ($CDCl_3$): δ 5.07 (d, 1H, $J_{1'2'} = 1.5$ Hz, H-1'), 4.78 (d, 1H, $J_{1'',2''} = 1.5$ Hz, H-1''), 3.65 (s, 3H, OCH_3), 2.28 (t, 2H, $J = 7.5$Hz, CH_2COO), 2.07 (s, 3H each, 2Ac), 1.26 (d, 3H, $J = 6.5$ Hz, H-6''), 1.24 (d, 3H, $J = 6.5$ Hz, H-6'); ^{13}C NMR: δ 174.27 ($COOCH_3$), 170.21, 169.99($COCH_3$), 101.57 (C-1, $^1J_{CH} = 153.3$ Hz), 99.56 (C-1', $^1J_{CH} = 171.6$ Hz), 97.77 (C-1'', $^1J_{CH} = 170.2$ Hz), 51.43 (OCH_3), 34.09 (CH_2COO), 21.13, 20.97 ($COCH_3$). Anal. Calcd for $C_{74}H_{90}O_{18}$: C, 70.10; H, 7.15. Found: C, 70.00; H, 7.18.

8-Methoxycarbonyloctyl 3,6-di-O-(6-deoxy-α-D-mannopyranosyl)-β-D-mannopyranoside (4). Compound **9** (52.5 mg, 0.04 mmol) was dissolved in dry methanol (5 mL) containing sodium methoxide (~ 0.01 M) and the resulting solution was stirred for 15 h at room temperature. Neutralization with Amberlite IR-120 (H^+), followed by removal of the resin and solvent evaporation provided the syrupy diol which was directly dissolved in 98 % ethanol (5 mL) and hydrogenated over 5 % palladium on charcoal (26 mg) at atmospheric pressure for 15 h. Removal of the catalyst by filtration, followed by evaporation and lyophilization provided a white powder (25 mg, 94 %). $[\alpha]_D = +1.22$ ° (*c* 0.8, water), R_f 0.61 (dichloromethane:methanol:water, 60:35:6). Key NMR data are collected in Table 1. Anal. Calcd. for $C_{28}H_{50}O_{16}\cdot 2H_2O$: C, 50.90; H, 8.23. Found: C, 50.65; H, 8.18.

Enzyme preparation and kinetics

Human milk GlcNAcT-I was partially purified by defatting, precipitation of the enzyme with $(NH_4)_2SO_4$ followed by chromatography on CM-Sephadex and UDP-hexanolamine agarose as previously described.[8] The resulting enzyme preparation had a specific activity of 0.48 mU/mL with **1** as the substrate. A unit (U) is defined as the quantity of enzyme producing 1.0 μmol/min of product at 37 °C under saturating conditions of substrate.

Rabbit liver GlcNAcT-I and GlcNAcT-II were prepared from acetone powder by detergent solubilization in 1 % Triton X-100 followed by a linear NaCl gradient on UDP-hexanolamine agarose as previously described.[10] Early fractions (0.2 M NaCl) were dialyzed against 50 mM Na cacodylate, 1 % Triton X-100, and 10 mM $MnCl_2$ pH 6.5 (buffer C) to provide GlcNAcT-II with a specific activity of 0.36 mU/mL using **2** as the acceptor. Dialysis against buffer C of late fractions (3 M NaCl) provided GlcNAcT-I with a specific activity of 0.32 mU/mL. For preparative reactions the rabbit liver GlcNAcT-I was ultrafiltered to provide a sample with 20-fold higher specific activity.

Kinetic constants were determined by a Sep–Pak assay.[25] In a 0.5 mL microcentrifuge tube the acceptors (40 μM–8 mM), UDP-GlcNAc (270 μM), and 1×10^6 dpm of UDP-^{3}H-GlcNAc were lyophilized. These tubes had 50 μL of the appropriate enzyme added and were incubated at 37 °C for 30 min. The reaction mixtures were added directly to C_{18} reverse phase cartridges in 5 mL H_2O. Unreacted UDP-^{3}H-GlcNAc was eluted with 10 mL H_2O and the radiolabelled product was eluted with 5 mL of MeOH and quantitated by liquid scintillation counting. The kinetic parameters collected in Table 1 were obtained by analysis using Wilkinson's method.[26]

Enzymatic synthesis of 5 and 6

Dideoxytrisaccharide **4** (3.65 mg, 5.7 μmol) and UDP-GlcNAc (7 mg, 9.5 μmol) were incubated with rabbit liver GlcNAcT-I (1.3 mU, 0.4 mL) at 37 °C for six days. At that point TLC on silica gel, using dichloromethane:methanol:water 60:35:6 as eluent, showed conversion of **4** (R_f 0.79) to tetrasaccharide **5** (R_f 0.58) along with the appearance of pentasaccharide **6** (R_f 0.33) due to the presence of GlcNAcT-II in the partially-purified enzyme preparation. The crude product was obtained following adsorption onto a C_{18} Sep–Pak cartridge as described above and further purified by chromatography on Bio-Gel P2 (1.5 × 45 cm, 10 % ethanol as solvent) to give **5** (2.9 mg, 3.4 μmol, 60 %). Tetrasaccharide **5** (2.9 mg, 3.4 μmol) and UDP-GlcNAc (9 mg, 12.2 μmol) were then incubated with GlcNAcT-II (0.36 mU, 2.0 mL) for 3 days at 37 °C by which time TLC showed complete conversion to **6** which was isolated by Sep-Pak adsorption and Bio-Gel chromatography as described for **5**. Compounds **1–6** were characterized by ^1H and ^{13}C NMR and FAB-MS with positive ion detection (Table 2).

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References

1. Schachter, H. *Biochem. Cell. Biol.* **1986**, *64*, 163.

2. Schachter, H. *Curr. Opin. Struct. Biol.* **1991**, *1*, 755.

3. Schachter, H. *Trends Glycos. Glycotechnol.* **1992**, *4*, 241.

4. Vella, G. J.; Paulsen, H.; Schachter, H. *Can. J. Biochem. Cell Biol.* **1984**, *62*, 409.

5. Srivastava, O. P.; Hindsgaul, O.; Shoreibah, M.; Pierce, M. *Carbohydr. Res.* **1988**, *179*, 137.

6. Khan, S. H.; Abbas, S. A.; Matta, K. L. *Carbohydr. Res.* **1989**, *193*, 125.

7. Brockhausen, I; Hull, E.; Hindsgaul, O.; Schachter, H.; Shah, R. N.; Michnick, S. W.; Carver, J. P. *J. Biol. Chem.* **1989**, *264*, 11211.

8. Srivastava, G.; Alton, G.; Hindsgaul, O. *Carbohydr. Res.* **1990**, *207*, 259.

9. Kaur, K. J.; Alton, G.; Hindsgaul, O. *Carbohydr. Res.* **1991**, *210*, 145.

10. Paulsen, H.; Reck, F.; Brockhausen, I. *Carbohydr. Res.* **1992**, *236*, 39.

11. Brockhausen, I.; Möller, G.; Yang, J.-M.; Khan, S. H.; Matta, K. L.; Paulsen, H.; Grey, A. A.; Shah, R. N.; Schachter, H. *Carbohydr. Res.* **1992**, *236*, 281.

12. Paulsen, H.; Meinjohanns, E. *Tetrahedron Lett.* **1992**, *33*, 7327.

13. Paulsen, H.; van Dorst, J. A. L. M.; Reck, F.; Meinjohanns, E. *Liebigs Ann. Chem.* **1992**, 513.

14. Paulsen, H.; Wilkens, R.; Reck, F.; Brockhausen, I. *Liebigs Ann. Chem.* **1992**, 1303.

15. Möller, G.; Reck, F.; Paulsen, H.; Kaur, K.J.; Sarkar, M.; Schachter, H.; Brockhausen, I. *Glycoconjugate J.* **1992**, *9*, 180.

16. Kaur, K. J.; Hindsgaul, O. *Carbohydr. Res.* **1992**, *226*, 219.

17. Khan, S. H.; Crawley, S. C.; Kanie, O.; Hindsgaul, O. *J. Biol. Chem.* **1993**, *268*, 2468.

18. Paulsen, H.; Reck, F.; Brockhausen, I. *Carbohydr. Res.* **1992**, *236*, 39.

19. Paulsen, H.; Meinjohanns, E.; Reck, F.; Brockhausen, I. *Liebigs Ann. Chem.* **1993**, 721.

20. Paulsen, H.; Meinjohanns, E.; Reck, F.; Brockhausen, I. *Liebigs Ann. Chem.* **1993**, 737.

21. Kanie, O.; Crawley, S. C.; Palcic, M. M.; Hindsgaul, O. *Carbohydr. Res.* **1993**, *243*, 139.

22. Palcic, M. M.; Ripka, J.; Kaur, K. J.; Shoreibah, M.; Hindsgaul, O.; Pierce, M. *J. Biol. Chem.* **1990**, *265*, 6759.

23. Hindsgaul, O.; Kaur, K. J.; Srivastava, G.; Blaszczyk-Thurin, M.; Crawley, S. C.; Heerze, L. D.; Palcic, M. M. *J. Biol. Chem.* **1991**, *266*, 17858.

24. Lemieux, R. U.; Bundle, D. R.; Baker, D. A. *J. Am. Chem. Soc.* **1975**, *97*, 4076.

25. Palcic, M. M.; Heerze, L. D.; Pierce, M.; Hindsgaul, O. *Glycoconjugate J.* **1988**, *5*, 49.

26. Wilkinson, G. N. *Biochem. J.* **1961**, *80*, 324.

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