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Synthesis of a di-*O*-methylated pentasaccharide for use in the assay of *N*-acetylglucosaminyltransferase III activity ☆

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Abstract

The biantennary oligosaccharide analogue β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 3)-[β-D-Glc*p*NAc-(1 → 2)-α-D-Man*p*-(1 → 6)]-β-D-Man*p*-O$(CH_2)_8$COOMe (**3**) is a potential substrate for *N*-acetylglucosaminyltransferases (GlcNAcTs) III–V which are present in mammalian cells. The di-*O*-methylated analogue of **3**, β-D-Glc*p*NAc-(1 → 2)-[4-*O*-methyl-α-D-Man*p*]-(1 → 3)-[β-D-Glc*p*NAc-(1 → 2)-[6-*O*-methyl-α-D-Man*p*]-(1 → 6)]-β-D-Man*p*-O-$(CH_2)_8$COOMe (**5**), was prepared by a block synthesis approach involving sequential addition of two *O*-methylated disaccharide donors to a protected central β-D-Man residue. The OH groups acted on by GlcNAcT-IV and -V are protected from glycosylation in **5** since they are present as their methyl ethers. Pentasaccharide **5** was found to be an excellent substrate for GlcNAcT-III (EC 2.4.1.144) from rat kidney with K_m = 0.15 mM. The product formed by incubation of *5* with a rat kidney extract, in the presence of UDP-GlcNAc, was isolated, structurally characterized by NMR spectroscopy and confirmed to be the expected di-*O*-methyl hexasaccharide where a β-D-Glc*p*NAc residue had been added to OH-4 of the central β-D-Man*p* unit.

Key words: Oligosaccharide; Pentasaccharide; *N*-Acetylglycosylaminyltransferase III; Block synthesis; Substrate

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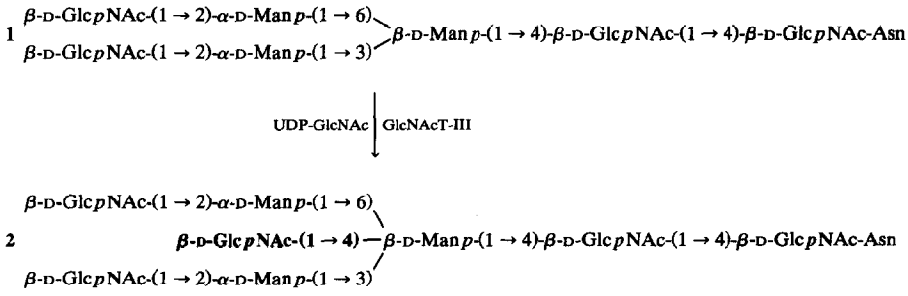
1. Introduction

N-Acetylglucosaminyltransferase III (EC 2.4.1.144) is the branching enzyme responsible for the synthesis of so-called bisected *N*-linked oligosaccharides [1,2]. It acts on glycopeptides such as **1** to which it transfers a GlcNAc residue, from UDP-GlcNAc, in a β-linkage to OH-4 of the central β-Man residue to produce **2**. The interest in developing rapid and specific assays for this enzyme activity follows reports of its increased activity in patients with liver disorders, especially hepatoma and cirrhosis [3,4]. The enzyme has recently been cloned [5].

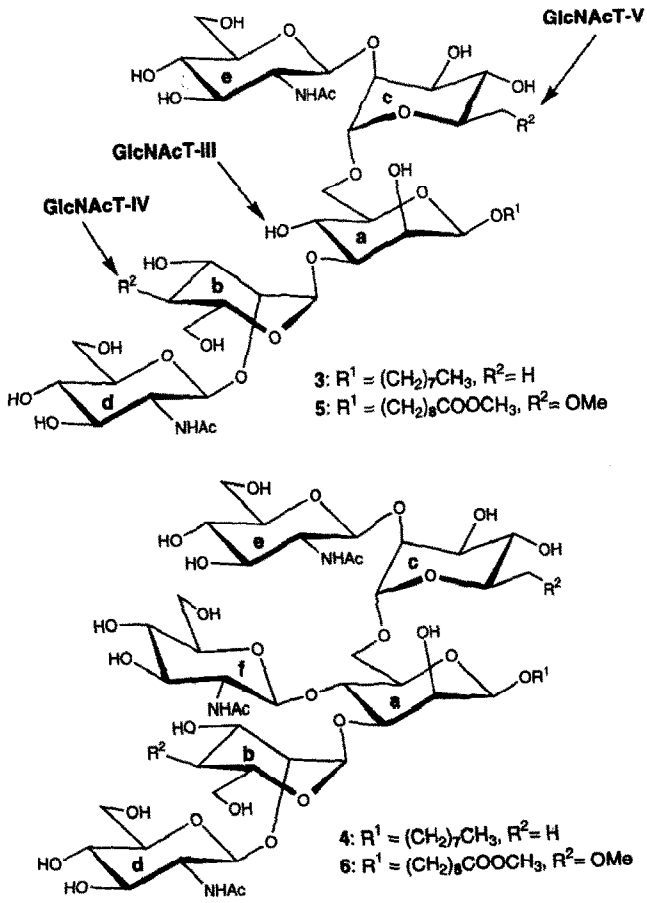
Three different approaches have been used to assay the activity of GlcNAcT-III in crude extracts of biological tissues. The earliest assays [2,3] involved incubation of isolated glycopeptides with radiolabeled UDP-[^{3}H or ^{14}C]-GlcNAc to produce a product quantitated by liquid scintillation counting. A serious drawback to such assays is the fact that the biantennary substrates most easily isolated are also substrates for other competing GlcNAc-transferases which therefore contribute to the labeling. The multiple products formed in an incubation consequently have to be separated. This separation could be effected by lectin-affinity chromatography on Con-A Sepharose [2,3].

A second approach uses similar isolated substrates but separates the products by HPLC [6–8]. The product specifically formed by GlcNAcT-III is then identified by comparing its retention time with that of an authentic standard. The product was initially quantitated by liquid scintillation counting. In a very useful modification of this approach, the acceptor oligosaccharide was fluorescently labelled with pyridylamine [9], allowing unlabeled UDP-GlcNAc to be used as the donor. The product was then simultaneously identified and quantitated by fluorescence detection in HPLC [9].

A third type of assay uses chemically synthesized acceptors designed to be specific for GlcNAcT-III by rendering inactive the hydroxyl groups to which the competing enzymes (GlcNAcT-IV and -V) would transfer [10,11]. Such compounds should in principle be monospecific for GlcNAcT-III, and the necessity for time-consuming separation of products should thereby be avoided. The single product formed in radioactive assays could therefore be readily quantitated. The challenge of the latter approach is to modify the natural acceptor substrates to



Scheme 1.



Scheme 2.

destroy cross-reactivity with interfering enzymes but not with the target GlcNAcT-III.

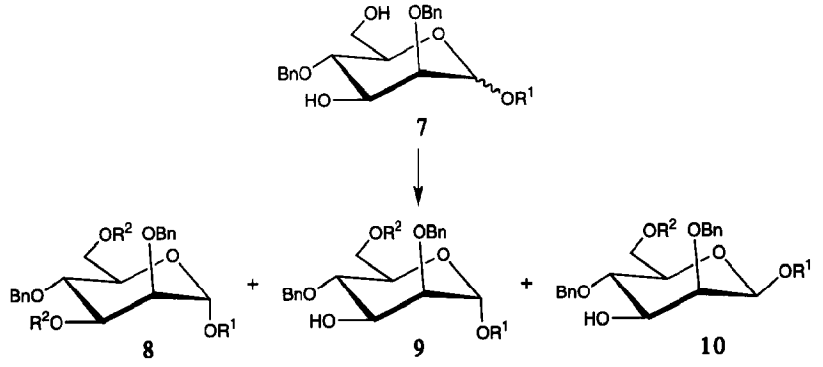
We recently reported [10] the combined chemical–enzymatic synthesis of a dideoxygenated pentasaccharide analogue of **1**, namely **3**, which was subsequently shown [11] to be active as a substrate for GlcNAcT-III producing **4**. We now report the synthesis of the corresponding di-*O*-methylated pentasaccharide **5**, a much more easily prepared acceptor substrate for this enzyme. We also show that the expected hexasaccharide **6**, synthesized by the action of GlcNAcT-III, is formed on incubation of rat-kidney extracts with this new substrate.

2. Results and discussion

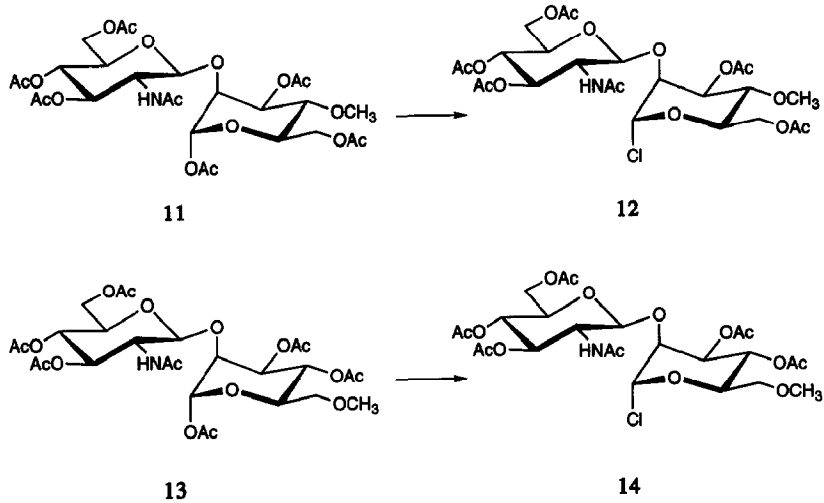
A retrosynthetic analysis of the target compound **5** suggested as a key intermediate the partially protected β-D-Man residue **10** to which two disaccharide donors

12 and **14** could be sequentially added in a direct block-synthesis approach commonly used [10,12,13] for the preparation of analogues of branched oligosaccharides such as **1** and **2**. The monosaccharide intermediate **10** was protected in a manner to first allow the glycosylation of C-3, followed by the liberation of the C-6 hydroxyl group for subsequent glycosylation.

Chloroacetylation of 8-methoxycarbonyloctyl 2,4-di-*O*-benzyl-α,β-D-mannopyranoside (**7**), available from previous work [14], proved to be a simple method for both resolving the anomers and for selectively protecting OH-6, thus providing the required β anomer **10** (19%). The required glycosyl donors **12** (73%) and **14** (93%) were prepared from **11** [15] and **13** [16], respectively. These donors were



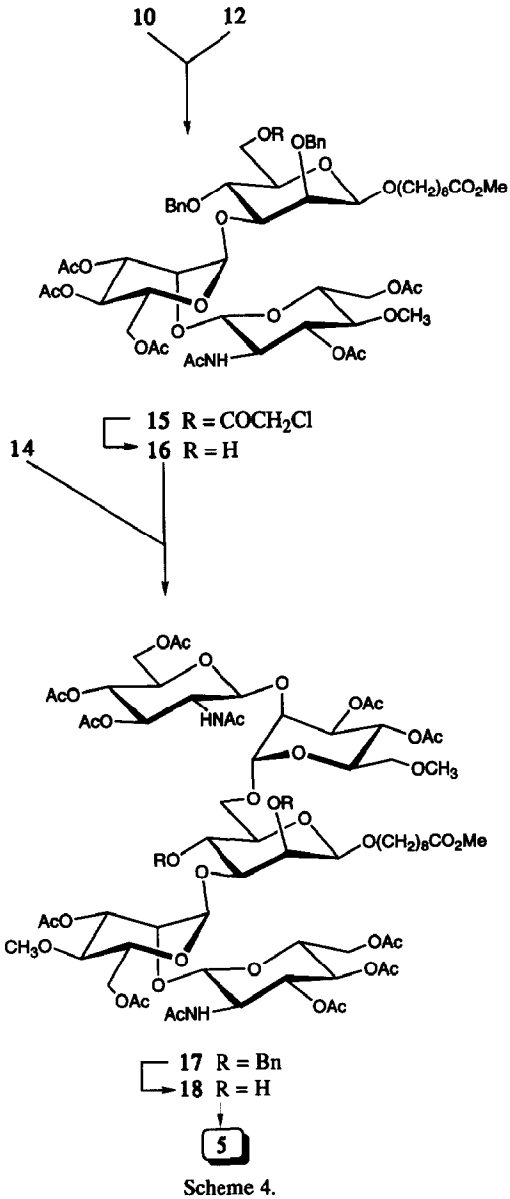
$R^1 = (CH_2)_8CO_2Me$; $R^2 = COCH_2Cl$



Scheme 3.

prepared as the stable glycosyl chlorides, in preference to the labile glycosyl bromides which have been reported earlier [15,16].

Glycosylation of acceptor **10** with **12** under Helferich conditions afforded the α-linked trisaccharide derivative **15** (55%). After selective cleavage of the chloroacetyl group by thiourea to give **16** (77%), the second disaccharide was introduced using donor **14**. The protected pentasaccharide **17** was obtained in 80%



Scheme 4.

Table 1
Selected ^{1}H NMR data for oligosaccharides **5** [a] and **6** [b]

Compound	^{1}H	Monosaccharide units **a**–**f**					
		a	**b**	**c**	**d**	**e**	**f**
5	H-1 ($J_{1,2}$)	4.661	5.111	4.912	4.553 (8.5)	4.571 (8.5)	–
	H-2 ($J_{2,3}$)	4.171 (3.5)	4.149 (3.5)	4.105 (3.0)	n.d.	n.d.	–
	CO_2CH_3	3.687	–	–	–	–	–
	CH_2CO_2	2.389 (7.5)	–	–	–	–	–
	OCH_3	–	3.526	3.402	–	–	–
	NAc	–	–	–	2.061	2.056	–
6	H-1 ($J_{1,2}$)	4.614	5.044	4.975	4.588 (8.3)	4.546 (8.0)	4.452 (9.0)
	H-2	4.064	4.216	4.192	n.d.	n.d.	n.d.
	CO_2CH_3	3.687	–	–	–	–	–
	CH_2CO_2	2.385 (7.5)	–	–	–	–	–
	OCH_3	–	3.522	3.439	–	–	–
	NAc	–	–	–	2.062	2.062	2.070

[a] Recorded at 300 MHz.
[b] Recorded at 500 MHz.

yield. Conventional deprotection of **17** proceeded via **18** to produce the desired target pentasaccharide **5** (64%).

Compound **5** was assayed as a substrate for GlcNAcT-III in a crude extract of rat kidney using a Sep-Pak assay [17], which quantitates the transfer of tracer-labeled [^{3}H]-GlcNAc from UDP-GlcNAc to this potential acceptor. The K_m value was found to be 0.15 ± 0.03 mM. This compares very favorably with the K_m value of 0.19 mM previously reported for the natural acceptor [9] and 0.65 mM for the dideoxypentasaccharide **3** [11]. The V_{max} value for **5** was 90% of that previously found [11] for **3**.

For the synthesis of hexasaccharide **6**, a partially purified preparation [9,11] of GlcNAcT-III was used in a preparative-transfer reaction using **5** (2.1 mg) as acceptor. After a 48-h incubation, analytical TLC and HPLC indicated ca. 30% conversion of **5** to a single, larger oligosaccharide that was isolated by preparative HPLC. The FAB-mass spectrum of the new product confirmed it to be formed by the addition of a GlcNAc residue {$[M + Na^+] = 1334$ (33%)} to **5**. The ^{1}H NMR data for this product are presented in Table 1, along with those for **5**. The peak corresponding to unreacted pentasaccharide was also collected and confirmed through its NMR data to be **5**. The new doublet (J 9.0 Hz) at the unusually high field shift of δ 4.452 ppm is characteristic [7,11,18,19] of bisected structures and thus confirms the identity of hexasaccharide **6**.

3. Experimental

General methods.—Optical rotations were measured at 22 ± 2°C with a Perkin–Elmer 241 polarimeter. TLC was conducted on glass plates precoated with

250-μm layers of Silica Gel 60-F_{254} (Whatman); the compounds were detected by quenching of fluorescence and/or by charring with aq 5% H_2SO_4. Column chromatography was performed on Silica Gel 60 (E. Merck, 40–63 μm). Iatrobeads refers to a bead silica gel (product no. 6RS-8060) from Iatron Laboratories (Tokyo). The following solvent systems (v/v) were used for chromatography: *A*, 4:1 hexane–EtOAc; *B*, 3:1 hexane–EtOAc; *C*, 5:1 CH_2Cl_2–acetone; *D*, 4:1 CH_2Cl_2–acetone; *E*, 10:4:1 toluene–EtOAc – MeOH; *F*, (2:1) EtOAc–hexane; *G*, 20:4:1 toluene–EtOAc–MeOH; *H*, 2:1 hexane–EtOAc; *I*, 19:1 CH_2Cl_2–MeOH; *J*, 5:4:1 toluene–EtOAc–MeOH; *K*, 13:6:1 $CHCl_3$–MeOH–H_2O. ^1H NMR spectra were recorded at 300 MHz (Bruker WH-300) or 500 MHz (Bruker AM-500) on solutions in $CDCl_3$ (internal Me_4Si, δ 0) or D_2O (external Me_4Si, δ 0) at ambient temperature. ^{13}C NMR spectra were recorded at 75.5 MHz (Bruker AM 300) on solutions in $CDCl_3$ (internal Me_4Si, δ 0) or D_2O (external 1% 1,4-dioxane in D_2O, δ 67.4). Only partial NMR data are reported, as the remaining 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 chemical shifts are tentative. Fast-atom bombardment mass spectra (FABMS) were obtained using an AEI MS-9 instrument with Xe as the bombarding gas with 5:1 1,4-dithiothreitol–1,4-dithioerythritol as matrix. Unless otherwise indicated, all reactions were carried out at ambient temperature, and in the workup, solutions in organic solvents were washed with equal volumes of aqueous solutions. Organic solutions were generally dried (anhyd Na_2SO_4) prior to concentration on a rotary evaporator under the vacuum of a water aspirator with a bath temperature of 40–50°C. Elemental analyses were performed on a Carlo Erba EA1108 analyzer.

Materials.—Millex-GV (0.22 μm) filter units were from Millipore (Mississauga, ON). EDTA, Triton X-100, and UDP-GlcNAc were obtained from Sigma Chemical Co. (St. Louis, MO). Liquid scintillation cocktail was either Ecolite(+) (ICN Biomedicals) or Scintiverse E (Fisher Scientific). Reversed-phase C_{18} Sep-Pak cartridges from Waters Associates (Mississauga, ON) were pre-equilibrated with MeOH (20 mL) and water (30 mL) before use. UDP-[6-^{3}H(N)]GlcNAc was obtained from American Radiolabeled Chemicals (St. Louis, MO). In order to reduce background values obtained in the radioassays, this material was lyophilized, passed through a C_{18} Sep-Pak cartridge preequilibrated with water, and then re-lyophilized and dissolved in 7:3 EtOH–H_2O for later use. Frozen rat kidneys (Pel Freez Biologicals, Rogres, AR) were used as the enzyme source.

8-(Methoxycarbonyl)octyl 2,4-di-O-benzyl-3,6-di-O-chloroacetyl-α-D-mannopyranoside (**8**), *8-(methoxycarbonyl)octyl 2,4-di-O-benzyl-6-O-chloroacetyl-α-D-* (**9**), *and -β-D-mannopyranoside* (**10**).—A solution of 8-(methoxycarbonyl)octyl 2,4-di-*O*-benzyl-α,β-D-mannopyranoside (**7**, 510 mg, 0.96 mmol) in *N,N*-dimethylformamide was cooled to −60°C, then chloroacetylchloride (76 μL, 0.96 mmol) was added dropwise. Stirring was continued for 1 h at −60°C, and the mixture was allowed to warm to room temperature and kept thereat for ~1.5 h. The mixture was diluted with MeOH (15 mL) and then concentrated to a syrup which was

purified by chromatography on Iatrobeads (solvent *A*) to give the following in order of elution.

Compound **8** (60 mg, 9%) as a syrup: $[\alpha]_D$ −28.6° (*c* 1.7, $CHCl_3$), R_f 0.43 (solvent *B*). ^1H NMR ($CDCl_3$): δ 7.40–7.24 (m, 10 H, Ar), 5.27 (dd, 1 H, $J_{2,3}$ 3.5, $J_{3,4}$ 9.5 Hz, H-3), 4.81 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 4.04, 3.79 (2 H, $COCH_2Cl$), 3.65 (CO_2CH_3), 2.30 (t, 2 H, *J* 7.5 Hz, CH_2CO_2); ^{13}C NMR ($CDCl_3$): δ 174.27 (CO_2CH_3), 167.07, 166.44 ($COCH_2Cl$), 137.76, 137.68 (quat. Ar), 97.61 (C-1), 74.95, 73.06 ($PhCH_2$),68.16 (OCH_2CH_2), 64.76 (C-6), 51.44 (CO_2CH_3), 40.73, 40.60 ($COCH_2Cl$), 34.09 (CH_2COCH_3), 29.32, 29.19, 29.15, 29.09, 26.08, 24.93 (octyl methylenes). Anal. Calcd for $C_{34}H_{44}Cl_2O_{10}$ (683.63): C, 59.74; H, 6.49. Found: C, 59.58; H, 6.02.

Compound **9** (220 mg, 37.7%) as a syrup: $[\alpha]_D$ +30.3° (*c* 1, $CHCl_3$), R_f 0.36 (solvent *B*). ^1H NMR ($CDCl_3$): δ 7.40–7.24 (m, 10 H, Ar), 4.86 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 4.01 (2 H, $COCH_2Cl$), 3.65 (CO_2CH_3), 2.51 (d, 1 H, $J_{3,OH}$ 10 Hz, OH), 2.29 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), ^{13}C NMR ($CDCl_3$): δ 174.14 (CO_2CH_3), 166.99 ($COCH_2Cl$), 138.02, 137.59,(quat. Ar), 96.62 (C-1), 74.73, 72.76 ($PhCH_2$), 67.77 (OCH_2CH_2), 65.03 (C-6), 51.33 (CO_2CH_3), 40.65 ($COCH_2Cl$). 33.94 (CH_2COCH_3), 29.23, 29.05, 29.02, 28.94, 25.98, 24.78 (octyl methylenes). Anal. Calcd for $C_{32}H_{43}ClO_9$ (607.15): C, 63.31; H, 7.14; Cl, 5.84. Found: C, 63.27; H, 7.20; Cl, 5.92.

Compound **10** (110 mg, 18.9%) as a syrup, $[\alpha]_D$ −30.5° (*c* 1.1, $CHCl_3$), R_f 0.26 (solvent *B*). ^1H NMR ($CDCl_3$): δ 7.41–7.27 (m, 10 H, Ar), 4.49 (d, 1 H, $J_{1,2} < 1$ Hz, H-1) 4.05 and 3.99 (each d, J_{gem} 15 Hz, $COCH_2Cl$), 3.66 (CO_2CH_3), 2.49 (d, 1 H, $J_{3,OH}$ 10 Hz, OH), 2.29 (t, 2 H, *J* 7.5 Hz, CH_2CO_2); ^{13}C NMR ($CDCl_3$): δ 174.26, (CO_2CH_3), 167.17 ($COCHD_2Cl$), 138.30, 138.04 (quat. Ar), 101.80 (C-1), 74.83, 73.65 ($PhCH_2$), 70.27 (OCH_2CH_2), 65.30 (C-6), 51.48 (CO_2CH_3), 40.83 ($COCH_2Cl$), 34.12 (CH_2COCH_3), 29.64, 29.22 (2 C), 29.11, 26.06, 24.96, (octyl methylenes). Anal. Calcd for $C_{32}H_{43}ClO_9$ (607.15): C, 63.31; H, 7.14, Cl, 5.84. Found: C, 62.92; H, 7.29; Cl, 5.91.

The last fraction that emerged from the column was unchanged **7** (100 mg).

*2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl-(1 → 2)-3,6-di-*O*-acetyl-4-*O*-methyl-α-*D*-mannopyranosyl chloride* (**12**).—Hydrogen chloride was passed for 15 min through a tube of Drierite into a solution of **11** (200 mg, 0.31 mmol) in CH_2Cl_2 (5 mL) at O°C. After 0.5 h, the solution was warmed to room temperature where it was kept for 18 h. The solution was then evaporated to dryness and the acetic acid byproduct was removed by coevaporation with toluene to give a residue which was chromatographed (solvent *C*) to give **12** (140 g, 73%) as a white solid: $[\alpha]_D$ +21° (*c* 0.7, $CHCl_3$), R_f 0.53 (solvent *D*); ^1H NMR ($CDCl_3$): δ 5.87 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 5.68 (d, 1 H, $J_{NH,2'}$ 8.5 Hz, NH), 5.26 (dd, 1 H, $J_{2',3'}$ 10.5, $J_{3',4'}$ 10 Hz, H-3′), 5.21 (dd, 1 H, $J_{2,3}$ 3.5, $J_{3,4}$ 9.5 Hz, H-3), 5.04 (t, 1 H, $J_{3',4'}$ $J_{4',5'}$ 10 Hz, H-4′), 4.68 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1′), 3.50 (s, 3 H, OCH_3), 2.16, 2.10, 2.09, 2.04, 2.02 (s, 3 H each, 5 OAc), 1.94 (s, 3 H, NAc); ^{13}C NMR ($CDCl_3$): δ 170.99, 170.85, 170.66, 170.46, 170.19, 160.40 ($COCH_3$),100.19 (C-1), 89.72 (C-1), 77.31 (C-2), 62.42 (C-6), 62.08 (C-6′), 60.62 (OCH_3), 54.49 (C-2′), 23.17, 21.93, 20.82, 20.67 (2

C), and 20.61 ($COCH_3$). Anal. Calcd for $C_{25}H_{36}ClNO_{15}$ (626.00): C, 47.96; H, 5.80; N, 2.24. Found: C, 47.67; H, 5.70; N, 2.21.

*2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl-(1 → 2)-3,4-di-*O*-acetyl-6-*O*-methyl-α-*D*-mannopyranosyl chloride* (**14**).—Hydrogen chloride was passed for 10 min through a tube of Drierite into a solution of **13** (100 mg, 0.15 mmol) in CH_2Cl_2 (4 mL) at O°C. After 0.5 h, the solution was warmed to room temperature where it was kept for 20 h. The solution was then evaporated to dryness and the acetic acid byproduct was removed by coevaporation with toluene to give a residue which was dissolved in a small amount of CH_2Cl_2. Addition of hexane caused the precipitation of **14** (90 mg, 93%) as an amorphous solid: $[\alpha]_D$ +21.8° (*c* 1.4, $CHCl_3$), R_f 0.3 (solvent *E*); ^{1}H NMR ($CDCl_3$): δ 5.98 (d, 1 H, $J_{1,2}$ 1.5 Hz, H-1), 5.57 (dd, 1 H, $J_{2',3'}$, $J_{3',4'}$ 9.5 Hz, H-3'), 5.12 (d, 1 H, $J_{1',2'}$ 8.5 Hz, H-1'), 5.04 (t, 1 H, $J_{3',4'}$ $J_{4',5'}$ 10 Hz, H-4'), 4.39 (dd, 1 H, $J_{2',3'}$ 3.5 Hz, H-2'), 3.34 (s, 3 H, OCH_3), 2.08, 2.07, 2.06, 2.02, 2.0 (s, 3 H each, 5 OAc), 1.94 (s, 3 H, NAc); ^{13}C NMR ($CDCl_3$): δ 171.10, 170.64, 170.51, 170.34, 169.55, 169.51 ($COCH_3$), 98.30 (C-1), 89.51 (C-1), 76.38 (C-2), 71.23 (C-6), 62.04 (C-6'), 59.36 (OCH_3), 55.81 (C-2'), 23.25, 20.78, 20.71 (3C), and 20.67 ($COCH_3$). Anal. Calcd for $C_{25}H_{36}ClNO_{15}$ (626.00): C, 47.96; H, 5.80; Cl, 5.66; N, 2.24. Found: C, 47.95; H, 5.80; Cl, 5.60; N, 2.21.

*8-(Methoxycarbonyl)octyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-gluco-pyranosyl-(1 → 2)-3,6-di-*O*-acetyl-4-O-methyl-α-*D*-mannopyranosyl-(1 → 3)-2,4-di-*O*-benzyl-6-*O*-chloroacetyl-β-*D*-mannopyranoside* (**15**).—To a stirred solution of 8-(methoxycarbonyl)octyl 2,4-di-*O*-benzyl-6-*O*-chloroacetyl-β-D-mannopyranoside **10** (29.3 mg, 0.048 mmol) in dry nitromethane (1 mL) containing powdered 4A molecular sieves (65 mg), mercuric cyanide (36.5 mg, 0.144 mmol) and mercuric bromide (51.9 mg, 0.144 mmol) was added dropwise a solution of glycosyl chloride 12 (90 mg, 0.144 mmol) in dry nitromethane (2 mL) at O°C under dry Ar. After stirring at O°C for 1 h, the reaction was slowly allowed to warm to room temperature and was stirred for 15 h. The mixture was then diluted with CH_2Cl_2 (50 mL) and filtered (Celite), the solids were thoroughly washed with CH_2Cl_2, and the filtrate and washings were combined. The solution was successively washed with water, M KI solution and water, dried, and concentrated. The resulting syrup was chromatographed (solvent *F*) to give first unchanged **10** (7.7 mg), followed by **15** (23.6 mg, 55% based on reacted **10**) isolated as a white solid: $[\alpha]_D$ +24.8° (*c* 1, $CHCl_3$), R_f 0.28 (solvent *G*). ^{1}H NMR ($CDCl_3$): δ 7.50–7.23 (m, 10 H, Ar), 4.93 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.91 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.48.(s, 1 H, H-1), 3.67 (s, 3 H, CO_2CH_3), 3.44 (s, 3 H, OCH_3), 2.30 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), 2.09, 2.08, 2.05, 2.02, 2.0 (s, 3 H each, 5 OAc), 1.86 (s, 3 H, NAc); ^{13}C NMR ($CDCl_3$): δ 174.30 (CO_2CH_3), 170.91, 170.84, 170.67, 170.31, 169.74, 169.32 ($COCH_3$), 167.24 ($COCH_2Cl$), 138.70, 138.14 (quat. Ar), 101.84 (C-1), 100.95 (C-1''), 99.77 (C-1'), 74.13, 73.52 ($PhCH_2$), 70.36 (OCH_2CH_2), 64.89 (C-6), 62.89 (C-6'), 61.96 (C-6''), 60.68 (OCH_3), 53.26 (C-2''), 51.49 (CO_2CH_3), 40.80 ($COCH_2Cl$), 34.12 (CH_2COCH_3), 29.67, 29.25 (2 C), 29.14, 26.11, 24.96 (octyl methylenes), 23.22, 21.05, 20.79, and 20.68 (2 C) ($COCH_3$). Anal. Calcd for $C_{57}H_{78}ClNO_{24}$ (1196.65): C, 57.21; H, 6.57; N, 1.17. Found: C, 57.57; H, 6.58; N, 1.27.

*8-(Methoxycarbonyl)octyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-gluco-pyranosyl-(1 → 2)-3,6-di-*O*-acetyl-4-*O*-methyl-α-*D*-mannopyranosyl-(1 → 3)-2,4-di-*O*-benzyl-β-*D*-mannopyranoside* (**16**).—A solution of **15** (20.9 mg, 0.017 mmol) and thiourea (21.7 mg) in 6:1 pyridine–EtOH (1 mL) was stirred at 80°C for 1.5 h. The mixture was diluted with water (30 mL) and extracted with CH_2Cl_2 (3 × 30 mL), and the combined organic layer was washed with satd aq $NaHCO_3$ and water, and concentrated. The resulting syrup was then chromatographed (solvent *F*) to furnish **16** (15 mg, 77%) as a white solid: $[\alpha]_D$ −36.5° (*c* 0.9, $CHCl_3$), R_f 0.31 (solvent *F*). ^{1}H NMR ($CDCl_3$): δ 7.50–7.21 (m, 10 H, Ar), 4.84 (d, 1 H, $J_{1',2'}$ 2.0 Hz, H-1'), 4.51 (s, 1 H, H-1), 3.66 (s, 3 H, CO_2CH_3), 3.44 (s, 3 H, OCH_3), 2.31 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), 2.09 (s, 6 H, 2 OAc), 2.05, 2.04, 1.99 (s, 3 H each, 3 OAc), 1.85 (s, 3 H, NAc); ^{13}C NMR ($CDCl_3$): δ 174.30 (CO_2CH_3), 170.89(2C), 170.69, 170.26, 169.69, 169.32 ($COCH_3$), 139.14, 138.09 (quat. Ar), 101.83 (C-1), 101.03 (C-1''), 99.60 (C-1'), 74.36, 73.42 (PhCH_2), 70.40 (OCH_2CH_2), 62.81 (C-6), 62.03, 61.97 (C-6', C-6''), 60.68 (OCH_3), 53.10 (C-2''), 51.49 (CO_2CH_3), 34.12 (CH_2COCH_3), 29.72, 29.28, 29.23, 29.13, 26.11, 24.96 (octyl methylenes), 23.20, 21.04, 20.79, and 20.68 (3 C) ($COCH_3$). Anal. Calcd for $C_{55}H_{77}NO_{23}$ (1120.18): C, 58.97; H, 6.93; N, 1.25. Found: C, 58.64; H, 6.91; N, 1.25.

*8-(Methoxycarbonyl)octyl 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-gluco-pyranosyl-(1 → 2)-3,6-di-*O*-acetyl-4-*O*-methyl-α-*D*-mannopyranosyl-(1 → 3)-*O*-[2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-*D*-glucopyranosyl]-(1 → 2)-3,4-di-*O*-acetyl-6-*O*-methyl-α-*D*-mannopyranosyl-(1 → 6)]-2,4-di-*O*-benzyl-β-*D*-mannopyranoside* (**17**).—To a stirred solution of **16** (23.6 mg, 0.021 mmol) in dry nitromethane (1 mL) containing powdered 4A molecular sieves (160 mg), mercuric cyanide (21.2 mg, 0.084 mmol), and mercuric bromide (30.3 mg, 0.084 mmol) were added dropwise a solution of glycosyl chloride **14** (52.6 mg, 0.0.84 mmol) in dry nitromethane (2 mL) at O°C under dry Ar. After stirring at O°C for 30 min, the reaction was slowly allowed to warm to room temperature and was stirred for 20 h. The mixture was then diluted with CH_2Cl_2 (50 mL) and filtered (Celite), the solids were thoroughly washed with CH_2Cl_2, and the filtrate and washings were combined and processed as described for **15** to give a residue which was chromatographed (solvent *H* → *I*) to give **17** (27.8 mg, 80%) as a white solid: $[\alpha]_D$ −6.5° (*c* 0.3, $CHCl_3$), R_f 0.23 (solvent *E*). ^{1}H NMR ($CDCl_3$): δ 7.51–7.20 (m, 10 H, Ar), 5.69 (d, 1 H, $J_{NH,2}$ 8.0 Hz, NH), 5.74 (dd, 1 H, $J_{2e,3e}$ 11, $J_{3e,4e}$ 9.0 Hz, H-3e), 4.86 (d, 1 H, $J_{1b,2b}$ 2.0 Hz, H-1b), 4.47 (s, 1 H, H-1a), 3.66 (s, 3 H, CO_2CH_3), 3.43 (s, 3 H, OCH_3-b), 3.26 (s, 3 H, OCH_3-c), 2.31 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), 2.09, 2.08, 2.03, 2.02, 2.01, (s, 3 H each, 5 OAc), 1.99 (s, 9 H, 3 OAc), 1.98 (s, 6 H each, 2 OAc), 1.80 and 1.75 (s, 3 H each, 2 NAc); ^{13}C NMR ($CDCl_3$): δ 174.26 (CO_2CH_3), 170.87 (2C), 170.74, 170.70 (2C), 170.38, 170.34, 170.26, 169.71, 169.53 (2 C), 169.30 ($COCH_3$), 139.04, 138.18 (quat. Ar), 101.81 (C-1a), 101.08 (C-1d), 99.65 (C-1e), 98.85 (C-1b), 97.66 (C-1c), 74.20, 73.35 (PhCH_2), 71.89 (C-6c), 70.35 (OCH_2CH_2), 66.56 (C-6a), 62.00, 61.95 (C-6d, C-6e), 60.64 (OCH_3-b), 59.21 (OCH_3-c) 55.66 (C-2e), 53.08 (C-2d), 51.49 (CO_2CH_3), 34.11 (CH_2COCH_3), 29.77, 29.37, 29.31, 29.17, 26.21, 24.98 (octyl methylenes), 23.38, 23.22, 21.04, 20.85, 20.82, 20.79, 20.76, 20.73 (2 C), 20.68(3C)

($COCH_3$). Anal. Calcd for $C_{80}H_{112}N_2O_{38}$ (1709.72): C, 56.20; H, 6.60; N, 1.64. Found: C, 56.22; H, 6.60; N, 1.69.

8-(Methoxycarbonyl)octyl 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1 → 2)-4-O-methyl-α-D-mannopyranosyl-(1 → 3)-[2-acetamido-2-deoxy-β-D-glucopyranosyl]-(1 → 2)-6-O-methyl-α-D-mannopyranosyl)-(1 → 6)]-β-D-mannopyranoside (**5**).—Compound **17** (12 mg, 0.007 mmol) was dissolved in 95% EtOH (2 mL) and glacial acetic acid (0.1 mL) containing 10% palladium-on-carbon (15 mg). The mixture was stirred under 1 atm of H_2 for 24 h, by which time TLC showed the complete disappearance of **17** to give one slower migrating spot, R_f 0.47 (solvent *J*), which was devoid of UV absorption in the TLC. The catalyst was removed by filtration (Celite) and washed with MeOH (Caution: fire hazard!), and the filtrate and washings were combined and concentrated to give a solid residue (containing **18**) which was dissolved in MeOH (2 mL) containing M NaOMe in MeOH (0.12 mL). After stirring overnight at room temperature, the base was neutralized with Amberlite IR-120 (H^+) cation-exchange resin, and removal of the resin and evaporation of the solvent gave a solid residue. A solution of the residue in water (15 mL) was passed through a Sep-Pak C_{18} cartridge which had been prewashed with 30 mL each of MeOH and water. The cartridge was then washed with water (30 mL), and the product was eluted with MeOH (30 mL). The MeOH was evaporated, and a solution of the residue was dissolved in water (5 mL) and filtered through a 0.22-μm Millex filter and then lyophilized to give **5** (5 mg, 64%) as a white powder: $[\alpha]_D$ −4.2° (*c* 0.3, H_2O), R_f 0.30 (solvent *K*). FABMS: *m/z* 1109 $[M + 1^+]$ and 1131 $[M + Na^+]$. ^1H NMR parameters are reported in Table 1; ^{13}C NMR (D_2O): δ 178.78 (CO_2CH_3), 175.65, 175.49 ($COCH_3$), 100.63 (C-1a), 100.47(C-1d), 100.20 (C-1e), 100.06 (C-1b), 97.57 (C-1c), 73.05(C-6c), 70.96 (OCH_2CH_2), 66.76 (C-6a), 63.38 (C-6b), 61.47 (2 C), (C-6d, C-6e), 61.16 (OCH_3-b), 59.21 (OCH_3-c) 56.22 (2 C), (C-2e, CO_2CH_3), 52.91 (C-2d), 34.55 (CH_2COCH_3), 29.48, 29.07, 28.94, 25.85, 25.11 (2 C) (octyl methylenes), and 23.19 (2 C), ($COCH_3$).

Evaluation of pentasaccharide **5** *as an acceptor for GlcNAc T-III.*—All isolation steps were carried out at 4°C. A crude extract of GlcNAc T-III was prepared as previously described [9,11]. Briefly, thawed rat kidneys (35 g) were homogenized in a Waring blender with 120 mL of 10 mM Tris-HCl buffer, pH 7.4, containing 150 mM NaCl. The homogenate was centrifuged at 900*g* for 15 min, and the supernatant was used for kinetic evaluations of pentasaccharide **5** as an acceptor substrate.

The GlcNAcT-III preparation (20 μL) and 120 mM MES buffer (40 μL), pH 6.3, containing 120 mM GlcNAc, 1% Triton X-100, 5 mM AMP, and 15 mM $MnCl_2$ were added to 0.5-mL microcentrifuge tubes that contained lyophilized acceptor, 0.7 μCi of UDP-[6-^{3}H]GlcNAc and unlabeled donor. The final donor concentration was 5.7 mM, and the acceptor concentrations ranged from 0.05 mM, to 3 mM. After incubating the tubes at 37°C for 3 h, the mixtures were diluted with 1 mL of 32 mM EDTA, and loaded onto Sep-Pak C_{18} cartridges. The cartridges were washed with water (ca. 60–100 mL) to remove unreacted donor and buffer components. After background counts were obtained in the aq washes, radiolabeled product was eluted with 2 × 3 mL of MeOH and quantitated by liquid

scintillation counting in a Beckman LS1801 instrument after the addition of 10 mL of scintillation cocktail. The kinetic data was analyzed by the Wilkinson method [20]. A milliunit of enzyme is defined as the amount of enzyme that catalyzes the formation of 1 nmol/min of product under the assay conditions above, using 5.7 mM donor and 0.3 mM acceptor **5**.

Preparative enzymatic glycosylation: conversion of compound **5** → **6**.—The crude extract contained esterases that hydrolyzed the methyl ester of the acceptor on prolonged incubation. Therefore, for preparative glycosylation, the crude extract (135 mL) was further purified by centrifugation at 100 000*g* for 60 min. The supernatant from this high-speed spin was fractionated by the addition of 113 g/L solid $(NH_4)_2SO_4$ to 20% saturation. After stirring slowly for 1 h, the mixture was centrifuged at 3000*g* for 30 min, and the supernatant was brought to 40% saturation by the addition of 123 g/L solid $(NH_4)_2SO_4$. After 2 h of slow stirring, centrifugation was repeated as above. To the 40% supernatant 132 g/L of $(NH_4)_2SO_4$ was added to 60% saturation. Activity was generally found in the fraction precipitating at 40–60% saturation. This was dissolved in a minimal volume of 0.1 M Tris-HCl buffer, pH 8.0, then dialyzed against 2 × 1 L of this buffer. The dialyzed extract (20 mL) was loaded onto a 2.5 × 10 cm DEAE-Sepharose Fast-Flow column equilibrated with 0.1 M Tris-HCl, pH 8.0, buffer. The column was washed with this same buffer until no further protein was eluted, then GlcNAcT-III activity eluted with 60 mL of 0.1 M Tris-HCl, pH 8.0 containing 0.2 M NaCl. Eluted enzyme was dialyzed against 50 mM MES buffer, pH 6.3, containing 20% glycerol, 5 mM $MnCl_2$, and 0.01% sodium azide. Any precipitate that formed during dialysis was removed by centrifugation at 7700*g* for 20 min. The supernatant was concentrated to 4 mL by ultrafiltration (Amicon) using a Diaflow PM-30 membrane for use in preparative glycosylation. The enzyme activity was found to be 2 mU/mL in the final concentrated solution.

The incubation mixtures for preparative glycosylation contained 2.1 mg of compound **5**, 18 mg UDP-GlcNAc, 1.5 mU of GlcNAcT-III in 1.2 mL of 80 mM MES buffer, pH 6.3, containing 13 mM $MnCl_2$, 1% Triton X-100, and 60 mM *N*-acetylglucosamine. After reaction at ambient temperature for 48 h, an additional 0.6 mU of enzyme were added to the mixture, and the reaction was continued for 48 h. The reaction progress was monitored by TLC on silica gel plates using 13:6:1 CH_2Cl_2–MeOH–H_2O as the solvent system with detection by spraying and charring with 5% H_2SO_4. The R_f is 0.33 for the starting material and 0.14 for the product. The mixture was then applied to a C_{18} reversed-phase Sep-Pak cartridge, which was washed with 100 mL of water. The product was eluted with 10 mL of MeOH and the eluate concentrated to dryness under reduced pressure. The residue was dissolved in 5 mL water, passed through a Millex-GV filter, and lyophilized. The residue was analyzed by HPLC on a Partisil-5-PAC column (0.47 × 11 cm) using 4:1 MeCN–H_2O as solvent at a flow rate of 1 mL/min with monitoring by UV absorption at 202 nm as previously described [11]. The retention times for **5** and **6** were 10.2 and 15.0 min, respectively. Repeated injections of the sample and collection of the individual fractions,

yielded sufficient material for analysis by ^{1}H NMR spectroscopy at 500 MHz. For selected ^{1}H NMR data, see Table 1.

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