Carbohydrate Research, 173 (1988) 263–272
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE TRISACCHARIDE β-D-GlcpNAc-(1→2)-α-D-Manp-(1→6)-β-D-Manp, AS ITS 8-METHOXYCARBONYLOCTYL GLYCOSIDE, IS AN ACCEPTOR SELECTIVE FOR *N*-ACETYLGLUCOSAMINYLTRANSFERASE V

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(Received December 18th, 1986; accepted for publication, February 13th, 1987)

ABSTRACT

Incubation of the trisaccharide acceptor, β-D-GlcpNAc-(1→2)-α-D-Manp-(1→6)-β-D-Manp-$O(CH_2)_8CO_2Me$ with sonicates of Rous sarcoma-transformed baby-hamster kidney cells, which contain *N*-acetylglucosaminyltransferase V activity, resulted in the production of β-D-GlcpNAc-(1→2)-[β-D-GlcpNAc-(1→6)]-α-D-Manp-(1→6)-β-D-Manp-$O(CH_2)_8CO_2Me$ (**4**). The product of the enzymic reaction was identified by comparison of its ^{1}H-n.m.r. spectrum with that of authentic **4** whose chemical synthesis is also described.

INTRODUCTION

Both polyoma[1,2] (a DNA papovavirus) and Rous sarcoma[2] (an RNA retrovirus) transformation of baby-hamster kidney (BHK) cells induce similar structural changes in the asparagine-linked oligosaccharide chains of membrane glycoproteins. These changes are characterized by an increase in the branching at the 6-position of the α-(1→6)-linked "core" D-mannose residue, resulting in the production of a larger proportion of tri- and tetra-antennary glycopeptides containing the sequence[1,2], →)-β-D-GlcpNAc-(1→6)-α-D-Manp-(1→6)-β-D-Manp-(→. Yamashita *et al.*[3] have shown that the increase in branching on polyoma-transformation correlates with a two-fold increase in the activity of *N*-acetylglucosaminyltransferase V (GnT-V), the enzyme that initiates this α-D-(1→6) branch[4]. In the biosynthesis of the asparagine (Asn)-linked carbohydrates, GnT-V catalyzes the transfer of a 2-acetamido-2-deoxy-β-D-glucopyranosyl group from uridine 5′-diphospho-*N*-acetylglucosamine (UDP-GlcNAc) to glycopeptides of structure **1** to produce more highly branched structures of type **2**.

The difficult and lengthy assays[3,4] required for the quantitation of GnT-V activity in crude cell-extracts prompted us to develop a new and rapid assay for this enzyme based on the synthetic trisaccharide acceptor[5] **3**, which was predicted to be

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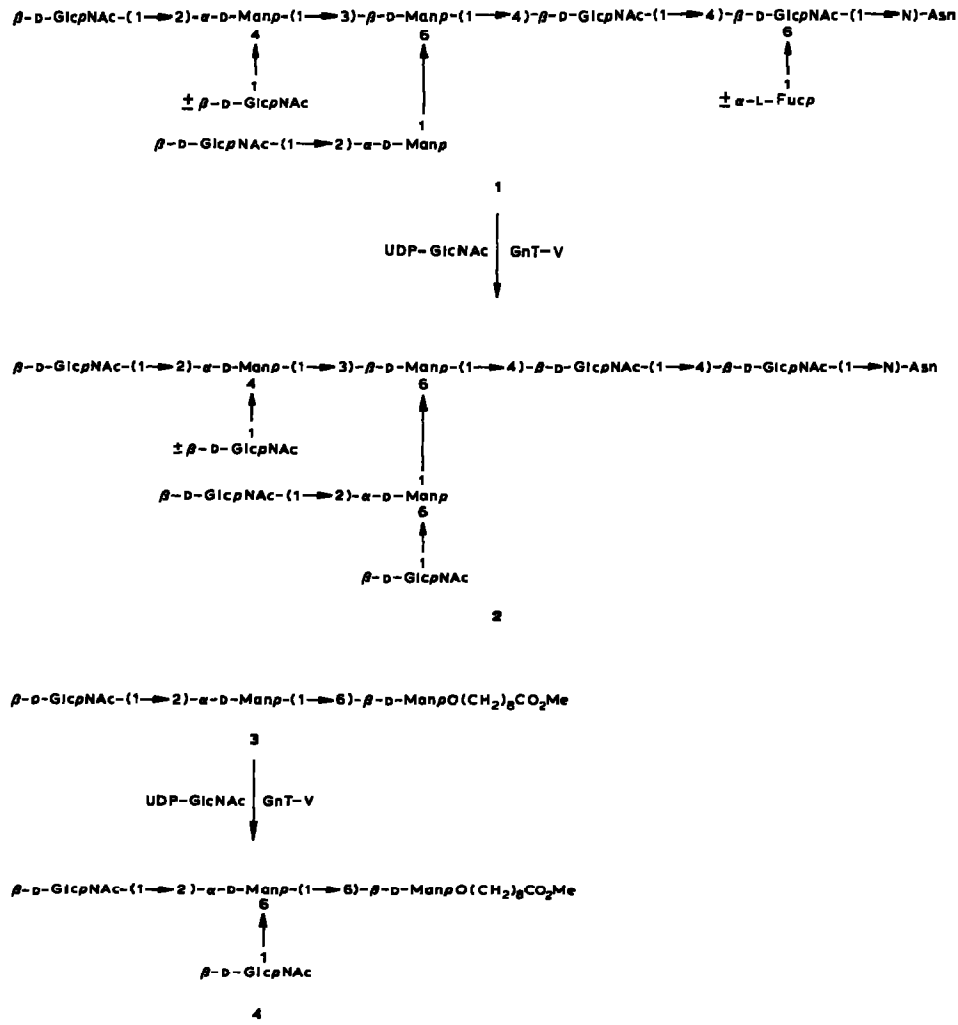

a selective substrate for GnT-V. This assay involves incubation of cell extracts with the synthetic acceptor **3** and radiolabelled UDP-GlcNAc, followed by isolation of the labelled product (presumably **4**), which is quantitatively adsorbed onto a reverse phase (C-18) cartridge by virtue of its hydrophobic linking-arm. Using this simple assay, we have recently shown[6] that GnT-V activity of Rous sarcoma-transformed BHK cells is also nearly two-fold elevated, when compared with the untransformed cells, and that GnT-V may therefore be a primary agent responsible for the observed changes in these Asn-linked oligosaccharide structures.

The aforementioned assay is based on the isolation and quantitation of labelled products having the chromatographic characteristics expected for **4**, but no definitive proof of the structure could be obtained. The possibility that trisaccharide **3** was either a substrate for a transferase other than GnT-V, for more than one

8 R = Ac
9 R = H
6 R = OAc
7 R = Br
5
10
NPhth = COC_6H_4CON
All = $CH_2—CH=CH_2$
11 R^1 = All, R^2 = Bn, R^3 = NPhth, R^4 = Ac
12 R^1 = H, R^2 = Bn, R^3 = NPhth, R^4 = Ac
13 R^1 = Ac, R^2 = Bn, R^3 = NHAc, R^4 = Ac
14 R^1 = H, R^2 = Bn, R^3 = NHAc, R^4 = H

GnT, or that the initially formed product **4** was a substrate for additional GnT's, could therefore not be rigorously excluded. In this report, we describe the chemical synthesis of the tetrasaccharide **4** and show that incubation of **3** with Rous sarcoma-transformed BHK cell sonicates, in the presence of UDP-GlcNAc, results in the production of **4** as the only detectable product.

RESULTS AND DISCUSSION

Compound **4** was prepared in seven steps (8% overall yield) from the β-D-mannoside **5** which was available from our earlier[5] preparation of **3**. The synthetic route was straightforward and is essentially identical to that used by Shah *et al.*[7] who have prepared the same tetrasaccharide sequence, but as the ($^2H_3$)methyl glycoside.

Condensation of **5** with the glycosyl bromide[8] **7**, freshly prepared from its acetate precursor[9] **6**, in the presence of silver trifluoromethanesulfonate and *N,N,N',N'*-tetramethylurea[10] furnished the expected α-D-linked disaccharide **8** (66%). The structure of **8** was evident from its ^{13}C-n.m.r. spectrum which showed two signals for anomeric carbon atoms at δ 101.71 ($^1J_{C,H}$ 153 Hz, C-1) and 97.76 ($^1J_{C,H}$ 172 Hz, C-1'). One-bond couplings of this magnitude are characteristic of β- and α-D-glycosidic linkages, respectively[11]. No evidence was obtained for the formation of any β-D-linked disaccharide which complicated the similar glycosylation reaction in the work of Shah *et al.*[7]. *O*-Deacetylation of **8** produced the diol **9**.

Reaction of diol **9** with excess 3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl bromide[12] (**10**), in the presence of silver trifluoromethanesulfonate and 2,4,6-trimethylpyridine in dichloromethane, gave the protected tetrasaccharide **11** (53%). Deprotection of **11** involved isomerization of the 3-allyl ether by tris(triphenylphosphine)rhodium(I) chloride[13], followed by hydrolysis of the vinyl ethers to give the alcohol **12** (85%) from which the phthalimido groups were removed[14] with hydrazine hydrate in refluxing methanol. Acetylation of the crude product and its purification by chromatography gave the di-*N*-acetyl derivative **13** in 40% yield. *O*-Deacetylation of **13**, followed by hydrogenation over 5% palladium-on-carbon, then furnished the target tetrasaccharide **4**.

Enzymic conversion of **3** *to* **4**. — Compound **3** (750 μg) was incubated with the supernatant from sonicates of 2×10^8 Rous sarcoma-transformed BHK cells, in the presence of UDP-[^{3}H]GlcNAc and 2-acetamido-2-deoxy-β-D-glucopyranosylamine[15] (to prevent product degradation by hexosaminidases[6,16]) for 6 h at 37°. The radioactive-labelled product of the reaction was isolated after sequential deionization, reverse-phase l.c., and preparative silica gel t.l.c., where it had the same chromatographic mobility as **4**. The product was then re-isolated on a reverse-phase cartridge, from which it was eluted with methanol. Approximately 0.3% of **3** had reacted, based on the radioactivity of the isolated product, which corresponds to the production of 2.7 μg of a labelled tetrasaccharide.

The partial, 360-MHz ^{1}H-n.m.r. spectrum of the aforementioned product is

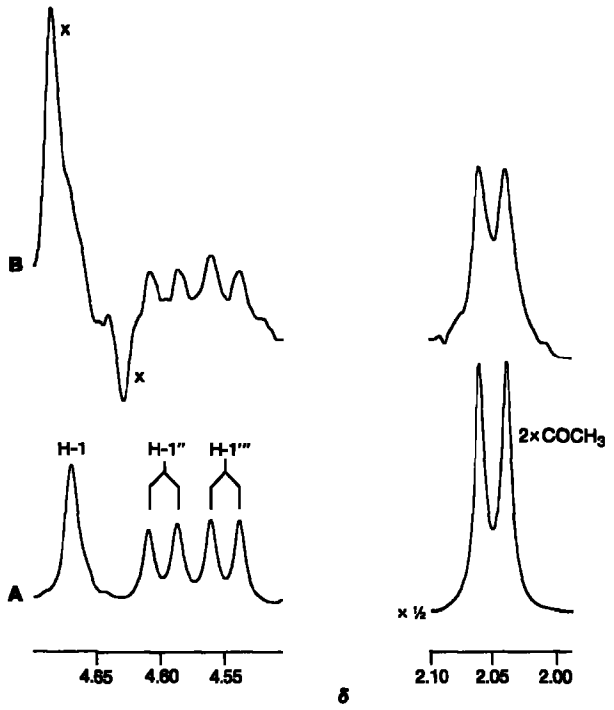

Fig. 1. Comparison of the partial 360-MHz ^{1}H-n.m.r. spectra of synthetic **4** (A) and the product isolated after incubation of **3** with BHK cell sonicates and UDP-GlcNAc (B). The spectra were recorded on solutions in D_2O at 296 K with acetone (δ 2.225) as internal standard. The sample in (B) contained ~2.7 μg of a tetrasaccharide and required an accumulation time of 24 h (40 000 scans). The residual DOH peak in B was suppressed by pre-irradiation, giving rise to the spectral artifacts labelled ×. A digital line-broadening of 2.0 Hz was applied to both spectra to improve the signal-to-noise ratio.

reproduced in Fig. 1, where it is compared with the spectrum of authentic **4** whose synthesis is described herein. The ^{1}H-n.m.r. spectrum revealed the sample to be substantially contaminated with lipid-related material, but the *N*-acetylmethyl (δ ~2.0) and β-anomeric (δ ~4.6) regions were fortunately not obscured. Fig. 1 clearly shows that only two acetamido groups are present, in a 1:1 ratio, with chemical shifts corresponding exactly to those for **4**. In addition, two anomeric signals for a β-linked 2-acetamido-2-deoxy-D-glucopyranosyl group are also evident, and are superimposable on those of authentic **4**. The signals for H-1 (δ 4.670) and H-1′ (δ 4.882) were obscured by spectral artifacts resulting from suppression of the residual HOD signal.

The superimpossibility of the partial ^{1}H-n.m.r. spectra shown in Fig. 1 is taken as conclusive evidence that **3** is an effective acceptor for only one BHK *N*-acetylglucosaminyltransferase which adds a 2-acetamido-2-deoxy-β-D-glucopyranosyl group to OH-6′ to produce **4**. This is the branch-specificity which characterizes GnT-V and, therefore, validates our use of **3** as a selective acceptor for

assaying this enzyme in the presence of other BHK *N*-acetylglucosaminyltransferases.

EXPERIMENTAL

General methods. — Optical rotations were measured with a Perkin–Elmer 241 polarimeter at ambient temperatures (22 ±2°). Solutions in organic solvents were dried over Na_2SO_4 before solvent removal on a rotary-evaporator at <40°. Unless otherwise noted, t.l.c. was performed on precoated plates of Silica Gel 60-F_{254} (E. Merck, Darmstadt) with detection by fluorescence or by charring after spraying with 5% H_2SO_4 in ethanol. Column chromatography was performed on Silica Gel Merck 60 (40–63 μm) with a loading of between 50:1 and 100:1 (w/w) silica gel/sample. ^{1}H-N.m.r. spectra were recorded at 300 MHz or at 360 MHz on Bruker instruments with either tetramethylsilane (Me_4Si, δ 0 in $CDCl_3$) or acetone (δ 2.225) as internal standards at ambient temperatures. ^{13}C-N.m.r. spectra were recorded at 75 MHz with either internal Me_4Si (δ 0) in $CDCl_3$ or external 1% 1,4-dioxane (δ 67.4) as reference standards. High-performance liquid chromatography (l.c.) used an LDC liquid chromatography pump and aliquots of the eluates were processed for liquid-scintillation counting with an LKB minivial Beta-Counter. The microanalyses were carried out by the Analytical Services Laboratory of this department.

Materials. — ADP, UDP-GlcNAc, 2-(*N*-morpholino)ethanesulfonic acid (MES), Triton X-100, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO). Dowex 1-X8 was from Bio-Rad (Richmond, CA) and UDP-[^{3}H]GlcNAc from American Radiolabelled Chemicals (St. Louis, MO). The ODS column and pellicular C18 mini-columns were from Phenomenex (Palos Verdes, CA). T.l.c. Silica Gel G plates were from Baker (Morristown, NJ).

8-Methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-6-O-(2,6-di-O-acetyl-3,4-di-O-benzyl-α-D-mannopyranosyl)-β-D-mannopyranoside (**8**). — Glycosyl bromide **7** (1.0 mmol, freshly prepared[7,8] from **6**) in dichloromethane (4 mL) was added to a mixture of **5** (482 mg, 0.845 mmol), silver trifluoromethanesulfonate (652 mg, 2.54 mmol), and *N,N,N′,N′*-tetramethylurea (392 mg, 3.38 mmol) stirred in dichloromethane (10 mL) at room temperature. After 30 min, additional **7** (1.0 mmol) in dichloromethane (4.0 mL) was added and stirring was continued for an additional 2 h. The mixture was then filtered on Celite, which was washed with additional dichloromethane, and the filtrate was washed successively with water, cold M HCl, and saturated $NaHCO_3$. Removal of the solvent left an oil which was purified by chromatography using a linear gradient of diethyl ether–hexane from 1:3 to 1:1 (1 L total) to provide **8** (558 mg, 66%), syrup, $[\alpha]_D^{22}$ −13° (*c* 1.25, chloroform), R_F (1:3 acetone–hexane) 0.37; ^{1}H-n.m.r. ($CDCl_3$): δ 5.865 (m, 1 H, H_2C=C*H*–CH_2), 5.468 (dd, 1 H, $J_{1',2'}$ 1.8, $J_{2',3'}$ 3.2 Hz, H-2′), 4.343 (d, H-1′), 4.875 (s, 1 H, H-1), 3.656 (s, OCH_3), 2.282 (t, 2 H, CH_2CO_2), 2.142 and 2.010 (each s, 3 H, $COCH_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 174.29 (CO_2CH_3), 170.77 and 170.11

($COCH_3$), 116.92 (CH_2=$CHCH_2$), 101.71 ($^1J_{C,H}$ 153 Hz, C-1), 97.76 ($^1J_{C,H}$ 172 Hz, C-1'), 67.12 (C-6), 63.32 (C-6'), 51.45 (OCH_3), 34.11 (CH_2CO_2), 21.08, and 20.84 ($COCH_3$).

Anal. Calc. for $C_{57}H_{72}O_{15}$: C, 68.66; H, 7.28. Found: C, 68.62; H, 7.31.

8-Methoxycarbonyloctyl 3-O-allyl-2,4-di-O-benzyl-6-O-(3,4-di-O-benzyl-α-D-mannopyranosyl)-β-D-mannopyranoside (**9**). — Compound **8** (507 mg, 0.51 mmol) was dissolved in dry methanol (20 mL) containing a trace of sodium methoxide and kept for 2 h at room temperature. Neutralization with Amberlite IR-120 (H^+) cation-exchange resin, resin removal, and solvent evaporation left a white foam (462 mg, 99%), $[\alpha]_D^{22}$ −6.2° (*c* 0.87, chloroform), R_F (1:2 acetone–toluene) 0.26; ^{1}H-n.m.r. ($CDCl_3$): δ 5.888 (m, 1 H, CH_2=C*H*–CH_2), 5.001 (d, 1 H, $J_{1',2'}$ 1.8 Hz, H-1'), 4.335 (s, 1 H, H-1), 4.122 (br., 1 H, H-2'), 3.652 (s, OCH_3), 2.68 and 2.30 (each br., D_2O-exchangeable, OH), and 2.286 (t, CH_2CO_2); ^{13}C-n.m.r. ($CDCl_3$): δ 174.30 (CO_2CH_3), 116.87 (CH_2=$CHCH_2$), 101.69 (C-1), 99.59 (C-1'), 66.53 (C-6), 61.90 (C-6'), 51.43 (OCH_3), and 34.06 (CH_2CO_2).

Anal. Calc. for $C_{53}H_{68}O_{13}$: C, 69.72; H, 7.51. Found: C, 69.43; H, 7.66.

8-Methoxycarbonyloctyl O-[3,4-di-O-benzyl-2,6-di-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-α-D-mannopyranosyl]-(1→6)-3-O-allyl-2,4-di-O-benzyl-β-D-mannopyranoside (**11**). — Glycosyl bromide **10** (1 mmol) in dichloromethane (2 mL) was added to a mixture of silver trifluoromethanesulfonate (1.25 g, 4.8 mmol), 2,4,6-trimethylpyridine (0.59 g, 4.8 mmol), molecular sieves 4A (4 g), and **9** (412 mg, 0.45 mmol), stirred in dichloromethane (10 mL) at −50°. After 0.5 h, the solution was warmed to room temperature where it was kept for 1 h. The mixture was then cooled to −50°, and more **10** (1.0 mmol) in dichloromethane (2 mL) was added. After 0.5 h at −50°, the mixture was warmed to room temperature where stirring was continued for 4 h. It was then diluted with dichloromethane (100 mL), filtered through Celite, and the filtrate washed sequentially with water, M HCl, saturated aqueous $NaHCO_3$, and taken to dryness. The residual foam was purified by chromatography, first with a linear dichloromethane–methanol gradient from 140:1 to 110:1 (2.0 L total), then with 2:1 ethyl acetate–toluene to provide **11** (416 mg, 53%), white foam, $[\alpha]_D^{22}$ −3.3° (*c* 1.0, chloroform), R_F (2:1 ethyl acetate–toluene) 0.25; ^{1}H-n.m.r. ($CDCl_3$): δ 5.859 (m, 1 H, CH_2=C*H*CH_2), 5.794 (dd, 1 H, $J_{2'',3''}$ 9.0, $J_{3'',4''}$ 10.5 Hz, H-3''), 5.511 (d, $J_{1'',2''}$ 7.8 Hz, H-1''), 5.497 (dd, $J_{2''',3'''} \approx J_{3''',4'''}$ 9–10 Hz), 4.548 (d, $J_{1''',2'''}$ 7.5 Hz, H-1'''), 4.548 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.275 (s, H-1), 3.672 (s, OCH_3), 2.323 (t, 2 H, CH_2CO_2), 2.090, 2.034, 2.007, 1.997, 1.867, and 1.830 (each s, 3 H, 6 $COCH_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 174.28 (CO_2CH_3), 116.72 (CH_2=$CHCH_2$), 102.05 (C-1), 98.67 (C-1'), 97.32 and 96.47 (C-1'',1'''), 83.15 (C-3), 66.25 (C-6), 62.38 and 62.06 (C-6'',6'''), 54.59 and 54.37 (C-2'',2'''), 51.46 (OCH_3), and 34.11 (CH_2CO_2).

Anal. Calc. for $C_{93}H_{106}N_2O_{31}$: C, 63.90; H, 6.11; N, 1.60. Found: C, 64.07; H, 6.21; N, 1.80.

8-Methoxycarbonyloctyl O-[3,4-di-O-benzyl-2,6-di-O-(3,4,6-tri-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranosyl)-α-D-mannopyranosyl]-(1→6)-2,4-di-O-

*benzyl-β-*D*-mannopyranoside* (**12**). — A solution of **11** (177 mg, 0.101 mmol, tris(triphenylphosphine)rhodium(I) chloride (6.6 mg, 7 μmol) and diazabicyclo-[2.2.2]octane (6 mg, 0.02 mmol) was refluxed in 3:7:1 benzene–ethanol–water for 20 h. The solvent was evaporated and the residue dissolved in acetone (6 mL) containing HgO (5 mg), and $HgCl_2$ (200 mg) in 9:1 acetone–water (5 mL) was added. After 0.5 h, the solvent was removed and the residues dissolved in dichloromethane and washed with 30% aqueous KBr and water. Solvent evaporation left a yellow oil which was purified by chromatography with 1:2 ethyl acetate–toluene as eluent to provide **12** (148 mg, 85%), white foam, $[\alpha]_D^{22}$ −2.1° (*c* 0.86, chloroform); ^{1}H-n.m.r. ($CDCl_3$): δ 5.794 (dd, 1 H, $J_{2'',3''}$ 9.0, $J_{3'',4''}$ 10.5 Hz, H-3″), 5.512 (d, *J* 7.8 Hz, H-1″), 5.508 (dd, $J_{2''',3'''}$ 9.0, $J_{3''',4'''}$ 10.5 Hz, H-3‴), 5.215 (dd, 1 H, $J_{4'',5''}$ 10.0 Hz, H-4″), 4.964 (dd, 1 H, $J_{4''',5'''}$ 10.0 Hz, H-4‴), 4.904 (d, 1 H, $J_{1''',2'''}$ 7.8 Hz, H-1‴), 4.542 (d, 1 H, *J* 1.6 Hz, H-1′), 4.369 (s, H-1), 2.338 (d, *J* 9.0 Hz, D_2O-exchangeable, OH), 2.310 (t, CH_2CO_2), 2.063, 2.055, 2.032, 2.008, 1.874, and 1.833 (each s, 3 H, 6 $COCH_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 174.11 (CO_2CH_3), 102.07 (C-1), 98.68 (C-1′), 97.13 and 96.41 (C-1″,1‴), 66.27 (C-6), 62.31 and 62.25 (C-6″,6‴), 54.68 and 54.56 (C-2″,2‴), 51.33 (OCH_3), and 34.08 (CH_2CO_2).

Anal. Calc. for $C_{90}H_{102}N_2O_{31}$: C, 63.30; H, 6.02; N, 1.64. Found: C, 63.59; H, 6.13; N, 1.87.

8-Methoxycarbonyloctyl O-[*2,6-di-*O-*(2-acetamido-3,4,6-tri-*O-*acetyl-2-deoxy-β-*D*-glucopyranosyl)-3,4-di-*O-*benzyl-α-*D*-mannopyranosyl]-(1→6)-3-*O-*acetyl-2,4-di-*O-*benzyl-β-*D*-mannopyranoside* (**13**). — Compound **12** (150 mg, 0.088 mmol) was *O*-deacetylated as described for the preparation of **9** to provide a product having R_F (10:1 dichloromethane–methanol) 0.29. This material was dissolved in methanol (5 mL), 85% hydrazine hydrate (0.1 mL) was added, and the solution refluxed for 2 h. After solvent evaporation, the residue was kept for 2 h under high vacuum, dissolved in 1:1 acetic anhydride–pyridine (3 mL), and stirred overnight. Excess acetic anhydride was removed by the addition of water (2 mL) at 0°, dichloromethane (50 mL) added, and the solution washed with water, M HCl acid, saturated aqueous $NaHCO_3$, and taken to dryness. The residue was purified by chromatography with 9:1 ethyl acetate–hexane as eluent to provide **13** (55 mg, 40%), white foam, $[\alpha]_D^{22}$ −27° (*c* 0.20, chloroform), R_F (9:1 ethyl acetate–hexane) 0.30; ^{1}H-n.m.r. ($CDCl_3$): δ 7.064 (d, 1 H, $J_{2''',NH'''}$ 7.5 Hz, D_2O-exchangeable, NH‴), 5.822 (dd, 1 H, *J* 9.2, 10.5 Hz, H-3‴), 5.417 (d, 1 H, $J_{2'',NH''}$ 8.5 Hz, D_2O-exchangeable, NH″), 5.269 (d, 1 H, $J_{1''',2'''}$ 8.5 Hz, H-1‴), 4.98 (H-1″), 4.847 (H-1′), 4.497 (s, 1 H, H-1), 4.371 (dd, 1 H, $J_{1',2'}$ 1.8, $J_{2',3'}$ 3.2 Hz, H-2′), 3.82 (H-2″), 3.664 (s, OCH_3), 3.254 (ddd, 1 H, H-2‴), 2.304 (t, 2 H, CH_2CO_2), 2.054, 2.046, 2.038, 2.024, 2.014, 1.982, 1.969, 1.929, and 1.839 (each s, 3 H, 9 $COCH_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 174.32 (CO_2CH_3), 101.66 (C-1), 101.55 (2 C, C-1″,1‴), 97.50 (C-1′), 55.07 and 54.13 (C-2″,2‴), 51.46 (OCH_3), and 34.10 (CH_2CO_2).

Anal. Calc. for $C_{80}H_{104}O_{30}N_2$: C, 61.06; H, 6.66; N, 1.78. Found: C, 60.83; H, 6.61; N, 1.76.

8-Methoxycarbonyloctyl O-[*2,6-di-*O-*(2-acetamido-2-deoxy-β-*D*-glucopyrano-*

*syl)-α-*D*-mannopyranosyl]-(1→6)-β-*D*-mannopyranoside* (**4**). — *O*-Deacetylation of **13** (19.1 mg, 0.012 mmol), as described for the preparation of **9**, gave a product which was homogeneous by t.l.c., R_F (5:1 dichloromethane–methanol) 0.28. This material was not further characterized but was stirred under H_2 (0.1 MPa), in the presence of 5% Pd–C (25 mg) in 95% ethanol (4 mL) for 6 days. The catalyst was removed by filtration, the solvent evaporated, and the residue passed through Bio-Gel P-2 (2 cm i.d. × 60 cm) with 10% ethanol as eluent. Lyophilization of the carbohydrate-containing fractions gave **4** (7.8 mg, 70%), $[\alpha]_D^{22}$ −19° (*c* 0.10, water), R_F (6:1 2-propanol-water) 0.65; ^{1}H-n.m.r.: δ 4.882 (d, 1 H, $J_{1',2'}$ 1.5 Hz, H-1'), 4.670 (s, 1 H, H-1), 4.598 (d, 1 H, $J_{1'',2''}$ 8.0 Hz, H-1''), 4.550 (d, 1 H, $J_{1''',2'''}$ 8.4 Hz, H-1'''), 4.118 (dd, 1 H, $J_{2',3'}$ 3.0 Hz, H-3'), 3.689 (s, OCH_3), 2.390 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), 2.061 and 2.039 (each s, 3 H, 2 $COCH_3$); ^{13}C-n.m.r. (D_2O): δ 178.79 (CO_2CH_3), 175.47 and 175.17 ($COCH_3$), 102.40 (C-1), 100.89 (C-1''), 100.50 (C-1'''), 97.83 (C-1'), 56.45 and 56.38 (C-2'',2'''), 52.92 (OCH_3), 34.56 (CH_2CO_2), 23.34 and 23.17 ($COCH_3$).

The quantity of material obtained was considered too small for elemental analysis.

Enzymic synthesis of **4**. — The incubation medium contained the following reagent concentrations in a final volume of 100 μL: 4mM ADP, 5mM 2-acetamido-2-deoxy-β-D-glucopyranosylamine[15], 5mM UDP-GlcNAc (3.4 c.p.m./pmol), and compound **3** (265 nmol). The reagents were added to a 0.75-mL microcentrifuge tube and evaporated to dryness under reduced pressure.

Rous sarcoma virus-transformed BHK (baby hamster kidney) cells were grown as described[2]. About 5 × 10^7 cells were scraped from plates and collected by centrifugation. The cell pellet was resuspended in ice-cold MES buffer (1 mL) and subjected to 20-strokes in a Dounce homogenizer. The cells were centrifuged for 4 min in the cold in a microfuge over a 75% (w/w) sucrose cushion to remove nuclei, lysosomes, and unbroken cells. The supernatant was then centrifuged for 1 h at 100 000*g* and the pellet redissolved in 0.1M MES buffer (pH 7.5, 0.20 mL) containing 1.0% (w/w) Triton X-100 and 10 mg/mL of BSA. To begin the reaction, the dried contents of the incubation tube was first dissolved in MES buffer and detergent mixture (50 μL). The redissolved membrane pellet (50 μL) was then added to the assay tube to bring the final volume to 0.100 mL, and the contents of the tube mixed. The amount of protein in each assay tube was measured with the Bradford assay[17] and determined to be 1.51 mg. Four enzyme incubations were prepared and incubated for 6 h at 37°. Under these conditions, production of product was shown to be linear with time[6]. To terminate and process the products of the assay, water (0.20 mL) was added to each tube and each was filtered through a 0.2-μm filter disk with a centrifuge-filter device. Over the filter was Dowex 1 anion-exchange resin (HCO_2^-; 0.25 mL) that bound >90% of the unused UDP-GlcNAc. The filtrate was then subjected to l.c. using a 10-cm ODS column equilibrated in, and eluted with, water. The substrate (**3**) and product (**4**) glycosides bound and were co-eluted with 100% methanol. Each incubation was chromato-

graphed separately, and the four methanol eluents were then pooled. The glycosides were concentrated by evaporation and applied to a Silica Gel G t.l.c. plate, which was developed in 6:2:1 dichloromethane–methanol–water. Assays on small sections of the plate located the radioactive product having R_F 0.33, while unreacted **3** had R_F ~0.8 on this plate. The plate was scraped, the radioactively labelled material extracted with water, and the product concentrated by applying the aqueous extracts to a pellicular C18 column (1-mL bed volume) and eluting the product with methanol. Methanol was evaporated and the sample lyophilized from 100% D_2O prior to ^1H-n.m.r. analysis. The yield of isolated product was 2.7 μg based on its radioactivity. The product also had the same retention volume as **4** on Bio-Gel P-2.

ACKNOWLEDGMENTS

The authors thank the Natural Sciences and Engineering Research Council of Canada and the Alberta Heritage Foundation for Medical Research for ongoing financial support. This work was also supported by a grant from the National Cancer Institute, National Institutes of Health (CA-35377).

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