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TRANSFER OF D-GALACTOSYL GROUPS TO 6-*O*-SUBSTITUTED 2-ACETAMIDO-2-DEOXY-D-GLUCOSE RESIDUES BY USE OF BOVINE D-GALACTOSYLTRANSFERASE

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ABSTRACT

Bovine D-galactosyltransferase was found to transfer D-galactose from UDP-galactose to 6-*O*-substituted 2-acetamido-2-deoxy-β-D-glucopyranosides. The resulting 6-*O*-substituted *N*-acetyllactosamines were readily synthesized in milligram amounts and conveniently isolated on a reverse-phase support when prepared as the 8-methoxycarbonyloctyl glycosides. The 6-*O*-substitution tolerated by the enzyme include an α-L-fucopyranosyl group and the methyl ester of α-linked *N*-acetylneuraminic acid, but not the free acid itself. The product trisaccharides were characterized by ^{1}H-n.m.r. spectroscopy and fast-atom-bombardment mass spectrometry.

INTRODUCTION

Increasing interest in the availability of structurally well-characterized oligosaccharides has led us to examine the usefulness of glycosyltransferases, especially the readily available D-galactosyltransferase[1] (UDP-D-galactose:2-acetamido-2-deoxy-D-glucose 4-β-D-galactosyltransferase; EC 2.4.1.90) as an adjunct to chemical synthesis, for the preparation of branched-chain complex oligosaccharides. Galactosyltransferase is known to transfer β-D-galactopyranosyl groups from UDP-D-galactose to OH-4 of terminal (nonreducing) 2-acetamido-2-deoxy-β-D-glucose (β-D-GlcNAc) unit resulting in the synthesis of nonsubstituted *N*-acetyllactosamine-terminated oligosaccharides. The enzymic reaction has been frequently used[1] for the modification of naturally occurring oligosaccharides, both isolated and on the cell surface, and has been employed in preparative-scale syntheses of *N*-acetyllactosamine[2] and the human H-type 2 determinant, α-L-Fuc*p*-(1→2)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc[3].

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The acceptor specificity of galactosyltransferase has been studied in great detail by many groups and has been reviewed[1]. Recent studies[4,5] have been concerned with the ability of the enzyme to recognize and distinguish the terminal β-D-Glc*p*NAc groups on naturally occurring glycoproteins and glycolipids with a view to understanding the controlling mechanisms of glycoconjugate biosynthesis. Other work[6,7] has concentrated on achieving a description of the acceptor site, at the molecular level, by use of mostly acceptor analogs including epimers and deoxysugars.

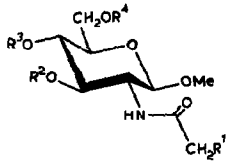
It is widely recognized that although the enzyme does recognize the aglyconic groups of acceptor β-D-Glc*p*NAc residues, a very large variety of aglycons, both natural and synthetic, are readily tolerated. We are aware of only two cases, however, where D-Glc*p*NAc residues substituted at positions other than O-1 have been reported to be acceptors. Berliner *et al.*[7] reported that 2-amino-2-deoxy-D-glucose compounds bearing a variety of *N*-acyl groups, from propionyl to dodecanoyl, act as excellent acceptors for the enzyme. Other *N*-acyl groups are also tolerated[8]. Schanbacher and Ebner[6] have also reported that *N*-acetyl-D-muramic acid, the 3-*O*-lactyl ether of D-GlcNAc, can also function as a galactosyl acceptor. We report herein that galactosyltransferase can, in appropriate instances, also use 6-*O*-glycosylated β-D-Glc*p*NAc derivatives as substrates and is, therefore, useful for the synthesis of 6-branched *N*-acetyllactosamine sequences.

RESULTS AND DISCUSSION

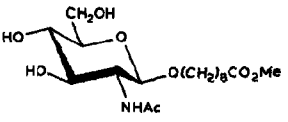
In order to rapidly assess the feasibility that substituted β-D-GlcNAc units might serve as substrates for galactosyltransferase, the kinetic parameters V_{max} and K_m for the series of simple methylated derivatives **1–5** were determined (Table I). These analogs were all prepared as the methyl β-D-glycosides in order to avoid the use of anomeric mixtures of acceptors since only the β-D anomer is a substrate in *N*-acetyllactosamine synthesis.

In agreement with the results of Berliner *et al.*[7], the *N*-propionyl derivative **2** was an excellent acceptor but the 3-methyl ether **3** was a much poorer substrate with a 60-fold elevated K_m. The 4-methyl ether **4**, where the reactive OH-4 is masked, is of course not a substrate but neither is it inhibitory and is, therefore, not bound by the enzyme. The fact that the 6-methyl ether **5** had a K_m elevated only 3-fold over that of the parent glycoside **1** suggested that more complex 6-*O*-substituted β-D-Glc*p*NAc residues might retain sufficient activity to also function as galactosyl acceptors. The maximum velocities (V_{max}) of the enzymic reactions can be seen, from Table I, to be qualitatively similar for those compounds that undergo galactosylation.

In Table II, we report the relative velocities, under a standard set of experimental conditions, for a number of 6-*O*-substituted β-D-Glc*p*NAc derivatives and their parent glycosides. With the methyl β-D-glycoside **1** taken as the reference and assigned a relative velocity of 100, 6-methylation to produce **5** could be seen to



	R^1	R^2	R^3	R^4
1	H	H	H	H
2	Me	H	H	H
3	H	Me	H	H
4	H	H	Me	H
5	H	H	H	Me



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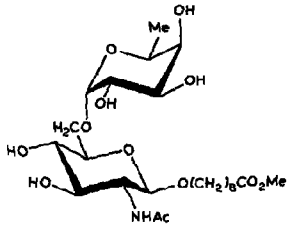

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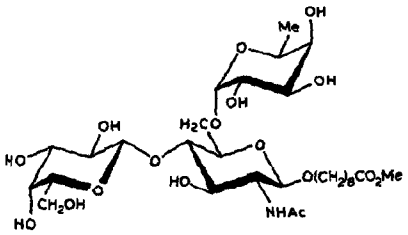

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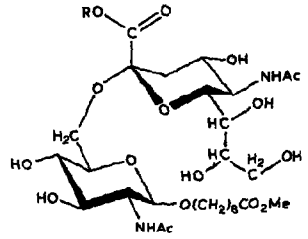

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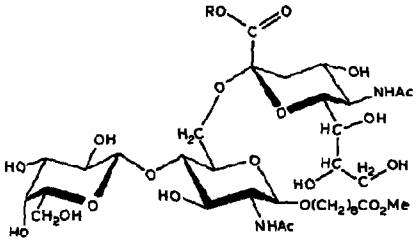
10 R = Me



11 R = Me
12 R = H

TABLE I

METHYL 2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSIDE ANALOGS TESTED AS GALACTOSYL ACCEPTORS[a]

Compound	K_m *(mM)*	V_{max} *(nmol/min)*
1	1.3 ±0.1	3.3 ±0.1
2	1.3 ±0.4	3.7 ±0.5
3	77 ±7	2.4 ±0.1
4[b]		
5	4.0 ±0.2	1.1 ±0.03

[a]Rates determined by use of the coupled enzyme assays of either Pierce *et al.*[20] or Fitzgerald *et al.*[21] measuring the production of UDP in 100mM cacodylate buffer (pH 7.5), 9.3mM $MnCl_2$, and 94μM UDP-Gal at 37°. Products were not isolated and characterized. [b]Neither substrate nor inhibitor.

TABLE II

RELATIVE RATES OF D-GALACTOPYRANOSYL GROUP TRANSFER TO SUBSTITUTED 2-ACETAMIDO-2-DEOXY-β-D-GLUCOPYRANOSYL RESIDUES[a]

Substrate	*Relative rate*
1	100
5	20
6	263
7	48
9	0
10	4

[a]Rates were determined by use of the coupled enzyme assay of Fitzgerald *et al.*[21] measuring UDP formation in 100mM cacodylate buffer, (pH 7.5), 9.3mM $MnCl_2$, and 94μM UDP-Gal at 37°. The products formed from **1**, **5**, and **6** were not isolated and characterized.

cause a 5-fold decrease in the rate of the enzymic reaction. With 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**6**) as galactosyl acceptor the enzymic reaction proceeded 2.6 times faster than with **1**. Although kinetic parameters were not determined for **6**, this observation is consistent with the decrease in K_m that has been observed for β-D-Glc*p*NAc glycosides of hydrophobic aglycons[8]. The disaccharide, 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-6-*O*-α-L-fucopyranosyl-β-D-glucopyranoside (**7**), could be seen to retain very good acceptor activity. Interestingly, both methylation and the much more sterically demanding α-L-fucosylation could be seen to cause a similar 5-fold decrease in the rate of galactosyl transfer when compared with the corresponding nonsubstituted parent glycoside. The product of the galactosylation of **7**, namely, 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-6-*O*-α-L-fucopyranosyl-4-*O*-β-D-galactopyranosyl-β-D-glucopyranoside (**8**), could be conveniently isolated from a preparative-scale reaction by virtue of its hydrophobic linking-arm using absorption onto a reverse-phase support and elution with 1:1 methanol–water after extensive aqueous washing. Fast-atom-bombardment (f.a.b.) mass-spectrometry showed **8** to be a tri-

saccharide [M + Na^+ = 722 (100%)] with the characteristic[9,10] fragmentation pattern observed in the positive-ion detection mode: M − $O(CH_2)_8CO_2CH_3$ = 512 (6%) and M − $O(CH_2)_8CO_2CH_3$ − $C_6H_{10}O_4$ (anhydrofucose) = 366 (12%). This latter fragment confirmed the presence of both nonsubstituted hexose (galactose) and deoxyhexose (fucose) and thus established the order of attachment of the sugar residues. The ^{1}H-n.m.r. spectrum showed the expected signals for α-L-linked fucose (H-1″, δ 4.950, $J_{1'',2''}$ 3.8 Hz) as well as two β-D-linked pyranosides (δ 4.549, $J_{1',2'}$ 7.5 Hz; and δ 4.539, $J_{1,2}$ 8.0 Hz). The doublet at δ 4.549 could be assigned to H-1′ (of the β-D-galactosyl group) by its coupling with H-2′ (δ 3.541, $J_{2',3'}$ 9.5 Hz). The f.a.b.–m.s. and ^{1}H-n.m.r. data conclusively showed that the β-D-galactopyranosyl group is attached to the β-D-Glc*p*NAc residue and we are assuming, without rigorous proof, that they are present in the expected (1→4) linkage. The carbohydrate sequence of **8**, β-D-Gal*p*-(1→4)-[α-L-Fuc*p*-(1→6)]-β-D-Glc*p*NAc, has been found[11] in oligosaccharides isolated from the urine of fucosidosis patients and may also occur in asparagine-linked oligosaccharide chains[12].

No reaction was observed with the disaccharide **9**, where the β-D-Glc*p*NAc residue is substituted with an α-(2→6)-linked *N*-acetylneuraminic acid (Neu5Ac) unit, even when the concentrations of both the enzyme and UDP-Gal were increased 10-fold over those described in Table II. This result was unexpected since a sterically demanding L-fucosyl group was so readily tolerated by the enzyme. The possibility that the charged carboxylate group of **9** might be responsible for this complete abolition of activity, likely by interaction with negatively charged groups either on UDP-Gal or on the enzyme, was examined by preparation, from **9**, of the methyl ester **10** according to the methylation procedure of Handa and Nakamura[13]. The neutral disaccharide **10** proved to be a weak acceptor (Table II) but still retained sufficient activity to allow the convenient preparation of **11** in 5–10-mg quantities. The ^{1}H-n.m.r. spectrum of **11** showed the expected signals for β-D-linked Glc*p*NAc (δ 4.492, $J_{1,2}$ 7.8 Hz) and *N*-acetylneuraminic acid (H-3*e*, δ 2.788, $J_{3a,3e}$ −12.5, $J_{3e,4}$ 4.5 Hz; and H-3*a*, δ 1.811, $J_{3a,4}$ 12.0 Hz), as well as the expected signals for the β-D-linked galactopyranosyl group (H-1, δ 4.507, $J_{1,2}$ 7.5 Hz, coupled to H-2, δ 3.496, $J_{2,3}$ 9.5 Hz). The major peaks of the f.a.b. positive-ion mass spectrum of **11** were assigned (Fig. 1). These data, again, leave no doubt as to the order of attachment of the individual sugar units and we assume that the enzymically synthesized product has the naturally occurring β-D-Gal*p*-(1→4)-D-Glc*p*NAc linkage. The methyl ester of the *N*-acetyl-D-neuraminyl residue could be selectively cleaved, in the presence of the aglyconic methoxycarbonyloctyl ester, with 1:2:2 triethylamine–methanol–water at 0° for 36 h to produce the *N*-acetyl-6-*O*-sialyllactosamine glycoside **12**. The β-D-Gal*p*-(1→3)-[α-Neu*p*5Ac-(2→6)]-β-D-Glc*p*NAc sequence is known to occur in human milk oligosaccharides and on glycoproteins[12,14,15]. The carbohydrate sequence of **12**, β-D-Gal*p*-(1→4)-[α-Neu*p*5Ac-(2→6)]-β-D-Glc*p*NAc, has not, to our knowledge, been described in naturally occurring structures.

In conclusion, we have demonstrated that 6-*O*-glycosylated 2-acetamido-2-

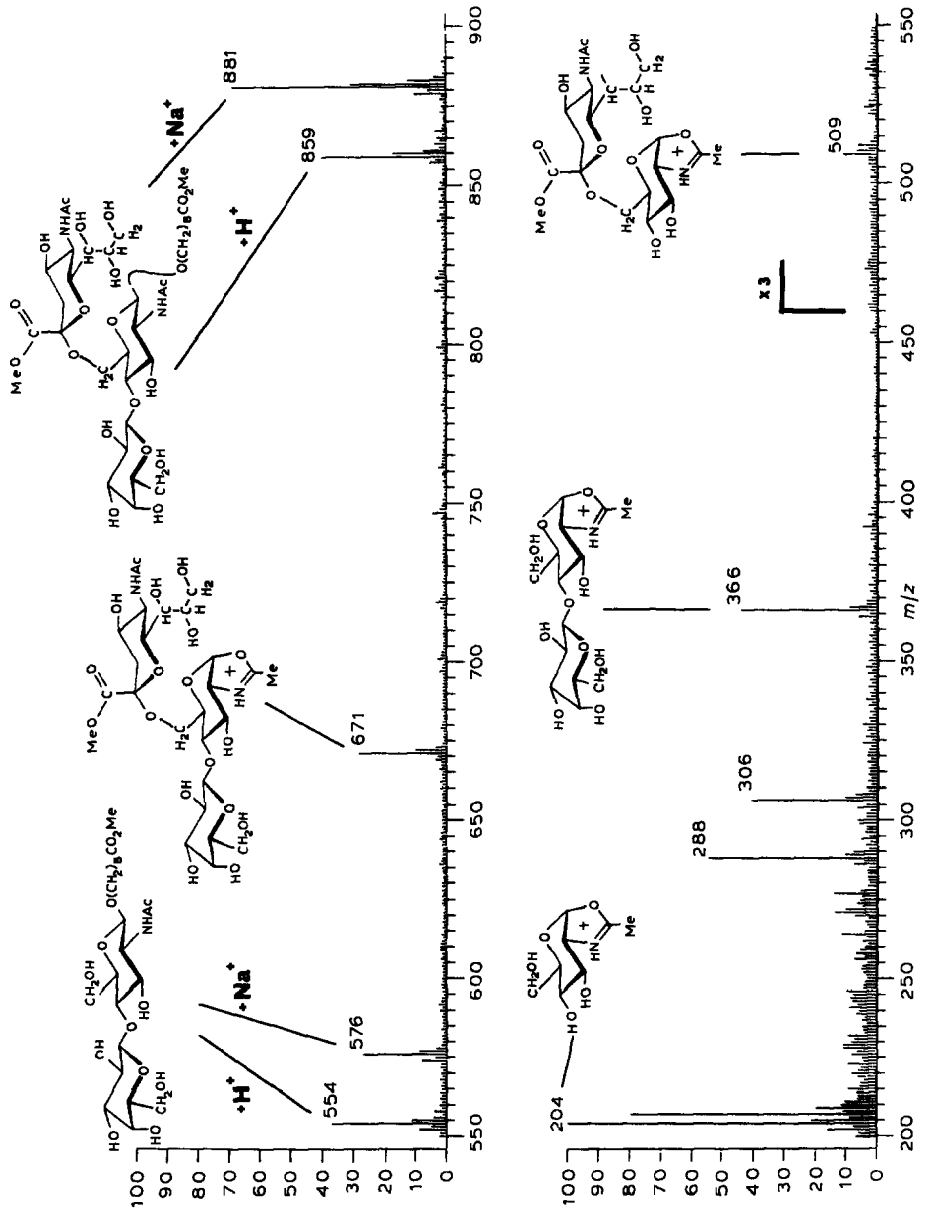


Fig. 1. Fast-atom-bombardment mass spectrum of the enzymically produced trisaccharide **11**.

deoxy-β-D-glucopyranosides can serve as acceptors for bovine galactosyltransferase, which is commonly regarded as functioning only in the synthesis of terminal, nonsubstituted *N*-acetyllactosamine sequences. Such "unnatural" reactions of glycosyltransferases should be useful for the rapid production of milligram quantities of oligosaccharides for use in biological testing, especially where the synthetic oligosaccharide substrates are readily available.

EXPERIMENTAL

General methods. — ^{1}H-N.m.r. spectra were recorded at 360 MHz with a Bruker WM-360 instrument and acetone (δ 2.225) as an internal standard in D_2O at ambient temperature (295 ±2°K). F.a.b.–mass spectra were recorded with an AEI MS-9 mass spectrometer using xenon as the bombarding gas and a recently described[16] fast-atom gun. Samples were run in a glycerol matrix. Thin-layer chromatography was performed on precoated plates of Silica gel 60-F254 (E. Merck, Darmstadt) with detection by charring after spraying with 5% H_2SO_4 in ethanol. Because only small amounts of material were biosynthesized, no elemental analysis was performed.

Materials. — Bovine milk galactosyltransferase (EC 2.4.1.90), nucleoside-diphosphate kinase (EC 2.7.4.6), UDP-glucose pyrophosphorylase (EC 2.7.7.9), UDP-glucose dehydrogenase (EC 1.1.1.22), pyruvate kinase (EC 2.7.1.40), L-lactate dehydrogenase (EC 1.1.1.27), UDP-galactose, α-D-glucopyranosyl phosphate, β-NAD, β-NADH, and phosphoenolpyruvate were obtained from Sigma Chemical Co. Methyl 2-acetamido-2-deoxy-3-*O*-methyl-, -4-*O*-methyl-, and -6-*O*-methyl-β-D-glucopyranosides (**3**, **4**, and **5**) were prepared as described by Allen *et al.*[17], and methyl 2-deoxy-2-propionamido-β-D-glucopyranoside (**2**) was prepared by the method of Neuberger[18]. 8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**6**) was prepared according to Lemieux *et al.*[19]. 8-Methoxycarbonyloctyl disaccharides **7** and **8** were synthesized by Dr. Z. A. Abbas of Chembiomed Ltd. (Edmonton, Alberta, Canada).

Initial velocities of the enzymic reactions were determined by measuring the rate of UDP formation at 37° with the coupled spectrophotometric assays of Pierce *et al.*[20] or of Fitzgerald *et al.*[21]. All reactions were carried out in 100mM cacodylate buffer (pH 7.5) with fixed concentrations of $MnCl_2$ (9.3mM) and UDP-Gal (94μM). Reaction was initiated by the addition of galactosyltransferase. Initial rate data were analyzed by the statistical method of Wilkinson[22] using a program provided by Mr. S. Hagen of Chembiomed Ltd. For the preparative-scale enzymic syntheses of **8** and **11**, the reactions were conducted at ambient temperatures, below the optimum temperature for the reaction, in order to minimize the rate of denaturation of the enzyme.

8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-6-O-α-L-fucopyranosyl-4-O-β-D-galactopyranosyl-β-D-glucopyranoside (**8**). — A solution of 8-methoxycarbonyloctyl 2-acetamido-2-deoxy-6-*O*-α-L-fucopyranosyl-β-D-glucopyranoside (**7**; 3.0 mg, 5.6 μmol), galactosyltransferase (0.1 unit), and UDP-galactose (30 μmol)

in 100mM sodium cacodylate (300 μL; pH 7.5) containing 20mM $MnCl_2$ was kept at ambient temperatures for 12 h. T.l.c. examination of the mixture in 60:35:6 chloroform–methanol–water showed quantitative conversion of **7** (R_F 0.55) into a new product (R_F 0.33). The mixture was diluted with water (10 mL) and passed directly onto a Sep-Pak C-18 reverse-phase cartridge (Waters Associates) which had been prewashed with 20 mL each of methanol, 1:1 chloroform–methanol methanol, 1:1 methanol–water, and finally water. The cartridge was washed with water (30 mL) and the product (**8**) was eluted with 1:1 methanol–water (20 mL). Evaporation of the aqueous washings and t.l.c. examination of the residue showed that no **8** had been eluted from the cartridge by the washing procedure. Evaporation of the methanol–water eluate, followed by lyophization, gave **8** as a white powder; ^{1}H-n.m.r. (D_2O): δ 4.950 (d, 1 H, $J_{1'',2''}$ 3.8 Hz, H-1″), 4.549 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1′), 4.539 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 3.694 (s, 3 H, CO_2CH_3), 3.541 (dd, 1 H, $J_{2',3'}$ 9.5 Hz, H-2′), 2.396 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), 2.040 (s, 3 H, $COCH_3$), and 1.236 (d, 3 H, $J_{5'',6''}$ 6.5 Hz, H-6″).

*8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-6-*O*-[methyl (5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosyl)onate]-β-*D*-glucopyranoside* (**10**). — The sodium salt of 8-methoxycarbonyloctyl 2-acetamido-6-*O*-(5-acetamido-3,5-dideoxy-α-D-*glycero*-D-*galacto*-2-nonulopyranosylonic acid)-2-deoxy-β-D-glucopyranoside (**9**, 7.5 mg) was dissolved in dimethyl sulfoxide (1.0 mL), and iodomethane (100 μL) was added. After 15 min at ambient temperature, t.l.c. (60:35:6 chloroform–methanol–water) showed the quantitative conversion of **9** (R_F 0.16) into a less polar product (R_F 0.42). The mixture was diluted with water (20 mL) and separated into two portions. Each portion was separately loaded onto a C-18 Sep-Pak cartridge and isolated as described for **8** to give **10** as a white lyophilized powder (8.0 mg); ^{1}H-n.m.r. (D_2O): δ 4.472 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 3.879 (s, 3 H, Neu CO_2CH_3), 3.684 (s, 3 H, $CH_2CO_2CH_3$), 2.736 (dd, 1 H, $J_{3'a,3'e}$ −12.5, $J_{3'e,4'}$ 4.5 Hz, H-3′*e*), 2.386 (t, 2 H, *J* 7.5 Hz, $CH_2CO_2CH_3$), 2.033 and 2.026 (each s, 3 H, $NHCOCH_3$), and 1.845 (dd, 1 H, $J_{3'a,4'}$ 12.0 Hz, H-3′*a*).

*8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-4-*O*-β-D-galactopyranosyl-6-*O*-[methyl (5-acetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosyl)onate]-β-D-glucopyranoside* (**11**). — UDP-galactose (10 μmol) was added every 6–12 h to a solution of **10** (7.0 mg, 10 μmol) and galactosyltransferase (0.1 unit) in sodium cacodylate buffer (500 μL; pH 7.5) containing 20mM $MnCl_2$. The progress of the reaction was monitored by t.l.c. (60:35:6 chloroform–methanol–water) by the disappearance of **10** (R_F 0.42) and the concomitant appearance of a new more polar product (R_F 0.26). Completion of the reaction required a total of seven additions of UDP-galactose (7 equiv. total) over a period of 60 h. The product of the reaction was isolated on two Sep-Pak cartridges, as described for the purification of **10**, to give **11** as a white lyophilized powder (9 mg) in essentially quantitative yield; ^{1}H-n.m.r. (D_2O): δ 4.507 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1′), 4.492 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1), 3.875 (s, 3 H, Neu CO_2CH_3), 3.681 (s, 3 H, $CH_2CO_2CH_3$), 3.496 (dd, 1 H, $J_{2',3'}$ 9.5 Hz, H-2′), 2.788 (dd, 1 H, $J_{3''a,3''e}$ −12.5, $J_{3''e,4''}$ 4.5 Hz, H-3″*e*), 2.384 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), 2.030 and 2.022 (each s, 3 H, $NHCOCH_3$), and 1.811 (dd, 1 H, $J_{3''a,4''}$ 12.0 Hz, H-3″*a*).

CH_2CO_2), 2.030 and 2.022 (each s, 3 H, $NHCOCH_3$), and 1.811 (dd, 1 H, $J_{3'a,4'}$ 12.0 Hz, H-3′*a*).

*8-Methoxycarbonyloctyl 2-acetamido-2-deoxy-6-O-(3,5-diacetamido-3,5-dideoxy-α-*D*-glycero-*D*-galacto-2-nonulopyranosylonic acid)-4-O-β-*D*-galactopyranosyl-β-*D*-glucopyranoside* (**12**). — Compound **11** (1 mg) was dissolved in 2:2:1 methanol–water–triethylamine (1.0 mL) and kept at 0° for 36 h. Solvent evaporation, followed twice by addition and evaporation of ethanol (5 mL), left a pale-yellow residue which was passed through Dowex 50-X8 (Na^+) cation-exchange resin (2 mL) in water and lyophilized to a pale yellow powder; ^{1}H-n.m.r. (D_2O): δ 4.575 (d, 1 H, $J_{1',2'}$ 7.5 Hz, H-1′), 4.506 (d, 1 H, $J_{1,2}$ 7.5 Hz), 3.687 (s, 3 H, $CH_2CO_2CH_3$), 3.501 (dd, 1 H, $J_{2',3'}$ 9.5 Hz), 2.785 (dd, 1 H, $J_{3'a,3'e}$ −12.0, $J_{3'e,4'}$ 4.5 Hz, H-3′*e*), 2.388 (t, 2 H, *J* 7.5 Hz, CH_2CO_2), 2.033 and 2.026 (each s, 3 H, $NHCOCH_3$), 1.694 (dd, 1 H, $J_{3'a,4'}$ 12.0 Hz, H-3′*a*); the f.a.b.m.s. showed the expected intense signal at *m/z* 867 (**12** + Na^+).

ACKNOWLEDGMENTS

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