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# Chemoenzymic synthesis of sialylated and fucosylated oligosaccharides having an *N*-acetyllactosaminyl core

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## ABSTRACT

Several sialylated and fucosylated oligosaccharides, based upon the *N*-acetyllactosaminyl core structure, have been synthesized from a single trisaccharide glycoside, β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-$OCH_2(CH_2)_7CO_2CH_3$, by the sequential use of several glycosyltransferases and one sialidase. In these chemoenzymic syntheses, selective internal monofucosylation of a dimeric *N*-acetyllactosaminyl tetrasaccharide is achieved via two routes. It is demonstrated that the pentasaccharide β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-$OCH_2(CH_2)_7CO_2CH_3$ is an acceptor for the rat liver β-D-Gal-(1 → 3/4)-D-GlcNAc α2,3- and β-D-Gal-(1 → 4)-D-GlcNAc α2,6-sialyltransferases. Among the structures obtained is the terminal hexasaccharide of the CD-65/VIM-2 epitope.

## INTRODUCTION

Some interactions of various subsets of leukocytes with endothelial cells are mediated by oligosaccharide moieties of the cell surface glycolipids or glycoproteins[1]. In particular, sialylated and fucosylated *N*-acetyllactosaminyl oligosaccharides appear to be important as cell adhesion molecules[2], hematopoietic cell differentiation markers[2,3] and tumor antigens[4]. The availability of synthetic oligosaccharides of this series and their conjugates facilitates studies of their biological relevance. Sialyl Lewis[a] and sialyl Lewis[x] structures, for example, have been prepared earlier by chemoenzymic synthesis[5–7].

A number of fucosyltransferases can transfer L-fucose from GDP-fucose to the *N*-acetylglucosamine moiety of acceptors containing the β-D-Gal-(1 → 3)-β-D-GlcNAc (type I) or the β-D-Gal-(1 → 4)-β-D-GlcNAc (type II) sequences[8]. Several of these enzymes have been cloned, and at least two have been employed for

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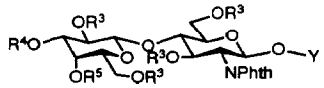
synthetic purposes[6,9]. A fucosyltransferase preparation, easily obtainable from human milk[5], is particularly useful. In this preparation, the β-D-Gal-(1 → 3/4)-D-GlcNAc α1,3/4-fucosyltransferase and a β-D-Gal-(1 → 4)-D-GlcNAc α1,3-fucosyltransferase co-purify[10,11], and the mixture can be used to efficiently transfer L-fucose to type I and type II structures[5]. Neither of these two enzymes transfers fucose to terminal *N*-acetylglucosamine[10]. Interestingly, the transfer of L-fucose also occurs to acceptors in which the 2- and the 3-hydroxyls of the terminal β-galactose have been substituted with other sugars[11,12]. For acceptors possessing unsubstituted repeating *N*-acetyllactosaminyl units, preferential fucosylation of one of the *N*-acetylglucosamine moieties has been reported to occur in some cases[13–15], although the reaction is not completely selective.

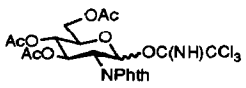
In this communication, we report that several sialylated and fucosylated oligosaccharides, based upon the *N*-acetyllactosaminyl core, can be obtained from the trisaccharide glycoside β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-OR, where $R = CH_2(CH_2)_7CO_2CH_3$ (**10**), by glycosyltransferase-assisted synthetic routes. In particular, internally monofucosylated structures, such as the hexasaccharide determinant of the CD-65/VIM-2 epitope, α-Neu5Ac-(2 → 3)-β-D-Gal-(1 → 4)-β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-[α-L-Fuc-(1 → 3)]-β-D-GlcNAc-OR[16,17] and the corresponding α2,6-sialylated hexasaccharide, can be obtained selectively. These results extend those presented in an earlier communication[18].

## RESULTS AND DISCUSSION

*Synthesis of trisaccharide* **10** *and tetrasaccharide* **11**.—The 8-(methoxycarbonyl)octyl group was chosen as the aglycone in order to facilitate the isolation of

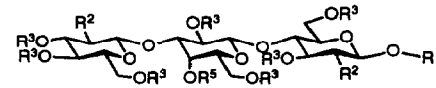



| | Y | $R^3$ | $R^4$ | $R^5$ |
|---|---|---|---|---|
| **1** | Ac | Ac | Ac | Ac |
| **2** | R | Ac | Ac | Ac |
| **3** | R | H | H | H |
| **4** | R | H | -Ipd- | |
| **5** | R | Ac | -Ipd- | |
| **6** | R | Ac | H | H |

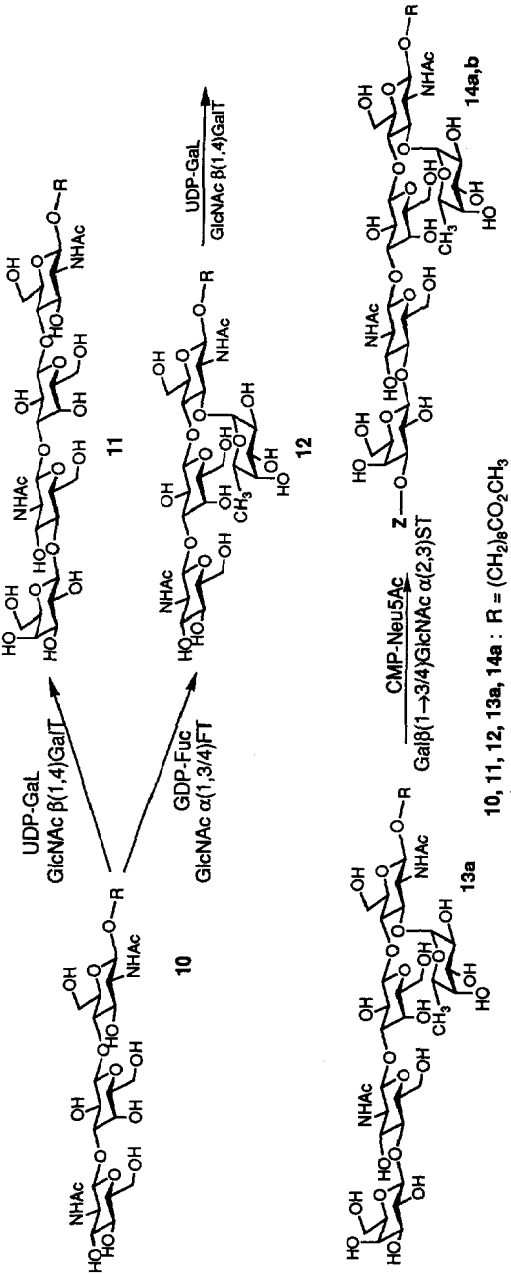



**7**

R = $(CH_2)_8CO_2CH_3$



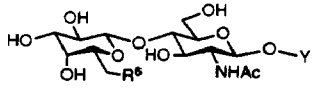

| | $R^2$ | $R^3$ | $R^5$ |
|---|---|---|---|
| **8** | NPhth | Ac | H |
| **9** | NHAc | Ac | Ac |
| **10** | NHAc | H | H |



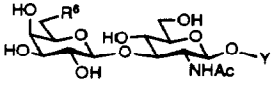

Scheme 1. Synthesis of the CD-65/VIM-2 hexasaccharide **14a,b** from trisaccharide **10** (FT = fucosyltransferase, GalT = galactosyltransferase, ST = sialyltransferase).

the reaction products in the enzymatic reactions[5], as well as to enable the synthesis of conjugates of the products obtained. Trisaccharide **10** was synthesized according to the procedure described by Alais et al.[19], with the exception of the change in the aglycone. Briefly, trimethylsilyl trifluoromethanesulfonate-catalyzed glycosylation of 8-(methoxycarbonyl)octanol by the disaccharide donor **1** provided glycoside **2**. Disaccharide **2** was transformed into the 3′,4′-diol **6** as described[19]. Glycosylation of the diol **6** by the imidate **7**, catalyzed by trimethylsilyl trifluoromethanesulfonate, provided trisaccharide **8**. This compound was deprotected by treatment with hydrazine acetate in refluxing ethanol, following which peracetylation and O-deacetylation provided trisaccharide **10**. Finally, enzymic transfer of D-galactose to **10** with the bovine milk D-GlcNAc β1,4-galactosyltransferase provided tetrasaccharide **11** (75%).

*Synthesis of hexasaccharide* **14a,b** *from trisaccharide* **10** *(Scheme 1).*—The relative rate of transfer of L-fucose from GDP-fucose to trisaccharide **10** by the milk fucosyltransferase was found to be 125% relative to β-D-Gal-(1 → 4)-β-D-GlcNAc-OR (**15**) at 2 mM concentration as 100%. As a result the preparative fucosylation of **10** proceeded smoothly to provide tetrasaccharide **12** in 79% yield. The assumption that fucosylation occurred on the reducing *N*-acetylglucosamine residue was supported by the values of the shifts of H-1 (δ 5.09) and H-5 (δ 4.81), which are characteristic of an α1,3-linked fucosyl residue in a Lewis$^x$ structure[20]. In an assay reaction, a Lewis-type fucosyltransferase extracted from Colo 205 cells had been found to transfer to the related trisaccharide glycolipid acceptor β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-Glc-(1 → 1)-ceramide[14]. Tetrasaccharide **12** proved to be a good substrate for the bovine milk D-GlcNAc β1,4-galactosyltransferase, providing pentasaccharide **13a** (82%). Studies of the specificity of this enzyme for modified acceptors have indicated a substantial decrease in activity correlated with modifications at the C-3 position of the terminal *N*-acetylglucosamine[21]. However, a fucosyl residue at the 3-position of the reducing moiety, as in **12**, is readily tolerated by the enzyme.



**15** $R^6 = OH$ $Y = R$
**16** $R^6 = H$ $Y = (CH_2CH_2O)_2CH_2CO_2CH_3$
$R = (CH_2)_8CO_2CH_3$



**17** $R^6 = H$ $Y = CH_3$
**18** $R^6 = OH$ $Y = R$

TABLE I

Apparent kinetic parameters for the transfer of *N*-acetylneuraminic acid to various acceptors by β-D-Gal-(1 → 3/4)-D-GlcNAc α2,3-sialyltransferase

| Acceptor | $K_m$ (mM) | rel $V_{max}$ | (rel $V_{max}$)/$K_m$ (1/mM) |
|---|---|---|---|
| **15** | 0.90 ± 0.27 | 1.0 | 1.11 |
| **13a** | 2.51 ± 0.20 | 1.6 | 0.63 |
| **11** | 5.52 ± 0.60 | 6.2 | 1.13 |

It has been shown that an α-fucosyl residue linked to the 3- or 4-position of the *N*-acetylglucosamine prevents the transfer of *N*-acetylneuraminic acid by the β-D-Gal-(1 → 3/4)-D-GlcNAc α2,3-sialyltransferase to the neighboring β-linked galactose[22–24] unit of type I and type II acceptors. Therefore, the accepted biosynthetic pathway leading to sialyl Lewis$^a$ and sialyl Lewis$^x$ structures proceeds by sequential sialylation and fucosylation. A similar route has been used for synthetic purposes[5–7]. The CD-65/VIM-2 epitope has also been proposed to result from the selective internal monofucosylation of an α2,3-sialylated dimeric *N*-acetyllactosaminyl structure[16]. The apparent kinetic parameters $K_m$ and $V_{max}$, measured for the transfer of *N*-acetylneuraminic acid from CMP-Neu5Ac to disaccharide **15**, tetrasaccharide **11**, and pentasaccharide **13a** by the affinity-purified rat liver β-D-Gal-(1 → 3/4)-D-GlcNAc α2,3-sialyltransferase, are reported in Table 1. The values of $K_m$ and $V_{max}$ indicate that the internally fucosylated pentasaccharide is a good acceptor for this transferase. As a result, preparative sialylation of **13a** provided the CD-65/VIM-2 hexasaccharide **14a,b** in an almost quantitative yield (occasional partial cleavage of the methyl ester group of the aglycon was observed during some of these enzymic reactions).

*Synthesis from the tetrasaccharide* **11** *(Scheme 2).*—Type I and type II disaccharide analogues modified by deoxygenation at the 2-(ref 25), the 3-, or the 4-position* of the β-galactose moiety are good acceptors for the milk fucosyltransferase preparation. This indicates that these hydroxyls are not specifically required for productive binding to the enzyme. However, the low activity of analogues such as 6-deoxy-β-D-Gal-(1 → 4)-β-D-GlcNAc-O$(CH_2CH_2O)_2CH_2CO_2CH_3$ (**16**, 9%) and 6-deoxy-β-D-Gal-(1 → 3)-β-D-GlcNAc-$OCH_3$ (**17**, 10%) shows that the 6-hydroxyl of the β-galactose is important for efficient transfer. It is well known that some fucosyltransferases do not transfer L-fucose to the *N*-acetylglucosamine moiety of type II acceptors in which the 6-hydroxyl of the β-galactose is substituted by an *N*-acetylneuraminyl residue[13,26]. However, this may be due to steric hindrance, or to the conformational preference of the α-(2 → 6)-linked sialic acid moiety[27].

The sialylation of tetrasaccharide **11** with the rat liver β-D-Gal-(1 → 4)-D-GlcNAc α2,6-sialyltransferase provided pentasaccharide **19a,b** (50%). Enzymic fucosylation of **19b** then gave hexasaccharide **20b** (94%). The $^1$H NMR data for **20b** are in

* K.B. Wlasichuk, M.A. Kashem, P.V. Nikrad, and A.P. Venot, unpublished data.

Scheme 2. Synthesis of pentasaccharide **13a** from tetrasaccharide **11**.

TABLE II

Apparent kinetic parameters for the transfer of N-acetylneuraminic acid to various acceptors by β-D-Gal-(1 → 4)-D-GlcNAc α2,6-sialyltransferase

| Acceptor | $K_m$ (mM) | rel $V_{max}$ | (rel $V_{max}$)/$K_m$ (1/mM) |
|---|---|---|---|
| **15** | 0.40 ± 0.05 | 1.0 | 2.50 |
| **13a** | 8.28 ± 0.87 | 1.8 | 0.22 |
| **11** | 2.17 ± 0.14 | 1.0 | 0.50 |

agreement with the proposed monofucosylated structure. Moreover, desialylation with immobilized *Clostridium perfringens* neuraminidase provided **13a,b**. Reaction of **13b** with diazomethane in methanol then provided **13a**, as evidenced by its $^1$H NMR spectrum, identical with that of the pentasaccharide obtained above.

The kinetic parameters $K_m$ and $V_{max}$, measured for the transfer of *N*-acetylneuraminic acid to disaccharide **15**, tetrasaccharide **11**, and pentasaccharide **13a** by the affinity purified β-D-Gal-(1 → 4)-D-GlcNAc α2,6-sialyltransferase from rat liver are reported in Table II. The results show that pentasaccharide **13a** is also an acceptor for this transferase, although with a 4-fold increase in $K_m$ and a 2.3-fold decrease in the kinetic efficiency parameter, as compared to the nonfucosylated structure **11**.

## CONCLUSION

The biosynthesis of sialylated and fucosylated type I and II oligosaccharides has been demonstrated to proceed via sequential core elongation, terminal sialylation and final fucosylation[13,22–24]. Such a route has also been proposed to account for internally monofucosylated dimeric *N*-acetyllactosaminyl structures, such as that of the CD-65/VIM-2 epitope[16]. A myeloid fucosyltransferase that generates such structures has been identified[8] and cloned[28]. In other cases, the biosynthetic route to some asialo, internally fucosylated structures, such as those identified on granulocytes[29] or the tumor antigen recognized by antibody ACFH-18[30], remains an open question.

In the current work a single trisaccharide has been used as starting material in enzymic syntheses which employ a combination of glycosyltransferases, and lead to internally fucosylated, or sialylated and fucosylated type II oligosaccharides. For these syntheses, two different pathways have been used: (*i*) a sequential route in which selective internal monofucosylation precedes core elongation and final α2,3-sialylation of the terminal galactose and (*ii*) a route which proceeds through core elongation, α2,6-sialylation of the terminal galactose, internal monofucosylation, desialylation, and final α2,3-sialylation. The success of the first route demonstrates that, in vitro and with small molecular weight acceptors, internal monofucosylation can precede terminal α2,3- or α2,6-sialylation. Although the $K_m$ values

TABLE III

Selected $^1$H NMR data for the synthesized oligosaccharides

| Sugar unit (no.) [a] and H atom (coupling) | Chemical shifts in ppm ($J$ values in Hz) [b] | | | | | | |
|---|---|---|---|---|---|---|---|
| | **10** | **11** | **12** | **13a** | **14a** | **19a** | **20a** |
| β-GlcNAc (1) | | | | | | | |
| 1 ($J_{1,2}$) | 4.51(7.5) | 4.52(7.5) | 4.53(7.7) | 4.53(7.8) | 4.53(8.0) | 4.52(7.5) | 4.53(8.0) |
| β-Gal (2) | | | | | | | |
| 1 ($J_{1,2}$) | 4.45(8.0) | 4.48 [c](8.0) | 4.44(7.7) | 4.44 [c](7.7) | 4.44(8.0) | 4.46 [c](7.8) | 4.44 [c](7.8) |
| 4 ($J_{3,4}$) | 4.15(3.0) | 4.16(3.0) | 4.09(3.5) | 4.10(3.0) | 4.10(3.0) | 4.16(3.4) | 4.10(3.0) |
| β-GlcNAc (3) | | | | | | | |
| 1 ($J_{1,2}$) | 4.66(8.5) | 4.70(8.2) | 4.68(8.2) | 4.70(8.2) | 4.69(8.2) | 4.73(7.7) | 4.73(7.7) |
| β-Gal (4) | | | | | | | |
| 1 ($J_{1,2}$) | | 4.49 [c](8.0) | | 4,48 [c](7.7) | 4.56(7.7) | 4.49 [c](8.0) | 4.46 [c](7.8) |
| 3 ($J_{2,3}$, $J_{3,4}$) | | | | | 4.12(10.6; 3.0) | | |
| α-Fuc | | | | | | | |
| 1 ($J_{1,2}$) | | | 5.09(4.0) | 5.10(3.8) | 5.09(3.8) | | 5.10(3.7) |
| 5 ($J_{5,6}$) | | | 4.82(6.5) | 4.82(6.5) | 4.81(6.5) | | 4.82(6.5) |
| 6 | | | 1.15 | 1.15 | 1.15 | | 1.15 |
| α-Neu5Ac(2 → 3) | | | | | | | |
| 3eq ($J_{3eq,4}$, $J_{3eq,3ax}$) | | | | | 2.76(4.5,13.0) | | |
| 3ax ($J_{3ax,4}$) | | | | | 1.80(12.0) | | |
| α-Neu5Ac(2 → 6) | | | | | | | |
| 3eq ($J_{3eq,4}$, $J_{3eq,3ax}$) | | | | | | 2.67 (4.15,12.5) | 2.67(4.5,12.5) |
| $3_{ax}$($J_{3ax,4}$) | | | | | | 1.72(12.0) | 1.72(12.0) |
| Other | | | | | | | |
| NAc | 2.03, 2.02 | 2.03 (two) | 2.03, 2.02 | 2.03, 2.02 | 2.03 (three) | 2.06, 2.03 (two) | 2.05, 2.03, 2.02 |
| $CH_2CO_2(J)$ | 2.39(7.5) | 2.39(7.5) | 2.39(7.5) | 2.39(7.5) | 2.39(7.5) | 2.39(7.5) | 2.39(7.5) |
| $CO_2CH_3$ | 3.68 | 3.69 | 3.69 | 3.69 | 3.69 | 3.69 | 3.69 |

[a] The sugar residues of the core structure are sequentially numbered starting from the reducing unit. [b] Measured at 300 or 500 MHz in $D_2O$ with acetone ($\delta$ 2.225) as reference. All compounds show multiplets centered at 1.58 (4 H) and 1.30 (8 H) for the methylene protons. [c] Interchangeable.

measured for the transfer of *N*-acetylneuraminic acid to the fucosylated substrate **13a** by the rat liver β-D-Gal-(1 → 3/4)-D-GlcNAc α2,3- and β-D-Gal-(1 → 4)-D-GlcNAc α2,6-sialyltransferases are slightly higher than those measured for the nonfucosylated tetrasaccharide **11**, the transfer proceeds well, particularly for the first enzyme.

All the enzymic reactions performed in this work proceeded to completion and gave products of high purity. The results further establish the versatility of the chemoenzymic approach to the synthesis of oligosaccharides, providing a variety of compounds from a single precursor.

## EXPERIMENTAL

*General.*—Precoated silica gel plates (Merck, 60-$F_{254}$) were used for analytical TLC, and spots were detected by charring after spraying with 5% $H_2SO_4$ in EtOH. Reagent grade chemicals were employed, and solvents used in chemical reactions were distilled, if necessary, and dried according to usual procedures. Unless otherwise noted, mixtures from chemical steps were processed by dilution with $CH_2Cl_2$, and successive washing with water, a dilute solution of $NaHCO_3$, and water. After drying over $MgSO_4$, the solvents were removed by evaporation under vacuum at a bath temperature of 35°C (or lower if necessary). Silica Gel 60 (Merck, 230–400 mesh) was used for column chromatography. Iatrobeads (6RS-8060) were from Iatron Laboratories, Tokyo. They were used with 65:35:3 (*A*), 65:35:5 (*B*), 65:35:8 (*C*), or 65:40:10 (*D*) $CHCl_3$–MeOH–water for chromatography of the final products. Bulk $C_{18}$ silica gel was from Waters Associates. Compounds **16** and **17** were generous gifts from Dr. R.U. Lemieux, University of Alberta. GDP-fucose and disaccharides **15** and **18** were available from Chembiomed Ltd., Edmonton, Canada. Microanalyses were performed by the Analytical Services of the Department of Chemistry, University of Alberta, Edmonton.

For $^1H$ NMR, tetramethylsilane (in $CDCl_3$) and acetone (δ 2.225 in $D_2O$) were used as internal standards. Deprotected oligosaccharides were freeze-dried twice from 99.96% $D_2O$, and spectra were run either at 300 MHz on a Bruker AM-300, or at 500 MHz on a Varian Unity spectrometer (University of Alberta), at 298 K. Only partial NMR data are presented, and coupling constants (observed splittings) are reported as if they were first order. All the spectra of compounds obtained by enzymic synthesis as 8-(methoxycarbonyl)octyl glycosides show a singlet at δ ~ 3.69 ($CO_2CH_3$) and a triplet at ~ 2.39 (*J* 7.5 Hz, $CH_2CO_2CH_3$). The spectra of compounds isolated as the 8-carboxyoctyl glycosides differ from the spectra of the corresponding 8-(methoxycarbonyl)octyl glycosides only by the absence of the singlet due to $CO_2CH_3$, and the shift of the α-methylene triplet to δ ~ 2.31.

*Fucosyltransferase preparation.*—The fucosyltransferase was purified from the milk of Lewis-positive donors according to the reported procedure[5]. For kinetic studies, as well as for synthetic purposes, the enzyme was further purified by

affinity chromatography on GDP–hexanolamine Sepharose, and assayed as indicated[5]. One unit of enzyme activity is defined as the amount of enzyme transferring 1 μmol of fucose/min.

*Fucosyltransferase kinetics.*—Determination of the relative rates was performed following the reported procedure[5]. The radiolabelled products were isolated by the Sep-Pak method[5] for acceptors with the hydrophobic 8-(methoxycarbonyl)octyl aglycon, or by ion-exchange chromatography on Dowex 1-X8 ($HPO_4^{2-}$) for acceptors with other aglycons. Activities are expressed relative to that of **15** at 2 mM as 100%.

*Preparative fucosylation.*—Preparative enzymic fucosylations were performed as described[5]. For example, trisaccharide **10** (15 mg, 0.020 mmol), $MnCl_2$ (10 mM), ATP (1.6 mM), $NaN_3$ (1.6 mM), the fucosyltransferase (56 mU), and GDP-fucose (33 mg) in sodium cacodylate buffer (100 mM, pH 6.5, 2.0 mL) were incubated 48–72 h at 37°C. The final mixture was diluted with water (10 mL) and applied to a column of $C_{18}$ silica gel (1 × 20 cm). After washing with water (100 mL), the products were eluted with MeOH (100 mL). After evaporation of the solvents, the residue was dissolved in a small amount of solvent *A* and applied to a column of Iatrobeads (3.5 g). Successive elution with the solvent mixtures *A*, *B*, and *C* provided **12** (14.0 mg, 79%). After freeze-drying, the recovered material was run through a small column of AG 50W-X12 ion exchange resin (Bio-Rad, 100–200 mesh, $Na^+$ form, 0.5 × 3 cm), and the eluate freeze-dried in vacuo. In cases where the enzymic reaction resulted in the partial cleavage of the methyl ester group of the aglycon, the two glycosides were always separated and further identified by $^1H$ NMR.

*Sialyltransferase preparation.*—The α2,3-sialyltransferase (EC 2.4.99.6) was purified and separated from the α2,6-sialyltransferase (EC 2.4.99.1) according to a published procedure[31]. Briefly, the α2,3- and α2,6-sialyltransferases were extracted from rat liver using Triton CF-54 as described by Weinstein et al.[32]. The detergent extracts containing both sialyltransferases were concentrated and partially purified on Cibacron Blue F3GA-Sepharose 6B, using NaCl (2.0 M) for elution, by a modification of the procedure of Sticher et al.[33]. The partially purified enzymes were separated on an affinity column prepared by covalently linking the hapten β-D-Gal-(1 → 3)-β-D-GlcNAc-$O(CH_2)_8CO_2H$, activated as its succinimidyl ester, to aminated epichlorohydrin-activated Sepharose 6B[34]. The α2,3-sialyltransferase, which binds strongly to the affinity column in the presence of cytidine diphosphate (CDP, 2.5–5 mM), was eluted using a buffer (10 mM sodium cacodylate) containing lactose (0.2 M), but no CDP. This preparation, about 82000-fold purified to a specific activity of 2.5 U/mg protein, was found devoid of α2,6-sialyltransferase activity when the product of preparative sialylation using **15** as an acceptor was analyzed by $^1H$ NMR spectroscopy. The α2,6-sialyltransferase, contained in the flow-through of the foregoing affinity column, was further purified to ~ 100000-fold (35 U/mg protein) by affinity chromatography on a CDP–hexanolamine Sepharose column as reported[32].

*Enzyme assays.*—The activities of the α2,3- and the α2,6-sialyltransferases were determined with their appropriate acceptors, compounds **18** and **15** respectively, following standard procedures[32]. Incubation mixtures contained 9 nmol CMP-[$^{14}$C]Neu5Ac (3,340 cpm/nmol), 2 mM acceptor substrate, 1 mg/mL bovine serum albumin, and the enzyme (0–0.2 mU) in 25 mM sodium cacodylate (pH 6.5) containing 0.5% Triton CF-54, in a total volume of 60 μL. After incubation at 37°C (10–30 min), the radioactive product was isolated as previously described[5]. One unit of enzyme activity is defined as the amount forming 1 μmol of product/min at saturating substrate concentrations. Protein concentrations were estimated using the method of Bradford[35].

*Kinetic studies.*—The apparent kinetic parameters of the α2,3- and the α2,6-sialyltransferases for action on synthetic acceptors were determined under similar assay conditions using a saturating concentration of CMP-[$^{14}$C]Neu5Ac[36]. Assays were performed in duplicate using 360 μU of enzyme. The oligosaccharide acceptor concentration was varied about the $K_m$ value (5–7 different concentrations), while the concentration of CMP-[$^{14}$C]Neu5Ac was kept constant at 560 μM (30 000 cpm). The time of incubation was varied to limit the CMP-[$^{14}$C]Neu5Ac consumption to 10–15%, and the reaction was terminated by the addition of 50 μL of 10 mM CTP. The radiolabelled product was isolated by the Sep-Pak method. The kinetic parameters, $K_m$ and $V_{max}$, were calculated using a computer program based on the method of Wilkinson[37].

*Preparative sialylation.*—Preparative sialylations using the β-D-Gal-(1 → 3/4)-D-GlcNAc α2,3- and β-D-Gal-(1 → 4)-D-GlcNAc α2,6-sialyltransferases were performed at 37°C in plastic tubes, with mixtures containing sodium cacodylate buffer (50 mM, pH 6.5), Triton CF-54 (0.5%), bovine serum albumin (1 mg/mL), and calf intestine alkaline phosphatase[38]. Recovery and purification of the reaction products proceeded as indicated above.

*Preparative galactosylation.*—The bovine milk D-GlcNAc β1,4-galactosyltransferase (EC 2.4.1.22, specific activity 6.5 units/mg of protein) and UDP-Gal were obtained from Sigma. The enzymic reactions were carried out at 37°C in plastic tubes, in a sodium cacodylate buffer (100 mM, pH 7.5) containing 20 mM manganese dichloride. The products were purified as indicated above for preparative fucosylation.

*8-(Methoxycarbonyl)octyl O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-(1 → 4)-3,6-di-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside* (**2**).—A solution of trimethylsilyl trifluoromethanesulfonate (0.614 mL, 2.6 mmol) in $CH_2Cl_2$ (4 mL) was added dropwise to a mixture of the disaccharide donor **1** (ref 19) (2.0 g, 2.6 mmol), drierite (4.0 g, crushed), and 8-methoxycarbonyloctanol (1.9 g, 10.1 mmol) in $CH_2Cl_2$ (30 mL) at 4°C. After being stirred for 0.5 h at 4°C, the mixture was slowly warmed up to room temperature and held for 1 h. After recooling to 4°C a further amount of the catalyst (0.307 mL, 1.3 mmol) in $CH_2Cl_2$ (2 mL) was added. The mixture was again slowly warmed up, and stirred at room temperature for 1 h prior to the addition of some triethylamine. The crude product, recovered by the

standard workup (see under *General*, above), was dried in vacuo and acetylated in 2:1 pyridine–$Ac_2O$. After the addition of MeOH the mixture was again worked up by the standard procedure, and the residual solvents coevaporated with an excess of toluene. The nonvolatile residue was chromatographed on silica gel using 2:1 toluene–EtOAc as eluent to provide **2** (1.40 g, 60%): $[\alpha]^{20}$ + 9.1° (*c* 0.73, $CHCl_3$); $^1$H NMR ($CDCl_3$): δ 7.90–7.70 (m, 4 H, aromatic), 5.75 (dd, 1 H, $J_{2,3}$ 11.0, $J_{3,4}$ 8.7 Hz, H-3), 5.34 (m, 2 H, H-1,4′), 5.13 (dd, 1 H, $J_{1',2'}$ 8.0, $J_{2',3'}$ 10.5 Hz, H-2′), 4.96 (dd, 1 H, $J_{3',4'}$ 3.2 Hz, H-3′), 3.67 (s, 3 H, $CO_2CH_3$), 2.22 (t, 2 H, *J* 7.5 Hz, $CH_2CO_2$), 2.14, 2.13, 2.07, 2.04, 1.97, 1.91 (6 s, 18 H, 6 OAc), 1.45 and 1.08 (2 m, 4 H and 8 H, $CH_2$). Anal. Calcd. for $C_{42}H_{55}NO_{20}$: C, 56.44; H, 6.16; N 1.56. Found C, 56.24; H, 6.19; N, 1.57.

*8-(Methoxycarbonyl)octyl O-(2,6-di-O-acetyl-β-D-galactopyranosyl)-(1 → 4)-3,6-di-O-acetyl-2-deoxy-2-phthalimido-β-D-glucopyranoside* (**6**).—A 1 M solution of NaOMe in MeOH (0.200 mL) was added to a solution of the disaccharide **2** (1.40 g, 1.56 mmol) in MeOH (40 mL), cooled to 4°C. After 1.5 h at 4°C the solution was deionized using IRC-50 resin ($H^+$ form). Filtration and evaporation of the solvent left the crude product **3** (1.0 g, 94%). A solution of crude **3** (0.776 g, 1.2 mmol) and *p*-toluenesulfonic acid monohydrate (60 mg) in dry acetone (60 mL) was refluxed for 3 h. After neutralization with $Et_3N$ the solvent was evaporated and the residue was chromatographed on silica gel (100:1 EtOAc–MeOH), providing **4** (0.575 g, 70%): $^1$H NMR ($CD_3OD$, reference DOH at δ 4.80): δ 7.80–7.60 (m, 4 H, aromatic), 5.10 (d, 1 H, $J_{1,2}$ 8.0 Hz, H-1), 4.38 (m, 2 H, H-1,3), 3.70 (s, 3 H, $CO_2CH_3$), 2.31 (*t*, *J* 7.5 Hz, $CH_2CO_2$), 1.65–1.00 [m, incl. 2 s, at 1.57 and 1.45, for $C(CH_3)_2$].

Compound **4** (0.575 g, 0.84 mmol) was acetylated in 2:1 pyridine–$Ac_2O$ for 24 h at 22°C. After the addition of MeOH and a standard work-up the remaining solvents were coevaporated with an excess of toluene, and the residue was chromatographed on silica gel (100:3 $CHCl_3$–MeOH), providing **5** (0.646 g, 90%): $[\alpha]_D$ + 13.8° (*c*, 1, $CHCl_3$); $^1$H NMR ($CDCl_3$): δ 7.90–7.70 (m, 4 H, aromatic), 5.74 (dd, 1 H, $J_{2,3}$ 10.5, $J_{3,4}$ 8.5 Hz, H-3), 5.34 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.88 (dd, 1 H, $J_{1'2'} \cong J_{2',3'}$ 6.5 Hz, H-2′), 3.67 (s, 3 H, $CO_2CH_3$), 2.23 (t, *J* 7.5 Hz, $CH_2CO_2$), 2.14, 2.13, 2.10, 1.91 (4 s, 12 H, 4 OAc), 1.30–1.54 [m, incl. 2 s, at 1.53 and 1.32, for $C(CH_3)_2$].

Compound **5** (0.575 g, 0.68 mmol) in 90% acetic acid (12 mL) was heated at 80°C for 2 h. After dilution with $CH_2Cl_2$ the solution was washed with water, aq $NaHCO_3$, and water. After drying over $MgSO_4$ the solvents were evaporated in vacuo, and the residue chromatographed on silica gel, providing **6** (0.452 g, 82%): $[\alpha]_D$ +12.1° (*c* 1.03, $CHCl_3$); $^1$H NMR ($CDCl_3$): δ 7.70–7.40 (m, 4 H, aromatic), 5.75 (m, 1 H, H-3), 5.35 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 4.86 (dd, 1 H, $J_{1',2'}$ 8.0, $J_{2',3'}$ 10.0 Hz, H-2′), 4.39 (d, 1 H, H-1′), 3.66 (m, incl. s for $CO_2CH_3$), 2.23 (t, 2 H, *J* 7.5 Hz, $CH_2CO_2$), 2.15, 2.14, 2.11, 1.90 (4 s, 12 H, 4 OAc), 1.40 and 1.10 (2 m, 4 H and 8 H, $CH_2$). Anal. Calcd. for $C_{38}H_{51}NO_{18}$: C, 56.36; H, 6.35; N, 1.72. Found: C, 55.76; H, 6.37; N, 1.70.

*8-(Methoxycarbonyl)octyl* O-*(3,4,6-tri-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranosyl)-(1 → 3)-*O*-(2,6-di-*O*-acetyl-β-*D*-galactopyranosyl)-(1 → 4)-3,6-di-*O*-acetyl-2-deoxy-2-phthalimido-β-*D*-glucopyranoside* (**8**).—Trimethylsilyl trifluoromethanesulfonate (0.036 mL, 0.060 mmol) in $CH_2Cl_2$ (0.5 mL) was added to a solution of the diol **6** (0.100 g, 0.123 mmol) in $CH_2Cl_2$ (5 mL). A solution of the imidate **7** (ref 39) (0.102 g, 0.176 mmol) in $CH_2Cl_2$ (4 mL) was slowly added to the above solution cooled to −70°C. After stirring at that temperature for 0.5 h. a further portion of the catalyst (0.018 mL, 0.030 mmol) in $CH_2Cl_2$ (0.5 mL) was added. After another 0.5 h at −70°C the reaction was stopped by addition of $Et_3N$, and the mixture worked up. The recovered residue was chromatographed on silica gel (100:2 $CHCl_3$–MeOH), providing the product **8** (0.120 g, 80%): $[\alpha]^{20}$ +20.0° (*c* 0.73, $CHCl_3$): $^1$H NMR ($CDCl_3$): δ 7.95–7.60 (m, 8 H, aromatic), 5.75 (dd, 1 H, $J_{2'',3''}$ 10.5, $J_{3'',4''}$ 9.2 Hz, H-3''), 5.61 (dd, 1 H, $J_{2,3}$ 10.5, $J_{3,4}$ 8.5 Hz, H-3), 5.47 (d, 1 H, $J_{1'',2''}$ 8.5 Hz, H-1''), 5.26 (d, 1 H, $J_{1,2}$ 8.5 Hz, H-1), 5.14 (dd, 1 H, $J_{4'',5''}$ 9.5 Hz, H-4''), 4.90 (dd, 1 H, $J_{1',2'}$ 8.5, $J_{2',3'}$ 9.5 Hz, H-2'), 3.68 (s, $CO_2CH_3$), 2.22 (t, *J* 7.5 Hz, $CH_2CO_2$), 2.12 (6 H), 2.10, 2.04, 1.86, 1.85, 1.57 (6 s, 21 H, 7 OAc), 1.40 and 1.20 (2 m, 4 H and 8 H, $CH_2$). Anal. Calcd. for $C_{58}H_{70}N_2O_{27}$: C, 56.77; H, 5.75; N, 2.28. Found: C, 56.20; H, 5.68; N, 2.25.

*8-(Methoxycarbonyl)octyl* O-*(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 3)-*O*-β-*D*-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**10**).—Hydrazine acetate (1.27 g, 13.8 mmol) was added to trisaccharide **8** (0.120 g, 0.098 mmol) in anhyd EtOH (15 mL). The mixture was refluxed for 18 h then concentrated, and the remaining solvents coevaporated with an excess of toluene. The residue was dried in vacuo and acetylated in 2:1 pyridine–$Ac_2O$ for 48 h. After quenching of the excess $Ac_2O$ with some MeOH, the mixture was worked up by the standard procedure. The remaining solvents were coevaporated with an excess of toluene. The residue was chromatographed on silica gel (100:9 $CHCl_3$–MeOH), providing the peracetylated trisaccharide intermediate **9**: $[\alpha]^{20}$ +4.3° (*c* 0.83 $CHCl_3$); $^1$H NMR ($CDCl_3$): δ 5.64 (d, 1 H, $J_{2,NH}$ 9.2 Hz, N*H*), 5.40–5.53 [m, 2 H, incl. 5.48 (t, *J* 9.5 and 10.0 Hz) and 5.45 (d, $J_{2,NH}$ 7.8 Hz, N*H*)], 5.34 (d, 1 H, $J_{3',4'}$ 3.5 Hz, H-4'), 3.68 (s, $CO_2CH_3$), 2.30 (t, *J* 7.5 Hz, $CH_2CO_2$), 2.12 (6 H), 2.11, 2.09, 2.05, 2.02, 2.01, 1.95, 1.91 (8 s, 27 H, 7 OAc and 2 NAc).

This material was O-deacetylated in anhyd MeOH (5 mL) in the presence of 0.2 M NaOMe in MeOH (0.200 mL). After reaction overnight at 22°C, deionization with Dowex 50 W X −8 ($H^+$ form), and filtration, the solvent was evaporated in vacuo. The recovered product was chromatographed on Bio-Gel P-2 with 1:1 water–EtOH as eluent, which provided the pure trisaccharide **10** (0.044 g, 60%): $[\alpha]_D$ −4.8° (*c* 0.83, $H_2O$; $^1$H NMR: see Table III.

*8-(Methoxycarbonyl)octyl* O-β-*D*-*galactopyranosyl-(1 → 4)-*O*-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl-(1 → 3)-*O*-β-*D*-galactopyranosyl-(1 → 4)-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**11**).—Trisaccharide **10** (20.0 mg, 0.027 mmol), UDP-Gal (22.0 mg), and D-GlcNAc β1,4-galactosyltransferase (5 U) were incubated for 24 h in the galactosylation buffer described above (3.6 mL). The usual isolation proce-

dure provided tetrasaccharide **11** (18.1 mg, 75%): $[\alpha]_D$ −9.4° (*c* 0.5, $H_2O$); $^1H$ NMR: see Table III.

*8-(Methoxycarbonyl)octyl* O-*(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 3)*-O-*β-*D*-galactopyranosyl-(1 → 4)*-O-*[α-*L*-fucopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**12**).—Trisaccharide **10** (15 mg, 0.020 mmol), GDP-fucose (33 mg), and the milk fucosyltransferase (56 mU) were incubated for 72 h in the fucosylation buffer (4 mL) as indicated above. Isolation and purification provided the tetrasaccharide **12** (14.0 mg, 79%): $[\alpha]_D$ −43.0° (*c*, 0.51, $H_2O$); $^1H$ NMR: see Table III.

*8-(Methoxycarbonyl)octyl* O-*β-*D*-galactopyranosyl-(1 → 4)*-O-*(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 3)*-O-*β-*D*-galactopyranosyl-(1 → 4)*-O-*[α-*L*-fucopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**13a**) *and the derived 8-carboxyoctyl glycoside* (**13b**)—*Method a:* Tetrasaccharide **12** (14.0 mg, 0.015 mmol), UDP-Gal (25 mg), and D-GlcNAc β1,4-galactosyltransferase (14.5 U, Sigma) were incubated for 48 h in the galactosylation buffer described above (3.2 mL). The usual isolation and purification procedure provided the pentasaccharide **13a** (13.2 mg, 82%): $[\alpha]_D$ −2.6° (*c* 0.52, $H_2O$); $^1H$ NMR: see Table III. The chemical synthesis of the heptasaccharide determinant recognized by antibody ACFH-18 has been reported[40].

*Method b:* A mixture of hexasaccharides **20a** and **20b** (1.7 mg) was incubated with *Clostridium perfringens* neuraminidase immobilized on agarose (Sigma, 1 U) in a sodium cacodylate buffer (50 mM, pH 5.2, 2 mL) at 37°C. After 24 h the mixture was diluted with water (10 mL) and filtered through an Amicon PM-10 membrane. The filtrate and washings were lyophilized, and the residue was dissolved in water (3 mL) and applied to two $C_{18}$ Sep-Pak cartridges. Each cartridge was washed with water (10 mL) prior to elution with MeOH (20 mL). After evaporation of the solvent, the residue was chromatographed on Iatrobeads (210 mg) giving **13a** (0.8 mg) and **13b** (0.7 mg). Compound **13b** was dissolved in dry MeOH and treated with diazomethane until TLC indicated complete conversion into **13a**. The $^1H$ NMR of this compound was identical to that of the product obtained by method a.

*8-(Methoxycarbonyl)octyl* O-*(5-acetamido-3,5-dideoxy-*D*-glycero-α-*D*-galacto-2-nonulopyranosylonic acid)-(2 → 3)*-O-*β-*D*-galactopyranosyl-(1 → 4)*-O-*(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 3)*-O-*β-*D*-galactopyranosyl-(1 → 4)*-O-*[α-*L*-fucopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-β-*D*-glucopyranoside* (**14a**) *and the 8-carboxyoctyl derived glycoside* (**14b**).—Compound **13a** (13.2 mg, 0.0012 mmol), CMP-Neu5Ac (20.0 mg), β-D-Gal-(1 → 3/4)-D-GlcNAc α2,3-sialyltransferase (63.2 mU), and alkaline phosphatase (30 U) were incubated in the sialylation buffer (3.3 mL) for 72 h. Isolation and purification of the products gave **14a** and **14b** (13.3 mg and 3.3 mg, 97%): $[\alpha]_D$ −17.0° (*c* 0.25, $H_2O$) for **14a**; $^1H$ NMR: see Table III.

*8-(Methoxycarbonyl)octyl* O-*(5-acetamido-3,5-dideoxy-*D*-glycero-α-*D*-galacto-2-nonulopyranosylonic acid)-(2 → 6)*-O-*β-*D*-galactopyranosyl-(1 → 4)*-O-*(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-(1 → 3)*-O-*β-*D*-galactopyranosyl-(1 → 4)-2-acetamido-*

*2-deoxy-β-D-glucopyranoside* (**19a**) *and the carboxyoctyl derived glycoside* (**19b**)—Compound **11** (21.2 mg, 0.02 mmol), CMP-Neu5Ac (25 mg), β-D-Gal-(1 → 4)-D-GlcNAc α2,6-sialyltransferase (228 mU), and alkaline phosphatase (50 U) were incubated for 72 h in 5 mL of the sialylation buffer. Isolation and purification provided **19a** and **19b** (8.4 mg and 5.8 mg, 50%, some loss of material occurred during recovery of the product): $[\alpha]_D$ −28.0° (*c* 0.42, $H_2O$) for **19a**; $^1H$ NMR: see Table III.

*8-(Methoxycarbonyl)octyl O-(5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid)-(2 → 6)-O-β-D-galactopyranosyl-(1 → 4)-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1 → 3)-O-β-D-galactopyranosyl-(1 → 4)-O-[α-L-fucopyranosyl-(1 → 3)]-2-acetamido-2-deoxy-β-D-glucopyranoside* (**20a**) *and the 8-carboxyoctyl derived glucoside* (**20b**).—Compound **19b** (5.8 mg, 0.005 mmol), GDP-fucose (10.3 mg) and milk fucosyltransferase (28 mU) were incubated for 68 h in the fucosylation buffer (2.5 mL). Isolation and purification provided **20b** (6.2 mg, 94%): $[\alpha]_D$ −38.9° (*c* 0.47, $H_2O$); In a preparative fucosylation of **19a**, a mixture of **20a** and 20b was obtained. $^1H$-NMR of **20a**: see Table III. The $^1H$ NMR spectra of natural glycolipids possessing terminal oligosaccharide moieties similar to those of **13a,b** and **20a,b**, determined in $(CD_3)SO–D_2O$, have already been reported [41,*].

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* The product of the enzymic fucosylation β-D-GlcNAc-(1 → 3)-β-D-Gal-(1 → 4)-β-D-Glc-(1 → 1)Cer has the L-fucose linked to the 3 position of the reducing Glc[42]. The purification of the α2,3-sialyltransferase by a procedure similar to that outlined in this manuscript has been reported in a footnote of a recent publication[43].

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