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Enzymatic synthesis of site-specifically (α1-3)-fucosylated polylactosamines containing either a sialyl Lewis x, a VIM-2, or a sialylated and internally difucosylated sequence

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Abstract

By using two different reaction pathways, we generated enzymatically three sialylated and site-specifically α1-3-fucosylated polylactosamines. Two of these are isomeric hexasaccharides Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc and Neu5Ac(α2-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)GlcNAc, containing epitopes that correspond to VIM-2 and sialyl Lewis x, respectively. The third one, nonasaccharide Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc, is a sialylated and internally difucosylated derivative of a trimeric *N*-acetyllactosamine. All three oligosaccharides have one fucose-free *N*-acetyllactosaminyl unit and can be used as acceptors for recombinant α1-3-fucosyltransferases in determining the biosynthesis pathways leading to polyfucosylated selectin ligands. © 1998 Elsevier Science Ltd.

Keywords: Enzymatic synthesis; Polylactosamine; (α1-3)-Fucosyltransferase; Sialyl Lewis x; VIM-2

Abbreviations: AEC, anion-exchange chromatography; FucT, (α1-3)-fucosyltransferase; GPC, gel-permeation chromatography; HPAEC, high-pH anion-exchange chromatography; Lex, Lewis x; LN, *N*-acetyllactosamine; MALDI-TOF MS, matrix-assisted laser desorption ionization/ time-of-flight mass spectrometry; SA, sialic acid (*N*-acetylneuraminic acid); sLex, sialyl Lewis x; WEFT, water eliminated Fourier transformation

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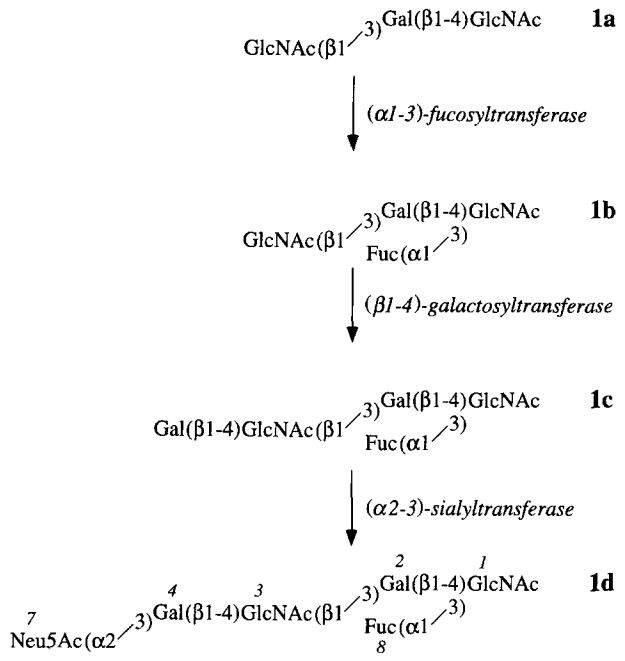
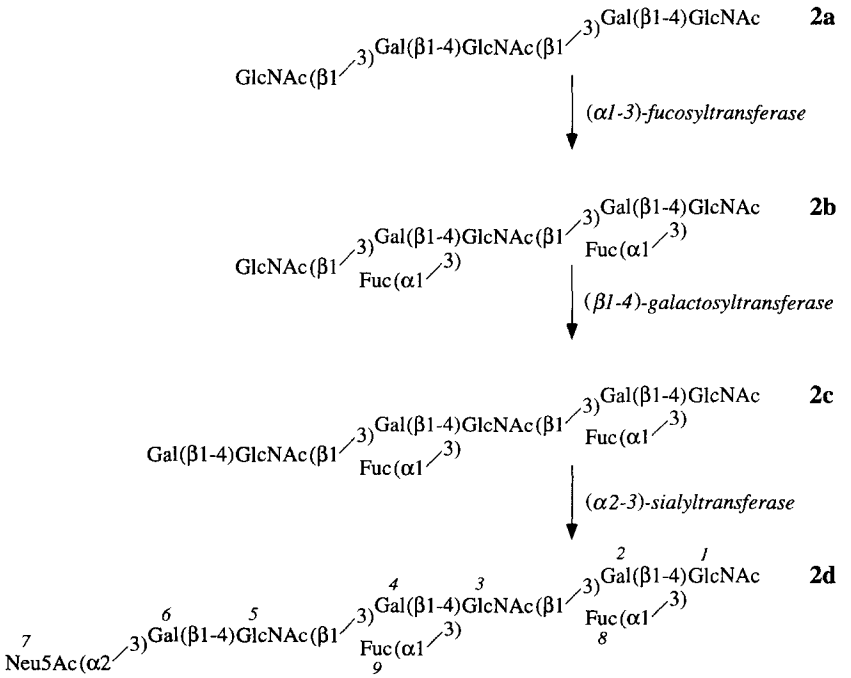
1. Introduction

The binding between selectins and their carbohydrate containing counterreceptors is crucial in the initial attachment of leukocytes to endothelium lining the blood vessels [1–6]. All selectins interact with the mucin-like glycoprotein PSGL-1 expressed on leukocytes [7–14]. Recently, PSGL-1 has been shown to carry a sialyl trimeric Lewis x epitope, consisting of a sialyl Lewis x (sLex) tetrasaccharide on top of a doubly fucosylated polylactosamine backbone [15]. The biosynthetic pathway for the polyfucosylated PSGL-1 glycan is not known. Of the five cloned human (α1-3)-fucosyltransferases, FucTIV and FucTVII are transcribed in leukocytic cell lines that express selectin ligands [16–21]. To investigate the relative roles of FucTIV and FucTVII in the control of polyfucosylated selectin ligand expression, we constructed three site-specifically but only partially prefucosylated oligosaccharide acceptors using (α1-3/4)-fucosyltransferase(s) from human milk [22,23].

Two different synthetic routes were used to direct the enzymatic transfer of (α1-3)-linked fucose to specific positions in oligomeric *N*-acetyllactosaminyl saccharides. In the first one, the synthesis of 'internally' (α1-3)-fucosylated sialyloligosaccharides involved (α1-3)-fucosylation of primer oligosaccharides, that carried a terminal β1-3-bonded residue. This GlcNAc does not serve as an acceptor site for human milk (α1-3/4)-fucosyltransferase(s) [24–26]. The products were then (β1-4)-galactosylated and terminally (α2-3)-sialylated as in Ref. [25]. One of the final products (**1d** in Scheme 1) thus obtained is a hexasaccharide corresponding to the VIM-2 epitope originally found on gangliosides of human myeloid cells [27]. The other one is an internally difucosylated nonasaccharide (**2d** in Scheme 2). A methyl glyco-



Scheme 1. Synthesis of the VIM-2 hexasaccharide (**1d**) and denotation of its monosaccharide residues.



Scheme 2. Synthesis of the internally difucosylated nonasaccharide (**2d**) and denotation of its monosaccharide residues.

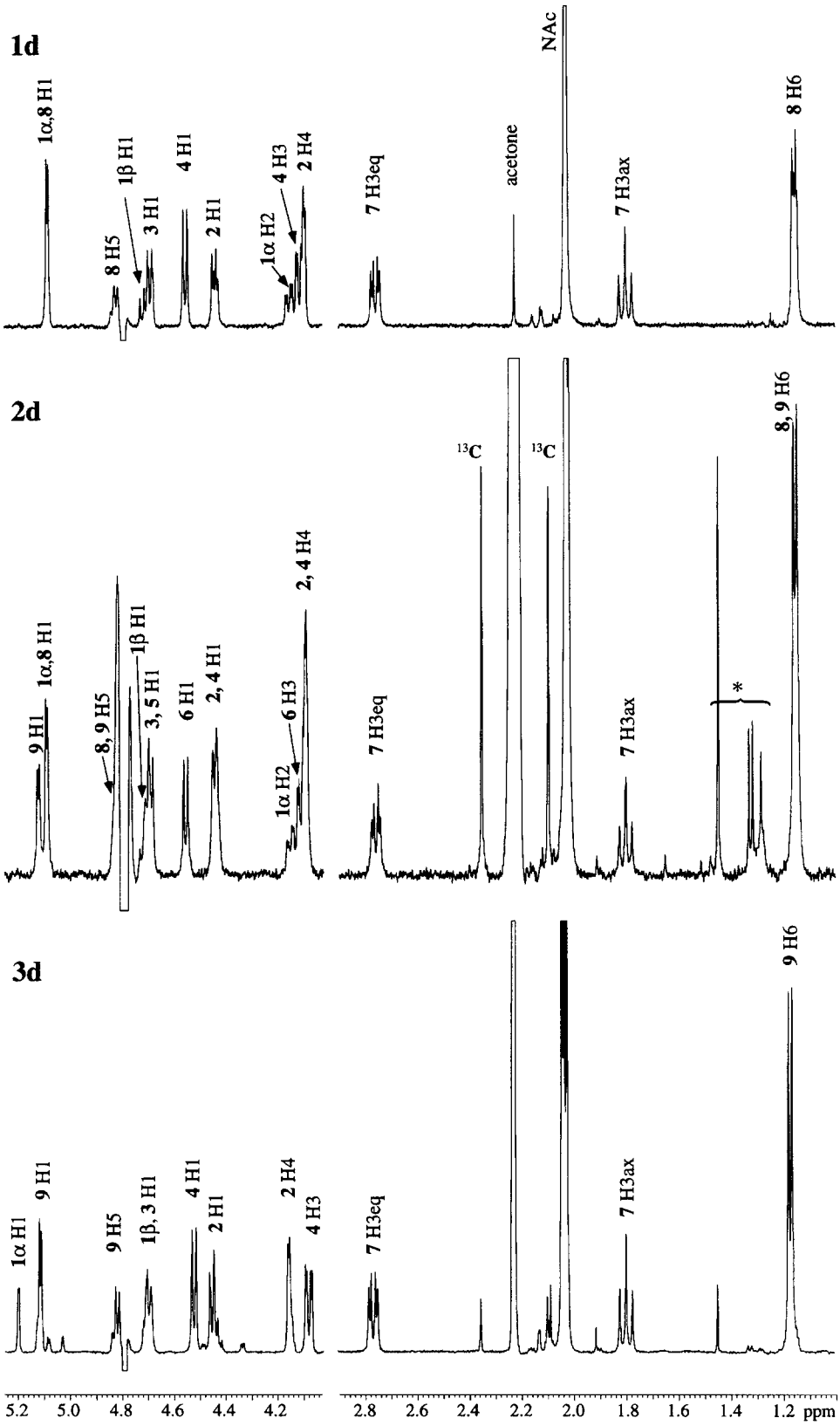


Fig. 1. Expansions of ^{1}H NMR spectra of the product saccharides **1d**, **2d** and **3d** at 23 °C. The denotation of the monosaccharide residues is shown in Scheme 1–3. The signals marked by an asterisk (*) arise from unknown contaminants and the resonances marked by ^{13}C are the ^{13}C satellites of acetone. The low intensity peaks seen in the spectrum of **3d** are due to reducing end ManNAc epimers of **3d**. For exact chemical shifts, see Table 1.

side analog of the latter one has been purified as an intermediate reaction product from a reaction mixture generated by human milk (α1-3/4)-fucosyltransferase(s) [28], but the first site-specific synthesis is described in the present experiments.

The second synthesis route, leading to the sLex-terminating hexasaccharide (**3d** in Scheme 3), started from the pentasaccharide Gal(β1-4)GlcNAc(β1-3)[GlcNAc(β1-6)]Gal(β1-4)GlcNAc (**3a**). We have recently shown that human milk (α1-3/4)-fucosyltransferase(s) do not react with inner *N*-acetyllactosaminyl units that carry a (β1-6)-linked *N*-acetylglucosaminyl branch at the Gal [26,29]. Here, we used this observation to synthesize the sialyl Lewis x hexasaccharide (**3d**) by directing the enzymatic (α1-3)-fucosylation to the distal *N*-acetyllactosaminyl unit of a sialylated dimer of *N*-acetyllactosamine (**3a**). After enzymatic (α2-3)-sialylation and (α1-3)-fucosylation, the protecting (β1-6)-GlcNAc was removed with β-*N*-acetylhexosaminidase.

2. Results

Synthesis of the hexasaccharide **1d** (Scheme 1).— The primer trisaccharide GlcNAc(β1-3)Gal(β1-4)GlcNAc (**1a**) was incubated with GDP-Fuc and (α1-3/4)-fucosyltransferase(s) from human milk. The reaction mixture was desalted by a column of Dowex AG 50 (H^+) and Dowex AG 1 (OAc^-) and the reaction product **1b** was purified by gel-permeation chromatography (GPC). The retention time of the product in GPC was 15.5 min (not shown). Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) of the product gave a major peak at *m/z* 755.6 [calculated *m/z* 755.7 for $(M + Na)^+$ of $dHex_1Hex_1HexNAc_2$].

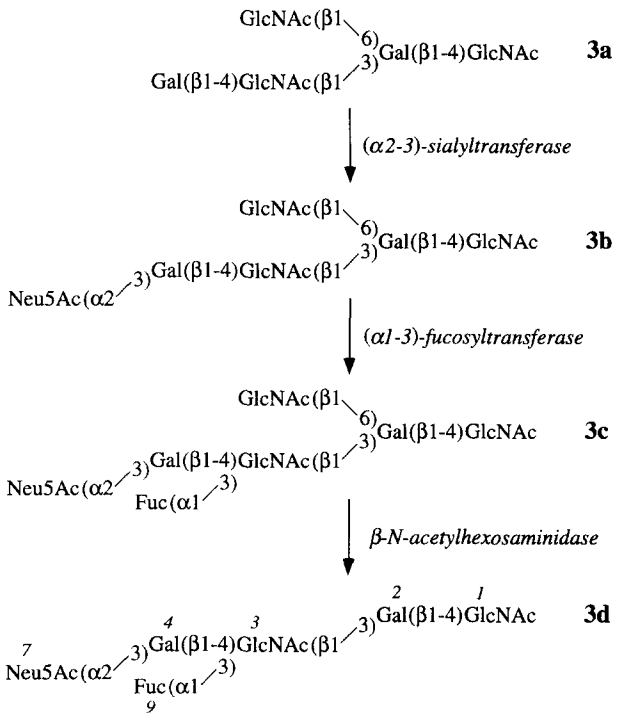
The tetrasaccharide **1b** was then incubated with UDP-Gal and bovine milk (β1-4)-galactosyltransferase. The product **1c** was desalted and purified by GPC; the retention time of the product was 14.6 min. MALDI-TOF MS showed that the reaction was complete; the product gave a peak at *m/z* 917.9 [calculated *m/z* 917.8 for $(M + Na)^+$ of $dHex_1Hex_2HexNAc_2$].

The pentasaccharide **1c** (670 nmol) was incubated with CMP-Neu5Ac and human placental microsomes containing α2-3-sialyltransferase activity. In anion-exchange chromatography (AEC) the major product eluted at the position of monosialyloligosaccharide; after desalting by GPC (retention time 13.4 min), the amount of the product **1d** was 540 nmol.

Table 1
^{1}H NMR chemical shifts (ppm) of structural reporter groups of glycans at 500 MHz, 23 °C

Reporter group	Residue[a]	Glycans				
		SA–LN–LN–LN[c]	**SA–LN–Lex–Lex–Me**[d]	**1d**	**2d**	**3d**
H-1	1α	5.204	–	5.090	5.090	5.205
	1β	4.718	4.472	4.725	4.726	4.718
	2[b]	4.465	4.431	4.440/4.447	4.437/4.445	4.458/4.462
	3[b]	4.701	4.707	4.693/4.697	4.705/4.709	4.702/4.706
	4	4.465	4.450	4.559	4.448	4.530
	5	4.695	4.698	–	4.693	–
	6	4.558	4.558	–	4.558	–
	8	–	5.086	5.090	5.090	–
	9	–	5.122	–	5.121	5.120
H-2	1α	n.d.	–	4.157	4.157	n.d.
H-3	4	n.d.	n.d.	4.117	n.d.	4.086
	6	4.116	n.d.	–	4.116	–
H-3ax	7	1.798	1.797	1.798	1.798	1.793
H-3eq	7	2.758	2.759	2.758	2.758	2.764
H-4	2	4.158	n.d.	4.098	4.097	4.163
	4	4.158	n.d.	n.d.	4.097	n.d.
H-6	8[b]	–	1.145	1.149/1.155	1.151	–
	9	–	1.152	–	1.151	1.167

[a] For numbering of the residues, see Scheme 1–3.
[b] If two values are given, they correspond to the two anomeric forms of residue 1.
[c] Present synthesis; the residues are numbered as in Scheme 2.
[d] Data from Ref. [28], acquired at 400 MHz, 300 K. The residues are numbered as in Scheme 2.
n.d., not determined; –, not applicable.



Scheme 3. Synthesis of the sLex-terminating hexasaccharide (**3d**) and denotation of its monosaccharide residues.

The ^{1}H NMR spectrum (Fig. 1 and Table 1) confirms that the sialylated product is the VIM-2 hexasaccharide **1d**. The spectrum reveals reporter group signals from all six monosaccharides in appropriate strength. The chemical shifts of the 'reporter groups' observed for **1d** are quite similar to those of the VIM-2 glycoside Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc–O-$CH_2(CH_2)_7CO_2CH_3$ reported by Kashem et al. [30]. The resonances originating from the reducing end area of **1d** resemble closely those of pentasaccharide Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc (Niemelä et al., in preparation) while the signals from the non-reducing area are similar to those of the fucose-free heptasaccharide Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc (see Table 1). In particular, the H-1 and H-3 resonances of Gal(4) and the H-3ax and H-3eq of Neu5Ac(7) are typical of the fucose-free Neu5Ac(α2-3)Gal(β1-4)GlcNAc sequence. On the other hand, the H-4 signal of Gal(2) at 4.098 ppm is characteristic to the fucose-containing reducing end Lex group in Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)[Fuc(α1-3)]GlcNAc (Niemelä et al., in preparation). The characteristic chemical shift for GlcNAc(1) H-2α [[23,31], Niemelä et al., in preparation] indicates also that the fucose is linked to GlcNAc(1).

Synthesis of the nonasaccharide **2d** (Scheme 2).— The primer pentasaccharide GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc (**2a**) was α1-3-fucosylated using GDP-[U-^{14}C]Fuc and human milk (α1-3/4)-fucosyltransferase(s) as reported by Niemelä et al. (in preparation). The major product of the reaction (87% of radioactivity) represented a heptasaccharide that was isolated by paper chromatography.

The heptasaccharide product **2b** was (β1-4)-galactosylated. In MALDI-TOF MS, the desalted product **2c** (Fig. 2) revealed a major peak at *m/z*

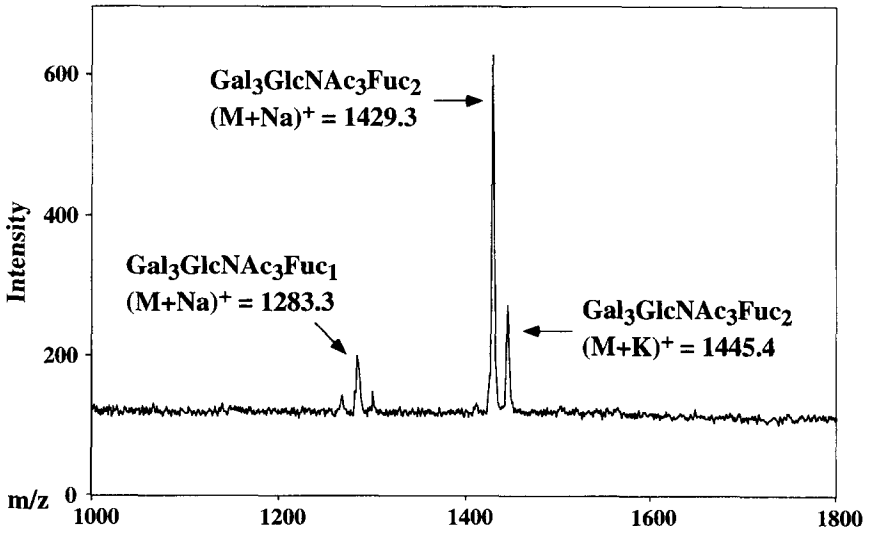


Fig. 2. MALDI-TOF mass spectrum of the octasaccharide **2c**. The two major peaks at *m/z* 1429.3 and 1445.4 correspond to $(M + Na)^+$ and $(M + K)^+$ of **2c**, respectively. The signal at *m/z* 1283.3 is assigned to $(M + Na)^+$ of analogous heptasaccharide which have lost one fucosyl residue.

1429.3 that was assigned to $(M + Na)^+$ of $dHex_2Hex_3HexNAc_3$ (calculated *m/z* 1429.3). The signal at *m/z* 1445.4 is assigned to $(M + K)^+$ of $dHex_2Hex_3HexNAc_3$ (calculated *m/z* 1445.3). The signal at *m/z* 1283.3 is assigned to $(M + Na)^+$ of $dHex_1Hex_3HexNAc_3$ (calculated *m/z* 1283.1); its size corresponds to 14% of total polylactosamines [32]. Degradation of **2c** to the monofucosylated compounds may have occurred during the mass spectrometric analysis.

The difucosylated saccharide **2c** (200 nmol) was sialylated as above. The reaction was terminated by running the reaction mixture on a column of Dowex AG 50 (H^+) and Dowex AG 1 (OAc^-); the sialylated product was eluted with CH_3COOH [33]. The retention time of the product in AEC was typical of monosialylated oligosaccharides. The yield of desalted **2d** was 160 nmol.

The ^1H NMR spectrum of **2d** (Fig. 1 and Table 1) reveals reporter group signals of nine monosaccharides, many of the resonances resembling closely those of the methyl glycoside of **2d** [28]. Besides the resonances originating from the hexasaccharide backbone, signals of two fucoses and one *N*-acetylneuraminic acid are present in the spectrum of **2d**. The spectrum shows typical signals of a reducing end Lex unit (Niemelä et al., in preparation), together with resonances of a non-reducing end sialyl-*N*-acetyllactosaminyl unit (see Table 1). Hence, the second (α1-3)-bonded fucosyl residue must be located at the middle *N*-acetyllactosaminyl unit. Taken together, the NMR-data confirm the structure of **2d**.

Synthesis of the hexasaccharide **3d** (Scheme 3).— The primer pentasaccharide Gal(β1-4)GlcNAc(β1-3)[GlcNAc(β1-6)]Gal(β1-4)GlcNAc (**3a**) was sialylated and desalted as above and the product **3b** was purified by AEC. From two runs, the major peak eluting at the position of monosialyloligosaccharides was pooled. The retention time of the pooled acidic saccharide **3b** in GPC was 13.1 min.

The sialylated hexasaccharide **3b** was further incubated with GDP-Fuc and human milk α1-3/4-fucosyltransferase. The reaction was terminated by injecting the reaction mixture into a Superdex Peptide HR 10/30 column in four batches; the peak at 12.9 min was pooled. The product **3c** was purified by AEC, where it behaved like monosialylsaccharides; it was then desalted by GPC. A small sample of **3c** was run in high-pH anion-exchange chromatography (HPAEC). A major peak was observed at 18.5 min and a minor peak at 22.4 min (not shown). The former represented **3c** and the latter probably the reducing end ManNAc epimer. The fucosylation was complete as no detector response was observed at the position of the nonfucosylated acceptor **3b**, at 32 min. The remarkable shift in retention time in HPAEC is typical for (α1-3)-fucosylation [34].

The (β1-6)-linked *N*-acetylglucosaminyl branch of the fucosylated heptasaccharide **3c** (580 nmol) was removed with β-*N*-acetylhexosaminidase from jack bean. The reaction product **3d** was purified by consecutive GPC, AEC and GPC runs. The retention time of the product **3d** in GPC was 13.3 min, which indicates a loss of a GlcNAc from the molecule. The yield of the putative hexasaccharide **3d** was 520 nmol.

Comparison with the ^1H NMR spectra of **1d** and **2d** (Fig. 1 and Table 1) shows that in **3d** the GlcNAc(1) is not fucosylated. On the other hand, the reporter group signals originating from the non-reducing end area are similar to those reported for sLex-glycans [35–39]. In addition, the resonances from the reducing end area are identical with those of Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)GlcNAc [29].

3. Discussion

In the present report, we show that sLex-terminating polylactosamines, free from VIM-2 type isomers or polyfucosylated products, can be synthesized by using human milk (α1-3/4)-fucosyltransferase(s) and acceptors in which the 'inner' *N*-acetyllactosaminyl units are temporarily blocked by (β1-6)-linked *N*-acetylglucosaminyl branches. The blocking *N*-acetylglucosaminyl branches can be transferred to the 'inner' galactoses by using a (β1-6)-*N*-acetylglucosaminyltransferase activity that is present in serum of mammals [40–42]. After the site-specific (α1-3)-fucosylation at the unblocked distal sialylated *N*-acetyllactosaminyl unit, the blocking *N*-acetylglucosaminyl residues can be removed by β-*N*-acetylhexosaminidase. In an earlier study, we used this approach to convert the neutral tetrasaccharide Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc to neutral pentasaccharide Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-3)Gal(β1-4)GlcNAc in a site-specific manner [29]. Analogous temporary blocking has been used in directing the fucosylation away from the non-reducing area by using a temporary (α2-6)-bonded *N*-acetylneuraminyl residue located at the terminal non-reducing end galactose [30].

The 'internally' (α1-3)-fucosylated sialyloligosaccharides were synthesized in our present study by using a similar synthesis route as reported by Kashem et al. [25], simply by consecutively (α1-3)-fucosylating, (β1-4)-galactosylating and (α2-3)-sialylating the primer oligosaccharides, that contained a terminal (β1-3)-bonded GlcNAc residue. This reaction sequence is based on the facts that (i) the unsubstituted (β1-3)-linked *N*-GLcNAc at the non-reducing terminus is not an acceptor for the fucosyltransferases of human milk [24–26] and (ii) the 'internal' fucosyl residue does not prevent the galactosylation by bovine milk β1-4-galactosyltransferase [25,30]. Successful sialylation of **1c** and **2c** by (α2-3)-sialyltransferase of human placenta shows that an (α1-3)-fucosylated, 'inner' *N*-acetyllactosaminyl unit of the acceptor does not prevent the reaction.

Many (α1-3)-fucosylation variants of unbranched, terminally (α2-3)-sialylated *N*-acetyllactosaminyl saccharides can now be obtained in acceptable yields by combining the two synthetic routes used in the present study. The action of human milk fucosyltransferase(s) can be directed to specific *N*-acetyllactosaminyl units in polylactosamines (i) by elongating the backbone after the fucosyltransferase reaction, and (ii) by using (β1-6)-GlcNAc branches to block the reaction at some sites. The identity of the milk enzymes that are responsible for these findings has not been very clearly established, but it appears possible that in milk the Lewis enzyme or FucTIII (EC 2.4.1.65) is accompanied by the plasma-type enzyme or FucTVI (EC 2.4.1.152) [28].

Terminally (α2-3) sialylated polylactosaminoglycans having several α1-3-fucosyl residues are putative high-affinity ligands for E-selectin [43–45]. The presence of the trifucosylated glycan (sialyl-triLex) of PSGL-1 [15] suggests that even P-selectin binds avidly to sialylated and multifucosylated polylactosaminoglycans. The availability of synthetic saccharides of this type will be valuable in future studies concerning the biological relevance of multiple (α1-3)-fucosylation.

The contributions of the two leukocytic (α1-3)-fucosyltransferases, FucTIV and FucTVII, in the biosynthesis of polyfucosylated selectin ligands is an important and largely unresolved issue. FucTIV-transfected CHO– and Jurkat cells show high levels of anti-VIM-2 reactivity concomitantly to low levels of sLex expression [17,46]. On the other hand, FucTVII overexpression in Jurkat cells induces high levels of sLex-related epitopes but eliminates the VIM-2 epitope [46]. These and other data have suggested that FucTIV and FucTVII may collaborate in synthesizing polyfucosylated selectin ligands. This hypothesis can now be elucidated by using the newly synthesized, partially prefucosylated oligosaccharides as acceptors for single species of recombinant (α1-3)-fucosyltransferases.

4. Experimental

Oligosaccharides and monosaccharide nucleotides.—The primer oligosaccharides were synthesized as described earlier: GlcNAc(β1-3)Gal(β1-4)GlcNAc (**1a**) [47]; GlcNAc(β1-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)GlcNAc (**2a**) [40]; Gal(β1-4)GlcNAc(β1-3)[GlcNAc(β1-6)]Gal(β1-4)GlcNAc (**3a**) [29,42]. The heptasaccharide Neu5Ac(α2-3)Gal(β1-4)GlcNAc(β1-3)Gal(β1-4)-GlcNAc(β1-3)Gal(β1-4)GlcNAc was obtained by (β1-4)-galactosylating and (α2-3)-sialylating the pentasaccharide **2a** [40]. CMP-Neu5Ac, GDP-Fuc, UDP-Gal and UDP-GlcNAc were from Sigma Chemical, St. Louis, MO. GDP-[U-^{14}C]Fuc was from Amersham (Buckinghampshire, UK).

Enzymatic methods.—The human milk (α1-3)-fucosyltransferase (EC 2.4.1.37) reactions were performed essentially as described [23,48], but in some reactions additional GDP-Fuc and fresh enzyme were added during the reaction.

The galactosylation with bovine milk (β1-4)-galactosyltransferase (EC 2.4.1.90; Sigma) was carried out as described [49,50].

Human placental microsomes, containing (α2-3)-sialyltransferase activity [51] (EC 2.4.99.4), were prepared as in Ref. [38]. Acceptor saccharide and 10-fold molar excess of the donor CMP-Neu5Ac were dried in a vacuum centrifuge and the (α2-3)-sialyltransferase reactions were started by adding 1 μL of microsome suspension (80 μg of protein) in 0.1 M Tris–maleate (pH 6.7) per 4 nmol of acceptor. The reactions were terminated after 19–24 h incubation at 37 °C.

Digestion with β-*N*-acetylhexosaminidase from jack bean (EC 3.2.1.30; Sigma) was carried out in 1.4 mL of 50 mM sodium citrate (pH 4.0) containing 5.1 U of enzyme and 10.5 mg/mL of γ-galactonolactone [52]. The reaction mixture was incubated at 37 °C overnight.

Chromatographic methods.—Filtration through a column of Dowex AG 50 (H^+) and Dowex AG 1 (OAc^-) was used for desalting of oligosaccharides. The neutral oligosaccharides were eluted with water

and the monosialylated saccharides were eluted with 0.5 M CH_3COOH as in Ref. [33].

Paper chromatography was performed as described [53] with the upper phase of *n*-butanol:CH_3 COOH:water 4:1:5 by volume.

GPC on a column of Superdex Peptide HR 10/30 (syntheses of **1d** and **3d**) or HR 10/75 (synthesis of **2d**; Pharmacia, Sweden) was used for desalting and quantitation of oligosaccharides [38]. The elution was performed at a flow rate of 1 mL/min using water for neutral oligosaccharides and 50 mM ammonium hydrogen carbonate for anionic oligosaccharides. The effluent was monitored with an UV detector at 214 or 205 nm, and the amount of oligosaccharide in each peak was quantified by reference to an external calibrant (GlcNAc and Neu5Ac).

HPAEC with pulsed amperometric detection was carried out with a Dionex 4500i HPLC system (Dionex, CA) equipped with a CarboPac PA-1 column (4 × 250 mm) at a flow rate of 1 mL/min [54]. The anionic saccharides were eluted with a linear gradient of 25–200 mM NaOAc in 100 mM NaOH over 150 min.

AEC was carried out on a Mono Q (5/5) column (Pharmacia) at a flow rate of 1 mL/min. The elution was performed isocratically with water for 4 min, then with linear gradients of 0–0.025 M NaCl over 8 min, 0.025–0.25 M NaCl over 8 min, and finally, 0.25–0.5 M NaCl over 4 min. The effluent was monitored with an UV detector at 214 or 205 nm.

^{1}H NMR spectroscopy.—Prior to NMR experiments, the saccharides were repeatedly dissolved in ^2H_2O and evaporated to dryness. The samples were then dissolved in 600 μL of ^2H_2O (99.996 atom %; Cambridge Isotope Laboratories, Woburn, MA, USA) and the NMR experiments were performed on a Varian Unity 500 spectrometer at 23 °C. A modified WEFT sequence was used for water suppression [55]. The ^{1}H chemical shifts were referenced to internal acetone (2.225 ppm).

MALDI-TOF MS.—MALDI-TOF MS in the reflector mode was performed with a BIFLEX™ mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany), using a 337-nm nitrogen laser. The sample solution (5–10 pmol of saccharide in 0.5–1.0 μL water) and the matrix solution (20 μg of 2,5-dihydroxybenzoic acid in 2 μL water) were mixed on the target plate and dried with a gentle stream of air. Dextran standard 1000 from *Leuconostoc mesenteroides* (Fluka Chemica–Biochemica) was used as an external calibrant.

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