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Improved enzymatic synthesis of a highly potent oligosaccharide antagonist of L-selectin

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Abstract The polylactosamine sLexβ1–3′(sLexβ1–6′)LacNAcβ1–3′(sLexβ1–6′)LacNAcβ1–3′(sLexβ1–6′)LacNAc (7) (where sLex is Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc and LacNAc is Galβ1–4GlcNAc) is a nanomolar L-selectin antagonist and therefore a potential anti-inflammatory agent (Renkonen et al. (1997) Glycobiology, 7, 453). Here we describe an improved synthesis of 7. The octasaccharide LacNAcβ1–3′LacNAcβ1–3′LacNAcβ1–3′LacNAc (4) was converted into the triply branched undecasaccharide LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)-LacNAc (5) by incubation with UDP-GlcNAc and the midchain β1,6-GlcNAc transferase activity of rat serum. Glycan 5 was enzymatically β1,4-galactosylated to LacNAcβ1–3′(LacNAcβ1–6′)LacNAcβ1–3′(LacNAcβ1–6′)LacNAcβ1–3′(LacNAcβ1–6′)-LacNAc (6). Combined with the enzymatic conversion of 6 to 7 (Renkonen et al., loc. cit.) and the available chemical synthesis of 4, our data improve the availability of 7 for full assessment of its anti-inflammatory properties.

Key words: Enzymatic synthesis; Tetravalent sLex glycan; L-Selectin antagonist; Midchain β1,6-GlcNAc transferase

1. Introduction

Lymphocyte extravasation to rejecting graft is initiated by interactions between L-selectin and saccharides that carry epitopes related to the tetrasaccharide Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc, which is known as the sialyl Lewis x (sLex) determinant [1,2]. Inflammatory stimuli induce the expression of the sLex type saccharides on the surface of the endothelium [3] to attract L-selectin-expressing lymphocytes to the graft. We have shown previously that a triply branched poly-*N*-acetyllactosamine, sLexβ1–3′(sLexβ1–6′)LacNAcβ1–3′(sLexβ1–6′)LacNAcβ1–3′(sLexβ1–6′)LacNAc (**7**) (where LacNAc is the disaccharide Galβ1–4GlcNAc), inhibits efficiently lymphocyte adhesion to the endothelium in vitro [4,5]. The presence of exogenous glycan **7** at 1 nM was shown to reduce lymphocyte adhesion to the rejection-activated endothelium in cardiac transplants by 50%, probably through competition for L-selectin with endothelial sLex saccharides. This property makes glycan **7** an interesting anti-inflammatory drug candidate.

Here, we describe a simplified and upscalable synthesis route to the L-selectin antagonist **7**. In the key step of the new synthesis, the linear octasaccharide LacNAcβ1–3′LacNAcβ1–3′LacNAcβ1–3′LacNAc (**4**) was incubated with UDP-GlcNAc and the midchain β1,6-GlcNAc transferase activity present in rat serum [6,7]. The product, LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc (**5**), was then enzymatically β1,4-galactosylated to yield the tetradecasaccharide LacNAcβ1–3′(LacNAcβ1–6′)LacNAcβ1–3′(LacNAcβ1–6′)LacNAcβ1–3′(LacNAcβ1–6′)LacNAc (**6**). To complete the novel synthesis of the L-selectin antagonist **7**, glycan **6** is sialylated and fucosylated as described already in our previous report [4].

2. Materials and methods

2.1. Synthesis of the heptasaccharide 1

Details of the synthesis and characterization of the heptasaccharide LacNAcβ1–3′(Fucα1–3)LacNAcβ1–3′LacNAc (**1**) will be described elsewhere (Niemelä et al., in preparation). Briefly, GlcNAcβ1–3′LacNAcβ1–3′LacNAc [8], was α1,3-fucosylated partially by using α1,3/4 fucosyltransferase(s) of human milk [9]. The fraction of monofucosylated products was isolated by paper chromatography and the hexasaccharide GlcNAcβ1–3′(Fucα1–3)LacNAcβ1–3′LacNAc was isolated from this fraction by WGA-agarose chromatography (Niemelä et al., loc. cit.) and converted to radiolabeled glycan **1** by enzymatic β1,4-galactosylation.

The difucosylated glycan LacNAcβ1–3′(Fucα1–3)LacNAcβ1–3′(Fucα1–3)LacNAc was obtained in an analogous fashion, starting from an exhaustively α1,3-fucosylated sample of GlcNAcβ1–3′LacNAcβ1–3′LacNAc.

2.2. Enzymatic methods

The β1,3-GlcNAc transferase (EC 2.4.1.149) reaction and the β1,4-Gal transferase (EC 2.4.1.90) reaction were carried out essentially as described in [10] and [11], respectively. The multiple midchain β1,6-GlcNAc transferase reaction was performed by incubating the acceptor with UDP-GlcNAc and the β1,6-GlcNAc transferase activity present in rat serum that was concentrated 4–5 fold by ultrafiltration [7].

2.3. Acid hydrolysis

The fucose residue was removed from neutral polylactosamine backbones by mild acid hydrolysis as described by Renkonen et al. [12].

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Abbreviations: Fuc, L-fucose; Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; HexNAc, *N*-acetyl-D-glucosamine or *N*-acetyl-D-mannosamine; HPAEC, high pH anion exchange chromatography; LacNAc, *N*-acetyllactosamine or Galβ1–4GlcNAc; MALDI-TOF-MS, matrix-assisted laser desorption/ionization mass spectrometry with time-of-flight detection; MH, maltoheptaose; MP, maltopentaose; Neu5Ac, *N*-acetyl-neuraminic acid; NMR, nuclear magnetic resonance; PAD, pulsed amperometric detection; sLex, sialyl Lewis x or Neu5Acα2–3Galβ1–4(Fucα1–3)GlcNAc

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Fig. 1. Outline of the present enzymatic synthesis of primer **4**. The fucose residue in the midchain of the primer, heptasaccharide **1**, was required to inhibit the branching reactions [18] that would have occurred in our system at the non-fucosylated acceptor because of the presence of a contaminating midchain β1,6-GlcNAc transferase activity [7,8].

2.4. Chromatographic methods

Descending paper chromatography was carried out as described [7] using *n*-butanol/acetic acid/water (10:3:7 v/v) as the solvent.

Gel permeation chromatography was performed as in [4].

HPAE chromatography with either pulsed amperometric detection or liquid scintillation counting was carried out as described [7]. The oligosaccharide content of individually pooled peaks was estimated by Superdex 75 HR 10/30 chromatography with UV-detection [4].

2.5. MALDI-TOF-MS

MALDI-TOF mass spectrometry was performed as described [7].

Table 1
^{1}H-NMR chemical shifts of structural reporter groups of glycans **2**, **3**, **4**, **5** and **6** at 23°C

Reporter group	Residue	Glycan				
		2	**3**	**4**	**5**	**6**
H-1	1	5.204 (α)	5.203 (α)	5.204 (α)	5.212 (α)	5.210 (α)
		4.720 (β)	n.d.[a]	4.720 (β)	4.730 (β)	4.726 (β)
	2	4.460	4.46	4.465	4.456	4.460
	3	4.712	4.712	4.701/4.698[b]	4.702/4.696	4.701/4.697
	4	4.445	4.445	4.465	4.456	4.460
	5	4.696	4.695	4.701	4.696	4.697
	6	4.467	4.467	4.465	4.456	4.460
	7	4.680	4.700	4.701	4.696	4.697
	8	–[c]	4.479	4.479	4.479	4.480
	9	–	–	–	4.585	4.625/4.619
	10,11	–	–	–	4.593	4.639
	12,13,14	–	–	–	–	4.464
	15	5.115	5.115	–	–	–
H-4	2	4.154	4.157	4.156	4.146	4.148
	4	4.098	4.098	4.156	4.146	4.148
	6	4.154	4.157	4.156	4.146	4.148
H-6	15	1.151	1.151	–	–	–

Numbering of the residues is shown in Figs. 1 and 5.
[a]n.d., not determined.
[b]The two chemical shifts given arise from signals representing the two anomeric forms of the glycan.
[c]–, not appropriate.

2.6. ^{1}H-NMR spectroscopy

^{1}H-NMR spectroscopy was performed as described by Maaheimo et al. [13].

3. Results

3.1. Enzyme-assisted synthesis of the linear octasaccharide primer LacNAcβ1–3′LacNAcβ1–3′LacNAcβ1–3′LacNAc (4)

Glycan **4**, which is involved in the key step in the present synthesis of glycan **7**, has been synthesized chemically [14,15]. We synthesized it enzymatically. For this, the heptasaccharide LacNAcβ1–3′(Fucα1–3)LacNAcβ1–3′LacNAc (**1**) was first elongated by the β1,3-GlcNAc transferase activity of human serum [16,17]. The fucosylated derivative of LacNAcβ1–3′LacNAcβ1–3′LacNAc was used as acceptor instead of the fucose-free hexasaccharide itself because the fucosyl residue prevents the action of the midchain β1,6-GlcNAc transferase activity of serum [18], which catalyzes the formation of GlcNAc-branches at the inner galactose residues of the hexasaccharide.

The resulting glycan **2** (see Fig. 1 for the structure) was purified by gel filtration, followed by HPAE-chromatography (not shown), and in some experiments by paper chromatography (R_{MP} = 0.74, R_{MH} = 1.10). In three separate experiments, the actual yields of purified glycan **2** averaged 33%. The MALDI-TOF mass spectrum (not shown) confirmed that the product had the molecular mass of $Gal_3GlcNAc_4Fuc$; a major peak assigned to $(M+Na)^+$ was observed at *m/z* 1485.4 (calc. *m/z* 1485.5) and an accompanying signal, assigned to $(M+K)^+$ was seen at *m/z* 1501.4 (calc. *m/z* 1501.6). An 'impurity peak' (16%) of the spectrum at *m/z* 1282.4 was assigned to $(M+Na)^+$ of $Gal_3HexNAc_3Fuc$. As this spectrum was obtained from a HPAE-chromatographically purified sample, this species may represent a reducing-end ManNAc epimer of the acceptor saccharide (**1**) [19]. Prompt fragmentation of HexNAc units has not been observed with this type of glycans in the MALDI-TOF system used. The ^{1}H-NMR spectrum confirmed the structure of **2** (Table 1). As expected, no resonances were observed around 4.58–4.59 ppm, in the area of the reported H-1 signals of β1,6-bonded GlcNAc residues of polylactosamines [7,20,21]. The successful terminal β1,3-*N*-acetylglucosaminylation of glycan **1** at its LacNAcβ1–3′(Fucα1–3)LacNAc determinant in the present experiments is sharply contrasted by the unreactivity of Fucα1–3LacNAc with the serum β1,3-GlcNAc transferase [16].

Glycan **2** was β1,4-galactosylated to give glycan **3**. The product was purified by gel filtration and in some experiments by paper chromatography (R_{MP} = 0.53, R_{MH} = 0.78). The yields of the purified product in two experiments averaged 66%. MALDI-TOF-MS of $Gal_4GlcNAc_4Fuc$ (not shown) had an abundant peak appropriate for the $(M+Na)^+$ at *m/z* 1647.6 (calc. *m/z* 1647.6) and a low abundance $(M+K)^+$ at *m/z* 1663.4 (calc. *m/z* 1663.7). The ^{1}H-NMR spectrum of glycan **3** (Table 1) confirmed the structure shown in Fig. 1.

Glycan **3** was converted into glycan **4** by removing the fucose residue by mild acid hydrolysis; the product was purified by gel filtration using two consecutive Superdex 75 HR 10/30 columns. The yields of purified **4** in two experiments averaged 44%. MALDI-TOF-MS confirmed that the product was essentially fucose-free. The spectrum showed a major peak at *m/z* 1502.3, and an accompanying signal at *m/z* 1518.3 (Fig. 2A); these were assigned to $(M+Na)^+$ and $(M+K)^+$, respectively, of $Gal_4GlcNAc_4$ (calc. *m/z* 1502.3 and 1518.4, respectively). The ^{1}H-NMR spectrum also confirmed the postulated structure of glycan **4** (Table 1, Fig. 3A). Overall, the structural reporter group region of glycan **4** resembled closely that obtained from the linear hexasaccharide LacNAcβ1–3′LacNAcβ1–3′LacNAc [7].

Glycan **4** was succesfully synthesized also from the difucosylated glycan LacNAcβ1–3′(Fucα1–3)LacNAcβ1–3′(Fucα1–3)LacNAc in experiments analogous to those described above. Radiolabeled glycan **4** was obtained by using UDP-[^{14}C]Gal in the final β1,4-galactosylation reaction.

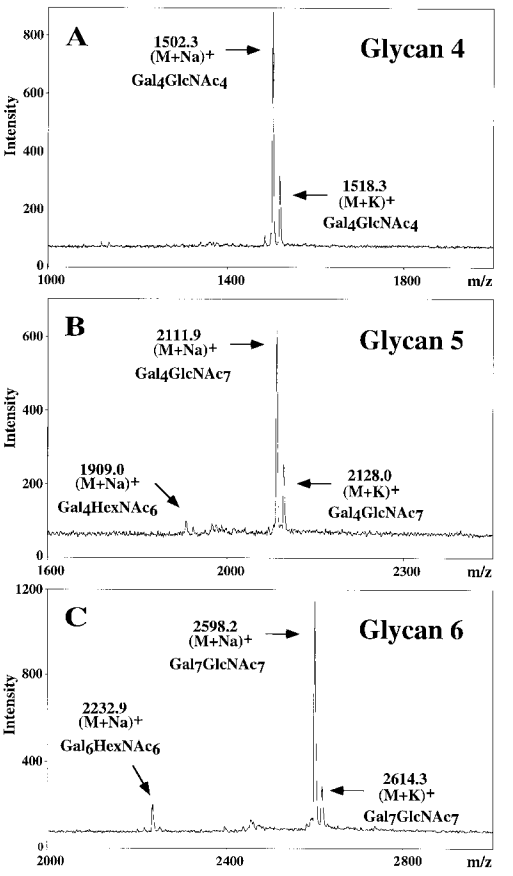
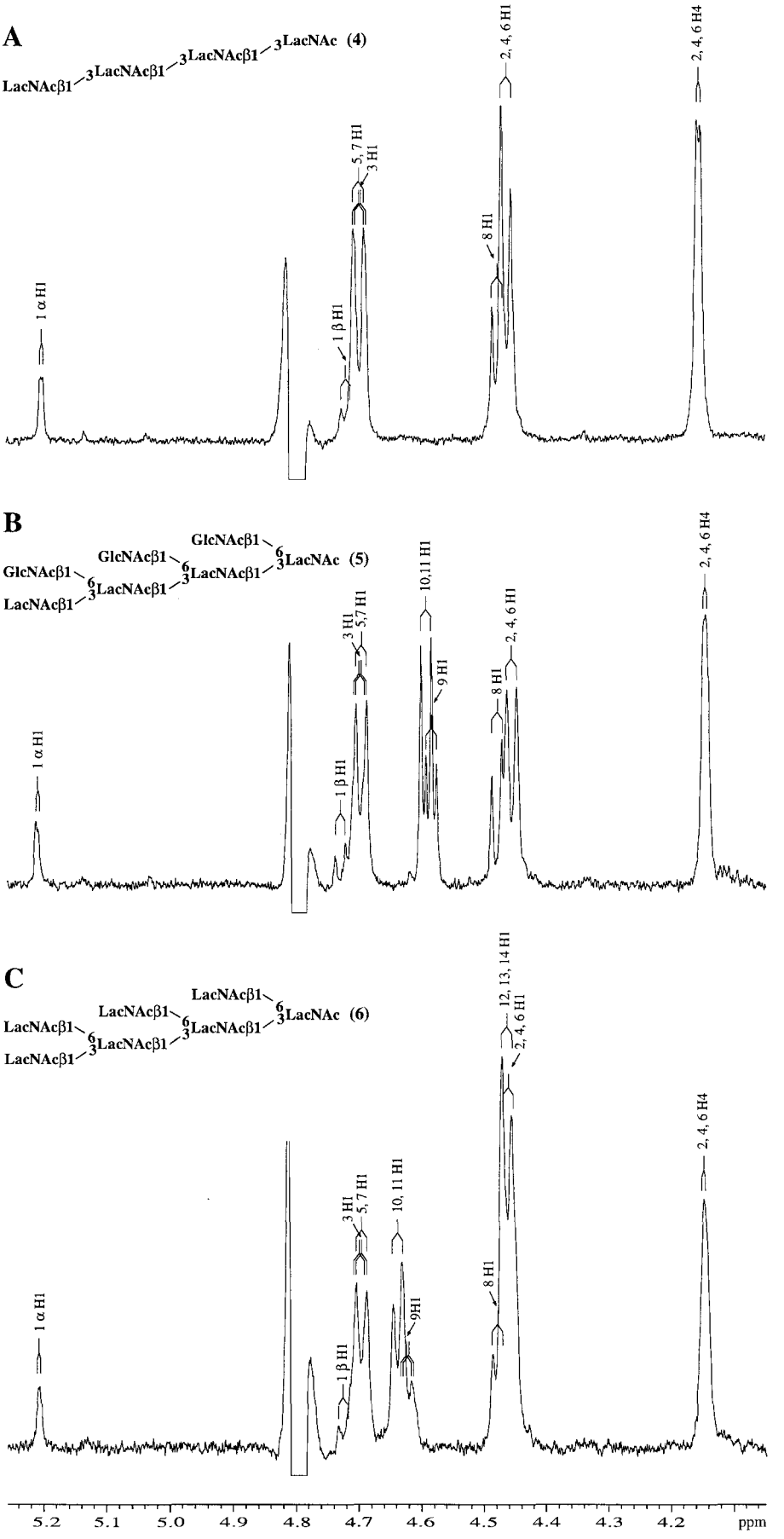


Fig. 2. MALDI-TOF mass spectrum of (A) the primer glycan **4**, (B) the triply branched glycan **5** and (C) the galactosylated glycan **6**. The minor signal in panel C, observed at *m/z* 2232.9, was assigned to $(M+Na)^+$ of $Gal_6HexNAc_6$ (calc. *m/z* for $Gal_6HexNAc_6$ 2233.0). This impurity could have been formed from a small amount of $Gal_4HexNAc_6$ in the acceptor glycan which was observed in the MALDI-TOF mass spectrum of **5**.

Fig. 3. Anomeric regions of ^{1}H-NMR spectra of (A) the primer glycan **4**, (B) the triply branched glycan **5** and (C) the galactosylated glycan **6** of the present synthesis route. For numbering of the monosaccharide residues, see Fig. 5.


A
LacNAcβ1-3LacNAcβ1-3LacNAcβ1-3LacNAc (4)
1 α H1
1 β H1
5, 7 H1
3 H1
8 H1
2, 4, 6 H1
2, 4, 6 H4
B
GlcNAcβ1-6
GlcNAcβ1-6
GlcNAcβ1-6
LacNAcβ1-3LacNAcβ1-3LacNAcβ1-3LacNAc (5)
1 α H1
1 β H1
3 H1
5, 7 H1
10, 11 H1
9 H1
8 H1
2, 4, 6 H1
2, 4, 6 H4
C
LacNAcβ1-6
LacNAcβ1-6
LacNAcβ1-6
LacNAcβ1-3LacNAcβ1-3LacNAcβ1-3LacNAc (6)
1 α H1
1 β H1
3 H1
5, 7 H1
10, 11 H1
9H1
8 H1
12, 13, 14 H1
2, 4, 6 H1
2, 4, 6 H4
5.2
5.1
5.0
4.9
4.8
4.7
4.6
4.5
4.4
4.3
4.2
ppm

3.2. *Enzyme-assisted conversion of glycan 4 into the triply branched undecasaccharide LacNAcβ1–3′(GlcNAcβ1–6′)-LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc (5)*

Incubation of the enzymatically synthesized glycan **4** (40.3 nmol) with UDP-GlcNAc and the midchain β1,6-GlcNAc transferase activity present in rat serum [6,7], gave several products that were separated by HPAE chromatography (Fig. 4). MALDI-TOF mass spectrum of the principal product (peak 7 in Fig. 4) showed a major signal at *m/z* 2111.9 that was assigned to $(M+Na)^+$ of $Gal_4GlcNAc_7$ (calculated *m/z* 2111.9) (Fig. 2B); an accompanying signal at *m/z* 2128.0 was assigned to the corresponding $(M+K)^+$ (calculated *m/z* 2128.0). Hence, the major product of the branching reaction was an undecasaccharide that contained three newly transferred GlcNAc residues. The ^{1}H-NMR spectrum of the principal product (Fig. 3B; Table 1) revealed the presence of three new protons resonating at 4.585–4.593 ppm, in the area characteristic to H1's of β1,6-bonded GlcNAc-residues. The reporter group signals originating from the main chain of the product resembled very closely their counterparts found in the spectra of the branched pentasaccharide LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc [13,21] and the doubly branched octasaccharide LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAc [7]. These data establish the structure of the undecasaccharide product as LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(GlcNAcβ1–6′)LacNAcβ1–3′(-GlcNAcβ1–6′)LacNAc (**5**). The yield of glycan **5** was 10.6 nmol (26%). Another branching experiment with UDP-GlcNAc and concentrated rat serum was performed with a 3 nmol/25 800 cpm-sample of ^{14}C-labeled glycan **4**. The radioactivity profile (not shown) resulting from HPAE chromatography was remarkably similar to the PAD-profile of Fig. 4. The analog of peak 7/Fig. 4 contained 6000 cpm of glycan **5** (23% yield) that showed in MALDI-TOF-MS the same signals as peak 7 of Fig. 4.

The numerous side-products in Fig. 4 were analyzed by MALDI-TOF-MS (not shown). Peak 2 represented fucosylated glycans and peak 3 the unreacted glycan **4**. The singly branched glycans appeared in peak 4, while peaks 5 and 6 probably represented doubly branched products and peak 8 their ManNAc epimers. Peak 9 represented a triply branched product $Gal_4HexNAc_7$, most likely the reducing-end ManNAc epimer of glycan **5**. Peaks 3–6 gave also signals of ions missing one galactose. Taken together, the data suggest that improvements in the yield of glycan **5**/peak 7 will be possible if (i) the branching reaction can be forced closer to completion and (ii) a purification method can be used that exposes the glycans to less basic conditions than those prevailing in HPAE chromatography, which catalyses partial epimerization at the unprotected reducing-end [19].

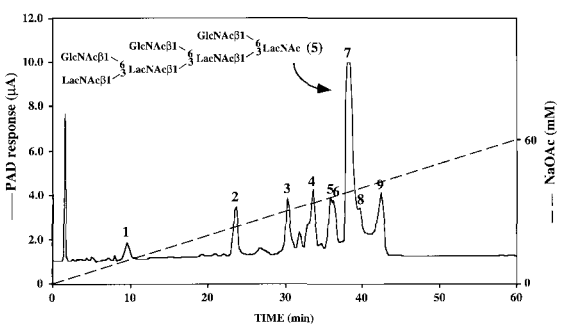


Fig. 4. HPAE chromatography of the oligosaccharides resulting from the incubation of glycan **4** with UDP-GlcNAc and concentrated rat serum. Peak 3 consists of glycan **4** while peak 7 represents glycan **5** and peak 1 is internal lactose marker. MALDI-TOF-MS experiments suggested that peaks 4–9 represent derivatives of the primer and/or its reducing-end ManNAc epimer containing one, two or three β1,6-bonded GlcNAc branches. The large number of peaks probably reflects also partial separation of branch isomers [7].

3.3. *Enzyme-assisted synthesis of the tetradecasaccharide LacNAcβ1–3′(LacNAcβ1–6′)LacNAcβ1–3′(LacNAcβ1–6′)-LacNAcβ1–3′(LacNAcβ1–6′)LacNAc (6)*

Enzymatic β1,4-galactosylation of glycan **5** gave a major product, glycan **6**, with the yield of 85%. MALDI-TOF-MS of the product (Fig. 2C) showed a major peak at *m/z* 2598.2 that was assigned to $(M+Na)^+$ of $Gal_7GlcNAc_7$ (calc. *m/z* 2598.3); an accompanying signal assigned to $(M+K)^+$ of $Gal_7GlcNAc_7$ was seen at *m/z* 2614.3 (calc. *m/z* 2614.4). The structure of glycan **6** was confirmed by ^{1}H-NMR spectroscopy (Fig. 3C). The 4.464-ppm signals assigned to H1's of the newly transferred branch galactoses *12*, *13* and *14* in the tetradecasaccharide **6** were identical to the analogous signals in the decasaccharide LacNAcβ1–3′(LacNAcβ1–6′)LacNAcβ1–3′(LacNAcβ1–6′)LacNAc [7] and the hexasaccharide LacNAcβ1–3′(LacNAcβ1–6′)LacNAc [22]. Glycan **6**, synthesized enzymatically via another route in our previous experiments [4], gave a proton NMR-spectrum (not shown) that was identical with that of Fig. 3C.

4. Discussion

The present data represent distinct improvements in the enzyme-assisted synthesis of glycan **7** (for the structural formula see Fig. 5), a tetravalent sialyl Lewis x (sLex) glycan that is a nanomolar inhibitor of L-selectin-mediated adhesion of lymphocytes to the inflammation-activated endothelium of rejecting cardiac transplants of rats [4]. The increased availability of glycan **7**, in turn, will lead to better assessment of the anti-inflammatory potential of this oligosaccharide in different in vivo inflammation models using experimental animals. The putative roles of glycan **7** and related saccharides as potential antagonists of E- and P-selectins will also merit a study when these glycans become available in sufficient amounts.

The major improvement in the synthesis of glycan **7** consists of the use of the octasaccharide LacNAcβ1–3LacNAcβ1–3LacNAcβ1–3LacNAc (**4**) rather than the hexasaccharide LacNAcβ1–3LacNAcβ1–3LacNAc as the primer, and four rather than six enzymatic steps to convert the primer into the tetravalent sLex-saccharide sLexβ1–3′(sLexβ1–6′)LacNAcβ1–3′(sLexβ1–6′)LacNAcβ1–3′(sLexβ1–6′)LacNAc (**7**) as shown in Fig. 5. The key reaction in the novel synthesis is the conversion of the linear octasaccharide **4** into the triply branched undecasaccharide **5** in a single-step transformation catalyzed by the midchain β1,6-GlcNAc transferase activity of rat serum. In the future this reaction will be catalyzed by the recombinant form of the midchain β1,6-GlcNAc transferase of embryonal carcinoma cells, which we have recently expressed in baculovirus-infected *Spodoptera frugiperda* (Sf9) insect cells and isolated in an active form (P. Mattila et al., in preparation). Another major advantage of the octasaccharide **4** is that this glycan is accessible in considerable amounts by chemical synthesis in solution [14,15]. It appears probable that

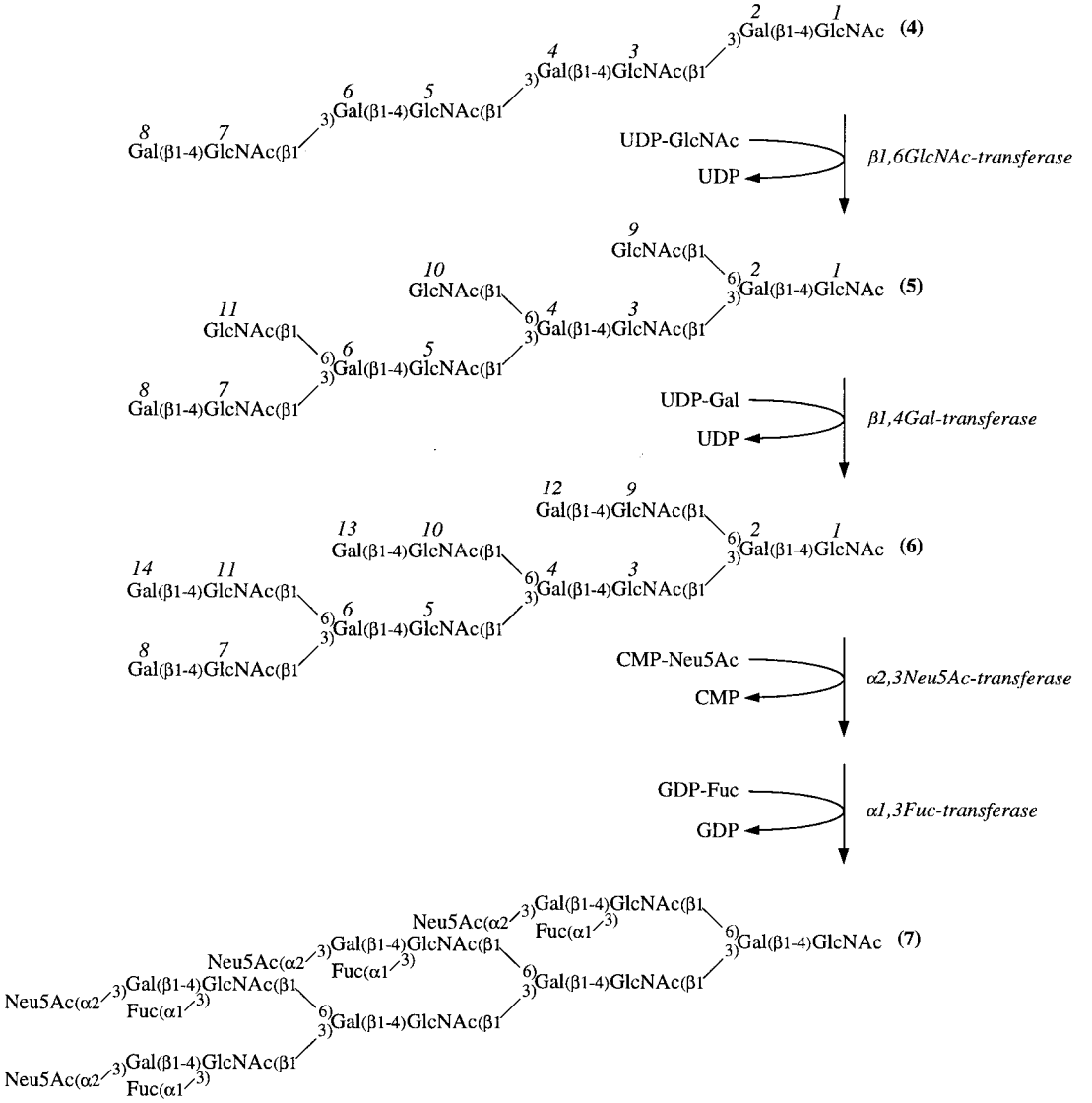


Fig. 5. The present, improved synthesis route to the tetravalent sialyl Lewis x glycan **7**.

also solid phase chemical synthesis of glycan **4** will soon become possible [23]. Its enzymatic synthesis, too, will probably develop rapidly when recombinant forms of the β1,3-GlcNAc transferase become available.

The triply branched undecasaccharide **5** was readily galactosylated by β1,4-galactosyl transferase of bovine milk, yielding the branched array of seven *N*-acetyllactosamine units that is shown as glycan **6** in Fig. 5. Glycan **6** of the present experiments was identical with a sample constructed in our early experiments via another route [4]; both samples revealed similar molecular weights in MALDI-TOF-MS and gave very similar 1D ^{1}H-NMR spectra, confirming the postulated structure of this oligosaccharide. Chemical synthesis of glycan **6** has not been described yet, but related syntheses have been presented [24,25], suggesting that in the future glycan **6** will be accessible also by chemical synthesis.

The enzymatic conversion of glycan **6** to the tetravalent sLex glycan **7** has been described before [4]; no improvements are described in the present report to the two reactions involved.

It remains to be seen whether chemical, enzymatic or hybridized chemo-enzymatic approaches will prove to be the most efficient ones in providing branched poly-*N*-acetyllactosamine backbones for construction of multivalent sLex saccharides and related glycans.

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