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

Heidi Salminen, Katja Ahokas, Ritva Niemelä, Leena Penttilä, Hannu Maahemo, Jari Helin, Catherine E. Costello, Ossi Renkonen

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')-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 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')LacNAc (3) (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'LacNAcβ1→3'LacNAcβ1→3'LacNAcβ1→3'LacNAcβ1→3'LacNAcβ1→3'LacNAc (6) 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.
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 \( \beta 1,6 \)-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].

2.5. MALDI-TOF-MS

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

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<th>3</th>
<th>4</th>
<th>5</th>
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<td>5.203 (( \alpha ))</td>
<td>5.204 (( \alpha ))</td>
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<td>4.695</td>
<td>4.701/4.698(^b)</td>
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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.

\(^\sim\), 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’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 LacNAcb1-3’(Fucocl-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 [2,7,13].

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 ($R_{GP}=0.74$, $R_{PM}=1.10$). 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$_3$GlcNAc$_3$Fuc; 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$_2$HexNAc$_2$Fuc. 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’(Fucocl-3)LacNAc determinant in the present experiments is sharply contrasted by the unreactivity of Fucocl-3-LacNAc determinant in the present experiments.

Glycan 2 was β1,4-galactosylated to give glycan 3. The product was purified by gel filtration and in some experiments by paper chromatography ($R_{GP}=0.53$, $R_{PM}=0.78$). The yields of the purified product in two experiments averaged 66%. MALDI-TOF-MS of Gal$_3$GlcNAc$_3$Fuc (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$_2$HexNAc$_2$Fuc (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 successfully synthesized also from the difucosylated glycan LacNAcβ1-3’(Fucocl-3)LacNAcβ1-3’(Fucocl-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.](image-url)
Fig. 1. NMR spectra of 2-deoxy-L-

A 6-N-acetyl-

B 6-N-acetyl-

C 6-N-acetyl-

lacNAc β 3 lactose (2, 4, 6-H)

lacNAc β 3 lacNAc β 1 lacNAc β 1 lacNAc (4)

lacNAc β 1 lacNAc β 1 lacNAc (4)

lacNAc β 1 lacNAc β 1 lacNAc (5)

lacNAc β 1 lacNAc β 1 lacNAc (5)

lacNAc β 3 lacNAc β 1 lacNAc β 1 lacNAc (6)

lacNAc β 1 lacNAc β 1 lacNAc β 1 lacNAc (6)
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 Galα1GlcNAc (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 1H-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. An unreacted 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 of Fig. 4 contained 6000 cpm of glycan 5 and peak 8 the unreacted glycan 4. The singly branched undecasaccharide 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 14C-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 of 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 Galα1HexNAcβ1, 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 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.

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 Galα1GlcNAc (calculated m/z 2598.3); an accompanying signal assigned to (M+K)+ of Galα1GlcNAc was seen at m/z 2614.3 (cal. m/z 2614.4). The structure of glycan 6 was confirmed by 1H-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')-LacNAcβ1-3'(LacNAcβ1-6')LacNAc (7).

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 LacNAcβ1-3(LacNAcβ1-3LacNAcβ1-3LacNAcβ1-3LacNAc (4) rather than the hexasaccharide LacNAcβ1-3(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 (SPF) 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 1H-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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References


