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Note

Site-directed enzymatic α-(1 → 3)-L-fucosylation of the tetrasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc at the distal *N*-acetyllactosamine unit

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Glycans containing Lewis *x*, Galβ(1 → 4)[Fucα(1 → 3)]GlcNAcβ1 → , and related structures at their chain termini, are considered to be ligands for E-, L- and P-selectins [1–3]. Synthesis of Lewis *x* glycans, in sialylated or sulfated form, is of great interest, because of their potential as anti-inflammatory agents. The last step in the enzymatic synthesis of selectin ligand saccharides consists of α-(1 → 3/4)-L-fucosylation, a reaction that can take place at all *N*-acetyllactosamine units of linear poly-*N*-acetyllactosamines [4], also in the tetrasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc (**1**) that reacts with human milk transferases (Niemelä et al., unpublished).

We have recently shown that human milk α-(1 → 3)-L-fucosyltransferase(s) do not react appreciably with *N*-acetyllactosamine residues that carry a β-(1 → 6)-linked GlcNAc branch at the galactose unit [5]. Here, this observation has been used to direct the enzymatic α-(1 → 3)-L-fucosylation of the divalent acceptor tetrasaccharide Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc (**1**) solely at the distal *N*-acetyllactosamine unit (see Fig. 1). The process involves (i) protection of the *N*-acetyllactosamine unit at

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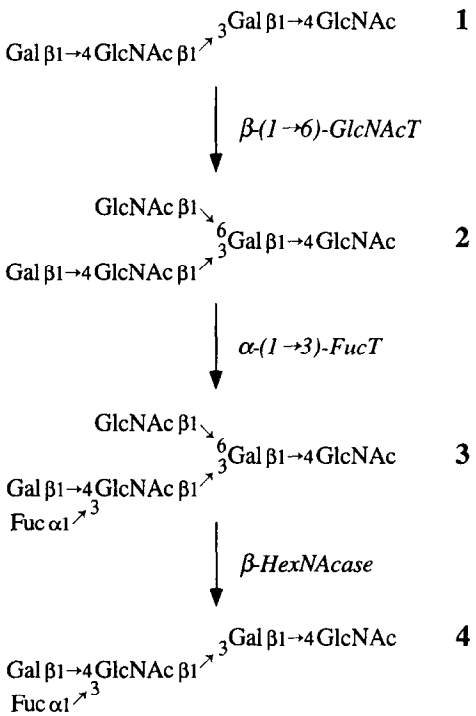


Fig. 1. The three reaction procedure leading to the site-directed α-(1 → 3)-L-fucosylation of glycan **1** to yield the Lewis *x* pentasaccharide **4**. Abbreviations: β-(1 → 6)-GlcNAcT, β-(1 → 6)-D-*N*-acetylglucosaminyltransferase; α-(1 → 3)-FucT, α-(1 → 3)-L-fucosyltransferase; β-HexNAcase, β-D-*N*-acetylhexosaminidase.

the reducing end of **1** by mid-chain β-(1 → 6)-D-*N*-acetylglucosaminylation that generates Galβ(1 → 4)GlcNAcβ(1 → 3)[GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc (**2**), (ii) site-specific fucosylation of **2** by human milk α-(1 → 3)-L-fucosyltransferase(s) at the distal *N*-acetyllactosamine residue of **2**, and (iii) removal of the protecting β-(1 → 6)-branched GlcNAc group with β-D-*N*-acetylhexosaminidase.

1. Results and discussion

Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc (**1**) was converted to Galβ(1 → 4)GlcNAcβ(1 → 3)[GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc (**2**) as described by Leppänen et al. [6], but by using the β-(1 → 6)-D-*N*-acetylglucosaminyltransferase of rat serum [7] instead of human serum. The resulting pentasaccharide co-chromatographed on paper with authentic **2** [8]. Its ^{1}H-NMR spectrum (Fig. 2B and Table 1) revealed the H-1 resonance of the incoming β-(1 → 6)-linked GlcNAc as one proton doublet at 4.585 ppm. The H-1 and H-4 resonances of the galactose residue 2 (for residue numbering, see Table 1) have both shifted to a higher field (compared to those of glycan

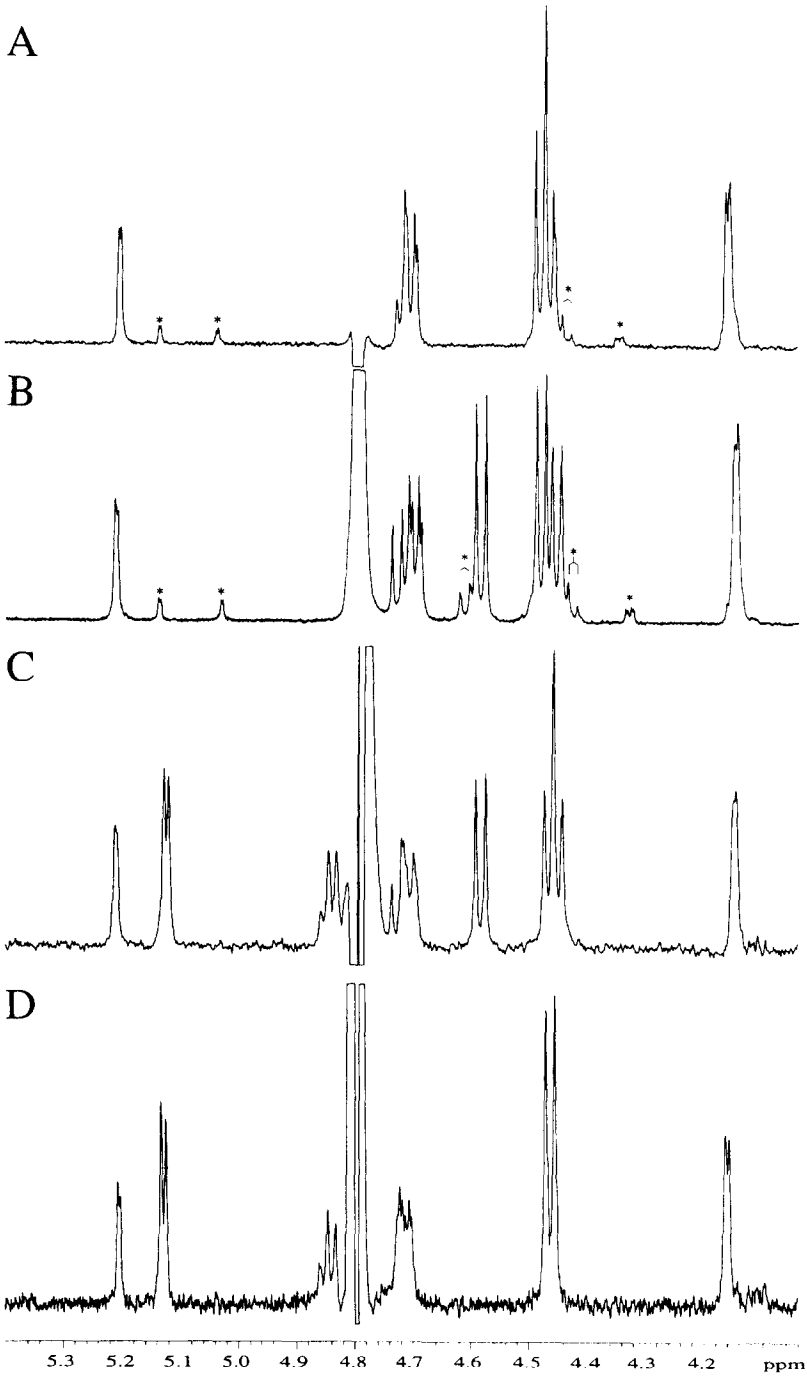
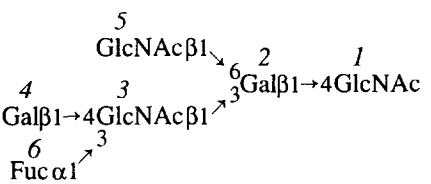


Fig. 2. Expansions of the 500 MHz ^{1}H-NMR spectra of (A) glycan **1**, (B) glycan **2**, (C) glycan **3** and (D) glycan **4**. The signals marked by an asterisk arise from the reducing end epimers of glycans **1** and **2**.

Table 1
^{1}H chemical shifts of structural reporter groups of saccharides **1–4** at 295 K

Reporter group	Residue [a]	Saccharides			
		1	**2**	**3**	**4**
H-1	*1*	5.205 (α)	5.212 (α)	5.211 (α)	5.204 (α)
		4.721 (β)	4.731 (β)	4.729 (β)	4.720 (β)
	2 [b]	4.465/4.462	4.454	4.450	4.462
	3 [b]	4.707/4.703	4.701/4.696	4.709/4.704	4.715/4.710
	4	4.480	4.481	4.465	4.462
	5	–	4.585	4.584	–
	6	–	–	5.125	5.128
H-4	*2*	4.159	4.149	4.148	4.159
H-5	*6*	–	–	4.840	4.840
H-6	*6*	–	–	1.175	1.175

[a] Numbering of the residues is as follows:



[b] The two chemical shift values given arise from signals representing the α- and β-pyranosic forms of the oligosaccharide.

1), indicating that this galactose is the site of substitution [9,10] and establishing the structure of the pentasaccharide as **2**. The structure of **2** was further confirmed by ^{1}H and ^{13}C 2D NMR experiments (Maaheimo et al., unpublished).

Glycan **2** was incubated with GDP-L-fucose and α-(1 → 3)-L-fucosyltransferase(s) from human milk. The reaction mixture was separated by HPAE chromatography on CarboPac PA-1 column (Fig. 3A), revealing a major product (peak 1), which proved to represent hexasaccharide Galβ(1 → 4)[Fucα(1 → 3)]GlcNAcβ(1 → 3)[GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc (**3**); in addition, the reducing end epimer (peak 2), unreacted glycan **2** (peak 3) and its reducing end epimer (peak 4) were observed. The major peak migrated faster than glycan **2**; this is characteristic to α-(1 → 3)-fucosylated products [11]. The structure of **3** was established by ^{1}H-NMR (Fig. 2C and Table 1). The spectrum reveals the H-1, H-5 and H-6 resonances of the incoming fucose at 5.125 ppm, 4.840 and 1.175 ppm (not shown), respectively. These chemical shifts are typical for α-(1 → 3)-linked fucose [12]. The integrals of these signals indicated that there was only one fucose in glycan **3**. The anomeric signals of GlcNAc residue 3 had shifted to somewhat lower field, while the H-1 signals of the reducing end GlcNAc were unaffected. Since also the anomeric signal of the galactose 4 experiences a notable upfield shift, the NMR data indicate that the fucose was bound to GlcNAc residue 3 rather than to residue 1. The structure of the glycan **3** was also supported by the MALDI-TOF mass spectrum (Fig. 4): a major peak assigned to $(M + Na)^+$ was

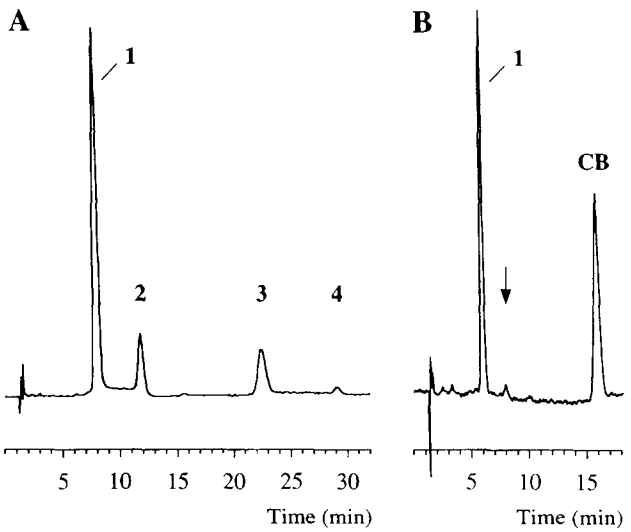


Fig. 3. High-pH anion exchange chromatograms. (A) The desalted and gel filtrated purified products of a α-(1 → 3)-L-fucosyltransferase reaction of glycan **2**. Peak 1 represents the fucosylated glycan **3**, while peak 3 is the unreacted glycan **2**. Peaks 2 and 4 are the reducing end epimers of glycans **3** and **2**, respectively. Elution system: 10 min isocratic with 100 mM NaOH, then linear gradient of increasing concentration of NaOAc (rate of increase 1 mM/min). (B) A small aliquot from β-D-*N*-acetylhexosaminidase digest of glycan **3** after gel filtration. Peak 1 is the Lewis *x* saccharide **4** and CB internal marker, cellobiose. Arrow shows the elution position of glycan **3**. Elution with 100 mM NaOH. No reducing end epimer of glycan **4** was formed, probably because the enzymatic cleavage reaction was performed at pH 4.0.

observed at *m/z* 1120.7 (calculated *m/z* 1121.0), while a minor peak assigned to $(M + K)^+$ was at *m/z* 1136.8 (calculated *m/z* 1137.0).

Hydrolysis of glycan **3** with β-D-*N*-acetylhexosaminidase converted it into Galβ(1 → 4)[Fucα(1 → 3)]GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc (**4**). This is shown by a run of small aliquot of the reaction product(s) in HPAE chromatography (Fig. 3B), where peak 1 is the glycan **4**, while the arrow is showing the position of glycan **3**. When

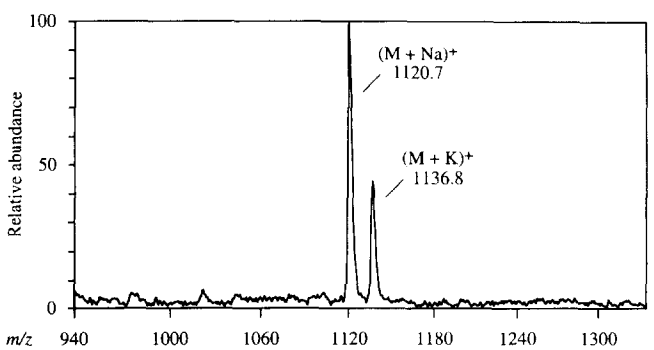


Fig. 4. MALDI-TOF mass spectrum of glycan **3** in the mass range of *m/z* 940–1360.

compared to the NMR spectrum of **3**, the most striking feature in the spectrum of **4** (Fig. 2D and Table 1) is the complete absence of doublet at 4.584 ppm, which is the H-1 resonance of the β-(1 → 6)-linked GlcNAc (residue 5). The H-1 signals of the β-(1 → 3)-linked GlcNAc (residue 3) have shifted to lower field and are now overlapping with those of the β-anomer of the reducing end GlcNAc; the two galactose H-1s resonate now at identical frequencies at 4.462 ppm. Comparison of the spectrum of **4** with that of **1** reveals that the anomeric signal of GlcNAc residue 3 has shifted to a lower field, while the H-1 signal of GlcNAc residue 1 is unshifted, further establishing that the fucose is linked to GlcNAc residue 3. MALDI-TOF mass spectrum of glycan **4** revealed that one *N*-acetylhexosamine residue had been hydrolyzed from the glycan **3**; a peak assigned to $(M + Na)^+$ (calculated m/z 917.8) was observed at m/z 917.6. The structure of glycan **4** was further established by β-D-galactosidase digestion, which left the glycan intact (data not shown), as expected, because β-D-galactosidase does not hydrolyze Lewis *x* structures [13].

The present three-reaction-procedure bears close resemblance to a similar process where the α-(1 → 3)-L-fucosylation is directed away from the distal *N*-acetyllactosamine unit by temporary α-(2 → 6)-sialylation [14]. The sialyl group at 6′-position of the 'outer' *N*-acetyllactosamine unit prevents the fucosylation at this *N*-acetyllactosamine [5,15].

The success of the present protection–reaction–deprotection procedure depends on the specific mode of action of two enzymes: (i) β-(1 → 6)-D-*N*-acetylglucosaminyltransferase of rat serum, that does not act on the distal *N*-acetyllactosamine units but rather on the inner chain units [7]; (ii) α-(1 → 3)-L-fucosyltransferase that does not act on branching *N*-acetyllactosamine units but acts on linear ones [5]. The value of this process lies in the site-specific formation of a pure Lewis *x* structure containing glycan **4**, carrying the α-(1 → 3)-L-fucosylated *N*-acetyllactosamine unit solely at the distal position. The process can also be used to synthesize pure sialyl Lewis *x* (sLex) glycans; we have synthesized branched structures like NeuAcα(2 → 3)Galβ(1 → 4)GlcNAcβ(1 → 3)[NeuAcα(2 → 3)Galβ(1 → 4)GlcNAcβ(1 → 6)]Galβ(1 → 4)GlcNAc, which are exclusively fucosylated at the distal *N*-acetyllactosamine units, rather than at the branching *N*-acetyllactosamine [16]. Hence, pure Lewis *x* structures and their sialyl derivatives, free from VIM-2 type isomers or difucosylated products, can be obtained by the present process.

Other methods to obtain the same goal appear to be emerging: (i) Howard et al. [17] have reported that nLc_6, Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)Glcβ(1 → 1)Cer, is fucosylated mainly at the external *N*-acetyllactosamine unit by the α-(1 → 3)-L-fucosyltransferase II of CHO-LEC12 cells. (ii) With glycolipids as acceptors, the proper choice of detergent can also lead to external α-(1 → 3)-L-fucosylation [18], but it is not clear whether sterically unhindered, non-micellar acceptors would react similarly. (iii) A chromatographic method capable of separating glycan **4** from its isomer Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)[Fucα(1 → 3)]GlcNAc has been developed in our laboratory (Niemelä et al., unpublished). Hence, glycan **4** can be directly isolated from the monofucosylated products generated by partial α-(1 → 3)-L-fucosylation of glycan **1** with human milk α-(1 → 3)-L-fucosyltransferase(s).

2. Experimental

Oligosaccharide primer.—The synthesis of Galβ(1 → 4)GlcNAcβ(1 → 3)Galβ(1 → 4)GlcNAc (**1**) was performed essentially as described in [19], using four-fold molar excess of UDP-D-galactose (Sigma, MO, USA).

Enzymatic methods.—α-(1 → 3)-L-Fucosyltransferase(s) (human milk) reactions were performed as described in [5,20]. β-(1 → 6)-D-*N*-acetylglucosaminyltransferase reactions were performed in the presence of 20 mM EDTA, 200 mM D-galactose and 60 mM D-galactonic acid γ-lactone, essentially as described in ref. [6], with the exception that rat serum was used as enzyme source. Hydrolysis with β-D-galactosidase (Jack beans, EC 3.2.1.23; Sigma) [21] and β-D-*N*-acetylhexosaminidase (Jack beans, EC 3.2.1.23; Sigma) [6] was performed as described.

Chromatographic methods.—Gel filtration was performed in a column of Superdex 75 HR 10/30 (Pharmacia, Sweden), with water as the eluant at a flow rate of 1 mL/min. The eluant was monitored at 205 nm, and oligosaccharides were quantified against external 2-acetamido-2-deoxy-D-glucose (D-GlcNAc). High-pH anion exchange chromatography with pulsed amperometric detection (HPAEC-PAD) was carried out with a Dionex series 4500i HPLC system (Dionex, CA, USA) with a CarboPac PA-1 column (4 × 250 mm), as described [22]. The column was equilibrated with 100 mM NaOH and run as follows: either isocratic with 100 mM NaOH or first 10 min isocratic with 100 mM NaOH followed with linear gradient of NaOAc ending to 100 mM NaOAc–100 mM NaOH at 110 min. Peaks were collected manually, neutralized with 0.4 M acetic acid and dried in a vacuum lyophilizer. The oligosaccharides were desalted by filtration in water through AG-1 (AcO^-) and AG-50W (H^+) (Bio-Rad, CA, USA).

^{1}H-NMR spectroscopy.—The samples were twice lyophilized from D_2O, 99.96% D (Cambridge Isotope Laboratories, MA, USA), and finally dissolved in 600 μL of D_2O, 99.996% D (CIL). The spectra were recorded on Varian Unity 500 spectrometer at 295 K using a modification of the WEFT sequence [23] for suppression of the residual HDO signal. The chemical shifts were referenced to internal acetone, 2.225 ppm.

MALDI-TOF MS.—Matrix-assisted laser desorption/ionization time of flight mass spectrometry was performed in the positive ion mode with irradiation from a nitrogen laser (337 nm) and 2,5-dihydroxybenzoic acid as the matrix with the Vestec VT-2000 linear time-of-flight instrument operated at 30 kV accelerating voltage. External calibration was used; this method has an accuracy of ±0.1% (±2 u at m/z 2000).

Acknowledgements

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