ELSEVIER

Carbohydrate Research 266 (1995) 191–209

CARBOHYDRATE RESEARCH

Stepwise transfer of α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc sequences to 3-OH and 6-OH of distal galactose residues in bi-, tri-, and tetra-antennary asialo-glycans of N-linked complex type

Jari Helin [a], Hannu Maaheimo [a], Antti Seppo [a], Anita Keane [b], Ossi Renkonen [a,*]

[a] *Institute of Biotechnology and Department of Biochemistry, University of Helsinki, P.O. Box 45, FIN-00014 University of Helsinki, Finland*

[b] *Finnigan Mat Ltd, Paradise, Hemel Hempstead, Herts, HP2 4TG, United Kingdom*

Received 13 July 1994; accepted 22 August 1994

Abstract

The hydroxyl groups 3 and 6 of distal galactose units in bi-, tri-, and tetra-antennary asialo-glycans of N-linked complex type were substituted stepwise by transferase reactions with the sequence α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc. The products of each transferase reaction were purified chromatographically and the structures were confirmed by ^{1}H NMR spectroscopy. Molecular weights of the final products were determined by matrix-assisted laser-desorption mass spectrometry (MALDI-MS).

Keywords: Poly-(*N*-acetyllactosamino)glycan; Glycosyltransferase; In vitro synthesis; ^{1}H NMR; HPAEC; MALDI-MS

1. Introduction

Poly-(*N*-acetyllactosamino)glycans are large glycoconjugates composed of chains of repeating β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3) units and, frequently, short R-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6) substitutions on the backbone Gal units are included [1,2]. Interest in these glycans stems from their postulated roles in various biological

* Corresponding author.

SSDI 0008-6215(94)00272-X

processes: Marked changes in the expression of poly-(*N*-acctyllactosamino)glycans have been observed during differentiation of murine post-implantation embryos [3], embryonal carcinoma and teratocarcinoma cells [3–5], and leukemic cells [6,7], as well as during murine spermatogenesis [8] and activation of macrophages [9]. The presence of poly-(*N*-acetyllactosamino)glycans on mammalian sperm [10] and eggs [11,12], and the stimulation of lactosamine chain synthesis in the uterus by estrogen [13] suggest that they may be adhesion elements in fertilization and implantation. In glycosaminoglycans poly-(*N*-acetyllactosamino)glycan chains are present in sulfated form as keratan sulfates [14].

The nature of the endogenous receptors for poly-(*N*-acetyllactosamino)glycans is not completely established. Cell surface galactosyltransferase is suggested to function as a receptor for uterine epithelial poly-(*N*-acetyllactosamino)glycans [15]. In addition, calf heart agglutinin (or L-14) [16] and CBP30 of baby hamster kidney cells [17], members of the galectin family [18], bind these glycans. Interestingly, L-14 [19] and macrophage β-galactoside lectin Mac-2 [20,21] bind to laminin, and CBP30 to laminin and fibronectin [17]. In the latter case poly-(*N*-acetyllactosamino)glycans were presumably involved, implying a role for these glycans in cell adhesion to basement membranes as well.

The blood group Ii antigens represent linear (i) and branched (I) poly-(*N*-acetyllactosamino)glycan chains [22–24]. Other types of antigenic structures found on these glycans include ABH blood group antigens [25,26] and (sialyl) Lewis-X determinants [27,28]. Poly-(*N*-acetyllactosamino)glycan chains terminated by α-D-Gal-(1→3) units are also well documented [29].

In the present study, we investigated the synthesis of I-type structures on N-linked type oligosaccharides in vitro. As opposed to the asymmetric pattern of β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc repeats on *N*-glycans from biological sources [30–32], we were able to obtain products fully glycosylated at all branches by prolonged incubations with (1→3)- and (1→6)-*N*-acetyl-β-D-glucosaminyltransferases and subsequent (1→4)-β-D- and (1→3)-α-D-galactosyltransferase reactions.

2. Experimental

Enzymic methods.—(1→3)-*N*-Acetyl-β-D-glucosaminyltransferase (EC 2.4.1.149) reactions with human serum were carried out as described [33]. (1→4)-β-D-Galactosyltransferase (EC 2.4.1.38) reactions were performed with bovine milk *N*-acetyllactosamine synthase (Sigma, St. Louis, MO, USA) essentially as described [34]. (1→6)-*N*-Acetyl-β-D-glucosaminyltransferase (EC 2.4.1.148) reactions with hog gastric mucosal microsomes were carried out as described [33]; sucrose present as a protein stabilizer in the microsome preparation was removed by Bio-Gel P-2 chromatography. (1→3)-α-D-Galactosyltransferase (EC 2.4.1.87) was purified [35] from bovine thymus according to procedure of Blanken and van den Eijnden [36]; (1→3)-α-D-galactosylations were performed as in [35].

Digestions with jack bean β-D-galactosidase (EC 3.2.1.23) (Sigma) [37] and *B. fragilis* endo-β-D-galactosidase (EC 3.2.1.103) (Boehringer, Mannheim, Germany) [38] were performed as described.

All enzymic treatments were terminated by heating in a boiling water bath for 5 min and desalted by filtration through Dowex AG-1 (AcO^-) and Dowex AG-50 (H^+) resins and lyophilization.

Materials.—UDP-D-galactose (UDP-Gal), UDP-2-acetamido-2-deoxy-D-glucose (UDP-GlcNAc), 2-acetamido-2-deoxy-D-glucose, D-galactose, lactose, maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, and maltoheptaose were from Sigma. UDP-D-[6-^{3}H]galactose was from Amersham (Buckinghampshire, UK). Isomaltooctaose was from BioCarb Chemicals (Lund, Sweden). Glycans **1**, **7**, and **12** (see Figs. 1, 7, and 10 for saccharide structures) were generous gifts from Drs. G. Strecker and J. Montreuil (Université des Sciences et Technologies de Lille, France). A radiolabeled version of **1** was obtained by exhaustive digestion with jack bean β-D-galactosidase followed by (1→4)-β-D-galactosylation with UDP-D-[6-^{3}H]galactose and (1→4)-β-D-galactosyltransferase from bovine milk.

Chromatographic methods.—High-pH anion-exchange chromatography with pulsed amperometric detection (HPAEC–PAD) was carried out with a Dionex Series 4500i HPLC system (Dionex, CA, USA), equipped with a CarboPac PA-1 column (4×250 mm) preceded by a CarboPac PA-1 guard column, at a flow rate of 1 mL/min at ambient temperature. Pulsed amperometric detection worked with the following pulse potentials and durations: E_1 0.05 V/300 ms, E_2 0.65 V/60 ms, and E_3 −0.95 V/180 ms. The system used was as follows: the column was equilibrated with 0.1 M NaOH, and after sample injection, a linear gradient of NaOAc was applied to a final composition of 100 mM NaOAc–0.1 M NaOH at 100 min. Lactose and maltooligosaccharides were cochromatographed as internal standards with the mixtures, because drifting of the retention times was observed. Fractions (1 mL) were collected in tubes containing 0.5 mL of 0.4 M AcOH acid to immediately neutralize the eluent. Salts were removed by filtration through Dowex AG-1 (AcO^-) and Dowex AG-50 (H^+) resins and lyophilization. Because of the limited capacity of the CarboPac PA-1 column, the products of early steps in the synthesis series were chromatographed in several batches.

Paper chromatography on Whatman III Chr was carried out with 10:3:7 butanol–AcOH–water.

Gel permeation chromatography was carried out with a column of Superdex 75 HR 10/30 (Pharmacia, Sweden) with water as the eluent at a flow rate of 1 mL/min using a 2150 HPLC Pump (Pharmacia). The eluent was monitored at 195 nm and oligosaccharides were quantified against external 2-acetamido-2-deoxy-D-glucose assuming that each *N*-acetyl group in the oligosaccharides contribute equally to the absorption.

Nuclear magnetic resonance spectroscopy.—Prior to spectroscopic analysis, the samples were repeatedly dissolved in 99.96% ^2H_2O [Cambridge Isotope Laboratories (CIL), Woburn, MA, USA] at room temperature with intermediate lyophilization. Finally, the samples were dissolved in 600 mL of 99.996 atom% ^2H_2O (CIL). The spectra were recorded at 500 MHz with a Varian Unity 500 spectrometer. Chemical shifts are presented by reference to internal acetone (δ 2.225 ppm). Resolution enhancement was accomplished by use of a shifted sine-bell weighting function.

Matrix-assisted laser-desorption ionization-mass spectrometry.—The mass spectra were recorded in the positive-ion mode using a LASERMAT™ time-of-flight mass-analyzer (Finnigan MAT Ltd., UK). The oligosaccharides were dissolved in water, pre-mixed with

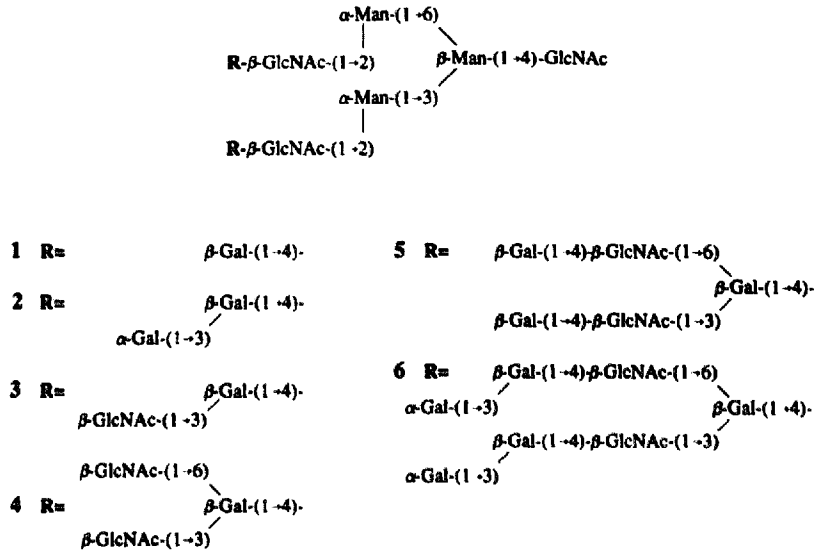


Fig. 1. Structures of the saccharides related to glycan **1**.

a 2,5-dihydroxybenzoic acid matrix, and 5 pmol of sample was loaded on a stainless steel target. Oligomannose 9 (M_w 1884 Da) and asialo tetraantennary *N*-glycan (M_w 2373 Da) (Oxford Glycosystems, UK) were used as external calibrants. Data from an average of 20–30 laser shots were acquired.

3. Results

Enzymic synthesis of the biantennary glycan **2**.—The biantennary glycan **1** (106 nmol, 8.4×10^5 dpm [^{3}H]) (see Fig. 1 for the structures of glycans **1**–**6** and Fig. 4 for the denotation of monosaccharide residues in this study) was (1→3)-α-D-galactosylated and the products were purified by paper chromatography (Fig. 2A). The single product obtained (83 nmol) represented glycan **2**. Its ^{1}H NMR spectrum (Fig. 3A) revealed, when compared to the NMR data of the acceptor glycan **1** [39] (see Table 1), features characteristic of α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4) units: a doublet at 5.146 ppm arising from H-1 of the α-D-Gal*p*-(1→3) units was evident, as were the H-4 signals of the α-D-Gal*p*-(1→3) units at 4.021 ppm [35,40,41]. In this case, the H-5 signals around 4.19 ppm [35,42] were severely overlapped by the β-D-Gal*p*-(1→4) H-4 and α-D-Man*p*-(1→3) H-2 signals. Moreover, the H-1 signals of the 6 and 6′ residues were shifted characteristically downfield to 4.541 ppm, and the H-4 signals of the (1→3)-substituted β-D-Gal*p* residues 6 and 6′ were found at 4.183 ppm. Spectral integration implied that the reaction product carried two α-D-Gal*p*-(1→3) residues.

In MALDI-MS this product gave a peak at *m/z* 1785.2 (Fig. 5A) which represents the sodiated species; the DHB matrix is known to produce mainly $(M+Na)^+$ signals for oligosaccharide samples [43,44]. The calculated mass for glycan **2** in the sodiated form is 1784.6 Da.

Enzymic synthesis of the tetraantennary glycan **6**.—When the biantennary glycan **1** (212 nmol, 2.15×10^6 dpm [^{3}H] at β-D-Gal*p* units 6 and 6′) was (1→3)-*N*-acetyl-β-D-glucos-

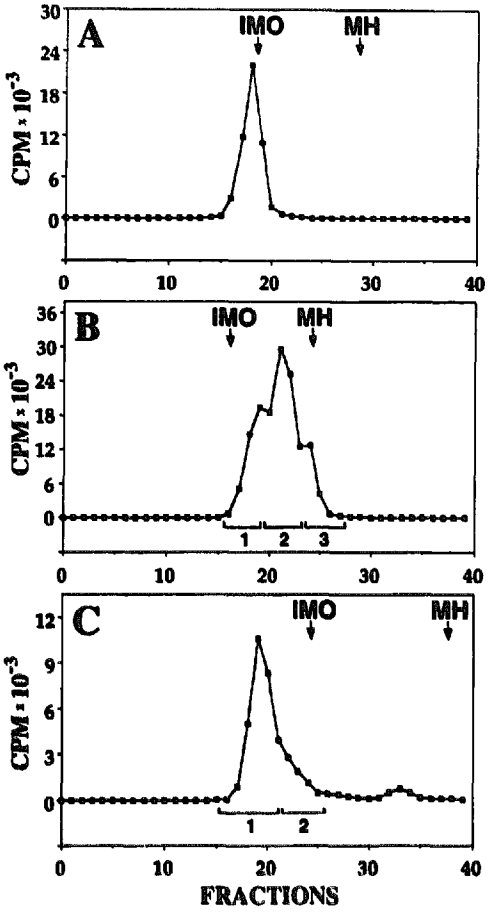


Fig. 2. Paper chromatographic separation of glycosyltransferase mixtures: (A) (1→3)-α-D-galactosylation of glycan **1** (see Fig. 1 for the structures); (B) (1→3)-*N*-acetyl-β-D-glucosaminylation of glycan **1**; (C) (1→6)-*N*-acetyl-β-D-glucosaminylation of glycan **3**. The positions of marker saccharides isomaltooctaose (IMO) and maltoheptaose (MH) are indicated.

aminylated, three components were obtained by paper chromatography (Fig. 2B). Peak 1 (55 nmol) represented the fully reacted product, glycan **3**; endo-β-D-galactosidase digestion liberated 96% of its [6-^{3}H]Gal label as β-D-Glc*p*NAc-(1→3)-D-[6-^{3}H]Gal, which was identified by paper chromatography (not shown). Peak 3 (25 nmol) exhibited a mobility similar to that of glycan **1**, while peak 2 (118 nmol) obviously contained mono-(1→3)-*N*-acetyl-β-D-glucosaminylated derivatives.

Re-(1→3)-*N*-acetyl-β-D-glucosaminylation of peak 2 in Fig. 2B yielded 76 nmol of glycan **3** and 17 nmol of unreacted substrate.

The structure of glycan **3** was fully confirmed by ^{1}H NMR spectroscopy (Table 1). The signals from the acceptor were accompanied by the following spectral features: the doublet at 4.680 ppm (stemming from two protons) is characteristic for H-1 of β-D-Glc*p*NAc-(1→3) units and the doublet at 4.154 ppm (two protons) represents H-4 of the (1→3)-substituted β-D-Gal*p* residues 6 and 6′ [45,46]. Interestingly, the H-1 signals of β-D-Glc*p*NAc-(1→2) units 5 and 5′ were separated, showing doublets at 4.578 and 4.582 ppm. In contrast, the H-1 signals of units 5 and 5′ coincide in glycans **1** and **2** as well as in the di-(α-D-Neu*p*5Ac-(2→3)) [47] and di-(α-D-Neu*p*5Ac-(2→6)) [39] derivatives of glycan **1**.

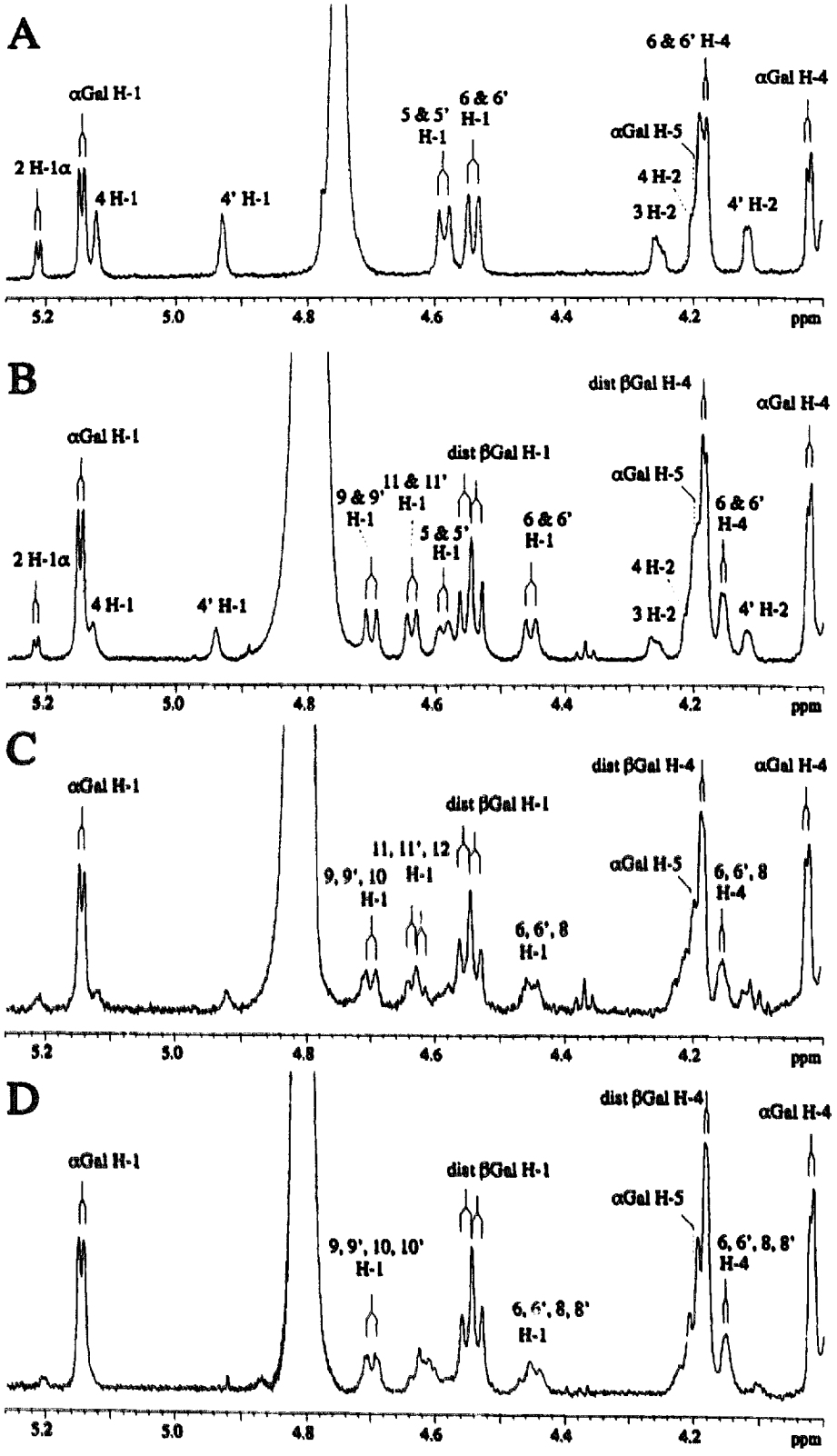


Fig. 3. The structural reporter group regions of ^{1}H NMR spectra of (1→3)-α-D-galactosylated glycans: (A) peak from Fig. 2A (glycan **2**); (B) peak 1 from Fig. 6B (glycan **6**); (C) peak 1 from Fig. 8D (glycan **11**); (D) peak 1 from Fig. 11D (glycan **16**). See Fig. 4 for the denotation system of the constituent monosaccharide residues.

Table 1
^{1}H NMR chemical shifts of structural reporter groups of monosaccharide residues in glycans **1–16**. See Fig. 4 for the notation system

	Glycan	1 [a]	2	3	4	5	6	7 [b]
Reporter group	Residue							
H-1	2	5.210(α)	5.213(α)	5.211(α)	5.213(α)	5.214(α)	5.210(α)	5.211(α)
	4	5.122	5.124	5.118	5.125	5.129	5.128	5.118
	4′	4/924	4.930	4.928	4.936	4.940	4.937	4.923(α) 4.925(β)
	5	4.582	4.587	4.578 [c]	4.583 [c]	4.585 [c]	4.58	4.569
	5′	4.582	4.587	4.582 [c]	4.588 [c]	4.588 [c]	4.58	4.584
	6	4.466	4.541	4.454	4.448	4.452	4.452	4.468
	6′	4.470(α) 4.472(β)	4.541	4.459	4.451	4.452	4.452	4.473(α) 4.471(β)
	7							4.546
	7′							
	8							4.463
	8′							
	9/9′			4.680	4.675	4.697	4.699	
	10/10′							
	11/11′				4.592	4.637	4.636	
	12/12′							
	distβGal					4.465/ 4.481	4.535/ 4.551	
	αGal		5.146				5.146	
H-4	6/6′		4.182	4.154	4.147	4.154	4.154	
	8/8′							
	distβGal						4.183	
	αGal		4.021				4.021	

When glycan **3** (75 nmol, 7.6×10^5 dpm [^{3}H]) was (1→6)-*N*-acetyl-β-D-glucosaminylated, the mixture yielded 41 nmol of glycan **4** (peak 1 in Fig. 2C) and 10 nmol of products carrying only one β-D-Glc*p*NAc-(1→6) unit (peak 2 in Fig. 2C). The acceptor glycan **3**, if present, would have migrated around fraction 29.

The ^{1}H NMR spectrum of glycan **4** (Table 1) revealed a doublet partially overlapping with the H-1 signals of units 5 and 5′ at 4.592 ppm; this value is characteristic for the H-1 signal of the β-D-Glc*p*NAc-(1→6) unit in the tetrasaccharide β-D-Glc*p*NAc-(1→3)[β-D-Glc*p*NAc-(1→6)]β-D-Gal*p*-(1→4)-D-GlcNAc [46,48] as well as in a large synthetic oligo-(*N*-acetyllactosamino)glycan [49]. In addition, small but clear upfield changes in the H-1 and H-4 signals of β-D-Gal*p* units 6 and 6′ were evident, characteristic of β-D-Glc*p*NAc-(1→6) substitution [46]. These data, together with spectral integration, confirms that peak 1 in Fig. 2C represented glycan **4**.

8	9	10	11	12[b]	13	14	15	16
5.211(α)	5.211(α)	5.210(α)	5.212(α)	5.205(α)	5.204(α)	5.206(α)	n.d.	n.d.
5.117	5.123	55.124	5.122	5.131(α)	5.130	5.132	n.d.	n.d.
				5.129(β)				
4/919	4.922	4.922	4.919	4.870	4.866	n.d.	n.d.	n.d.
4.566	4.571	4.571	n.d.	4.572	4.569	n.d.	n.d.	n.d.
4.581	n.d.[d]	4.587	n.d.	4.592	4.588	n.d.	n.d.	n.d.
4.457	4.444[c]	4.45	4.45	4.469	4.455	4.55	n.d.	n.d.
4.457	4.450[c]	4.45	4.45	4.469(α)	4.455	4.45	n.d.	n.d.
				4.471(β)				
4.542	4.547	4.545	n.d.	4.547	4.536	n.d.	n.d.	n.d.
				4.554	4.546	n.d.	n.d.	n.d.
4.451	4.444[c]	4.45	4.45	4.464	4.450	4.45	n.d.	n.d.
				4.480	4.469	4.46	n.d.	n.d.
4.686	4.674	4.696	4.700		4.681[c]	4.68	4.697[c]	4.70
4.686	4.674	4.696	4.700		4.686[c]	4.68	4.700[c]	4.70
	4.593[e]	4.637[c]	4.632[c]			4.591	4.625[e]	n.d.
	4.588[e]	4.626[c]	4.621[c]			4.591	4.640[e]	n.d.
		4.464/	4.536/				4.466/	4.538/
		4.480	4.552				4.481	4.553
			5.146					5.146
4.154	4.146	4.154	4.15		4.154	4.145	4.15	4.15
4.148	4.140	4.148	4..		4.154	4.145	4.15	4.15
			4.183					4.183
			4.021					4.020

[a] Values of Wieruszeski et al. [39].
[b] Values of Vliegenthart et al. [47].
[c] Values may have to be interchanged.
[d] n.d., Not determined.
[e] Values may have to be interchanged.

Enzymic (1→4)-β-D-galactosylation of glycan **4** gave a mixture that was separated by HPAE chromatography (Fig. 6A), revealing one major peak (peak 1; 25 nmol) in the oligosaccharide region. This material represented glycan **5**: as compared to the ^{1}H NMR spectrum of glycan **4** (Table 1), that of **5** revealed additional β-D-Gal*p* H-1 resonances of four extra protons. The two new doublets at 4.465 and 4.481 ppm probably originated from the β-D-Gal*p* H-1 units substituting the β-D-Glc*p*NAc-(1→3) units 9 and 9′ and the β-D-Glc*p*NAc-(1→6) units 11 and 11′, respectively [50–52]. The β-D-Glc*p*NAc-(1→3) H-1 signals showed clear shifts to lower field upon (1→4)-β-D-galactosylation (4.675–4.697 ppm). An even more pronounced downfield shift was evident for the H-1 signals of β-D-Glc*p*NAc-(1→6) units 11 and 11′ (4.592 ppm to 4.637 ppm), characteristic of β-D-Gal*p*-(1→4) substitution [49,51,53]; these signals are now clearly separated from the H-1 signals of β-D-Glc*p*NAc-(1→2) units 5 and 5′.

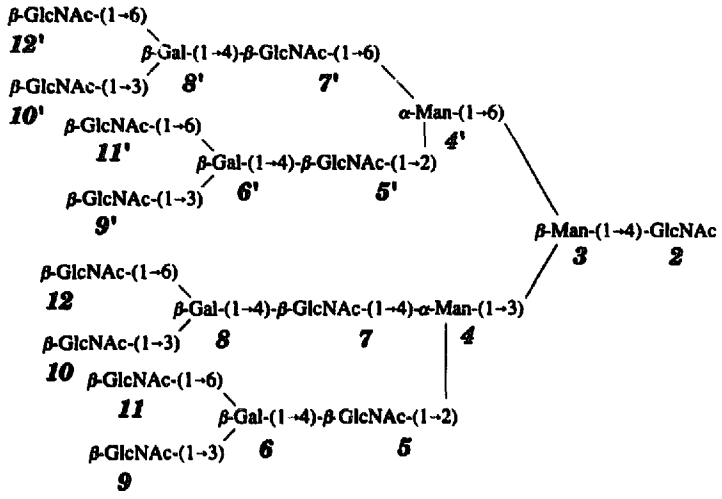


Fig. 4. The denotation system used in this study for the constituent monosaccharides. (1→4)-β-D-Gal*p* units substituting the β-D-Glc*p*NAc-(1→3/6) residues are referred to as dist βGal. Distal (1→3)-α-D-Gal*p* units are denoted as αGal.

The other peaks in Fig. 6A were not studied; some of them may represent undergalactosylated products and reducing end epimers.

The glycan **5** was finally (1→3)-α-D-galactosylated enzymically. Separation of the mixture by HPAE chromatography (Fig. 6B) gave a major product (peak 1; 10.5 nmol) representing glycan **6** as shown by ^{1}H NMR analysis (Fig. 3B and Table 1). The spectrum

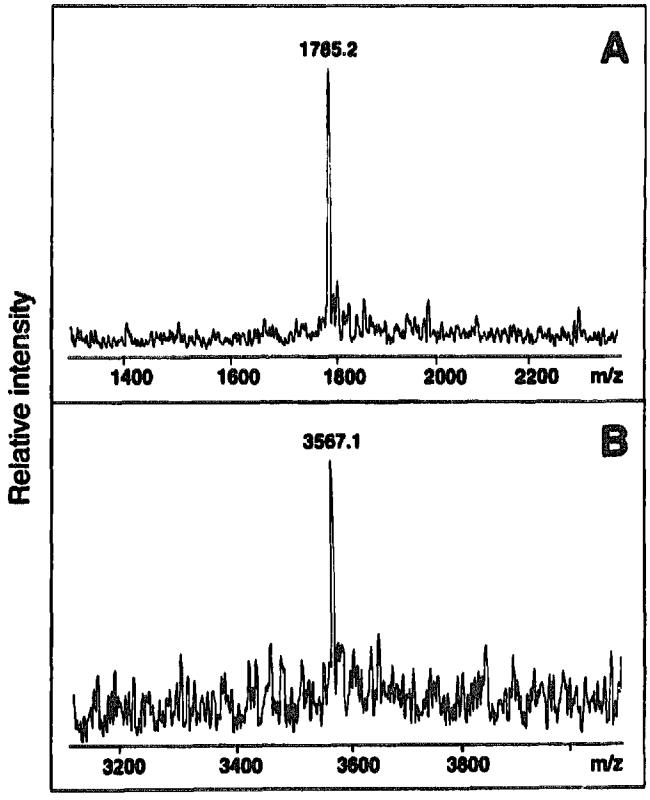


Fig. 5. Matrix-assisted laser-desorption mass spectrometric analysis of reaction products: (A) glycan **2**; (B) glycan **6**.

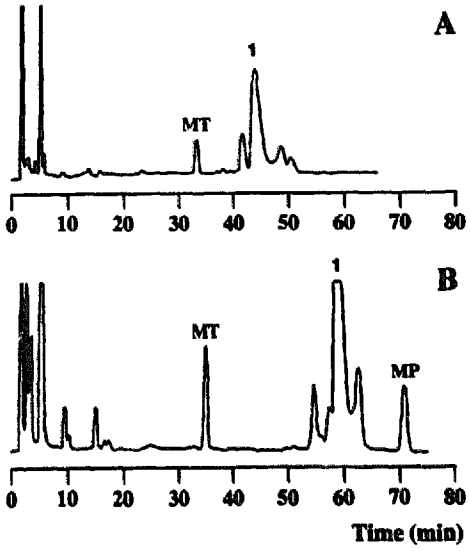


Fig. 6. HPAEC of glycosyltransferase reaction products: (A) (1→4)-β-D-galactosylation of glycan **4**; (B) (1→3)-α-D-galactosylation of glycan **5**. The elution peaks of internal marker saccharides maltotriose (MT) and maltopentaose (MP) are indicated.

of **6** revealed several specific signals for the distal α-D-Gal*p*-(1→3) units, similar to those in glycan **2**: H-1 and H-4 doublets at 5.146 and 4.021 ppm, respectively, and H-5 at ca. 4.19 ppm [obscured by the β-D-Gal*p*-(1→4) H-4 and α-D-Man*p*-(1→3) H-2 signals]. The 3-substitution of the distal β-D-Gal*p*-(1→4) units is visualized by a downfield shift of their H-4 signal to 4.183 ppm; these signals are clearly separated from the H-4 signals of β-D-Gal*p*-(1→4) units 6 and 6′, substituted at 3-OH and 6-OH by β-D-Glc*p*NAc units. In addition, the H-1 resonances of the distal β-D-Gal*p*-(1→4) units showed a pronounced shift to lower field, giving rise to doublets at 4.551 and 4.535 ppm; these probably originate from the distal β-D-Gal*p*-(1→4) units in the β-D-Glc*p*NAc-(1→3) (9 and 9′) and β-D-Glc*p*NAc-(1→6) (11 and 11′) branches, respectively [35]. Integration of the spectrum in Fig. 3B implied that it carried four α-D-Gal*p*-(1→3) units.

The molecular weight of the material in peak 1 of Fig. 6B was determined by MALDI-MS (Fig. 5B). The spectrum showed a peak at *m/z* 3567.1; the calculated mass for the sodiated species of **6** is 3570.2 Da.

Enzymic synthesis of the hexaantennary glycan **11**.—The triantennary glycan **7** (306 nmol) (see Fig. 7 for structures of glycans **7–11**) was (1→3)-*N*-acetyl-β-D-glucosaminylated as above. The oligosaccharide fraction of the mixture was isolated by Bio-Gel P-2 chromatography and subsequently subjected to HPAE chromatography (Fig. 8A). Peaks 1 and 2 were tentatively identified as di-(β-D-Glc*p*NAc-(1→3))- and tri-(β-D-Glc*p*NAc-(1→3)) products, respectively. Peaks 3 and 4 were not studied; they may represent ManNAc analogues of the main products. Peak 1 and material eluting between 33–37 min were re-(1→3)-*N*-acetyl-β-D-glucosaminylated. The HPAE chromatograms revealed a major product with similar relative mobility as peak 2 in Fig. 8A (not shown), implying that it indeed was the fully (1→3)-*N*-acetyl-β-D-glucosaminylated product, glycan **8**. The overall yield of **8** was 118 nmol.

The ^{1}H NMR spectrum of glycan **8** (Table 1) revealed characteristic H-1 signals of β-D-Glc*p*NAc-(1→3) units at 4.686 ppm, originating from three protons. The substitution

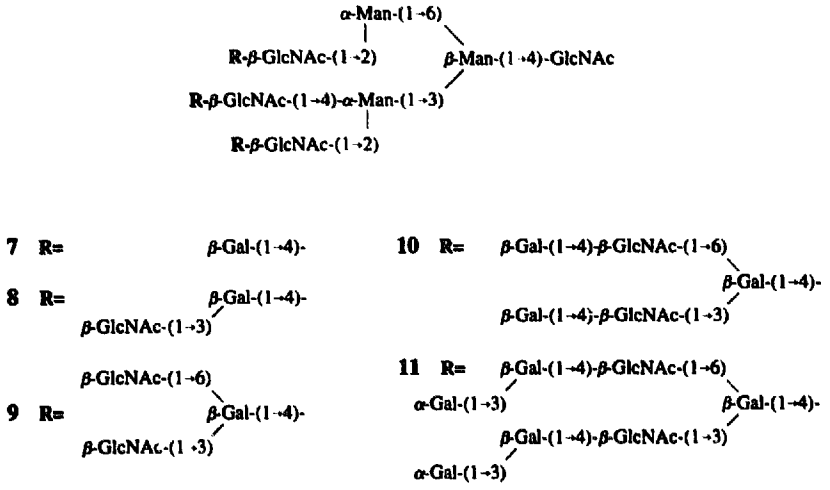


Fig. 7. Structures of the saccharides related to the triantennary glycan **7**.

of β-D-Gal*p* residues 6, 6′, and 8 by β-D-Glc*p*NAc-(1→3) units was clearly indicated by the appearance of the 6, 6′, and 8 H-4 signals around 4.15 ppm, equivalent to three protons. The NMR data thus confirmed the identity of peak 2 in Fig. 8A as glycan **8**.

Glycan **8** was enzymically (1→6)-*N*-acetyl-β-D-glucosaminylated as above. The reaction products were subjected to HPAE chromatography (Fig. 8B), showing one major product (peak 1; 53 nmol), which was identified as glycan **9** by ^{1}H NMR: a new pair of doublets originating from three protons was evident at 4.588 and 4.593 ppm, a typical value for H-1 signals of unsubstituted β-D-Glc*p*NAc-(1→6) units (see above). Also, the H-1 signals of the β-D-Gal*p* units 6, 6′, and 8 and both the β-D-Glc*p*NAc units 5, 5′, and 7 and 9, 9′, and 10 showed clear shifts to higher field, as did the H-4 signals of 6, 6′, and 8. All these changes are compatible with the structure of glycan **9**.

(1→4)-β-D-Galactosylation of the glycan **9** (47 nmol) gave a major product in HPAE chromatography (peak 1 in Fig. 8C; 21 nmol), which represented the glycan **10**. In the ^{1}H NMR spectrum of **10**, the distal β-D-Gal*p*-(1→4) units gave a pair of doublets at 4.464 and 4.480 ppm (Table 1); together with the H-1 signals of 6, 6′, and 8 the β-D-Gal*p* H-1 region now carried signals from nine protons. As in the case of glycan **5**, the H-1 signals of β-D-Glc*p*NAc-(1→6) units (11, 11′, and 12) experienced a pronounced shift to lower field upon β-D-galactosylation, and were separated from the H-1 signals of 5, 5′, and 7.

Finally, glycan **10** (19.2 nmol) was (1→3)-α-D-galactosylated as above. The major peak from the HPAEC separation of the mixture (Fig. 8D, peak 1; 7.6 nmol) represented glycan **11**. In the ^{1}H NMR spectrum of **11** (Fig. 3C and Table 1), α-D-Gal*p*-(1→3) H-1 and H-4 signals identical to those of glycan **6** were found; in addition, the H-1 signals of the distal β-D-Gal*p*-(1→4) units experienced characteristic shifts to lower field (see above). Integration of the spectrum implied that this material carried six α-D-Gal*p*-(1→3) units.

Glycan **11** was further analyzed by MALDI-MS (Fig. 9A). The spectrum revealed a major peak at *m/z* 4987.0; the calculated value for the glycan **11** in the sodiated form is 4990.5 Da.

Enzymic synthesis of the octaantennary glycan **16**.—The tetraantennary glycan **12** (300 nmol) (see Fig. 10 for the structures of glycans **12**–**16**) was (1→3)-*N*-acetyl-β-D-glucosaminylated enzymically as above. The mixture was separated by HPAEC, revealing three

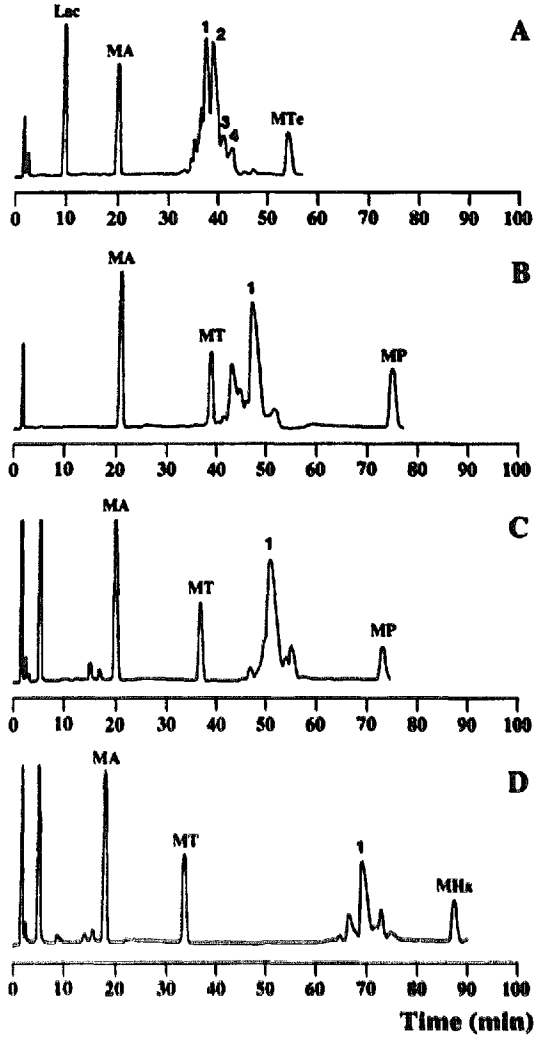


Fig. 8. HPAEC of glycosyltransferase reaction products: (A) (1→3)-*N*-acetyl-β-D-glucosaminylation of glycan **7**; (B) (1→6)-*N*-acetyl-β-D-glucosaminylation of glycan **8**; (C) (1→4)-β-D-galactosylation of glycan **9**; (D) (1→3)-α-D-galactosylation of glycan **10**. The elution peaks of internal marker saccharides lactose (Lac), maltose (MA), maltotriose (MT), maltopentaose (MP), and maltohexaose (MHx) are indicated.

products (Fig. 11A). Each peak showed characteristic features of (1→3)-*N*-acetyl-β-D-glucosaminylated products in the ^{1}H NMR spectra; by comparing the intensities of the H-1 signals of β-D-Glc*p*NAc-(1→3) units and H-4 signals of 3-substituted β-D-Gal*p*-(1→4) units, peaks 1 and 2 could be identified as di- and tri-(β-D-Glc*p*NAc-(1→3)) products, respectively (data not shown), and peak 3 as the fully reacted product, glycan **13**. The ^{1}H NMR spectrum of glycan **13** (Table 1) revealed characteristic H-1 signals of β-D-Glc*p*NAc-(1→3) units originating from four protons at 4.681/4.686 ppm. Substitution of β-D-Gal*p* residues 6, 6′, 8, and 8′ by β-D-Glc*p*NAc-(1→3) units was indicated by the appearance of the 6, 6′, 8, and 8′ H-4 signals at 4.154 ppm, equivalent to four protons.

The material from peaks 1 and 2 was re-(1→3)-*N*-acetyl-β-D-glucosaminylated, yielding material with a mobility similar to that of peak 3. The total yield of **13** was 138 nmol.

When glycan **13** (138 nmol) was (1→6)-*N*-acetyl-β-D-glucosaminylated, HPAEC analysis of the products revealed one major peak (Fig. 11B, peak 1; 18.8 nmol) which was

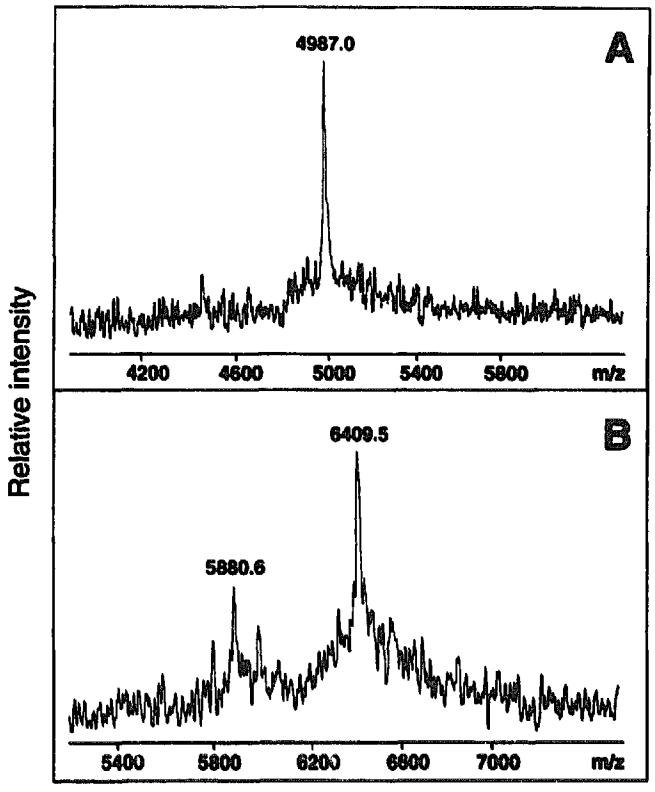
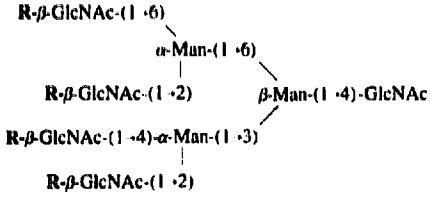


Fig. 9. Matrix-assisted laser-desorption mass spectrometric analysis of reaction products: (A) glycan **11**; (B) glycan **16**.

found to represent impure glycan **14**. In the ^{1}H NMR analysis, the H-1 signals of the newly attached β-D-Glc*p*NAc-(1→6) units were found at a characteristic position, 4.591 ppm, but integration of the spectrum suggested that one β-D-Glc*p*NAc-(1→3) unit was missing from about one half of the molecules. Hog gastric microsomal preparations were subsequently shown to contain *N*-acetyl-β-D-glucosaminidase activity [54].



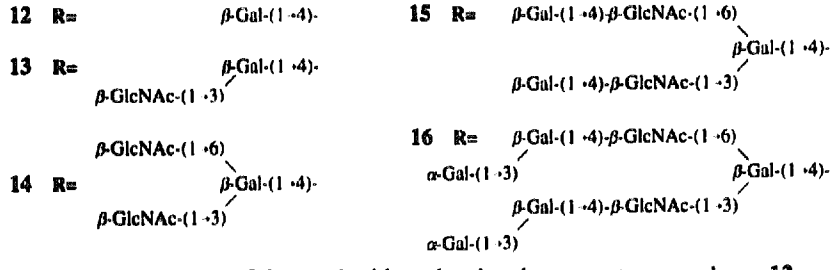


Fig. 10. Structures of the saccharides related to the tetraantennary glycan **12**.

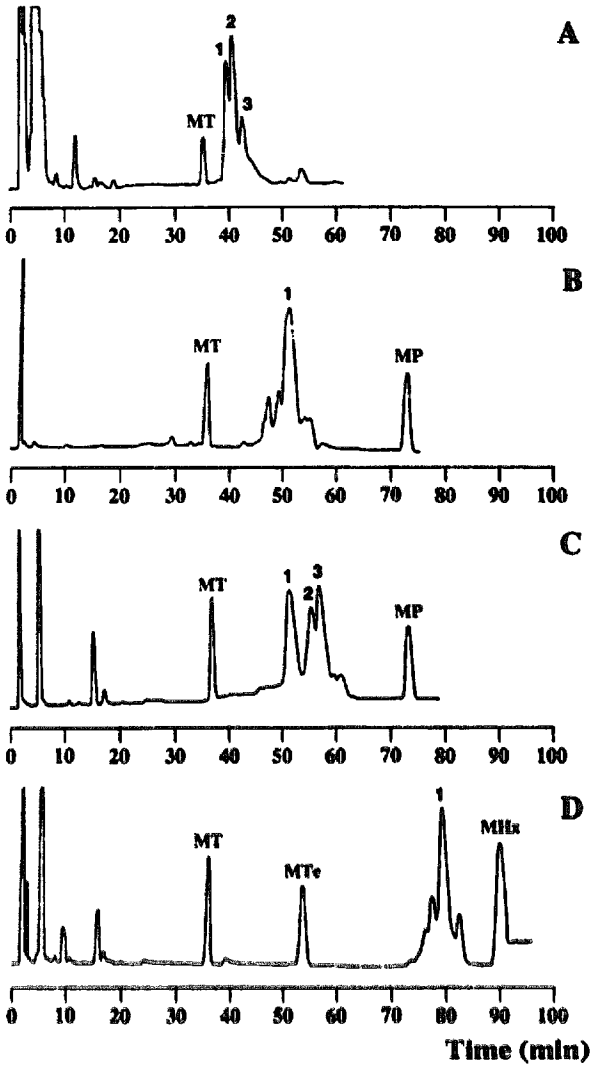


Fig. 11. HPAEC of glycosyltransferase reaction products: (A) (1→3)-*N*-acetyl-β-D-glucosaminylation of glycan **12**; (B) (1→6)-*N*-acetyl-β-D-glucosaminylation of glycan **13**; (C) (1→4)-β-D-galactosylation of glycan **14**; (D) (1→3)-α-D-galactosylation of glycan **15**. The elution peaks of internal marker saccharides maltotriose (MT), maltotetraose (MTe), maltopentaose (MP), and maltohexaose (MHx) are indicated.

Impure glycan **14** (18.3 nmol) was subjected to enzymic (1→4)-β-D-galactosylation without further attempts to purify the mixture. Three major products were obtained by HPAEC (Fig. 11C) of which peak 3 (7.7 nmol) proved to represent the octaantennary 28-mer, glycan **15**. ^{1}H NMR analysis of **15** revealed a pair of doublets at 4.466 and 4.481 ppm, originating from the newly attached (1→4)-β-D-Gal*p* units. In addition, H-1 signals of the β-D-Glc*p*NAc-(1→6) units 11, 11′, 12, and 12′ were characteristically shifted to lower field. Spectral integration implied that ca. twelve (1→4)-β-D-Gal*p* units were present. ^{1}H NMR analysis of peak 2 suggested that it was a mixture of fully galactosylated heptaantennary glycans [with three β-D-Glc*p*NAc-(1→3) units, see above] and undergalactosylated glycans (data not shown). Peak 1 appeared to be of non-carbohydrate origin (not shown).

Glycan **15** (7.4 nmol) was finally (1→3)-α-D-galactosylated. HPAE chromatography of the mixture (Fig. 11D) revealed one major product (peak 1; 6.1 nmol). ^{1}H NMR analysis

of this material showed characteristic signals for H-1 (5.146 ppm) and H-4 (4.020 ppm) of α-D-Gal*p*-(1→3) units (Fig. 3D). Moreover, H-1 and H-4 signals of the distal β-D-Gal*p*-(1→4) units were found at characteristic values (Table 1). Integration of the spectrum implied that ca. eight α-D-Gal*p*-(1→3) units were present. Hence, the NMR data suggested that the product represented glycan **16**.

Peak 1 of Fig. 11D was also analyzed by MALDI-MS (Fig. 9B). The spectrum revealed a major peak at *m/z* 6409.5. The calculated value for the glycan **16** in sodiated form is 6410.8 Da. An impurity was seen at *m/z* 5880.5; the mass difference (529 Da) suggested that it was a 33-mer lacking two hexose units and one HexNAc unit, probably a α-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc group of glycan **16**.

4. Discussion

Nonsialylated glycoproteins carrying terminal β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc units were initially found as good acceptors for (1→3)-*N*-acetyl-β-D-glucosaminyltransferases from Novikoff ascites tumor cells [55] and human serum [56,57]. Specifically, desialylated α_1-acid glycoprotein, carrying mainly tetraantennary *N*-glycans, was a better acceptor than asialofetuin or asialotransferrin [56–58]. Consistently, it was shown that N-linked type oligosaccharides carrying the β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)[β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)]α-D-Man*p*-(1→6) unit were more efficiently (1→3)-*N*-acetyl-β-D-glucosaminylated than structures lacking this feature [58]. Indeed, analysis of poly-(*N*-acetyllactosamino)glycans in *N*-glycans from biological sources indicated that the pentasaccharide unit described above is the preferred site of repeating *N*-acetyllactosamine sequences [31,32]. Interestingly, in cases where poly-(*N*-acetyllactosamino)glycan chains are present in biantennary type *N*-glycans, the α-D-Man*p*-(1→6) branch also seems to accommodate longer sequences [30,59].

Unlike natural poly-(*N*-acetyllactosamino)glycan chains, the glycans in the present experiments were fully (1→3)-*N*-acetyl-β-D-glucosaminylated bi-, tri-, and tetra-antennary N-linked type oligosaccharides. These structures were generated by prolonged incubations with human serum (1→3)-*N*-acetyl-β-D-glucosaminyltransferase, suggesting that the restricted location of the interlinked β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→3) residues in natural glycans may arise by kinetic selectivity of (1→3)-*N*-acetyl-β-D-glucosaminylation. Subsequently we generated blood group I-type structures capped with (1→3)-α-D-Gal units on the glycans, following the protocols established in the *de novo* synthesis of a 18-meric oligo-(*N*-acetyllactosamino)glycan [49]: The glycans were branched by use of (1→6)-*N*-acetyl-β-D-glucosaminyltransferase activity from hog gastric mucosal microsomes [33,60], (1→4)-β-D-galactosylated with bovine milk galactosyltransferase [34], and finally (1→3)-α-D-galactosylated by use of the bovine thymus enzyme [35,36].

Because of the crude nature of the (1→3)- and (1→6)-*N*-acetyl-β-D-glucosaminyltransferases used in this study, the yield of these steps was relatively small. Clearly, for efficient syntheses more pure enzymes would be advantageous. Recently, Bierhuizen et al. cloned a cDNA encoding a (1→6)-*N*-acetyl-β-D-glucosaminyltransferase [61]; when this cDNA was expressed in i-antigen presenting cells, I-antigens were formed, as was indicated by immunofluorecence with an anti-I antibody, endo-β-D-galactosidase digestion, and meth-

ylation analysis. In addition, Kawashima et al. purified a (1→3)-*N*-acetyl-β-D-glucosaminyltransferase from calf serum [62], which exhibited acceptor characteristics quite similar to those of human serum [56,57] and Novikoff ascites fluid transferases [58].

The use of the HPAEC–PAD system in the purification of large branched glycans, up to 36-mer, was to be found effective. In particular, the fully (1→3)-α-D-galactosylated tetra-, hexa-, and octa-antennary glycans **6**, **11**, and **16** eluted ca. 14, 20, and 24 min later, respectively, than the precursor glycans **5**, **10**, and **15**, implying that each inserted (1→3)-α-D-Gal*p* unit contributed ca. 3 min to the retention time. Addition of (1→4)-β-D-Gal*p* units resulted in considerably smaller increase in retention time, as exemplified by the pairs **9**/**10** and **14**/**15**. In general, glycans with α-glycosidic bonds seem to be more strongly retained on HPAE columns than their β-anomeric isomers, reflecting the stronger acidity of the α-linked monosaccharide units [63]. To our knowledge, the glycans in the present study represent the largest *N*-glycans analyzed by HPAEC; however, Koizumi et al. have reported separation of glucose polymers up to dp 50 [64].

A drawback in the use of the HPAEC–PAD system is the unpredictable relationship between molar amount and PAD response for different glycans. High-performance gel permeation chromatography on Superdex 75 HR with UV detection was used in the present study and proved to be efficient as a quantitation system and served as a final purification step before NMR analyses. Another drawback to HPAEC is the separation of the reducing end epimeric forms. In a separate study, we have established by ^{1}H NMR spectroscopy that triantennary *N*-glycans, liberated by peptide *N*-glycosidase F from asialofetuin, are separated by HPAEC into reducing end GlcNAc and ManNAc forms (A. Seppo et al., unpublished results). In the present experiments, the more slowly migrating ManNAc epimer constituted 10–15% of the whole glycan pool. Deficiently glycosylated ManNAc analogues may thus be present as contaminats in the main products of the present experiments.

In the ^{1}H NMR analyses, the structural reporter groups [47] could be used for verifying the nature of the transferred monosaccharide unit as well as the anomericity and the substitution pattern. Integration of the spectra was found to be quite reliable; only in the case of the 36-meric octaantennary glycan **16**, mass spectrometry revealed the presence of an impurity, a 33-mer that was not detected by NMR. The presence of the 33-mer was however somewhat unexpected, because HPAEC should have separated it from the 36-mer. The 33-mer probably did not originate from fragmentation in the mass spectrometric ionization process either, as neutral oligosaccharides have not been reported to undergo fragmentation in a linear MALDI system [44]; underivatized gangliosides however do produce fragment ions by loss of sialic acid residues [65].

Multivalent carbohydrate ligands bind to their receptors with higher affinity than the monovalent structures [66–69]. As α-D-Gal units in the O-linked glycans of mouse egg ZP3 glycoprotein form an essential part of the sperm binding element [70], some of the constructs obtained in the present study may prove useful in assessing the structural parameters involved in the adhesion process between mouse gametes. Although the natural adhesive carbohydrate structures are carried by the O-glycosidically linked glycans of zona pellucida protein ZP3 on mouse egg [71,72], there is data supporting the view that the distal parts of saccharides carry the binding elements and the O-glycosidic core structures are not essential for binding [70,72,73]. With the aid of the glycans obtained in the present study, we hope to get information about the valency of the binding elements in the mouse

gamete adhesion, as well as about the difference of O- and N-linked type oligosaccharides as adhesion inhibitors.

Acknowledgements

This work was supported in part by grants from the University of Helsinki, from the Academy of Finland, from the Jenny and Antti Wihuri Foundation, from the Finnish Cultural Foundation, from the Orion Corporation Research Foundation, from the Emil Aaltonen Foundation, and from the Leo and Regina Wainstein Foundation.

The authors gratefully acknowledge the kind donation of glycans **1**, **7**, and **12** by Drs. G. Strecker and J. Montreuil.

References

[1] M. Fukuda, *Biochim. Biophys. Acta*, 780 (1985) 119–150.
[2] A.H.L. Koenderman, PhD Thesis, 1987, Vrije Universiteit, The Netherlands.
[3] A. Kapadia, T. Feizi, and M.J. Evans, *Exp. Cell Res.*, 131 (1981) 185–195.
[4] T. Muramatsu, G. Gachelin, J.F. Nicolas, H. Condamine, H. Jakob, and F. Jacob, *Proc. Natl. Acad. Sci. U.S.A.*, 75 (1978) 2315–2319.
[5] D. Spillmann and J. Finne, *Eur. J. Biochem.*, 220 (1994) 385–394.
[6] A. Mizoguchi, S. Takasaki, S. Maeda, and A. Kobata, *J. Biol. Chem.*, 259 (1984) 11949–11957.
[7] M. Fukuda, S.R. Carlsson, J.C. Klock, and A. Dell, *J. Biol. Chem.*, 261 (1986) 12796–12806.
[8] B.A. Fenderson, D.A. O'Brien, C.F. Millette, and E.M. Eddy, *Dev. Biol.*, 103 (1984) 117–128.
[9] A.M. Mercurio and P.W. Robbins, *J. Immunol.*, 135 (1985) 1305–1312.
[10] Y. Tsuji, H. Clausen, E. Nudelman, T. Kaizu, S.-I. Hakomori, and S. Isojima, *J. Exp. Med.*, 168 (1988) 343–356.
[11] S. Noguchi and M. Nakano, *Eur. J. Biochem.*, 209 (1992) 883–894.
[12] C.H. Hokke, J.B.L. Damm, B. Penninkhof, R.J. Aitken, J.P. Kamerling, and J.F.G. Vliegenthart, *Eur. J. Biochem.*, 221 (1994) 491–512.
[13] A. Dutt, J.-P. Tang, and D.D. Carson, *J. Biol. Chem.*, 263 (1988) 2270–2279.
[14] G.M. Brown, T.N. Huckerby, H.G. Morris, B.L. Abram, and I.A. Nieduszynski, *Biochemistry*, 33 (1994) 4836–4846.
[15] A. Dutt, J.-P. Tang, and D.D. Carson, *Dev. Biol.*, 119 (1987) 27–37.
[16] R.K. Merkle and R.D. Cummings, *J. Biol. Chem.*, 263 (1988) 16143–16149.
[17] S. Sato and R.C. Hughes, *J. Biol. Chem.*, 267 (1992) 6983–6990.
[18] S.H. Barondes, V. Castronovo, D.N.W. Cooper, R.D. Cummings, K. Drickamer, T. Feizi, M.A. Gitt, J. Hirayabashi, C. Hughes, K.-I. Kasai, H. Leffler, F.-T. Liu, R. Lotan, A.M. Mercurio, M. Monsigny, S. Pillai, F. Poirer, A. Raz, P.W.J. Rigby, J.M. Rini, and J.L. Wang, *Cell*, 76 (1994) 597–598.
[19] D.N.W. Cooper and S.H. Barondes, *J. Cell Biol.*, 110 (1990) 1681–1691.
[20] H.-J. Woo, L.M. Shaw, J.M. Messier, and A.M. Mercurio, *J. Biol. Chem.*, 265 (1990) 7097–7099.
[21] B.J. Cherayil, S. Chaitovitz, C. Wong, and S. Pillai, *Proc. Natl. Acad. Sci. U.S.A.*, 87 (1990) 7324–7328.
[22] H. Niemann, K. Watanabe, S.-I. Hakomori, R.A. Childs, and T. Feizi, *Biochem. Biophys. Res. Commun.*, 81 (1978) 1286–1293.
[23] J. Koscielak, E. Zdebska, Z. Wilczynska, H. Miller-Podraza, and W. Dzierzkowa-Borodej, *Eur. J. Biochem.*, 96 (1979) 331–337.
[24] K. Watanabe, S. Hakomori, R.A. Childs, and T. Feizi, *J. Biol. Chem.*, 254 (1979) 3221–3228.
[25] J. Koscielak, H. Miller-Podraza, R. Krauze, and A. Piasek, *Eur. J. Biochem.*, 71 (1976) 9–18.
[26] J. Finne, T. Krusius, H. Rauvala, R. Kekomäki, and G. Myllylä, *FEBS Lett.*, 89 (1978) 111–115.

[27] E. Spoocer, M. Fukuda, J.C. Klock, J.E. Oates, and A. Dell, *J. Biol. Chem.*, 259 (1984) 4792–4801.
[28] M. Fukuda, E. Spooncer, J.E. Oates, A. Dell, and J.C. Klock, *J. Biol. Chem.*, 259 (1984) 10925–10935.
[29] H. Egge, M. Kordowicz, J. Peter-Katalinic, and P. Hanfland, *J. Biol. Chem.*, 260 (1985) 4927–4935.
[30] M. Fukuda, A. Dell, J.E. Oates, and M.N. Fukuda, *J. Biol. Chem.*, 259 (1984) 8260–8273.
[31] M.N. Fukuda, A. Dell, J.E. Oates, and M. Fukuda, *J. Biol. Chem.*, 260 (1985) 6623–6631.
[32] C.H. Hokke, J.P. Kamerling, G.W.K. van Dedem, and J.F.G. Vliegenthart, *FEBS Lett.*, 286 (1991) 18–24.
[33] A. Seppo, L. Penttilä, A. Makkonen, A. Leppänen, J. Jäntti, J. Helin, and O. Renkonen, *Biochem. Cell Biol.*, 68 (1990) 44–53.
[34] K. Brew, T.C. Vanaman, and R.L. Hill, *Proc. Natl. Acad. Sci. U.S.A.*, 59 (1968) 491–497.
[35] A. Seppo, L. Penttilä, A. Leppänen, H. Maaheimo, R. Niemelä, J. Helin, J.-M. Wieruszeski, and O. Renkonen, *Glycoconjugate J.*, in press.
[36] W.M. Blanken and D.H. van den Eijnden, *J. Biol. Chem.*, 260 (1985) 12927–12934.
[37] O. Renkonen, L. Penttilä, A. Makkonen, R. Niemelä, A. Leppänen, J. Helin, and A. Vainio, *Glycoconjugate J.*, 6 (1989) 129–140.
[38] O. Renkonen, L. Penttilä, R. Niemelä, and A. Leppänen, *Glycoconjugate J.*, 8 (1991) 376–380.
[39] J.-M. Wieruszeski, J.-C. Michalski, J. Montreuil, and G. Strecker, *Glycoconjugate J.*, 6 (1989) 183–194.
[40] L. Dorland, H. van Halbeek, and J.F.G. Vliegenthart, *Biochem. Biophys. Res. Commun.*, 122 (1984) 859–866.
[41] H. Debray, D. Dus, J.-M. Wieruszeski, G. Strecker, and J. Montreuil, *Glycoconjugate J.*, 8 (1991) 29–37.
[42] H. van Halbeek, J.F.G. Vliegenthart, H. Winterwerp, W.M. Blanken, and D.H. van den Eijnden, *Biochem. Biophys. Res. Commun.*, 110 (1983) 124–131.
[43] B. Stahl, M. Steup, M. Karas, and F. Hillenkamp, *Anal. Chem.*, 63 (1991) 1463–1466.
[44] K.K. Mock, M. Davey, and J.S. Cottrell, *Biochem. Biophys. Res. Commun.*, 177 (1991) 644–651.
[45] E.F. Hounsell, J. Feeney, P. Scudder, P.W. Tang, and T. Feizi, *Eur. J Biochem.*, 157 (1986) 375–384.
[46] A.H.L. Koenderman, P.L. Koppen, and D.H. van den Eijnden, *Eur. J. Biochem.*, 166 (1987) 199–208.
[47] J.F.G. Vliegenthart, L. Dorland, and H. van Halbeek, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.
[48] O. Renkonen, R. Niemelä, A. Leppänen, H. Maaheimo, A. Seppo, L. Penttilä, and A. Vilkman, *Glycoconjugate J.*, 8 (1991) 368–375.
[49] A. Seppo, L. Penttilä, R. Niemelä, H. Maaheimo, O. Renkonen, and A. Keane, submitted to *Biochemistry*.
[50] H. van Halbeek, L. Dorland, J.F.G. Vliegenthart, N.K. Kochetkov, N.P. Arbatsky, and V.A. Derevitskaya, *Eur. J. Biochem.*, 127 (1982) 21–29.
[51] E.F. Hounsell, J. Feeney, and P. Scudder, *Biochem. J.*, 250 (1988) 9–13.
[52] M.T. Tarrago, K.H. Tucker, H. van Halbeek, and D.F. Smith, *Arch. Biochem. Biophys.*, 267 (1988) 353–362.
[53] D.M. Whitfield, H. Pang, J.P. Carver, and J.J. Krepinsky, *Can. J. Chem.*, 68 (1990) 942–952.
[54] J. Helin, A. Seppo, A. Leppänen, L. Penttilä, H. Maaheimo, R. Niemelä, S. Lauri, and O. Renkonen, *FEBS Lett.*, 335 (1993) 280–284.
[55] D.H. van den Eijnden, H. Winterwerp, P. Smeeman, and W.E.C.M. Schiphorst, *J. Biol. Chem.*, 258 (1983) 3435–3437.
[56] A.D. Yates and W.M. Watkins, *Carbohydr. Res.*, 120 (1983) 251–268.
[57] F. Piller and J.-P. Cartron, *J. Biol. Chem.*, 258 (1983) 12293–12299.
[58] D.H. van den Eijnden, A.H.L. Koenderman, and W.E.C.M. Schiphorst, *J. Biol. Chem.*, 263 (1988) 12461–12471.
[59] E. Mori, S. Takasaki, J.L. Hedrick, N.J. Wardrip, T. Mori, and A. Kobata, *Biochemistry*, 30 (1991) 2078–2087.
[60] F. Piller, J.-P. Cartron, A. Maranduba, A. Veyrières, Y. Leroy, and B. Fournet, *J. Biol. Chem.*, 259 (1984) 13385–13390.
[61] M.F.A. Bierhuizen, M.-G. Mattei, and M. Fukuda, *Genes Dev.*, 7 (1993) 468–478.
[62] H. Kawashima, K. Yamamoto, T. Osawa, and T. Irimura, *J. Biol. Chem.*, 268 (1993) 27118–27126.
[63] Y.C. Lee, *Anal. Biochem.*, 189 (1990) 151–162.
[64] K. Koizumi, Y. Kubota, T. Tanimoto, and Y. Okada, *J. Chromatogr.*, 464 (1989) 365–373.
[65] P. Juhasz and C.E. Costello, *J. Am. Soc. Mass Spectrom.*, 3 (1992) 785–796.
[66] B.A. Fenderson, U. Zehavi, and S.-I. Hakomori, *J. Exp. Med.*, 160 (1984) 1591–1596.

[67] R.T. Lee and Y.C. Lee, *Glycoconjugate J.*, 4 (1987) 317–328.
[68] S.A. DeFrees, F.C.A. Gaeta, Y.-C. Lin, Y. Ichikawa, and C.-H. Wong, *J. Am. Chem. Soc.*, 115 (1993) 7549–7550.
[69] C. Unverzagt, S. Kelm, and J.C. Paulson, *Carbohydr. Res.*, 251 (1994) 285–301.
[70] J.D. Bleil and P.M. Wassarman, *Proc. Natl. Acad. Sci. U.S.A.*, 85 (1988) 6778–6782.
[71] H.M. Florman and P.M. Wassarman, *Cell*, 41 (1985) 313–324.
[72] D.J. Miller, M.B. Macek, and B.D. Shur, *Nature*, 357 (1992) 589–593.
[73] E.S. Litscher, P.M. Wassarman, K. Juntunen, A. Seppo, R. Niemelä, L. Penttilä, and O. Renkonen, *Mol. Biol. Cell*, 4 (1993) 140a.