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Isolation and Structural Characterization of Twenty-One Sialyloligosaccharides from Galactosialidosis Urine

An Intact *N,N'*-Diacetylchitobiose Unit at the Reducing End of a Diantennary Structure

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Summary: Galactosialidosis urine was fractionated by gel-permeation chromatography on Bio-Gel P-6. The obtained sialic acid-containing carbohydrate fractions were purified by reversed-phase chromatography and separated according to charge by medium-pressure anion-exchange chromatography on Mono Q. The Mono Q fractions, being mixtures of sialyloligosaccharides differing mainly in sialic acid-linkage type (α2–3/α2–6), were subfractionated by high-performance liquid chromatography on Lichrosorb-NH_2. The purified compounds were analysed by 500-MHz ^{1}H-NMR spectroscopy. Twenty-one fully and partially sialylated *N*-acetyllactosamine-type compounds include mono-, di-, tri- and tetra-antennary structures. All structures have the sequence Manβ1–4GlcNAc at the reducing terminus in common, except one diantennary structure bearing an intact *N,N'*-diacetylchitobiose unit at the reducing end, which is a new feature in human glycoproteinosis urine.

Isolierung und Strukturaufklärung von einundzwanzig Sialyloligosacchariden aus Galactosialidose-Urin. Intakte N,N'*-Diacetylchitobiose-Einheit am reduzierenden Ende einer diantennären Struktur*

Zusammenfassung: Urin von Galactosialidose-Patienten wurde durch Gelpermeations-Chromatographie an Bio-Gel P-6 fraktioniert. Die Sialinsäure-haltigen Kohlenhydrat-Fraktionen wurden durch Chromatographie mit umgekehrten Phasen gereinigt und entsprechend ihrer Ladung durch Mitteldruck-Anionenaustausch-Chromatographie an Mono Q getrennt. Die Mono-Q-Fraktionen, welche Gemische aus Sialyloligosacchariden – hauptsächlich durch den Sialinsäure-Bindungstyp (α2–3/α2–6) voneinander unterschieden – darstellen, wurden durch Hochdruck-Flüssigkeitschromatographie an Lichrosorb-NH_2 weiter aufgetrennt. Die gereinigten Verbindungen wurden mittels 500-MHz-^{1}H-NMR-Spektroskopie analysiert. 21 voll oder teilweise sialylierte Verbindungen vom *N*-Acetyllactosamin-Typ umfassen mono-, di-, tri- und tetraantennäre Strukturen. Alle Strukturen haben am reduzierenden Ende die Sequenz Manβ1–4GlcNAc, mit Ausnahme einer diantennären Struktur, die am reduzierenden Ende eine intakte *N,N'*-Diacetylchitobiose trägt, was ein neues Charakteristikum in menschlichem Glycoproteinose-Urin darstellt.

Key words: Galactosialidosis, sialyloligosaccharides, ^{1}H-NMR spectroscopy.

Enzymes:
β-Galactosidase, β-D-galactoside galactohydrolase (EC 3.2.1.23);
Sialidase, acylneuraminyl hydrolase (EC 3.2.1.18).

Abbreviations:
AU = absorption unit; HPLC = high-performance liquid chromatography; PNGase-F = peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (EC 3.5.1.52).

Oligosaccharidurias or glycoproteinoses belong to the group of lysosomal storage diseases. They are characterized by an excessive excretion of oligosaccharides in urine[1–3]. The study of the urinary oligosaccharides has led to an impressive knowledge of the various primary structures of carbohydrate chains derived from glycoproteins and to a deeper insight into their catabolism[3]. Sialidosis, caused by a sialidase deficiency, leads to the excretion of sialyloligosaccharides in urine[4,5]. Investigations have resulted in structural characterization of eight different monosialylated, four disialylated and two trisialylated oligosaccharides[6–11]. Another oligosaccharidosis, galactosialidosis, is caused by a combined deficiency of β-galactosidase and sialidase[12–14]. Recently, the accumulated sialyloligosaccharides from galactosialidosis fibroblasts[15] and placenta[16] were studied and only fully sialylated structures were found in both sources, indicating that the additional β-galactosidase deficiency is not reflected in the storage material. To complete the comparative studies, galactosialidosis urine was taken for investigation of excreted material.

Materials and Methods

Urine

Galactosialidosis urine was obtained from a man with a combined deficiency of sialidase and β-galactosidase with onset at juvenile age[17]. The urine was chilled at − 20 °C immediately after collection and was stored without preservatives.

Chromatographic procedures

Gel permeation chromatography was performed on a Bio-Gel P-6 (200–400 mesh, Bio-Rad) column (120 × 2.5 cm), eluted with 0.1M ammonia/acetic acid buffer, pH 5.5, at 4 °C and at a flow rate of 21 m*l*/h. The elution profiles were obtained by hexose measurements with the phenol/sulfuric acid method (492 nm)[18] and by sialic-acid determinations with the orcinol/conc. HCl/$FeCl_3$ method (570 nm)[19], using 25- and 100-μ*l* aliquots of the 5.0-m*l* fractions, respectively. The hexose and sialic acid contents were determined by using Glc and NeuAc, respectively, as standards.

Medium-pressure reversed-phase chromatography was carried out on a Pep RPC column (50 × 5 mm, Pharmacia) eluted with 6 m*l* water and subsequently with 10 m*l* of a gradient of 0–50% acetonitrile in water at a flow rate of 1.0 m*l*/min. The eluate was monitored at 214 nm with a Pharmacia UV-1/214 detector, operating at 2.0 AU sensitivity. Injection volumes were 0.5 m*l*.

Medium-pressure anion-exchange chromatography was performed on a Mono Q column (HR 5/5, Pharmacia), eluted with a gradient of 0–100mM NaCl in 10 m*l* water (Lichrosolv, Merck). The eluate (flow rate: 2.0 m*l*/min) was monitored at 214 nm with a Pharmacia UV-1/214 detector at a sensitivity of 2.0 AU[20]. The injection volumes for preparative separations were 0.5 m*l*.

The gradients for reversed-phase and anion-exchange chromatography were obtained by a Fast Protein Liquid Chromatography apparatus equipped with a Liquid Chromatography Controller LCC-500 and two P-500 pumps (Pharmacia).

High-performance liquid chromatography was carried out on a Kratos liquid chromatograph consisting of two Spectroflow 400 Solvent Delivery Systems, a Spectroflow 450 Solvent Programmer and a Rheodyne injection valve module. Separations were performed on a Lichrosorb-10-NH_2 column (4.6 × 250 mm, Chrompack). The monosialylated compounds were eluted isocratically with a mixture of acetonitrile/30mM KH_2PO_4, pH 4.7 (65 : 35, v/v)[21,22]. The elution of the higher-sialylated compounds was performed isocratically with a mixture of acetonitrile/30mM KH_2PO_4-K_2HPO_4 buffer, pH 7.0 (62.5 : 37.5, v/v). The eluate (flow rate: 2.0 m*l*/min) was monitored at 205 nm by a Spectroflow 783 Programmable Absorbance Detector (Kratos) connected with a Spectra Physics SP 4290 Integrator. Injection volumes of the samples, dissolved in the eluent, were about 50–70 μ*l*.

After each chromatographic procedure fractions were desalted on a Bio-Gel P-2 (100–200 mesh, Bio-Rad) column (15 × 1 cm) with distilled water as eluent and subsequently lyophilized.

Sugar analysis

Sugar analysis was carried out by gas-liquid chromatography on a capillary CP-Sil 5 WCOT fused silica column (25 m × 0.32 mm, Chrompack) using a Varian Aerograph 3700 gas chromatograph. The trimethylsilylated methyl glycosides were prepared by methanolysis (1.0M methanolic HCl, 24 h, 85 °C), *N*-reacetylation and trimethylsilylation[23].

500-MHz ^{1}H-NMR spectroscopy

Sialyloligosaccharides were repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, Aldrich) at p^{2}H ~ 7 with intermediate lyophilization. Resolution-enhanced 500-MHz ^{1}H-NMR spectra were recorded on a Bruker AM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen and Department of NMR spectroscopy, Utrecht University, The Netherlands) at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225 ppm in ^2H_2O at 27 °C)[24].

Results

Galactosialidosis urine (80 m*l*) was lyophilized and the residue, resuspended in 15 m*l* water, was subjected to Bio-Gel P-6 chromatography (Fig. 1). In the pooled fractions 1–5 the total amounts of NeuAc and hexose were 57 mg and 94 mg,

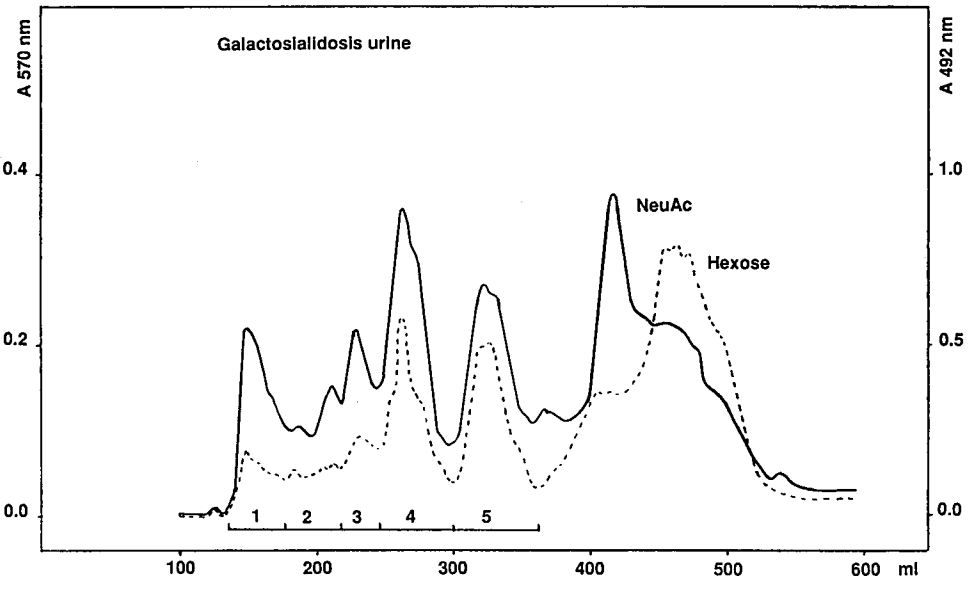


Fig. 1. Bio-Gel P-6 elution profile of galactosialidosis urine.

The column (120 x 2.5 cm) was eluted at 4 °C with 0.1M ammonia/acetic acid, pH 5.5, at a flow rate of 21 m*l*/h. The hexose profile is obtained by phenol/sulfuric acid determinations (492 nm; --------), using 25-μ*l* aliquots of the 5.0-m*l* fractions and the sialic acid profile is obtained by use of the orcinol/conc. HCl/$FeCl_3$ method (570 nm; ——), using 0.1 m*l* of the 5.0-m*l* fractions. Fractions **1–5** were pooled as indicated.

Table 1. Sugar analysis data of the pooled Bio-Gel P-6 fractions **1–5**.

The data are expressed as molar ratios relative to Man = 3.00.

Monosaccharide	1	2	3	4	5
Fuc	1.0	0.8	0.3	0.1	–
Man	3	3	3	3	3
Gal	8.7	5.4	3.5	2.5	2.0
GalNAc	5.0	1.6	0.5	0.3	0.5
GlcNAc	7.0	6.3	4.4	3.2	2.7
NeuAc	8.3	5.2	3.5	2.9	2.4

respectively. Sugar analysis of fractions 1–5 (Table 1) suggests the occurrence of sialylated carbohydrates derived from *N*- and *O*-glycosidic chains. 500-MHz ^{1}H-NMR spectroscopy indicated the presence of monosialylated monoantennary compounds in fraction **5**, disialylated diantennary compounds in **4** and trisialylated triantennary compounds in **3**. Fraction **2** seemed to contain tetraantennary compounds, but the spectrum of fraction **1** could not be unravelled. In all fractions a considerable heterogeneity with respect to the linkage of NeuAc to Gal (α2–3/α2–6) was observed. But, the larger the compounds the less NeuAc in α2–6 linkage could be detected. The occurrence of oligo-*N*-acetyllactosamine units in fractions 1–4 was obvious by some very characteristic chemical-shift values in the various ^{1}H-NMR spectra.

The Bio-Gel P-6 fractions were purified by reversed-phase chromatography, as they contained *O*-glycopeptides, suggested by the presence of GalNAc (Table 1), which is bound in a non-reducing position[25]. The non-retarded material of **2–5** which eluted merely with water, was subfractionated by medium-pressure anion-exchange chromatography on Mono Q (Fig. 2). Separation on Mono Q of fraction **1** was not possible under the applied conditions. The series of Mono Q subfractions were subjected to sugar analysis (Table 2) and 500-MHz ^{1}H-NMR spectroscopy and were finally separated by HPLC on Lichrosorb-NH_2.

Fraction *5*

Fractionation of **5** on Mono Q (Fig. 2D) afforded several subfractions of which **52** contained the highest amount of carbohydrate (about 10 μmol Man, Table 2). Separation of **52** by HPLC on Lichrosorb-NH_2 at pH 4.7, gave rise to the subfractions **521–526** (Fig. 3). The ^{1}H-NMR data of **523**, **525** and **526** indicated the presence of five different monosialylated monoantennary structures, as summarized in the scheme. The pairs of compounds **523a/523b** and **525a/525b** occurred in mixtures which could not be separated under the applied conditions. The chemical shift values of the structural-reporter groups of the isolated structures match exactly those of reference compounds isolated from sialidosis urine[7,24] and are given in Table 3. The ^{1}H-NMR spectra of the other HPLC fractions suggested the occurrence of *O*-glycosidic glycopeptides, but could not be unravelled completely.

The non-retarded Mono Q fraction **51** contained the main component of **52** (**525**), which has to

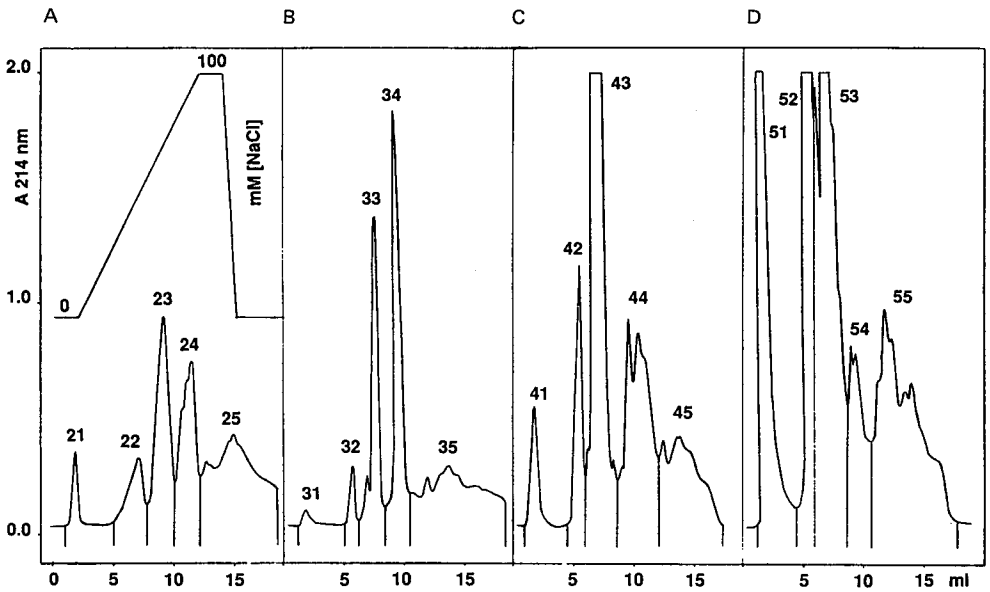


Fig. 2. Mono Q elution profiles of the Bio-Gel P-6 fractions **2**–**5** monitored at 214 nm.

(A) Fraction **2**; (B) fraction **3**; (C) fraction **4**; (D) fraction **5**. The column was eluted at a flow rate of 2.0 m*l*/min with a linear NaCl gradient as shown in (A). The Mono Q subfractions were pooled and designated as indicated.

Table 2. Sugar analysis data and total amounts of Man of the Mono Q subfractions.
The data are expressed as molar ratios relative to Man = 3.00 or to Man = 2.00 in **51**–**55**.

Monosaccharide	**21**	**22**	**23**	**24**	**25**	**32**	**33**	**34**	**35**	**41**	**42**
Fuc	1.8	0.6	0.4	0.5	0.8	–	–	–	–	1.4	0.2
Man	3	3	3	3	3	3	3	3	3	3	3
Gal	6.2	5.0	4.1	4.7	5.4	4.2	3.0	3.0	5.4	3.7	2.6
GalNAc	+	–	–	–	1.4	–	–	–	2.6	0.5	0.2
GlcNAc	5.6	6.1	5.5	6.1	5.9	5.8	4.4	3.9	5.1	3.0	3.2
NeuAc	1.4	2.6	3.4	4.6	5.6	1.4	2.7	3.3	6.5	0.7	1.4
Man [nmol]	65	283	789	469	300	133	682	1071	195	536	1500

Monosaccharide	**43**	**44**	**45**	**51**	**52**	**53**	**54**	**55**
Fuc	–	–	–	0.7	+	+	0.8	–
Man	3	3	3	2	2	2	2	2
Gal	2.0	5.2	8.8	1.5	1.1	2.2	5.5	11.1
GalNAc	–	2.0	5.4	0.2	0.1	0.9	3.0	6.7
GlcNAc	3.0	3.7	4.0	1.2	1.8	1.7	2.6	3.4
NeuAc	2.3	4.6	11.9	0.6	1.2	2.4	7.5	15.9
Man [nmol]	7500	517	125	1429	10,000	833	120	105

be considered as an overloading artifact of the analytical Mono Q column. The relatively high amount of Man in Mono Q fraction **53** (Table 2) is caused by an inappropriate collecting of fractions **52** and **53** (Fig. 2D). In the Mono Q fractions **54** and **55** only *O*-glycosidic material could be detected. Although structural analysis of the *O*-glycosidic sialic acid-containing glycopeptides is seriously hampered by the heterogeneity in the peptide part of the compounds[26], the following glycopeptide is suggested to occur in **55**:

NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc-peptide

The indications for the tetrasaccharide-peptide mixture[26], which is probably also present in **53** and **54** are as follows: the two sets of NeuAc H-3a and H-3e signals show characteristic values indicative of NeuAc in α2–3 and in α2–6 linkage in *O*-glycosidic structures (α2–3: H-3a/H-3e, δ 1.787/2.750; α2–6: H-3a/H-3e, δ 1.658/2.703). One broad peak at δ 2.028 represents NAc signals of NeuAc and GalNAc residues, being heterogeneous due to variations in the peptide part. Moreover, several signals indicating the heterogeneity in the peptide moiety can be observed for H-1 and H-4 of GalNAc and for H-1 and H-3 of Gal.

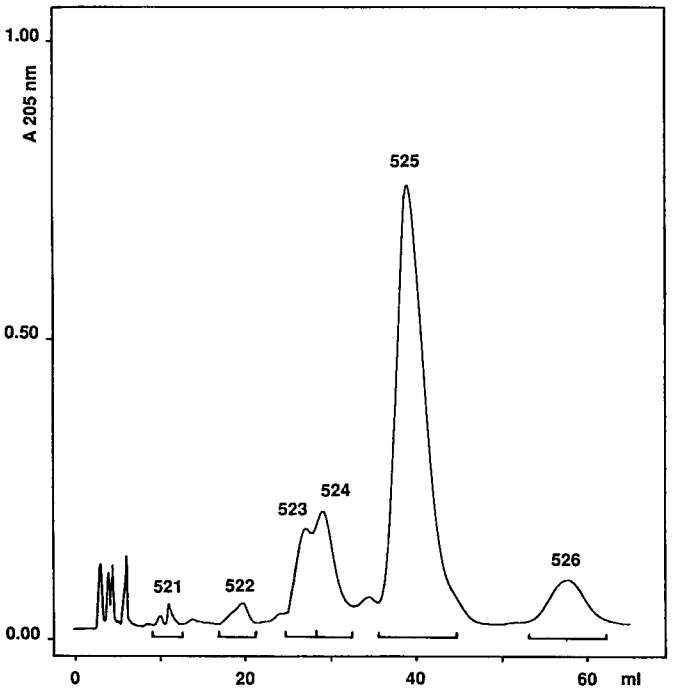


Fig. 3. HPLC elution profile of Mono Q fraction **52** monitored at 205 nm.

The Lichrosorb-NH_2 column was eluted isocratically with acetonitrile/30mM KH_2PO_4, pH 4.7, (65 : 35, v/v) at a flow rate of 2.0 ml/min. The HPLC subfractions were pooled and designated as indicated.

Table 3. ^{1}H-Chemical shifts of structural-reporter groups of the constituent monosaccharides of the monosialylated oligosaccharides present in the HPLC subfractions **523**, **525**, **526**, **423** and **424**, isolated from galactosialidosis urine.

Chemical shifts (δ) are expressed in ppm downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate in ^2H_2O at 27 °C acquired at 500 MHz, but were actually measured by reference to internal acetone (δ 2.225 ppm). For numbering of the monosaccharides and complete structures, see scheme. In the table heading, the structures are represented by short-hand symbolic notation: ■ = Gal; ● = GlcNAc; ♦ = Man; ○ = NeuAcα2–6; △ = NeuAcα2–3.

n.d. means not determined.

Reporter group	Residue	Chemical shift in							
		523a	**523b**	**525a**	**525b**	**526**	**423**	**424a**	**424b**
H-1	GlcNAc **2**α	5.206	5.216	5.207	5.220	5.214	5.213	5.213	5.213
	Man **3**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	4	5.124	–	5.141	–	5.138	5.121	5.138	5.121
	4'	–	4.923	–	4.945	4.921	4.928	4.930	4.948
	GlcNAc **5**	4.568	–	4.601	–	4.606	4.575	4.607	4.587
	5'	–	4.575	–	4.601	–	4.585	4.587	4.607
	Gal **6**	4.545	–	4.447	–	4.446	4.545	4.447	4.470
	6'	–	4.545	–	4.447	–	4.468	4.470	4.447
H-2	Man **3**α	4.242	4.090	4.248	4.090	4.269	4.258	4.265	4.265
	β	4.230	4.080	4.236	4.078	4.260		4.255	4.255
	4	4.192	–	4.199	–	4.199	4.193	4.198	4.198
	4'	–	4.108	–	4.110	< 4.0	4.116	4.116	4.116
H-3	Gal **6**	4.115	–	n.d.	–	n.d.	4.116	n.d.	n.d.
	6'	–	4.115	–	n.d.	–	n.d.	n.d.	n.d.
H-3a	NeuAc α2–6	–	–	1.721	1.721	1.727	–	1.722	1.722
	α2–3	1.798	1.798	–	–	–	1.796	–	–
H-3e	NeuAc α2–6	–	–	2.668	2.668	2.667	–	2.667	2.667
	α2–3	2.757	2.757	–	–	–	2.758	–	–
NAc	GlcNAc **2**α	2.041	2.063	2.041	2.060	2.059	2.062	2.062	2.062
	β		2.060			2.055			
	5	2.048	–	2.070	–	2.071	2.050	2.071	2.050
	5'	–	2.041	–	2.064	–	2.050	2.050	2.071
	NeuAc	2.029	2.029	2.029	2.029	2.030	2.030	2.030	2.030

Compounds	Fract. no.	Ref.
Monosialylated structures		
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3(Manβ1-4GlcNAc)-6,4	**523a**	7,16,24
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAc)-3,4	**523b**	16,24
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Manβ1-4GlcNAc)-6,4	**525a**	7,16,24
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Manβ1-4GlcNAc)-3,4	**525b**	16,24
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Manα1-6,Manβ1-4GlcNAc)-6,4	**526**	7,24
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6,Manβ1-4GlcNAc)-6,4	**423**	7,24
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3(Galβ1-4GlcNAcβ1-2Manα1-6,Manβ1-4GlcNAc)-6,4	**424a**	7,24
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(Galβ1-4GlcNAcβ1-2Manα1-3,Manβ1-4GlcNAc)-6,4	**424b**	24
Disialylated structures		
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3(NeuAcα2-3Galβ1-4GlcNAcβ1-4)-6,4,3,2	**431**	7,16,24
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6(NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3,Manβ1-4GlcNAc)-6,4	**432**	7,16,24
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3,Manβ1-4GlcNAc)-6,4	**434**	7,16,24
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3,Manβ1-4GlcNAc)-6,4	**435**	7,16,24
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6(NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3)-6,4,3,2,1-Manβ1-4GlcNAcβ1-4GlcNAc	**436**	

Scheme. Survey of the isolated sialyloligosaccharides from galactosialidosis urine, together with fraction numbers and references.

Compounds	Fract. no.	Ref.
Trisialylated structures		
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6\ Manβ1-4GlcNAc NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3/ / NeuAcα2-3Galβ1-4GlcNAcβ1-4	**341**	16
NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6\ Manβ1-4GlcNAc NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3/ / NeuAcα2-3Galβ1-4GlcNAcβ1-4	**343**	16
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6\ Manβ1-4GlcNAc NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3/ / NeuAcα2-3Galβ1-4GlcNAcβ1-4	**345**	7,16,24
NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6\ Manβ1-4GlcNAc NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3/ / NeuAcα2-6Galβ1-4GlcNAcβ1-4	**347**	16,24
8' 7' NeuAcα2-3Galβ1-4GlcNAcβ1-6 6' 5' \4' NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6\ 3 2 6 5 4 Manβ1-4GlcNAc NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3/	**342**	16
Tetrasialylated structures		
NeuAcα2-3Galβ1-4GlcNAcβ1-6 \ NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6\ Manβ1-4GlcNAc NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-3/ / NeuAcα2-3Galβ1-4GlcNAcβ1-4	**241**	16
NeuAcα2-3Galβ1-4GlcNAcβ1-6 \ NeuAcα2-3Galβ1-4GlcNAcβ1-2Manα1-6\ Manβ1-4GlcNAc NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3/ / NeuAcα2-3Galβ1-4GlcNAcβ1-4	**242**	16
NeuAcα2-3Galβ1-4GlcNAcβ1-6 \ NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-6\ Manβ1-4GlcNAc NeuAcα2-6Galβ1-4GlcNAcβ1-2Manα1-3/ / NeuAcα2-3Galβ1-4GlcNAcβ1-4	**243**	16

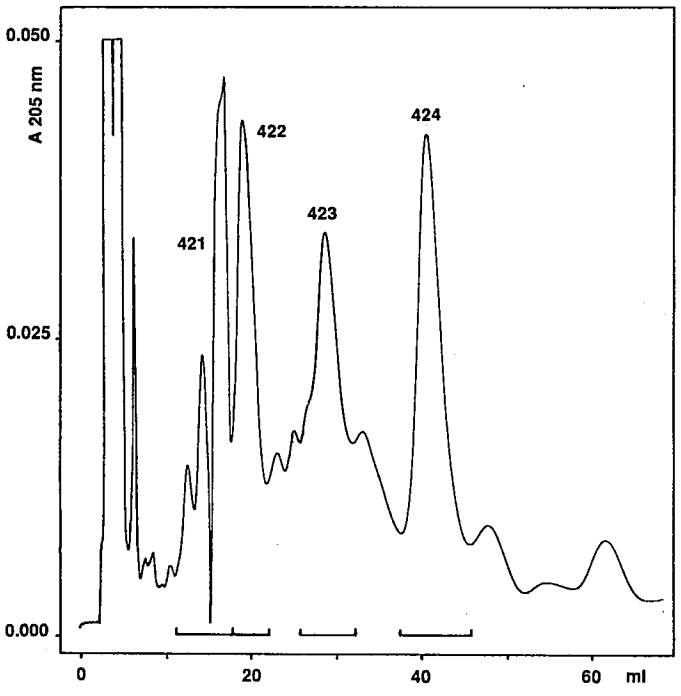


Fig. 4. HPLC elution profile of Mono Q fraction 42 monitored at 205 nm.

The Lichrosorb-NH_2 column was eluted isocratically with acetonitrile/30mM KH_2PO_4-K_2HPO_4 buffer, pH 7.0, (62.5 : 37.5, v/v) at a flow rate of 2.0 ml/min. The HPLC subfractions were pooled and designated as indicated.

Fraction *4*

Fraction 4 was separated on Mono Q in one major (43) and several minor peaks (Fig. 2C). The retention time on Mono Q and sugar analysis data of fraction 42 strongly suggested one sialic acid residue per molecule. HPLC of 42 on Lichrosorb-NH_2 at pH 7.0 resulted in three carbohydrate-containing fractions, 422–424 (Fig. 4). The ^{1}H-NMR spectrum of 424 showed the presence of a mixture of two monosialylated diantennary compounds with NeuAc in α2–6 linkage and that of 423 showed one similar compound with NeuAc in α2–3 linkage (see scheme). The ^{1}H-NMR parameters, which match completely those of reference compounds[7,24], are summarized in Table 3. The ^{1}H-NMR spectrum of 422 suggested the presence of monoantennary compounds bearing di-*N*-acetyllactosamine units sialylated with NeuAc in α2–3 linkage.

The disialylated diantennary compounds present in 43 were separated by HPLC on Lichrosorb-NH_2 at pH 7.0, resulting in six fractions, denoted 431–436 (Fig. 5). The ^{1}H-NMR spectra of the fractions 431, 432, 434 and 435 proved the presence of four different disialylated diantennary structures, as shown in the scheme. The chemical-shift values of these structures match completely those of reference compounds[7,24] and are given in Table 4.

The very minor fraction 436 eluted just after the major compound 435. The latter is a diantennary structure sialylated with two NeuAc residues in α2–6 linkage and with GlcNAc-2 at the reducing terminus. Inspection of the ^{1}H-NMR spectrum of 436 evidenced, besides contaminating 435, a product identical to the diantennary structure with two NeuAc residues in α2–6 linkage and with an intact *N,N'*-diacetylchitobiose unit, obtained from human serotransferrin after incubation with PNGase-F[27].

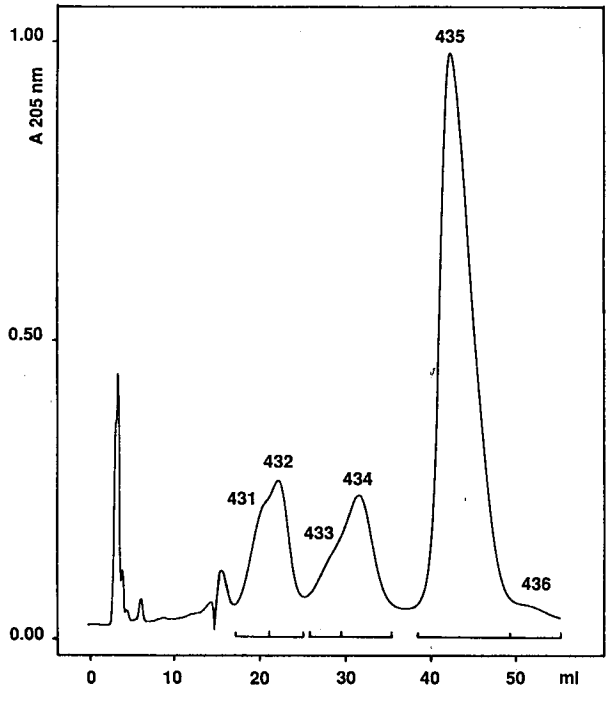


Fig. 5. HPLC elution profile of Mono Q fraction 43 monitored at 205 nm.

Details are as in Fig. 4.

436

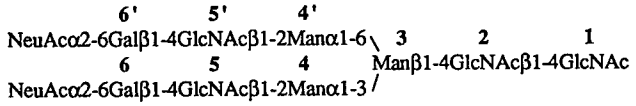
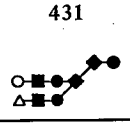
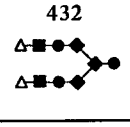
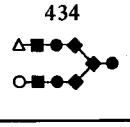
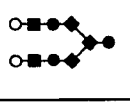
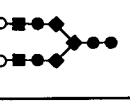


Table 4. ^{1}H-Chemical shifts of structural-reporter groups of the constituent monosaccharides of the disialylated oligosaccharides present in the HPLC subfractions **431**, **432**, **434**, **435** and **436**, isolated from galactosialidosis urine.
Details are as in Table 3.

Reporter group	Residue		Chemical shift in				
			431 	**432** 	**434** 	**435** 	**436** 
H-1	GlcNAc	1α	–	–	–	–	5.190
		2α	5.206	5.212	5.212	5.215	4.616[a]
	Man	3	n.d.	n.d.	n.d.	n.d.	n.d.
		4	5.133	5.117	5.134	5.137	5.135
		4′	–	4.923	4.925	4.955	4.948
	GlcNAc	5	4.592	4.574	4.606	4.608	4.606
		5′	–	4.574	4.578	4.608	4.606
		7	4.547	–	–	–	–
	Gal	6	4.444	4.545	4.444	4.447	4.444
		6′	–	4.545	4.545	4.447	4.444
		8	4.547	–	–	–	–
H-2	Man	3α	4.216	4.259	4.265	4.269	–
		β	4.198	4.249	4.255	4.259	4.255
		4	4.216	4.192	4.199	4.200	4.199
		4′	–	4.122	4.123	4.123	4.122
H-3	Gal	6	n.d.	4.113	n.d.	n.d.	n.d.
		6′	–	4.113	4.115	n.d.	n.d.
		8	4.116	–	–	–	–
H-3a	NeuAc	α2–6	1.722	–	1.721	1.721[b]	1.719[b]
		α2–3	1.800	1.799[b]	1.800	–	–
H-3e	NeuAc	α2–6	2.666	–	2.667	2.670[b]	2.668[b]
		α2–3	2.755	2.755[b]	2.756	–	–
NAc	GlcNAc	1	–	–	–	–	2.036
		2α	2.041	2.059	2.061	2.064	2.082
		β				2.060	
		5	2.069	2.049	2.070	2.072	2.068
		5′α	–	2.044	2.045	2.069	2.068
		β				2.067	
		7	2.074	–	–	–	–
	NeuAc		2.030[c]	2.030[c]	2.030[c]	2.030[c]	2.029[c]

[a] GlcNAc-2β signal at δ 4.606.
[b] Signal stemming from two protons.
[c] Signal stemming from two NAc groups.

The *N,N′*-diacetylchitobiose unit is characterized by the H-1α signal of GlcNAc-1 at δ 5.190 and the NAc signal at δ 2.036. The H-1 signals of GlcNAc-2 are present at δ 4.616 and 4.606 (α/β anomerization of GlcNAc-1) and the NAc signal can be observed at δ 2.082. The other structural reporter groups (Table 4) match those of structure **435**, except for the absence of an H-2α signal of Man-3, which indicates the absence of anomerization at H-1 of GlcNAc-2.

Fraction *3*

Chromatography of fraction 3 on Mono Q (Fig. 2B) resulted in three sharp peaks (32–34) at positions which suggest the presence of mono-, di- and tri-sialylated compounds, respectively (for sugar analysis, see Table 2).

The ^{1}H-NMR spectrum of the monosialylated fraction 32 showed relatively intense signals at the characteristic values for additional *N*-acetyl-lactos-

amine units (H-1/H-4 internal Gal, δ 4.467/4.152; H-1 GlcNAcβ1–3, δ 4.700), but the amount of material was too little for further investigation.

The ^{1}H-NMR spectrum of fraction 33 gave evidence for diantennary compounds containing a di-*N*-acetyllactosamine unit in one of the branches. The NeuAc H-3a and H-3e regions showed the presence of NeuAc residues in both α2–3 and α2–6 linkage. HPLC on Lichrosorb-NH_2 resulted in seven subfractions which are still under investigation.

HPLC of the trisialylated fraction 34 resulted in a series of eight peaks, denoted 341–348 (Fig. 6). ^{1}H-NMR spectroscopy of 341, 343, 345 and 347 showed that they all contain trisialylated triantennary compounds, differing from each other merely in the sialylation pattern, as shown in the scheme. The chemical shift data of these fractions, compiled in Table 5, match exactly the data of the trisialylated triantennary structures isolated from human galactosialidosis placental tissue[16]. The ^{1}H-NMR spectrum of fraction 342 gave evidence for a tri′-antennary compound bearing three NeuAc residues in α2–3 linkage (see scheme). The chemical shift values are given in Table 5 and match those of the tri′-antennary structure from galactosialidosis placenta[16]. The elution volumes of the

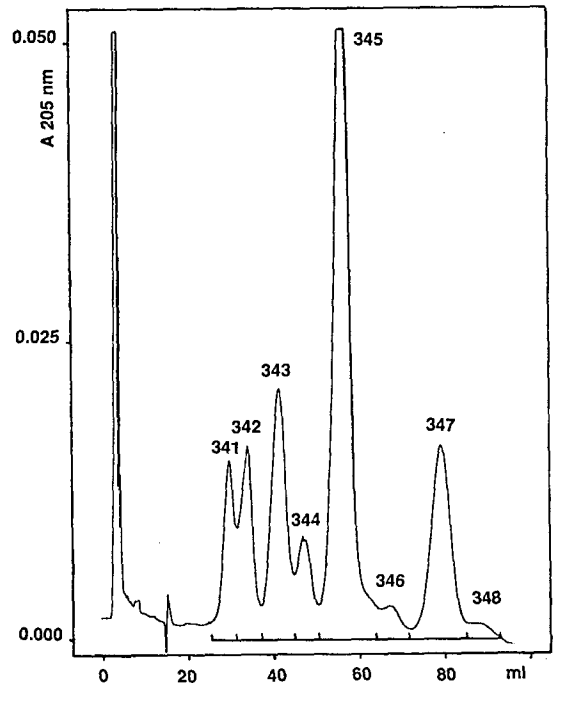


Fig. 6. HPLC elution profile of Mono Q fraction 34 monitored at 205 nm.

Details are as in Fig. 4.

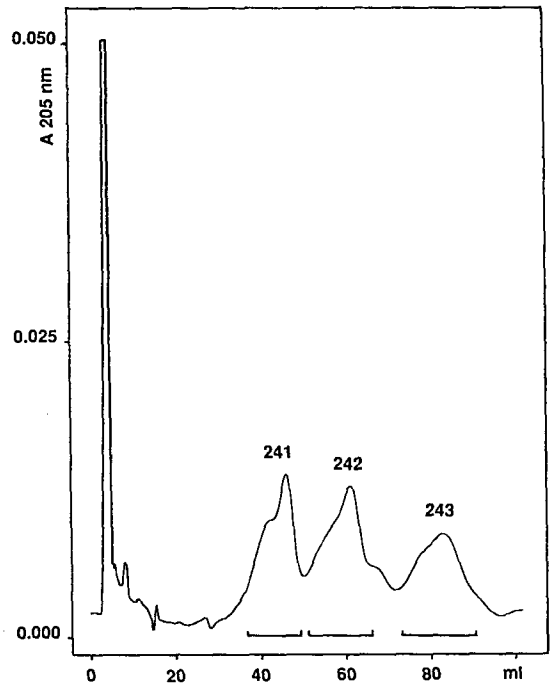


Fig. 7. HPLC elution profile of Mono Q fraction 24 monitored at 205 nm.

Details are as in Fig. 4.

triantennary structures present in 341, 343, 345 and 347 showed a direct correlation with the number of α2–6 linked NeuAc residues. On basis of this correlation, it can be anticipated that the subfractions 342, 344, 346 and 348 should contain tri′-antennary compounds with a similar sialylation pattern as detected for the trianntennary compounds. Unfortunately, the amounts of material present in the fractions 344, 346 and 348 were too small to get conclusive evidence by ^{1}H-NMR spectroscopy. Fraction 348 contained only α2–6 bound NeuAc residues, as evidenced by the NeuAc H-3a/H-3e regions and the NAc-region.

Fraction *2*

The Mono Q separation procedure applied to fraction 2 (Fig. 2A) afforded three relatively broad, but well separated peaks, 22, 23 and 24, suggesting the presence of di-, tri- and tetrasialylated compounds, respectively (for sugar analysis results, see Table 2).

The ^{1}H-NMR spectra of 22 and 23 suggested the occurrence of di- and tri-antennary compounds, respectively, and showed the presence of additional *N*-acetyllactosamine units. HPLC of 22 and 23 both resulted in several subfractions, again indicating the diversity of this type of compounds, but the fractions are still under investigation.

Table 5. ^{1}H-Chemical shifts of structural-reporter groups of the constituent monosaccharides of the tri- and tetra-sialylated oligosaccharides present in the HPLC subfractions **341–343**, **345**, **347** and **241–243**, isolated from galactosialidosis urine.
Details are as in Table 3.

Reporter group	Residue		Chemical shift in							
			341	**343**	**345**	**347**	**342**	**241**	**242**	**243**
H-1	GlcNAc	2α	5.212	5.212	5.214	5.213	5.208	5.204	5.204	5.206
	Man	3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
		4	5.119	5.133	5.132	5.137	5.127	5.132	5.138	5.139
		4′	4.911	4.912	4.943	4.941	4.878	4.863	4.864	4.889
	GlcNAc	5	4.563	4.593	4.595	4.595	4.57	4.560	4.600	4.595
		5′	4.576	4.574	4.604	4.604	4.58	4.593	4.582	4.605
	Gal	6	4.547	4.443	4.444	4.444	4.545	4.544	4.441	4.441
		6′	4.547	4.547	4.444	4.444	4.545	4.544	4.545	4.441
	GlcNAc	7	4.547	4.547	4.551	4.572	–	4.544	4.545	4.547
		7′	–	–	–	–	4.545[a]	4.544[a]	4.545[a]	4.547[a]
	Gal	8	4.547	4.547	4.546	4.444	–	4.544	4.545	4.547
		8′	–	–	–	–	4.555[a]	4.560[a]	4.560[a]	4.557[a]
H-2	Man	3	4.224	4.224	4.221	4.227	4.258	4.22	4.22	4.22
		4	4.224	4.224	4.221	4.227	4.200	4.22	4.22	4.22
		4′	4.115	4.116	4.119	4.121	4.109	4.107	4.107	4.107
H-3	Gal	6	4.115	n.d.	n.d.	n.d.	4.118	4.115	n.d.	n.d.
		6′	4.115	4.116	n.d.	n.d.	4.118	4.115	4.120	n.d.
		8	4.115	4.116	4.116	n.d.	–	4.115	4.120	4.115
		8′	–	–	–	–	4.118	4.115	4.120	4.115
H-3a	NeuAc	α2–6	–	1.719	1.719[b]	1.717[b]/1.705	–	–	1.719	1.718[b]
		α2–3	1.800[c]	1.801[b]	1.801	–	1.802[c]	1.800[d]	1.802[c]	1.801[b]
H-3e	NeuAc	α2–6	–	2.668	2.670[b]	2.670[c]	–	–	2.670	2.670[b]
		α2–3	2.756[c]	2.756[b]	2.756	–	2.756[c]	2.755[d]	2.757[c]	2.755[b]
NAc	GlcNAc	2	2.061	2.060	2.062	2.062	2.058	2.057	2.058	2.057
		5	2.046	2.068	2.068	2.068	2.055	2.050	2.070	2.071
		5′	2.046	2.045	2.068	2.068	2.039	2.038	2.038	2.069
		7	2.074	2.074	2.074	2.102	–	2.075	2.076	2.076
		7′	–	–	–	–	2.039	2.038	2.038	2.038
	NeuAc		2.031[e]	2.030[e]	2.030[e]	2.030[e]	2.030[e]	2.031[f]	2.031[f]	2.031[f]

[a] Assignments may have to be interchanged.
[b] Signal stemming from two protons.
[c] Signal stemming from three protons.
[d] Signal stemming from four protons.
[e] Signal stemming from three NAc groups.
[f] Signal stemming from four NAc groups.

HPLC of tetrasialylated fraction 24 (Fig. 7) resulted in three subfractions 241–243. ^{1}H-NMR spectroscopy of 241–243 indicated as main components three tetrasialylated tetraantennary compounds, differing from each other in the sialylation pattern (see scheme). The chemical shift values of the fractions 241, 242 and 243, given in Table 5, match those of the same compounds isolated from galactosialidosis placenta[16].

Discussion

Many sialyloligosaccharides from galactosialidosis urine were isolated by a combination of several chromatographic methods. The fully sialylated structures were formerly also isolated from the placenta of a human galactosialidosis fetus[16], including the tri-, tri′- and tetra-antennary compounds. The few partially sialylated structures, which were isolated from urine, are identical to some of the carbohydrate chains from sialidosis urine[6–11]. The relative amounts of the partially sialylated compounds compared to fully sialylated compounds are almost the same in urines from both diseases. Therefore, the sialidase deficiency in galactosialidosis appears to be the primary enzyme defect affecting degradation of carbohydrate chains of glycoproteins and determines the nature of the excreted oligosaccharides. The additional β-galactosidase deficiency is not reflected in the urinary

oligosaccharides, as only small amounts of oligosaccharides with terminal Gal residues were found. Also in galactosialidosis fibroblasts[15] and placenta[16] the additional β-galactosidase deficiency was not reflected in the storage material.

To our knowledge the disialylated diantennary compound **436** is the first reported structure in human lysosomal storage diseases with an intact *N,N'*-diacetylchitobiose unit at the reducing end. This structure, present in tiny amounts, indicates that *N*-glycosidically linked chains are detached from the protein core by cleavage of the GlcNAc-Asn linkage, before hydrolysis of the GlcNAcβ1–4GlcNAc linkage by an endoglucosaminidase. Our observation supports recent data on the sequential action of aspartylglucosaminidase and an endoglucosaminidase[28–30]. Comparable structures with an intact *N,N'*-diacetylchitobiose unit at the reducing terminus have been found as major compounds in urine of β-mannosidosis goats[31], GM_1-gangliosidosis dogs[32], α-mannosidosis cats[33,34] and swainsonine-intoxicated sheep[35,36], next to minor amounts of structures bearing just one GlcNAc residue at the end. The explanation for this difference between humans and the mentioned animals, all suffering from glycoproteinosis, has to be found in species dependent kinetics of the two afore-mentioned enzymes.

The occurrence of oligo-*N*-acetyllactosamine units is a new element in galactosialidosis urine, but has been reported before to be present in human GM_1-gangliosidosis urine[37,38]. From a structural point of view the extension of carbohydrate chains by *N*-acetyllactosamine units creates extra heterogeneity possibilities in structures of oligosaccharides. Moreover, it is to be expected that the additional units can be sialylated with NeuAc in α2–3 and α2–6 linkage, which gives an even greater variety of carbohydrate chains.

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