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Structural analysis of *O*-glycosidic type of sialyloligosaccharide-alditols derived from urinary glycopeptides of a sialidosis patient

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Sialidosis urine was fractionated by gel filtration on Bio-Gel P-6. All pooled fractions containing carbohydrates showed the presence of small amounts of GalNAc in non-reducing position, besides free *N*-acetyllactosamine type of oligosaccharides as major constituents. The fractions were subjected to reductive alkaline borohydride degradation, after which the major part of GalNAc was recovered as *N*-acetyl-D-galactosaminitol (GalNAc-ol). The GalNAc-ol-containing material was separated from the *N*-glycosidic oligosaccharides by a second gel-filtration step on AcA 202. Subsequently, the *O*-glycosidic sialyloligosaccharide-alditols were subfractionated by anion-exchange chromatography on Mono Q. Structural analysis by 500-MHz ^{1}H-NMR spectroscopy revealed two major components in all fractions, namely: NeuAcα2-3Galβ1-3GalNAc-ol and NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc-ol. Furthermore, NeuAcα2-3Galβ1-3[NeuAcα2-3Galβ1-4GlcNAcβ1-6]GalNAc-ol was found as a minor component in some of the fractions. The presence of these carbohydrate chains in Bio-Gel fractions differing in molecular mass suggested that they are derived from glycopeptides which are heterogeneous in their peptide part.

Sialidosis (mucolipidosis I) is a rare inherited metabolic disease, caused by an α-sialidase deficiency [1, 2]. Besides various severe clinical symptoms, the excretion of sialic-acid-containing carbohydrates in urine is a prominent feature of this lysosomal storage disease. The urinary material is mainly derived from an impaired catabolism of glycoproteins bearing *N*-glycosidic linked carbohydrate chains [3, 4], leading to *N*-acetyllactosamine type of sialyloligosaccharides, having the Manβ1-4GlcNAc sequence at the reducing terminus in common [5–10].

Also the catabolism of *O*-glycosidic chains seems to be disturbed leading to the excretion of sialylglycopeptides. In contrast with the accumulated *N*-glycosidic oligosaccharides, the urinary *O*-glycosidic material bears at least one amino acid residue [11, 12]. This finding points to a different pathway for the breakdown of *O*-glycosidic carbohydrate chains of glycoproteins in comparison to *N*-glycosidic chains. In our structural studies on sialyloligosaccharides from sialidosis urine, the presence of non-reducible GalNAc was noticed. In the present study the isolation and characterization of sialyloligosaccharide-alditols obtained after alkaline borohydride treatment of occurring glycopeptides is described.

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Abbreviations. Fuc, L-fucose; GalNAc, *N*-acetyl-D-galactosamine; NeuAc, *N*-acetylneuraminic acid; GlcNAc-ol, *N*-acetyl-D-glucosaminitol; GalNAc-ol, *N*-acetyl-D-galactosaminitol.

Enzyme. Sialidase (previously called neuraminidase) (EC 3.2.1.18).

MATERIALS AND METHODS

Preparation and isolation of sialyloligosaccharide-alditols

Sialidosis urine (100 ml) was lyophilized, redissolved in 8 ml of distilled water and applied to a column (140 × 2.6 cm) of Bio-Gel P-6 (200–400 mesh, Bio-Rad). The elution was carried out with 0.1 M ammonia/acetic acid buffer, pH 5.5, at room temperature and at a flow rate of 25 ml/h. The elution profile was obtained by hexose determinations with the phenol/sulfuric acid assay [13], using 50-µl aliquots of 5.0-ml fractions. A small part (1%) of the pooled and lyophilized fractions was used for sugar analysis.

The alkaline borohydride reduction was performed on 10% of the pooled Bio-Gel P-6 fractions **1**–**5**. Each sample was incubated with 1.0 ml 0.1 M NaOH containing 1 M $NaBH_4$ for 24 h at 37°C. Then, Dowex H^+ (50WX8, 100–200 mesh) was carefully added at 0°C, until the pH was slightly acidic. After filtration and lyophilization, boric acid was removed by co-evaporation with methanol under reduced pressure. Sugar analysis was repeated on 10% of the treated fractions.

The liberated *O*-glycosidic oligosaccharide-alditols in the fractions **2**–**5** were separated from the major amount of reduced *N*-glycosidic oligosaccharides on an AcA 202 column (140 × 1.6 cm, LKB) with 0.1 M ammonia/acetic acid buffer, pH 5.5, as eluent, and were fractionated by anion-exchange chromatography on Mono Q (HR 5/5, Pharmacia), eluted with a linear gradient of 0–100 mM NaCl in 10 ml water (Lichrosolv, Merck). Fraction **1** was directly fractionated on Mono Q, omitting the AcA 202 separation procedure. The eluate (flow rate: 2.0 ml/min) was monitored at 214 nm with a Pharmacia UV-1/214 detector [14]. Before structural investigations the fractions were desalted on a Bio-Gel P-2 column

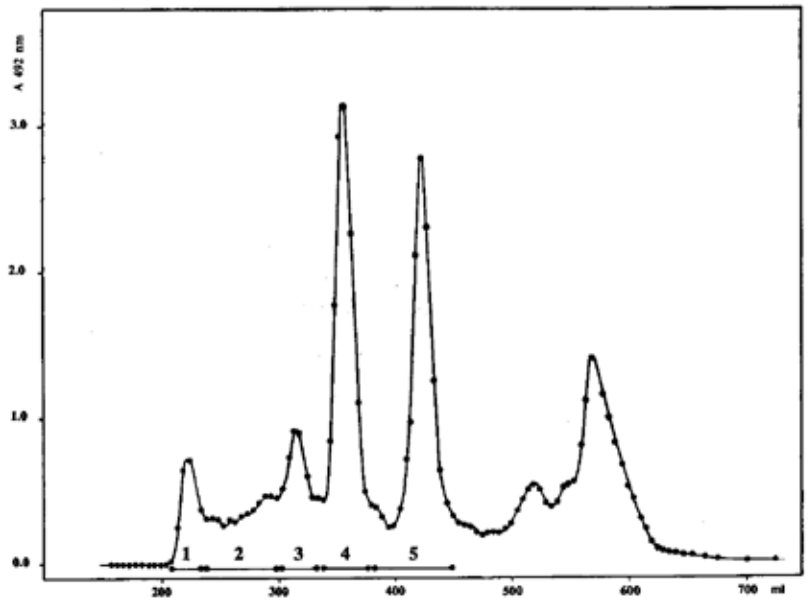

Fig. 1. *Bio-Gel P-6 elution profile of sialidosis urine, using a 0.1 M ammonia/acetic acid buffer, pH 5.5, as eluent at a flow rate of 25 ml/h.* The profile was obtained by hexose determinations at 492 nm, using 50-µl aliquots of 5.0-ml fractions. The fractions **1**–**5** were pooled as indicated

Table 1. *Sugar analysis of Bio-Gel P-6 fractions before and after alkaline borohydride treatment*
Molar ratios are calculated relative to 3.00 Man residues

Monosaccharide	P-6 fractions					P-6 fractions after β-elimination				
	1	**2**	**3**	**4**	**5**	**1**	**2**	**3**	**4**	**5**
Fuc	1.7	0.8	–	–	–	1.4	0.6	–	–	–
Man	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0
Gal	15.8	9.0	4.5	2.3	2.6	11.4	7.8	4.5	2.1	1.3
Glc	2.9	0.4	–	–	–	1.9	–	–	–	–
GalNAc	9.4	3.5	0.7	0.2	0.8	1.5	0.4	0.2	0.1	0.2
GalNAc-ol	–	–	–	–	–	4.8	1.6	0.8	0.2	0.1
GlcNAc	9.6	9.1	5.0	2.8	3.2	6.9	5.9	2.6	1.8	0.7
GlcNAc-ol	–	–	–	–	–	+	+	1.8	0.9	1.5
NeuAc	12.1	6.6	3.8	2.0	2.2	10.6	5.2	3.0	2.0	1.4

(100–200 mesh, 18 × 1 cm) with distilled water and subsequently lyophilized.

Sugar analysis

Sugar analysis was carried out by gas-liquid chromatography on a capillary CP-Sil5 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 (1.0 M methanolic HCl, 24 h, 85 °C), *N*-reacetylation and trimethylsilylation [15].

500-MHz ^{1}H-NMR spectroscopy

Sialyloligosaccharide-alditols were repeatedly exchanged in ^2H_2O (99.96 atom% ^{2}H, Aldrich) with intermediate lyophilization. Resolution-enhanced ^{1}H-NMR spectra were recorded on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands) operating at 500 MHz in the Fourier-transform mode and at a probe temperature of 27 °C. Chemical shifts (δ) are expressed in ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ 2.225 in ^2H_2O at 27 °C) [16].

RESULTS

The fractionation pattern of sialidosis urine on Bio-Gel P-6, as obtained by hexose determinations, is shown in Fig. 1. The sugar analysis data of the pooled fractions **1**–**5** are summarized in Table 1. The molar ratios of the monosaccharides relative to Man point to the presence of various sialylated *N*-acetyllactosamine type of structures in the fractions **1**–**5**. In addition, a variable amount of GalNAc is present.

A direct reduction with $NaBH_4$ on an aliquot of the Bio-Gel fractions gave rise to a partial conversion of GlcNAc into GlcNAc-ol, but GalNAc-ol could not be detected (data not shown). This indicates that the *N*-glycosidic type of compounds are present as free oligosaccharides, whereas GalNAc is bound in a non-reducing position. After alkaline borohydride reduction, sugar analysis of the treated fractions (Table 1) shows that in fractions **1**–**3** the major part of GalNAc is recovered as GalNAc-ol. For fractions **4** and **5**

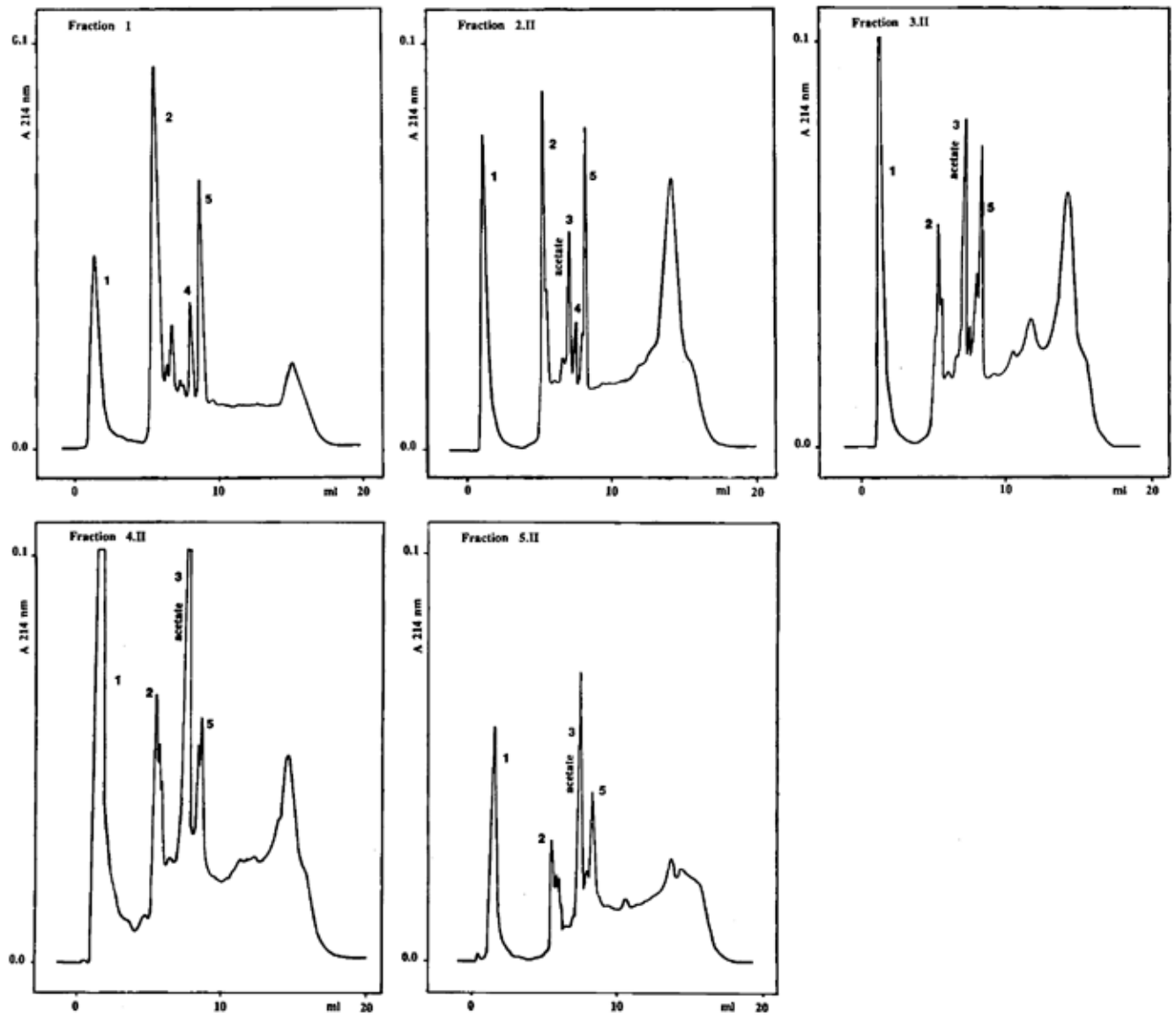

Fig. 2. *Mono Q elution profiles of the fractions 1, 2.II, 3.II, 4.II and 5.II at 214 nm, using a linear gradient of 0 – 100 mM NaCl from 2 to 12 ml.* (The gradient is not indicated in the figure.) The subfractions were pooled and designated as indicated

the percentage GalNAc-ol relative to GalNAc is lower. The finding of GalNAc-ol after the cleavage procedure with $NaOH/NaBH_4$ indicates that GalNAc is very likely bound to at least a dipeptide [17]. The ratios of GlcNAc-ol to GlcNAc after the reductive β-elimination procedure are similar to the ratios obtained after direct reduction.

Except for fraction **1**, the *O*-glycosidic type of oligosaccharide-alditols, which have been liberated from their peptide part, were separated from the major amount of *N*-glycosidic material by a second gel-filtration step on AcA 202. The fractions **2 – 5** yielded two subfractions of which the first one corresponded with the elution volume of the original untreated sample and the second one (designated by **II**) contained the remaining carbohydrate-positive fractions.

Subsequently, the fractions **1**, **2.II**, **3.II**, **4.II** and **5.II** were separated by anion-exchange chromatography on Mono Q. The elution patterns obtained by monitoring at 214 nm are shown in Fig. 2. Each of these fractions gave rise to a series of subfractions, designated as indicated, which were analyzed by 500-MHz ^{1}H-NMR spectroscopy. The first, non-retarded peak from each fraction contained non-carbohydrate material and the relatively large peaks **2.II.3**, **3.II.3**, **4.II.3** and **5.II.3** contained mainly acetate. The acetate peak did not occur in fraction **1**, as this particular one was not separated by AcA 202 gel-filtration chromatography.

^{1}H-NMR spectroscopy demonstrated that the fractions **1.2**, **2.II.2** and **4.II.2** all contained the following trisaccharide-alditol: NeuAcα2-3Galβ1-3GalNAc-ol.

The chemical shift values of the structural-reporter groups of the mentioned fractions match completely those of reference compound **B** [18]. The data of fraction **1.2** and compound **B** are given in Table 2. The following characteristic features may be mentioned. The NeuAc H-3*ax* (δ 1.800) and H-3*eq* (δ 2.770) signals are specific for NeuAc α2-3-linked to Gal. The *N*-acetyl proton region showed two signals, namely at δ 2.045 and 2.033 belonging to GalNAc-ol and NeuAc, respectively. It is very likely that also fractions **3.II.2** and **5.II.2** contained this trisaccharide-alditol, as the retention times of the fractions **3.II.2** and **5.II.2** were exactly the same as those of fractions **1.2**, **2.II.2** and **4.II.2**.

Table 2. *^{1}H-chemical shifts of structural-reporter group protons of constituent monosaccharides of the fractions 1.2, 1.4 and 1.5, together with those of reference compounds B, C [18] and* **IV** *[19].* The first superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. A second superscript is used to discriminate between identically linked residues, by indicating the type of linkage of the neighbouring residue in the sequence. 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). In the table heading, the structures are represented by shorthand symbolic notation: ◇ = GalNAc-ol; ■ = Gal; ● = GlcNAc; △ = NeuAcα2-3 and ○ = NeuAcα2-6. n.d. means not determined

Residue	Reporter group	Chemical shift in					
		B	**1.2**	**C**	**1.5**	**IV**	**1.4**
							
		ppm					
GalNAc-ol	H-2	4.390	4.386	4.378	4.377	4.387	4.397
	H-3	4.074	4.071	4.067	4.067	4.066	4.066
	H-4	3.498	3.495	3.524	3.525	3.441	3.442
	H-5	4.187	4.185	4.240	4.238	4.265	4.268
	H-6	3.68	n.d.	3.84	n.d.	3.928	3.928
	H-6′	3.65	n.d.	3.475	3.472	n.d.	n.d.
	NAc	2.046	2.045	2.042	2.042	2.065	2.064
Gal^3	H-1	4.547	4.544	4.541	4.540	4.529	4.531
	H-3	4.122	4.119	4.117	4.116	4.113	4.114
	H-4	3.931	3.929	3.927	3.925	3.928	3.928
GlcNAc	H-1	–	–	–	–	4.550	4.550
	H-6	–	–	–	–	4.004	4.004
	NAc	–	–	–	–	2.062	2.062
Gal^4	H-1	–	–	–	–	4.545	4.546
	H-3	–	–	–	–	4.113	4.114
	H-4	–	–	–	–	3.956	3.957
NeuAc^{3,3}	H-3*ax*	1.800	1.800	1.800	1.799	1.800	1.798
	H-3*eq*	2.774	2.770	2.774	2.772	2.775	2.776
	NAc	2.034	2.033	2.032	2.032	2.033	2.031
NeuAc^6	H-3*ax*	–	–	1.692	1.691	–	–
	H-3*eq*	–	–	2.723	2.722	–	–
	NAc	–	–	2.032	2.032	–	–
NeuAc^{3,4}	H-3*ax*	–	–	–	–	1.800	1.798
	H-3*eq*	–	–	–	–	2.755	2.751
	NAc	–	–	–	–	2.031	2.031

The ^{1}H-NMR spectra of the fractions **1.5**, **2.II.5** and **4.II.5** all showed the presence of the tetrasaccharide-alditol: NeuAcα2-3Galβ1-3[NeuAcα2-6]GalNAc-ol.

The chemical shift values of the compound present in fraction **1.5** are given in Table 2, together with those of reference compound **C** [18]. The NMR data of the other two fractions also match those of the reference compound. Briefly, two sets of NeuAc H-3 structural-reporter groups were observed. One set (H-3*ax*, δ 1.799; H-3*eq*, δ 2.772) is characteristic for NeuAc α2-3-linked to Gal, whereas the other (H-3*ax*, δ 1.691; H-3*eq*, δ 2.722) indicates a NeuAc α2-6-linkage. The *N*-acetyl proton region showed two signals in an intensity ratio of 2:1. The signal at δ 2.032 belongs to the NeuAc residues and that at δ 2.042 is assigned to GalNAc-ol. The fractions **3.II.5** and **5.II.5** showed the same retention times as fractions **1.5**, **2.II.5** and **4.II.5**. Therefore, it is reasonable to assume that the fractions **3.II.5** and **5.II.5** also contained the tetrasaccharide-alditol.

^{1}H-NMR spectroscopy of the fractions **1.4** and **2.II.4**, which eluted slighty earlier from the column than the tetrasaccharide-alditol, demonstrated the presence of the hexasaccharide-alditol: NeuAcα2-3Galβ1-3[NeuAcα2-3Galβ1-4GlcNAcβ1-6]GalNAc-ol.

The chemical shift values of the structural-reporter groups of the constituting monosaccharides of fractions **1.4** and **2.II.4** match completely those of compound **IV** [19]. The NMR data of fraction **1.4** and reference compound **IV** are given in Table 2. The hexasaccharide-alditol is characterized by the presence of two coinciding NeuAc H-3*ax* triplets (δ 1.798) and of two H-3*eq* signals of equal intensity (δ 2.776 and δ 2.751). The *N*-acetyl proton region showed three signals in an intensity ratio of 1:1:2. The signal with double intensity at δ 2.031 belongs to the NeuAc residues, that at δ 2.064 to GalNAc-ol and the one at δ 2.062 is assigned to GlcNAc.

In conclusion, after the β-elimination procedure all Bio-Gel fractions showed the presence of *O*-glycosidic tri- and tetra-saccharide-alditols. Moreover, fractions **1** and **2** contained in addition the *O*-glycosidic hexasaccharide-alditol. The glycopeptides from which the carbohydrate chains are derived, differ in apparent molecular mass from 1000 Da to at least 6000 Da, as has been deduced from the gel-filtration chromatography on Bio-Gel P-6. These molecular masses are

much larger than can be calculated from a single carbohydrate chain only.

DISCUSSION

The sialidase deficiency in sialidosis obviously leads to the excretion of *N*-glycosidic sialyloligosaccharides, having in common the Manβ1-4GlcNAc sequence at the reducing end [5–10]. Recently, the mechanism of the catabolism leading to oligosaccharides has been unraveled. A sequential action of a glycopeptidase and an endoglycosidase is responsible for an efficient removal of the asparagine and the GlcNAc-1 residue, respectively [20, 21].

Also the catabolism of sialic-acid-containing *O*-glycosidic chains is disturbed in sialidosis, resulting in the excretion of sialylglycopeptides [11, 12] (and this study). Previous investigations demonstrated the occurrence of non-characterized glycoserines [11] and of NeuAcα2-3Galβ1-3[NeuAcα2-6]-GalNAc coupled to Thr, to Ser or to Thr in the dipeptide Thr-Ser and to Ser in the dipeptide Ser-Thr [12]. Our studies on the urine of another sialidosis patient indicate that besides the earlier reported tetrasaccharide also NeuAcα2-3Galβ1-3[NeuAcα2-3Galβ1-4GlcNAcβ1-6]GalNAc and NeuAcα2-3Galβ1-3GalNAc can occur in accumulated glycopeptide material. Moreover, based on the gel-filtration profile, the latter material also contains much larger peptide chains than the reported one or two amino acids, or contains glycopeptides with more than one carbohydrate chain. It seems that an enzyme for splitting sialylated carbohydrate chains from *O*-glycosidic glycopeptides does not exist or, at least in case of sialidase deficiency, does not function or is inhibited by the presence of sialic acid relatively near to the place of action. The latter suggestion is supported by the observation that a bacterial endo-α-*N*-acetyl-galactosaminidase acts only upon (Galβ1-3GalNAc)-glycoproteins [22, 23]. Regarding the heterogeneity in the peptide part of the excreted glycopeptides [12] (and this study), protease activity can also depend on previous removal of the whole *O*-glycosidic carbohydrate chains or of sialic acid from such chains. It would be worthwhile investigating the possible presence of glycopeptides in other lysosomal storage diseases with, for example, galactosidase or hexosaminidase deficiencies, to learn more about the catabolism of *O*-glycosidic glycopeptides in the lysosomes.

The analyzed sialyloligosaccharides are known as normal constituents of human glycoproteins [24, 25] and represent a rather specific group of *O*-glycosidic carbohydrate chains. Carbohydrate chains of mucus glycoproteins with different branching patterns [26, 27] are not found in sialidosis urine.

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