Eur. J. Biochem. *140*, 415–420 (1984)

The structure of sialyl-glycopeptides of the *O*-glycosidic type, isolated from sialidosis (mucolipidosis I) urine

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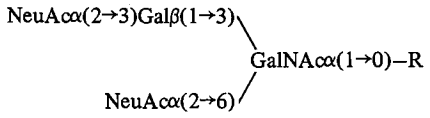
(Received October 4, 1983/January 2, 1984) – EJB 83 1099

Sialyl-glycopeptides containing an *O*-glycosidically linked tetrasaccharide chain were obtained from the urine of a patient suffering from mucolipidosis I. Isolation of these compounds was achieved by gel filtration, ion-exchange chromatography and preparative paper chromatography. Their structures were determined by a combination of carbohydrate and amino acid analysis, dansylation, periodate oxidation, methylation studies, enzymatic hydrolysis and ^{1}H-NMR spectroscopy, to be as follows:



wherein R = peptide linked through -Thr-, -Ser-, -Ser-Thr- or -Thr-Ser-.

The finding of these glycopeptides in urine shows that mucolipidosis I is characterized by a general "glycoprotein-specific" sialidase deficiency. The possibility of the existence of a human endo-α-*N*-acetylgalactosaminidase is discussed.

Mucolipidosis I (MLP-I, one of the forms of sialidosis) is an inherited metabolic disease characterized clinically by the presence of a cherry-red spot on the fundus, by elective overload of the Kupffer cells and by a variety of neurological syndroms. Chemical studies of this disease showed accumulation of abnormal amounts of sialic-acid-containing compounds in fibroblasts and leukocytes of the patients, due to a deficiency of sialidase activity in these cells. By consequence, an enhanced excretion of sialic-acid-containing oligosaccharides was observed in the urine of MLP-I patients; these oligosaccharides originate from the impaired catabolism of *N*-glycosidic glycoprotein carbohydrate chains [1–4].

However, the sialidase deficiency in MLP-I does not only affect the catabolism of the *N*-glycosidic type of glycoprotein oligosaccharides but, as previously reported by us [5] and others [6], also the excretion of the *O*-glycosidic type of glycoprotein carbohydrates is found increased about tenfold in MLP-I urine, as compared to normal. Structural studies on the excreted sialic-acid-containing compounds of the *O*-glycosidic type have been scarce until now; however, the occurrence of a glyco-aminoacid constituted of NeuAc, GalNAc and Ser has been reported [7].

The present work describes the structure determination of some sialyl-glycopeptides of the *O*-glycosidic type isolated from the urine of a patient suffering from MLP-I. The results provide further insight into the biochemical consequences of the enzyme deficiency. A preliminary account of the outcome of the structural studies has been presented [8].

MATERIALS AND METHODS

Materials

Urine of a patient (P.P.) with MLP-I, whose clinical characteristics have already been reported [9], was collected on merthiolate (10 ml/l) without any dietary restriction. Urine was kept frozen (−18 °C) until use.

Isolation and purification of sialyl-glycopeptides

After thawing, the urine (1.5 l) was concentrated by ultrafiltration over hollow-fiber Amicon HP-05. The ultrafiltrate was subjected to gel permeation chromatography over Sephadex G-25 (fine) (Pharmacia). The sialic-acid-containing material was pooled into fractions I, IIa and IIb as reported previously [5]. Fraction IIb was further fractionated by ion-exchange chromatography on a DEAE-Sephadex A-25 column (4.5 × 23 cm, acetate form, Pharmacia). The column was equilibrated in 0.05 M pyridine/acetate buffer pH 5.7 and eluted by a discontinuous gradient (0.05 M pH 5.7; 0.05 M pH 5.3; 0.1 M pH 5.3; 0.1 M pH 4.8; 0.2 M pH 4.8 and 0.2 M pH 4.5) of pyridine/acetate buffer.

Abbreviations. MLP-I, mucolipidosis I; ^{1}H-NMR, proton nuclear magnetic resonance; NeuAc, *N*-acetylneuraminic acid; Gal, galactose; GalNAc, *N*-acetylgalactosamine; 2,4,6-Me_3-Gal-ol, 2,4,6-tri-*O*-methyl-1,3,5-tri-*O*-acetyl-galactitol; 2,3,4,6-Me_4-Gal-ol, 2,3,4,6-tetra-*O*-methyl-1,5-di-*O*-acetyl-galactitol; 4-Me-GalNAc(Me)-ol, 2-deoxy-2-*N*-methylacetamido-4-mono-*O*-methyl-1,3,5,6-tetra-*O*-acetyl-galactitol; 4,6-Me_2-GalNAc(Me)-ol, 2-deoxy-2-*N*-methyl-acetamido-4,6-di-*O*-methyl-1,3,5-tri-*O*-acetyl-galactitol.

Enzymes. α-Neuraminidase (EC 3.2.1.18); β-D-galactosidase (EC 3.2.1.23).

The sialic-acid-containing material eluted by 0.1 M pyridine/acetate buffer pH 5.3 was passed over another DEAE-Sephadex A-25 column (1.7 × 13 cm, acetate form) equilibrated with 0.02 M pyridine/acetate buffer pH 5.3 and eluted with 150 ml of a linear gradient from 0.02 M to 0.2 M of the same buffer. The material eluted at 0.12–0.13 M was purified on a Sephadex G-10 column (1.8 × 100 cm) and was designated compound A.

The sialic-acid-containing material eluted by 0.2 M pyridine/acetate buffer pH 4.8 was also passed over another DEAE-Sephadex A-25 column (1.7 × 13 cm, acetate form) equilibrated with 0.2 M pyridine/acetate buffer pH 5.0. Elution was performed with 150 ml of a linear gradient from pH 5.0 to pH 4.5 of a 0.2 M pyridine/acetate buffer. The material eluted at pH 4.58–4.53 represented a major compound which was further purified by descending preparative paper chromatography on Schleicher & Schüll 2043-b paper in the solvent system ethyl acetate/pyridine/acetic acid/water (5/5/1/3, v/v). Material positive to ninhydrin and Schiff's reagent was subsequently purified on Bio-Gel P-2 (200–400 mesh, BioRad) in order to eliminate impurities from the paper; the resulting compound was denoted compound B.

The purity of compounds A and B was checked by thin-layer chromatography and high-voltage paper electrophoresis. Thin-layer chromatography was performed on silica gel plates (HPTLC, Merck) in the aforementioned solvent system. After development, the plates were sprayed either with ninhydrin or with α-naphthol/sulfuric acid reagent [10] and heated at 100 °C for 10 min. High-voltage paper electrophoresis was performed in a Gilson apparatus at 55 V/cm for 2 h, using Whatman 3MM paper with the following buffers: pH 1.9 formic acid/acetic acid/water (2.5/7.5/90, v/v) and pH 6.5 pyridine/acetic acid/water (25/1/1974, v/v). Papers were stained with ninhydrin and Schiff's reagent after periodate oxidation as described for paper chromatography.

Analytical methods

Sugar analysis was performed by gas-liquid chromatography of pertrimethylsilylated derivatives of methyl glycosides, formed by methanolysis in methanol/1.5 M HCl, 85 °C, 18 h [11]. A Hewlett-Packard 5750-G gas chromatograph was used, equipped with a glass column (3% w/w of SE-30 on chromosorb WAW-DMCS, 100–200 mesh).

Amino acids were determined on an amino acid analyzer (Jeol) after hydrolysis in 3 M HCl for 4 h or 6 M HCl for 7 h in vacuo.

NH_2-terminal amino acids were determined as dansyl derivatives according to Woods and Wang [12] and located on thin-layer chromatography under ultraviolet light.

Ninhydrin-degraded [13] borodeuteride-reduced compound A was permethylated as described by Stellner et al. [14] with potassium *tert*-butoxide as base [15]. The permethylated derivative was extracted with chloroform and purified on a silica gel column [16]. After hydrolysis, reduction and acetylation [14], the partially methylated alditol acetates were identified by gas chromatography coupled with mass spectrometry using a Hewlett-Packard 5993 B instrument operated with 5985 software. The injection port was kept at 270 °C, the capillary column was connected with the ion source of the mass spectrometer with a platinum restrictor which splits the flow at the entry of the ion source. The fused silica capillary column (CP-Sil-5 Chrompack, 25 m × 0.25 mm) was heated at a rate of 3 °C/min from 160 °C to 260 °C. The mass spectra were recorded at an ionization potential of 70 eV. The methylated alditol acetates were identified by a combination of gas chromatographic retention times, selected-ion recording and their mass spectra. The spectra were matched against a library of known standards.

Carbohydrates (60 μmol) were successively digested with the following enzymes: α-neuraminidase from *Vibrio cholerae* (Behringwerke) in 0.05 M sodium acetate containing 0.15 M NaCl and 0.009 M $CaCl_2$ pH 5.5 at 37 °C for 18 h; β-D-galactosidase from jack bean (Sigma, suspension in 3 M ammonium sulfate, 0.025 M sodium citrate buffer pH 5.5) at 37 °C for 8 h and from beef liver (Sigma grade III) in 0.01 M sodium phosphate buffer pH 7, 0.15 M NaCl, at 37 °C for 8 h. To all reaction mixtures, 1 drop of toluene was added to inhibit bacterial growth. Reactions were stopped by heating the reaction mixture in a boiling water bath for 5 min. Released sialic acid and galactose were quantified according to Warren [17] and Kornfeld et al. [18], respectively.

Alkaline borohydride treatment was performed as described by Carlson [19] on 200 μmol of glycans A and B. After destruction of excess borohydride, the solution was concentrated and borate was eliminated as methyl borate. Amino acids and carbohydrates were analyzed as reported above.

Periodate oxidation was carried out in 0.035 M sodium acetate pH 4.5 containing 0.035 M $NaIO_4$ for 24 h at 4 °C in the dark. Excess $NaIO_4$ was destroyed with ethyleneglycol. Remaining monosaccharides and amino acids were analyzed as described above.

Prior to ^{1}H-NMR spectroscopic analysis, the glycopeptide fractions A and B were repeatedly treated with ^2H_2O at p^{2}H 7 and room temperature, with intermediate lyophylization. Finally, the samples were redissolved in 0.4 ml ^2H_2O (99.96 atom % ^{2}H, Aldrich).

500-MHz ^{1}H-NMR spectroscopy was performed on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University, The Netherlands) operating in the Fourier-transform mode under control of an Aspect-2000 computer. For further experimental details, see [20]. For solvent-peak suppression, a water-eliminating Fourier-transform (WEFT) pulse sequence (composite 180° pulse-delay/–90° pulse-acquisition) [21] was used. The probe temperature was 27 °C. Chemical shifts (δ) are expressed downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate, but were actually measured by reference to internal acetone (δ = 2.225 ppm), with an accuracy of 0.002 ppm.

RESULTS

Both compounds A and B appeared to be homogeneous as judged by paper chromatography, thin-layer chromatography and high-voltage electrophoresis at two pH values. Finally, 6 mg of compound A and 2.5 mg of compound B were obtained.

Structure determination of compound A

The molar composition of compound A is given in Table 1. Gal, GalNAc, NeuAc and Thr, in molar ratios of 1.0:1.0:1.8:1.0, together accounted for 92% of the dry weight. No other monosaccharides were present. Traces of Ser, Asp, Glu, Pro, Gly and Ala were present after hydrolysis (total less than 8%). After dansylation, Thr was identified as the major dansylated amino acid by thin-layer chromatography.

Periodate oxidation destroyed completely the sialyl residues of the native glycopeptide A, whereas the Gal and GalNAc

residues remained intact. This result indicated that Gal was substituted at C-3 and GalNAc at C-3 and/or C-4. Thr was recovered in equimolar amount to Gal and GalNAc. Under similar conditions, the vicinal NH_2-CH-CHOH- functions of free Thr were found to be oxidized. Thus, the carbohydrate chain is linked to the hydroxyl group of Thr.

Glycopeptide A was resistant to alkaline borohydride treatment even if the NaOH concentration was raised to 0.3 M. Neither GalNAc-ol nor Gal-ol originated from this treatment, indicating that there are no free reducing termini in this compound. The resistance to alkaline borohydride treatment indicated that either the amino or the carboxyl group of Thr was unsubstituted [22]. A similar result has been reported previously for a urinary glycoside [23].

Table 1. *Amino acid and carbohydrate composition of compounds A and B isolated from MLP-I urine*
The molar ratios were calculated relative to one GalNAc residue. The amino acids were quantified on an amino acid analyzer after hydrolysis in 6 M HCl at 100 °C for 7 h. GalNAc was quantified on the same analyzer after hydrolysis in 3 M HCl at 100 °C for 4 h and also by gas-liquid chromatography

Component	Amount in compound	
	A	B
	mol/mol	
Gal	1.03	1.12
GalNAc	1.00	1.00
NeuAc	1.81	2.02
Thr	1.00	1.20
Ser	0.18	1.01
Asp	0.12	0.13
Glu	0.13	0.18
Pro	0.09	0.10
Ala	0.15	0.18
Gly	0.15	0.20
Other amino acids	< 0.05	< 0.06

The sugar sequence and the anomeric configuration of the glycosidic linkages in compound A were determined by stepwise hydrolysis with specific exoglycosidases. Removal of Gal by beef-liver β-D-galactosidase was effective (0.85 mol Gal liberated/mol A) only after the release of sialic acid (1.94 mol NeuAc liberated/mol A); under similar conditions, jack-bean β-D-galactosidase was inefficient. These results are in favour of a $\beta(1 \rightarrow 3)$-linkage between Gal and GalNAc rather than a $\beta(1 \rightarrow 6)$- or $\beta(1 \rightarrow 4)$-linkage [24, 25].

Methylation analysis was carried out on the ninhydrin-degraded borodeuteride-reduced compound A. Hydrolysis of the permethylated compound followed by $NaBH_4$ reduction and peracetylation gave 2,4,6-Me_3-Gal-ol and 4-Me-GalNAc(Me)-ol. These derivatives were identified by their chromatographic retention times and their mass spectra which showed fragmentation patterns similar to those reported for the corresponding methyl ethers of *N*-acetylglucosamine [26, 27]. Traces of 2,3,4,6-Me_4-Gal-ol and 4,6-Me_2-GalNAc(Me)-ol were also identified. These data in combination with the aforementioned results of periodate oxidation suggest that the sialyl residues are linked to C-3 of Gal (because of the finding of 2,4,6-Me_3-Gal-ol) and to C-6 of GalNAc [since 4-Me-GalNAc(Me)-ol was found], respectively. The other methyl ethers probably originate from partial loss of NeuAc during the processing of the samples.

The 500-MHz ^{1}H-NMR spectrum of sialyl-glycopeptide fraction A in ^2H_2O is depicted in Fig. 1. The chemical shifts of the "structural reporter-group" protons bearing the essential information on the primary structure of the carbohydrate moiety [20] are compiled in Table 2. For the spectral interpretation, advantage was taken of the ^{1}H-NMR data established for a similar sialic-acid-containing glycopeptide (α_2HS-

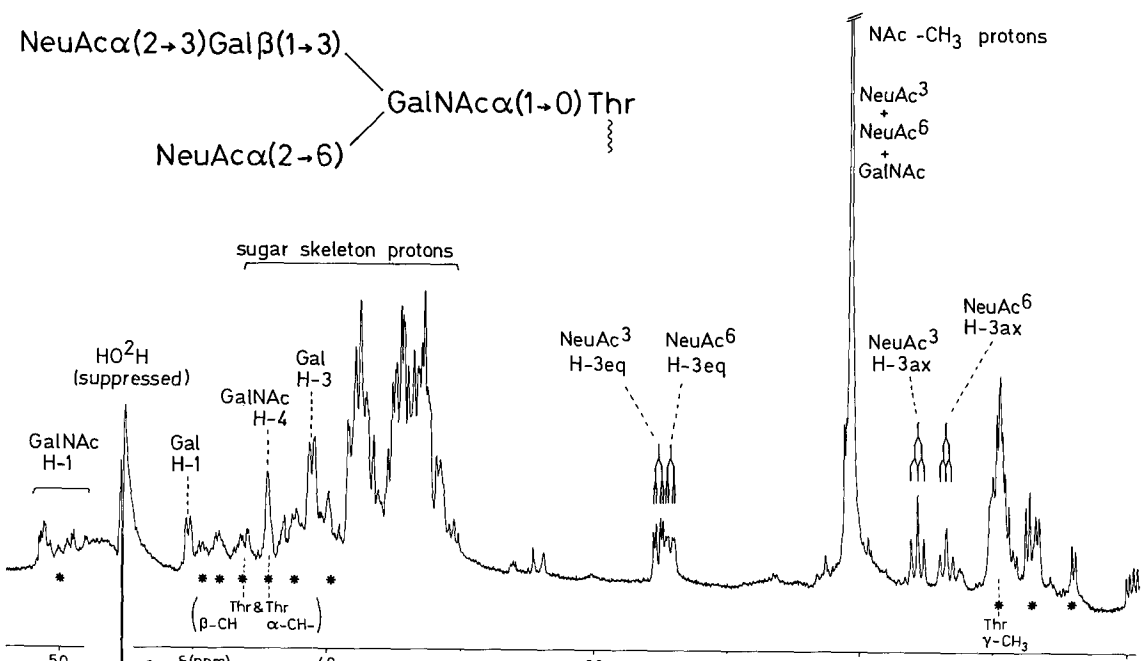
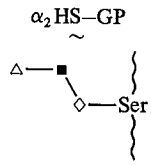
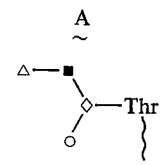
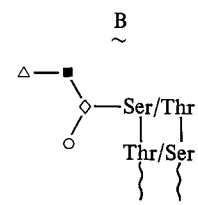


Fig. 1. *500-MHz ^{1}H-NMR spectrum of sialyl-glycopeptide A, obtained from mucolipidosis I urine.* The spectrum was recorded in ^2H_2O at p^{2}H 7 and 27 °C; the HO^{2}H signal was suppressed by a WEFT pulse sequence. Values are relative to internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. Asterisks indicate signals of amino-acid protons

Table 2. *^1H chemical shifts of structural-reporter-group protons of constituent monosaccharides for the urinary O-glycosidic glycopeptides A and B, obtained from an MLP-I patient, together with those for a reference glycopeptide (α_2HS-GP) [28]*
Chemical shifts were acquired at 500 MHz for neutral ^2H_2O solutions at 27 °C; they are expressed downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate. In the table heading, compounds are represented by schematic structures; ◇— = GalNAc; ■— = Gal; △— = NeuAcα(2→3); ○— = NeuAcα(2→6)

Reporter group	Residue	Chemical shift in		
		α_2HS–GP 	A 	B 
		ppm		
H-1	GalNAc	4.92[a]	5.04[a]	4.886/4.994
	Gal	4.564	4.521	4.522/4.542
H-3	Gal	4.070	4.054	4.062
H-3*ax*	NeuAcα(2→3)	1.797	1.782	1.785/1.790
	NeuAcα(2→6)	–	1.673	1.660/1.680
H-3*eq*	NeuAcα(2→3)	2.749	2.758	2.753
	NeuAcα(2→6)	–	2.711	2.706
H-4	GalNAc	4.230	4.223	4.248/4.254
	Gal	3.925	3.92	3.92
NAc	GalNAc	2.015[a]	2.03[a,b]	2.03[a,b]
	NeuAcα(2→3)	2.029	2.031	2.031
	NeuAcα(2→6)	–	2.031	2.031

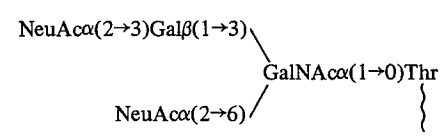
[a] Several signals are observed due to heterogeneity of the peptide moiety (see text).
[b] Signal is almost hidden under the relatively broad singlet from the *N*-acetyl methyl-protons of the NeuAc residues.

GP) being NeuAcα(2→3)Galβ(1→3)GalNAcα(1→O)Ser, derived from the B-chain of human plasma α_2HS-glycoprotein [28]; the data for α_2HS-GP have been included in Table 2.

From the presence of two NeuAc H-3*ax* signals, at 1.78 ppm and 1.67 ppm, and also from the two H-3*eq* signals at 2.76 ppm and 2.71 ppm (see Fig. 1), it can be concluded that compound A contains two NeuAc residues in different types of linkage. The former set of signals (H-3*ax* at 1.782 ppm, H-3*eq* at 2.758 ppm) belongs to NeuAc α(2→3)-linked to a Gal residue (compare α_2HS-GP in Table 2 and [20, 29]). The latter set (H-3*ax* at 1.673 ppm, H-3*eq* at 2.711 ppm) is a novel one; the resonance position of H-3*ax* is indicative of the α(2 → 6)-type of linkage, whereas the H-3*eq* chemical shift shows resemblance with that observed for NeuAc in α(2→6)-linkage to GalNAc-ol in mucin-type oligosaccharide-alditols, as well as to Gal in NeuAcα(2→6)Galβ(1→4)Glc [29]. This, together with the data of carbohydrate analysis (Table 1), suggests that the carbohydrate portion of compound A may be an extension of α_2HS-GP with NeuAc α(2→6)-linked to GalNAc. This conclusion is corroborated by the difference in chemical shift observed between H-1 of Gal in compound A and α_2HS-GP ($\Delta\delta \approx -0.04$ ppm). This value is in line with the effect upon δ for H-1 of Gal due to introduction of a NeuAc in α(2→6)-linkage to GlcNAc forming part of NeuAcα(2→3)Galβ(1→3)-GlcNAcβ(1→) structural element [30]. The relative broadness of the NeuAcα(2→6) H-3*ax* and H-3*eq* signals is most probably caused by heterogeneity of the peptide moiety. Furthermore, as is usually observed for glycopeptides [20], the chemical shifts of the reporter groups of the peptide-linked sugar, in this case GalNAc, are considerably affected by the composition of the peptide moiety (see Fig. 1 and compare compound A with α_2HS-GP, Table 2).

Nevertheless, the range of chemical shifts of H-1 of GalNAc (4.9 – 5.1 ppm) and their coupling constants ($J_{1,2} \approx 3.5$ Hz) point to an α-type of linkage to Thr. An average chemical shift value has been listed in Table 2 for GalNAc H-1 of compound A having GalNAc-Thr as the major glycopeptide linkage constituent. For the explanation of minor-intensity GalNAc H-1 signals at 4.90 ppm and 4.94 ppm see the information for compound B below. The *N*-acetyl signals of GalNAc, partly hidden under the high singlet of the NeuAc methyl protons, are shifted downfield, as compared to α_2HS-GP (Table 2). The substitution of Gal by NeuAc at C-3 is further evidenced by H-3 of Gal resonating at 4.05 ppm outside the bulk signal of most of the sugar skeleton protons [28, 29]. The $J_{1,2}$ value for Gal H-1 (≈ 8 Hz) points to a β-glycosidic linkage between Gal and GalNAc.

On the basis of these results, the following structure was derived for compound A:



Structure determination of compound B

The molar composition of compound B is included in Table 1. Gal, GalNAc, NeuAc, Thr and Ser, being present in a molar ratio of 1.1 : 1.0 : 2.0 : 1.2 : 1.0, together accounted for 93% of the dry weight. No other monosaccharides were present. Other amino acids accounted for 7% of the dry weight.

The carbohydrate composition of compound B is very similar to that of compound A. Furthermore, the results of

enzymatic hydrolysis and of periodate oxidation are identical for both compounds suggesting that the carbohydrate structure of compounds A and B is the same.

Thr and Ser were present in equimolar amounts, as compared to Gal and GalNAc after their release by acid hydrolysis. Dansylation produced both Dns-Ser and Dns-Thr as the major derivatives. This result suggests that compound B contains a mixture of glycopeptides, differing in their peptide chains. The major sequences might be Ser-Thr and Thr-Ser.

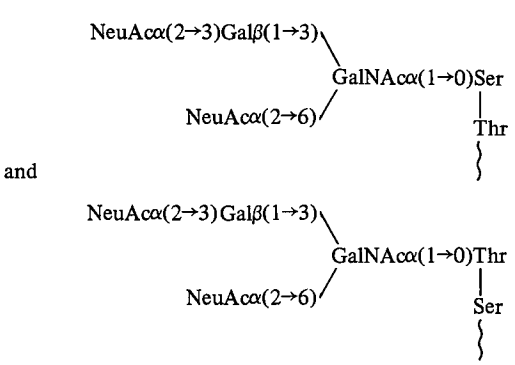
The site of attachment of the carbohydrate chain to the peptide was identified to be on the N-terminal amino acid on the basis of periodate oxidation: no loss of Thr or Ser was observed as compared to Gal or GalNAc whereas, in similar conditions, free Thr or Ser were completely destroyed.

Alkaline borohydride treatment again failed to release the carbohydrate chain from the peptide; stronger conditions only gave rise to destruction of the glycopeptides. The result is in line with the attachment of the carbohydrate chain to the N-terminal amino acid of the peptide chain [22] as demonstrated by periodate oxidation.

The 500-MHz ^{1}H-NMR spectrum of sample B illustrated, independently, that this fraction is a mixture of glycopeptides that differ not only in composition and length of their peptide moiety, but even in the nature of the glycosylated amino acid. The carbohydrate moiety of the components of sample B is identical to that of compound A, as is evident from the spectral data included in Table 2.

For the major set of constituents, the H-1 doublet of GalNAc is found at 4.886 ppm ($J_{1,2} \approx 3.5$ Hz); this is attributed to the compounds having GalNAc α-linked to Ser in the peptide portion, because the shift decrement as compared to GalNAc for α_2HS-GP ($4.92-4.886: \Delta\delta \approx -0.04$ ppm, see Table 2) is essentially the same as observed for Gal H-1 upon attachment of NeuAc in α(2→6)-linkage to this Gal [20, 29]. A similar shift effect is observed between H-1 of GalNAc for compound A as compared to the minor series of constituents of sample B (5.04 − 4.994 ppm), suggesting that the latter possess GalNAc in α-linkage to Thr. Doubling of many structural-reporter-group signals, like H-1 of Gal, and H-3*ax* of both NeuAcα(2→6) as well as NeuAcα(2→3), reflects this heterogeneity of the peptide moiety. The influence of the heterogeneity reaches farther than expected on the basis of observations for *N*-glycosidic glycopeptides [20]. It could be speculated that a specific interaction between the carbohydrate and the peptide moiety in compounds of the *O*-glycosidic type exists. It should be mentioned that the H-1 chemical shift values characteristic for GalNAc linked to Ser also apply to the minor component of fraction A which may provide an explanation for the observed heterogeneity (ratio Ser/Thr 0.18/1.0, see Table 1).

From the data given above, it is concluded that the glycopeptides present in fraction B possess the following structures:



From a structural point of view, it may be noted that the carbohydrate portion of both compounds A and B had been characterized previously by ^{1}H-NMR [31], in the form of the corresponding oligosaccharide-alditol. The present study shows that glycopeptides of the mucin type are also amenable for structure determination by ^{1}H-NMR spectroscopy.

DISCUSSION

The structure determination of the *O*-glycosidic sialyl-glycopeptides excreted in the urine of an MLP-I patient substantiates our previous assumptions that, in this patient, there is a general disturbance of the metabolism of sialyl-oligosaccharide chains of glycoproteins, not only of the *N*-glycosidic type but also of the GalNAc-Ser and/or GalNAc-Thr type [5]. It remains to be established whether this is a general feature of sialidosis, since this study deals with one patient only. The simultaneous accumulation of *O*- and *N*-glycosidic type of glycoprotein-derived sialyl-oligosaccharide chains in the urine of this patient confirms that in MLP-I the glycoprotein-specific sialidase is deficient [1, 2].

Our results do not provide conclusive evidence for an endo-α-*N*-acetylgalactosaminidase to be involved in the catabolism of *O*-glycosidically linked oligosaccharide chains of glycoproteins. However, the finding by Parkkinen and Finne [32] (which was published after submission of this paper) of the reducing tetrasaccharide NeuAcα(2→3)Galβ(1→3)[NeuAcα-(2→6)]GalNAc in normal urine can be considered as an indication for the existence of such an enzyme. The theoretical possibility that our patient lacks both glycoprotein-specific sialidase and endo-α-*N*-acetylgalactosaminidase at the same time is only likely if their activities are expressed together, like those of sialidase and β-galactosidase [33]. Indeed, an endo-α-*N*-acetylgalactosaminidase was found in bacteria [34, 35] but it was reported to act only upon asialoglycoproteins. This implies a difference in specificity between human and bacterial endo-enzyme.

It would be worthwhile investigating the existence and properties of human endo-α-*N*-acetylgalactosaminidase and how its activity is expressed in relation to sialidase.

This work was supported by grants from the *Institut National pour la Santé et la Recherche Médicale* (Unités 16 and 180, ATP 76-69 and CRL 81-30-30), the *Centre National de la Recherche Scientifique* (LA 293) and the *Faculté de Médecine Lariboisière-Saint-Louis*, Université Paris VII, by the Netherlands Foundation for Chemical Research (SON/ZWO), and by grant UUKC-OC 79 − 13 from the Netherlands Foundation for Cancer Research (KWF). The authors wish to thank Dr P. Maroteaux (*CHU Necker-Enfants Malades*, Paris XV) for the generous gift of the MLP-I urine sample.

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