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Sialyl-α2-6-mannosyl-β1-4-*N*-acetylglucosamine, a Novel Compound Occurring in Urine of Patients with β-Mannosidosis*

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Johannes van Pelt, Lambertus Dorland‡, and Marinus Duran

From the University Children's Hospital "Het Wilhelmina Kinderziekenhuis," Nieuwe Gracht 137, Utrecht NL-3512 LK, The Netherlands

Cornelis H. Hokke, Johannis P. Kamerling, and Johannes F. G. Vliegenthart

From the Department of Bio-organic Chemistry, Bijvoet Center, Utrecht University, Padualaan 8, Utrecht NL-3584 CH, The Netherlands

Human β-mannosidosis urine was fractionated by gel permeation chromatography on Bio-Gel P-2 and by high performance liquid chromatography on Partisil 10 SAX. Besides the disaccharide Manβ1-4GlcNAc as the major component, a sialic acid-containing compound was detected in an amount of 10% compared to that of Manβ1-4GlcNAc. Structural characterization of the oligosaccharide and of its reduced analogue by sugar composition analysis, methylation analysis, gas-liquid chromatography-mass spectrometry, and 500-MHz ^{1}H NMR spectroscopy gave conclusive evidence for a novel urinary constituent: NeuAcα2-6Manβ1-4GlcNAc. This linear trisaccharide can be considered as the result of an α2-6-sialylation of the major accumulating compound, Manβ1-4GlcNAc. The hitherto unknown linkage between sialic acid and mannose was shown to be susceptible to sialidase digestion.

β-Mannosidosis is an inherited disorder of glycoprotein catabolism caused by a deficiency of the enzyme β-mannosidase (EC 3.2.1.25). This lysosomal storage disease has been described in goats (1); and recently, the disorder has been discovered in man (2-4). In goats, β-mannosidosis leads to severe neonatal neurological deficits (1) and to an excessive urinary excretion of Manβ1-4GlcNAc and Manβ1-4GlcNAc-β1-4GlcNAc (5). The di- and trisaccharides also accumulate in various tissues (6, 7); and in addition, Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc, Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAc (compound a), and Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc (compound b) are found in the kidneys of affected goats (8). The heterogeneity in excreted material is partly caused by the existence of two catabolic pathways in goats. The activities of endo-β-*N*-acetylglucosaminidase and aspartylglucosaminidase result in oligosaccharides either with an *N*,*N*′-diacetylchitobiose unit or with just 1 GlcNAc residue at the reducing terminus (9-11). Compounds a and b are not known as glycoprotein constituents and can be considered as extensions of the primary di- and trisaccharides with an additional Manβ1-4GlcNAcβ1-4 moiety, but their origin is still unknown.

Human β-mannosidosis patients seem to be less affected, and they mainly excrete the disaccharide Manβ1-4GlcNAc in urine (2-4). Recently, Manβ1-4GlcNAcβ1-*N*-urea was isolated from human β-mannosidosis urine (12), and this conjugate also represents the unknown structural Man-βGlcNAc isomer[1] reported in Ref. 13. This paper describes the structural characterization of sialylated Manβ1-4GlcNAc, a compound occurring in urine of patients with β-mannosidosis. The origin of the novel compound with the hitherto unknown linkage between sialic acid and mannose is discussed.

EXPERIMENTAL PROCEDURES

Chromatographic Procedures—Urine (350 ml) from the elder of two brothers with β-mannosidosis (4) was lyophilized and redissolved in 17 ml of water. Gel permeation chromatography of one part (5 ml) was carried out on a Bio-Gel P-2 column (Bio-Rad, 200-400 mesh, 90 × 2.6 cm) using water as eluent at a flow rate of 24 ml/h. The fractions (6.0 ml) were analyzed for hexose at 492 nm by the phenol/H_2SO_4 assay (14). Pooled fractions were subfractionated by HPLC[2] on a Partisil 10 SAX column (Whatman, 250 × 4.6 mm). Depending on the aimed separation, elutions (flow rate, 2.0 ml/min) were carried out with mixtures of acetonitrile and 25 mM NH_4HCO_3, pH 7.2; acetonitrile and 30 mM KH_2PO_4, pH 4.9; or acetonitrile and water. The HPLC system (LKB 2150 and 2152) was connected to a diode array detector (LKB 2140 rapid spectral detector) used for UV detection in the wavelength trajectory of 190-220 nm. The HPLC subfractions obtained by chromatography with acetonitrile/phosphate buffer were desalted on a Bio-Gel P-2 column (200-400 mesh, 40 × 1 cm) using water as eluent (13 ml/h).

TLC was performed on Kieselgel 60 plastic or aluminum sheets (Merck, 0.2 mm, 10 × 10 cm) eluted twice with 1-butanol/acetic acid/water (2:1:1, v/v) (solvent system I) or with ethanol/1-butanol/pyridine/water/acetic acid (100:10:10:30:3, v/v), followed by 1-propanol/water (2:1, v/v) (solvent system II) (15). The plates were stained with orcinol/H_2SO_4 to detect hexose-containing compounds and with orcinol/$FeCl_3$/HCl to visualize sialic acid-containing compounds (16).

Monosaccharide Analysis—Samples were subjected to methanolysis (1.0 M methanolic HCl, 24 h, 85 °C) followed by gas-liquid chromatography of the trimethylsilylated and *N*-reacetylated (methyl ester) methyl glycosides on a capillary CP-Sil 5 WCOT fused silica column (Chrompack, Inc., 25 m × 0.32 mm, 0.11-μm film thickness) with a temperature program of 125-240 °C at 5 °C/min (17).

Preparation of Oligosaccharide-Alditols—The reduction of oligosaccharides to oligosaccharide-alditols was carried out in aqueous $NaBH_4$ or NaB^2H_4 (10 mg/ml, 2.5 h, 21 °C). After acidification to pH 5 with Dowex 50W-X8-H^+ (Fluka, 100-200 mesh) at 0 °C and filtration, the samples were evaporated under reduced pressure. Boric acid was removed by coevaporation with methanol.

Methylation Analysis—Methylation analysis of an oligosaccharide-alditol (0.9 mg) was carried out essentially as described (18). After permethylation and purification on a Sep-Pak C_{18} column, the derivatized material was hydrolyzed with 4 M trifluoroacetic acid (0.2 ml,

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‡ To whom correspondence should be addressed.

[1] L. Dorland and J. van Pelt, unpublished results.

[2] The abbreviations used are: HPLC, high performance liquid chromatography; GlcNAc-ol, *N*-acetylglucosaminitol.

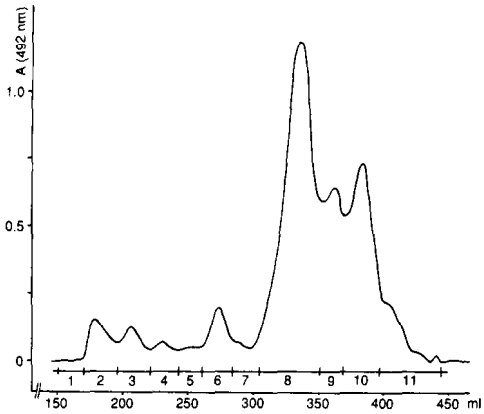

FIG. 1. **Elution profile of human β-mannosidosis urine on Bio-Gel P-2.** The column (90 × 2.6 cm) was eluted with water at a flow rate of 24 ml/h. The eluate was monitored by hexose determinations with phenol/sulfuric acid using 10-μl aliquots of the 6.0-ml fractions. Fractions 1–11 were pooled as indicated.

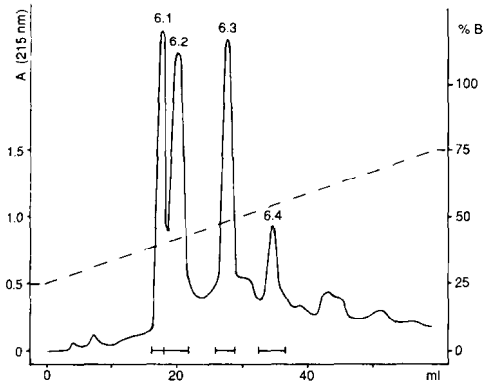

FIG. 2. **HPLC elution profile of Bio-Gel P-2 fraction 6 on Partisil 10 SAX.** The column (250 × 4.6 mm) was eluted with acetonitrile, 25 mM NH_4HCO_3, pH 7.2, in a linear gradient as indicated by *%B*. The eluate (flow rate, 2.0 ml/min) was monitored at 215 nm. Subfractions 6.1–6.4 were pooled as indicated.

TABLE I

Carbohydrate composition of Bio-Gel P-2 fractions 2–9

Fractions 1, 10, and 11 are not included as they contained no Man.

Monosaccharide	Fraction							
	2	3	4	5	6	7	8	9
Fuc[a]	0.7	0.7	0.3	0.3	0.3	+		
Man[b]	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
Gal	1.5	1.3	1.0	0.5	0.7	0.6		
Glc		0.2	0.2		0.5	0.7	0.1	3.4
GlcA						0.3		3.0
GalNAc	0.4					0.3		
GlcNAc	1.7	1.5	1.3	0.9	0.9	0.3	0.8	
NeuAc	1.2	1.1	1.1	0.9	1.3	0.4		
Amount (μmol)[c]	10.4	6.9	5.5	12.1	19.3	9.8	112.6	6.0

[a] Fuc, fucose; GlcA, glucuronic acid.
[b] Molar ratios calculated relative to Man = 1.0.
[c] Total amount of Man.

TABLE II

Carbohydrate composition of HPLC subfractions 6.1–6.4, 8.3, and 8.4

Monosaccharide	Subfraction					
	6.1	6.2	6.3	6.4	8.3	8.4
Man[a]	1.0	1.0	1.0	1.0	1.0	1.0
Gal	6.7	0.3	0.9	2.4		
Glc	5.3	0.2	2.0	3.2	0.1	
GlcNAc	6.0	0.9			0.8	0.9
NeuAc	5.3	1.1		0.8		
Amount (μmol)[b]	0.08	11.25	0.50	0.23	79.45	27.68

[a] Molar ratios calculated relative to Man = 1.0.
[b] Total amount of Man.

4 h, 100 °C). The obtained mixture of partially methylated monosaccharides was reduced with NaB^2H_4 (10 mg/ml) in water (1.5 ml, 2.5 h, 21 °C) and acetylated with acetic anhydride (0.15 ml, 3 h, 121 °C). The partially methylated alditol acetates were analyzed by gas-liquid chromatography and gas-liquid chromatography-mass spectrometry (19, 20).

Gas-Liquid Chromatography-Mass Spectrometry—This was performed on a Varian 6000 GC/Ribermag R 10-10 C Nermag mass spectrometer connected with a Digital PDP 11/23 computer. Conditions were as follows: electron energy, 70 eV; accelerating voltage, 2.7 kV; ionizing current, 100 μA; ion source temperature, 270 °C; capillary CP-Sil 19 fused silica column, 25 m × 0.32 mm (Chrompack, Inc.); and temperature program, 130 °C for 2.5 min and then 130–280 °C at 7 °C/min.

500-MHz ^1H NMR Spectroscopy—Resolution-enhanced ^1H NMR spectra were recorded on a Bruker AM-500 spectrometer (Department of NMR Spectroscopy, Utrecht University) operating at 500 MHz at probe temperatures of 300 and 310 K. Before analysis, the fractions were repeatedly exchanged in ^2H_2O (Aldrich, 99.96% ^2H) with intermediate lyophilization. Chemical shifts (δ) were measured relative to internal acetone (δ = 2.225 ppm) (21).

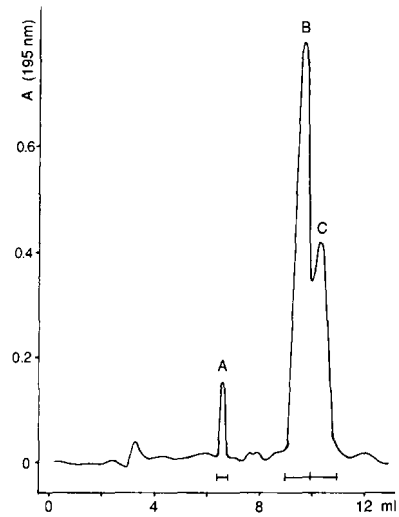

FIG. 3. **HPLC elution profile of subfraction 6.2 on Partisil 10 SAX.** The column (250 × 4.6 mm) was eluted isocratically with acetonitrile, 30 mM KH_2PO_4, pH 4.9 (3:2, v/v). The eluate (flow rate, 2.0 ml/min) was monitored at 195 nm. Subfractions A–C were pooled as indicated.

TABLE III

Carbohydrate composition of subfractions C, B, B-ol-1, and B-ol-2

Monosaccharide	Fraction			
	C	B	B-ol-1	B-ol-2
Man[a]	1.0	1.0		1.0
Gal		0.3	1.3	
Glc		0.3		
Glc-ol[a]			1.0	
GlcNAc	1.0	1.1		
GlcNAc-ol				0.8
NeuAc	1.0	1.4	1.0	1.0

[a] Molar ratios calculated relative to Man = 1.0 or to glucitol (Glc-ol) = 1.0 in fraction B-ol-1.

Sialidase Digestion—The characterized sialyloligosaccharide (21 μg) was dissolved in 50 μl of 50 mM sodium acetate, pH 5.2; and after addition of 10 milliunits of sialidase (from *Clostridium perfringens*, Boehringer Mannheim), it was incubated for 5 h at 37 °C. The incubation mixture was analyzed by TLC in solvent system I as described under "Chromatographic Procedures."

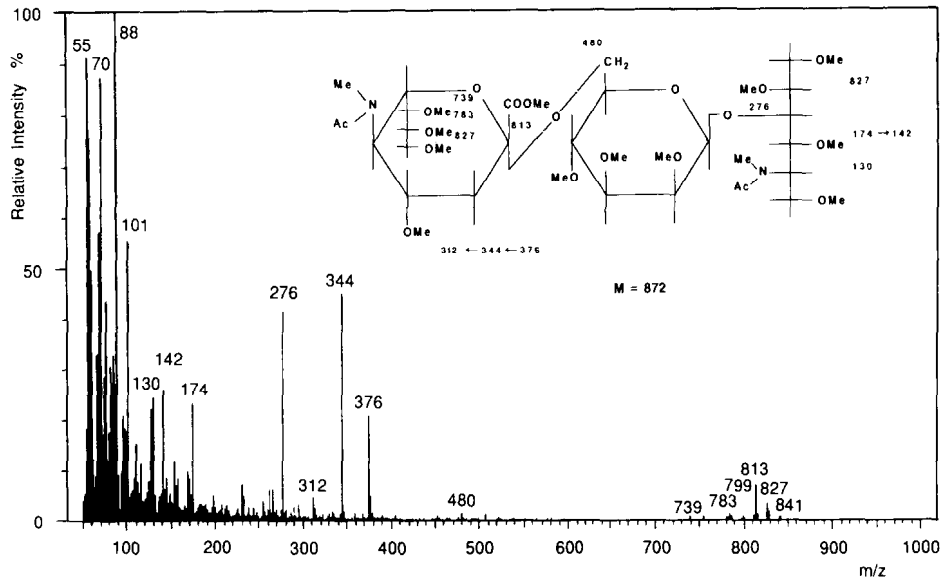

FIG. 4. **70-eV electron impact mass spectrum of reduced and permethylated fraction C.**

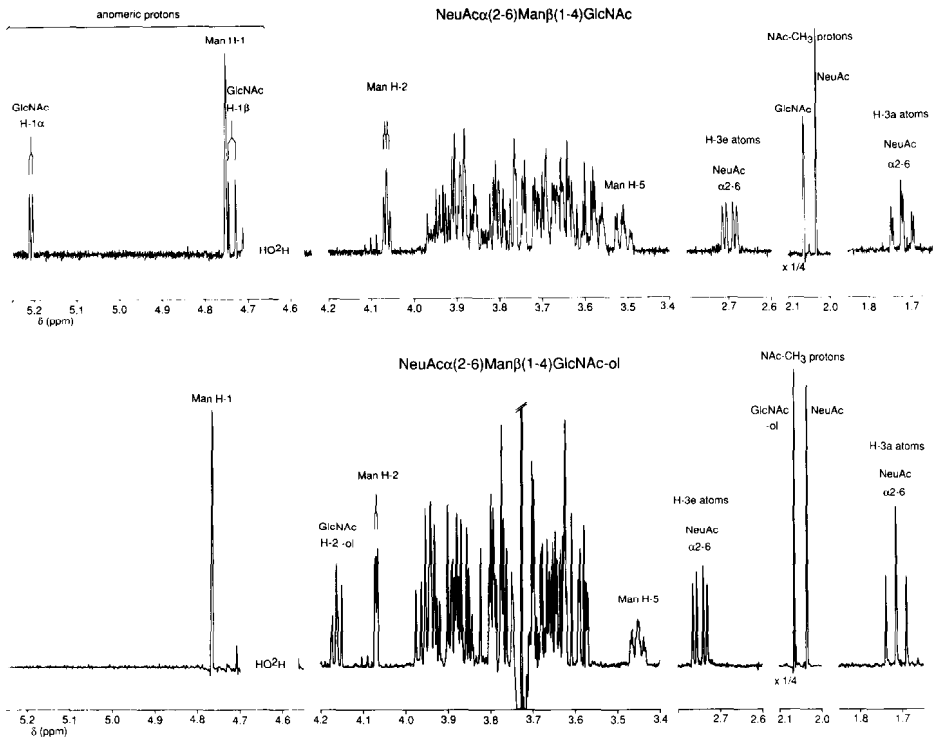

FIG. 5. **500-MHz ^{1}H NMR spectra of fractions C (*upper*) and C-ol (*lower*) recorded in ^2H_2O at 310 K.** The relative intensity scale of the *N*-acetyl regions differ from that of the other parts of the spectra as indicated.

RESULTS

TLC of human β-mannosidosis urine in solvent system I and staining with orcinol/H_2SO_4 revealed several faint bands and one clearly visible band between the origin and the very intense band of the major excreted compound, Manβ1-4GlcNAc. Gel permeation chromatography on Bio-Gel P-2 of lyophilized β-mannosidosis urine with detection at 492 nm (phenol/H_2SO_4 assay) resulted in several small peaks before elution of a huge amount of hexose-positive material (Fig. 1). A small part (1/100) of pooled fractions 1–11 was subjected to monosaccharide analysis (Table I), which showed the presence of a variety of sugars in most fractions. TLC of 1/200 of the fractions pointed to the presence of hexose-positive compounds in fractions 2–9, and at least one of the compounds in fractions 2–7 was also sialic acid-positive. Based on TLC and sugar analysis data, fractions 6 and 8 were selected for further investigation.

Fraction 8 was purified by HPLC on Partisil 10 SAX. Elution of the column with a mixture of acetonitrile, 25 mM NH_4HCO_3, pH 7.2, using a linear gradient from 4:1 to 1:1 (v/v) in 30 min and UV detection at 210 nm yielded several subfractions, denoted 8.1–8.9 (data not shown). Sugar analysis and TLC in solvent system II of the pooled subfractions revealed that the major amount of material was present in subfractions 8.3 and 8.4 (Table II). 500-MHz ^{1}H NMR spectroscopy proved that the compound present in subfractions 8.3 and 8.4 was Manβ1-4GlcNAc (12).

Fraction 6 was also subfractionated by HPLC on Partisil 10 SAX. Elution of the column with a mixture of acetonitrile, 25 mM NH_4HCO_3, pH 7.2, in a linear gradient from 3:1 to 1:3 (v/v) in 30 min and UV detection at 215 nm yielded four subfractions, denoted 6.1–6.4 (Fig. 2). Quantitative sugar analysis of a small part (1/50) of subfractions 6.1–6.4 (Table II) revealed that the major amount of carbohydrate was eluted in subfraction 6.2. The monosaccharide composition of the

TABLE IV

^1H chemical shift values of structural reporter group protons of the constituent monosaccharides of fractions C and C-ol together with those of reference compounds Manβ1-4GlcNAc (12) and Manβ1-4GlcNAc-ol

Residue	Reporter group	Manβ1-4-GlcNAc	Manβ1-4-GlcNAc-ol	Fraction	
				C	C-ol
GlcNAc	H-1α	5.211		5.207	
	H-1β	4.724		4.736	
	H-2-ol		4.264		4.162
	NAc	2.044	2.053	2.063	2.064
Man	H-1	4.769	4.797	4.752	4.765
	H-2	4.071/61[a]	4.071	4.067/60[a]	4.068
	H-5	3.438	3.375	3.509	3.45
NeuAc	H-3e			2.698	2.747
	H-3a			1.724/20[a]	1.714
	NAc			2.033	2.033

[a] Two signals were observed due to α/β-anomerization of GlcNAc.

latter fraction suggested the presence of a major component with Man, GlcNAc, and NeuAc in an equimolar ratio. 500-MHz ^1H NMR spectroscopy of subfraction 6.2 indicated the occurrence of at least two components as deduced from the structural reporter groups of NeuAc: a major compound (80%) with NeuAc in α2-6-linkage (H-3a and H-3e: δ 1.725/1.720 and 2.697) and a minor compound (20%) with NeuAc in α2-3-linkage (H-3a and H-3e: δ 1.794 and 2.761) (21, 22).

Subfraction 6.2 was further purified by HPLC on Partisil 10 SAX, eluted isocratically with a mixture of acetonitrile, 30 mM KH_2PO_4, pH 4.9 (3:2, v/v). The HPLC profile obtained by UV detection at 195 nm showed two major fractions, denoted B and C (Fig. 3). TLC analysis (solvent system II) of fraction C demonstrated the presence of one sialic acid- and hexose-containing band, and sugar analysis of fraction C (Table III) yielded Man, GlcNAc, and NeuAc in a molar ratio of 1.0:1.0:1.0. The electron impact mass spectrum of the reduced ($NaBH_4$) and permethylated compound in fraction C (Fig. 4) showed, in the high mass region, peaks at *m/z* 841 (M − OMe), *m/z* 827 (M − CH_2OMe), *m/z* 813 (M − COOMe), *m/z* 799 (M − NHAcMe), *m/z* 783 (M − $CH_2OMeCHOMe$), and *m/z* 739 (M − $CH_2OMeCHOMeCHOMe$) in accordance with a permethylated trisaccharide-alditol (M = 872) composed of sialic acid, hexose, and *N*-acetylhexosaminitol. The sequence NeuAc-Man-GlcNAc-ol was indicated by the peaks at *m/z* 480, 376, 344, and 276 (see Fig. 4). Evidence for the 1-4-linkage between Man and GlcNAc-ol was obtained from the presence of the peaks at *m/z* 174 (→142) and *m/z* 130 in combination with the complete absence of an ion at *m/z* 133 (5, 7, 8). Subsequent methylation analysis, including reduction with NaB^2H_4, yielded 1,5,6-tri-*O*-acetyl-2,3,4-tri-*O*-methylhexitol (1-$^2H_1$-labeled) and 4-*O*-acetyl-1,3,5,6-tetra-*O*-methyl-2-*N*-methylacetamido-2-deoxyhexitol, representing 6-substituted Man and 4-substituted GlcNAc-ol, respectively, and indicating a -6Man1-4GlcNAc structural element. Incubation of fraction C with sialidase (EC 3.2.1.18) from *C. perfringens* resulted in a complete conversion into Manβ1-4GlcNAc and NeuAc within 5 h, as demonstrated by TLC analysis. The ^1H NMR spectrum of fraction C (Fig. 5, *upper*) supported the various data mentioned above. It revealed the β-configuration for the Man unit and the chemical shift features of one NeuAc residue in α2-6-linkage (NAc, H-3e, and H-3a signals at δ 2.033, 2.698, and 1.724/1.720, respectively) (Table IV) (21–23). The attachment of NeuAc in α2-6-linkage to Manβ1-4GlcNAc gave rise to significant chemical shift alterations for Man H-1 (Δδ −0.017) and Man H-5 (Δδ +0.071) and for the acetamido group of GlcNAc (Δδ +0.019) (see Table IV). In conclusion, the analytical data obtained for the substance in fraction C demonstrated the presence of the following compound: NeuAcα2-6Manβ1-4GlcNAc.

For comparison purposes, the ^1H NMR spectrum of fraction C-ol (fraction C treated with NaB^2H_4) was recorded, showing the chemical shift features of reduced NeuAcα2-6Manβ1-4GlcNAc (Fig. 5, *lower*). The signals of GlcNAc H-1α/β had disappeared, and a characteristic signal of GlcNAc-ol H-2 could be noticed at δ 4.162 (Table IV). As a consequence of reduction, the effect of α/β-anomerization on the signals of Man H-2 and NeuAc H-3a had disappeared. The NeuAc H-3e signal is influenced significantly by the reduction (Δδ +0.049).

TLC analysis in solvent system II of fraction B showed two sialic acid- and hexose-positive compounds, of which the major one co-migrated with the compound present in fraction C and the minor one with NeuAcα2-3Galβ1-4Glc. HPLC of fraction B-ol (fraction B treated with NaB^2H_4) on Partisil 10 SAX eluted with a mixture of acetonitrile and 30 mM KH_2PO_4, pH 4.9 (65:35, v/v) gave two peaks, denoted B-ol-1 and B-ol-2, in an intensity ratio of ~1:5. The major fraction, B-ol-2, had the same HPLC retention time as fraction C-ol, and both fractions co-migrated on TLC in solvent system II. The minor fraction, B-ol-1, showed a smaller retention time on HPLC and a larger mobility on TLC, in comparison with fraction B-ol-2. Fractions B, B-ol-1, and B-ol-2 were subjected to sugar analysis (Table III) and 500-MHz ^1H NMR spectroscopy. The ^1H NMR spectrum of fraction B-ol-2 was identical to that of fraction C-ol, proving the structure NeuAcα2-6Manβ1-4GlcNAc-ol; and the ^1H NMR spectrum of fraction B-ol-1 indicated the presence of NeuAcα2-3Galβ1-4glucitol. The ^1H NMR spectrum of fraction B disclosed the signals belonging to a mixture of NeuAcα2-6Manβ1-4GlcNAc and NeuAcα2-3Galβ1-4Glc in a molar ratio of ~4:1. The latter compound is a common constituent in human urine (23, 24). The sugar analysis data of fractions B, B-ol-1, and B-ol-2 (Table III) are fully in accordance with the NMR results. The presence of NeuAcα2-6Manβ1-4GlcNAc in both HPLC fractions B and C was caused by a separation of the two anomeric forms on Partisil 10 SAX, which was confirmed by reinjection of both fractions B and C, demonstrating the same chromatographic profile.

DISCUSSION

A novel compound, NeuAcα2-6Manβ1-GlcNAc, characterized by a hitherto unknown glycosidic linkage between NeuAc and Man, was isolated from human β-mannosidosis urine. This compound can be considered as the result of the sialylation in α2-6-linkage of Manβ1-4GlcNAc, the major storage compound in human β-mannosidosis. Recently, we have shown (25) that *in vitro*, Galβ1-4GlcNAc α2-6-sialyltransferase can transfer sialic acid in α2-6-linkage to mannose in Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc. Therefore, it is likely that also *in vivo*, a sialyltransferase can accept Manβ1-4GlcNAc as substrate. However, normally, Manβ1-4GlcNAc will be degraded in the lysosomes and will not reach the *trans*-Golgi region, where sialyltransferases are located (26). But, in human β-mannosidosis, Manβ1-4GlcNAc is an abundantly occurring compound which may pass the Golgi complex sometimes or otherwise encounters sialyltransferase.

A similar event can clarify the occurrence of Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAcβ1-4GlcNAc in caprine β-mannosidosis kidneys (8). Both compounds can be synthesized by sequential addition of GlcNAc and Man in β1-4-linkages to Manβ1-

4GlcNAc and Manβ1-4GlcNAcβ1-4GlcNAc, respectively, the major storage compounds in goats. However, it is remarkable that so far, Manβ1-4GlcNAcβ1-4Manβ1-4GlcNAc was not found in our study of the human counterpart of the disease and that NeuAcα2-6Manβ1-4GlcNAc(β1-4GlcNAc) had not been reported to occur in caprine β-mannosidosis. Therefore, irrespective of the origin of these particular compounds, a species-dependent pathway is to be expected.

Also in human aspartylglucosaminuria, several compounds have been found which do not fit in the frame of normal *N*-glycosidic chains, *e.g.* NeuAcα2-3/6(Galβ1-4GlcNAcβ1-3)$_{0-2}$Galβ1-4GlcNAc-Asn (27–30). In a similar way, these compounds can have been synthesized by sequential action of glycosyltransferases with the major storage compound GlcNAc-Asn as a substrate.

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