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Different oligosaccharides accumulate in the brain and urine of a cat with α-mannosidosis: structure determination of five brain-derived and seventeen urinary oligosaccharides

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Five brain-derived and 17 urinary oligomannose-type oligosaccharides were isolated by ion-exchange chromatography on Mono Q or Dowex, followed by HPLC on Lichrosorb-NH_2 from a Persian cat suffering from α-mannosidosis. The structures of the carbohydrate chains were determined by 500- or 600-MHz ^{1}H-NMR spectroscopy. Different oligosaccharide patterns were found in brain and urine. 99% of the urinary oligosaccharides possess an α(1-6)-linked mannose residue attached to β-mannose, whereas only 5% of the brain-derived oligosaccharides contain such a residue. Furthermore, of the urinary carbohydrate chains 71% end with Manβ1-4GlcNAcβ1-4GlcNAc and 29% end with Manβ1-4GlcNAc, whereas the corresponding amounts are 23% and 77%, respectively, for the brain-derived oligosaccharides.

Keywords: α-mannosidosis (feline), ^{1}H-NMR, oligomannose-type oligosaccharides

Abbreviations: MLEV-17, composite pulse devised by M. Levitt; HOHAHA, homonuclear Hartmann–Hahn spectroscopy; TPPI, time-proportional phase incrementation; 2D, two dimensional; GlcNAc, *N*-acetylglucosamine; Man, mannose; Fuc, fucose.

α-Mannosidosis is a lysosomal storage disease resulting from a deficiency in lysosomal acidic α-D-mannosidase(s) (EC 3.2.1.24) [1]. The disease is characterized by the massive accumulation in cells and excretion in urine of mannose-rich oligosaccharides. The first case of α-mannosidosis was described in man [2], and afterwards in cattle [3] and cats [4]. A similar condition can also be induced in animals by ingestion of plants containing the α-mannosidase inhibitor swainsonine [5, 6].

Biochemically, feline α-mannosidosis shows both similarities to and differences from bovine and human α-mannosidosis [7]. Clinically, the extent and the appearance of the cells resemble more closely those in the severe human variant than those in the bovine disease [7]. Therefore, feline α-mannosidosis is a useful animal model for studying the human disease [8]. The accumulated oligosaccharides in different tissues in feline α-mannosidosis have been the subject of several investigations [9–12]. Oligosaccharides from feline α-mannosidosis brain have been partially characterized by chromatographic profiling, but no detailed structural work has been carried out [10]. The present study was undertaken to characterize the brain-derived oligosaccharides from a recently identified case of feline α-mannosidosis [13, 14]. For comparison, also the urinary oligosaccharides were studied. The results reported here differ significantly from those obtained by chromatographic profiling [10].

Materials and methods

Isolation of oligosaccharides from urine and brain

Urine (4 ml) of the cat was passed through a column (5 × 2 cm) containing equal layers of AG 50W-X8 (H^+-form, Bio-Rad, Richmond, CA, USA) and AG 1-X8 (acetate-form) equilibrated in water. The column was eluted with

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water and 10 ml fractions were collected. Carbohydrate-containing fractions, as indicated by orcinol/H_2SO_4 staining, were combined and evaporated to dryness.

Brain tissue (7 g wet weight) of the cat was cut into small pieces and homogenised with two volumes of water for 5 min at 4°C with a Potter-Elvehjem blender. After three freeze–thaw cycles and sonication for two periods of 30 s (Soniprep 150 MSE) at 4°C, the homogenate was centrifuged at 5000 × **g** for 15 min at 25°C. The supernatant was concentrated to 4 ml by rotary evaporation and passed through a column (180 × 1 cm) of Bio-Gel P-10 (200–400 mesh, Bio-Rad). The column was eluted with water, containing 0.02% (by weight) sodium azide, and 2 ml fractions were collected. Carbohydrate-containing fractions were identified by anthrone/H_2SO_4 staining [15], pooled, concentrated, and applied to a column (180 × 1 cm) of Bio-Gel P-2 (200–400 mesh, Bio-Rad) eluted with water. Carbohydrate-positive fractions were combined and evaporated to dryness.

Anion-exchange chromatography of the mixture of brain-derived oligosaccharides was performed on a Mono Q HR 5/5 column (50 × 5 mm, Pharmacia, Sweden) connected to a Fast Protein Liquid Chromatography (FPLC) apparatus (Pharmacia) equipped with two P-500 pumps and a LCC-500 control unit. The sample was dissolved in 1.0 ml water and 200 µl aliquots were injected. Elution of the sample was performed with 2 ml water followed by a linear gradient of 0–50 mM NaCl in 8 ml water, and finally followed by a steeper gradient from 50–500 mM NaCl in 10 ml water at a flow rate of 1 ml min^{-1}. The eluate was monitored at 214 nm using a Pharmacia UV-1 detector. Carbohydrate-containing fractions were identified by orcinol/H_2SO_4 staining, combined and evaporated to dryness.

HPLC

HPLC was carried out with a Kratos SF 400 system (ABI Analytical, Kratos Division, USA) equipped with a Lichrosorb-NH_2 10 µm column (250 × 4.6 mm, Chrompack, The Netherlands). The pool of urinary oligosaccharides was separated isocratically with water/acetonitrile, 35/65 by vol, at a flow rate of 1.5 ml min^{-1}. The pool of brain-derived oligosaccharides was fractionated isocratically with water/acetonitrile, 25/75 by vol, at a flow rate of 1 ml min^{-1}. The eluate was monitored by a Spectroflow 757 variable-wavelength recorder (ABI Analytical, Kratos Division) at 195 nm (brain-derived oligosaccharides) or 205 nm (urinary oligosaccharides). The molar ratio of oligosaccharides is determined on the basis of HPLC peak areas (corrected for the number of *N*-acetyl groups) [16]. For HPLC peaks containing more than one oligosaccharide, the ^1H-NMR spectra have also been used for determination of relative amounts of oligosaccharides.

500 and 600 MHz ^1H-NMR spectroscopy

Oligosaccharides were repeatedly exchanged in ^2H_2O (99.96 atom-% ^2H, Aldrich, USA) with intermediate lyophilization. Resolution-enhanced ^1H-NMR spectra were recorded on a Bruker AM-500 spectrometer (Department of Chemistry, Utrecht University) or a Bruker AM-600 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, Nijmegen University, The Netherlands) operating at 500 and 600 MHz, respectively, at probe temperatures of 27°C. Chemical shifts (δ) are expressed in ppm relative to internal acetone in ^2H_2O ($\delta = 2.225$ ppm) [17]. The two-dimensional homonuclear Hartmann-Hahn (2D HOHAHA) experiments were performed with a MLEV-17 mixing sequence [18], with total mixing times of 20–100 ms. Usually about 400 free-induction decays of 2 k data points, 32–96 scans each (depending on the amount of the respective sample), were collected. The 90° ^1H-pulse width was adjusted to 25–30 µs and the residual HO^2H signal was suppressed by presaturation during 1 s. The time-proportional phase incrementation (TPPI) was used for the t_1-amplitude modulation [19].

Results

The oligomannose-type oligosaccharides isolated from the urine and the brain of the cat suffering from α-mannosidosis can be divided into two groups. Group A contains oligosaccharides ending with Manβ1-4GlcNAc, and group B comprises oligosaccharides ending with Manβ1-4GlcNAcβ1-4GlcNAc. The presence of GlcNAc or *N*,*N*′-diacetylchitobiose at the reducing end can readily be deduced from characteristic structural-reporter-group signals in the ^1H-NMR spectrum of the corresponding oligosaccharide. Relevant ^1H-NMR parameters for the manno-oligosaccharides isolated in the present study are compiled in Tables 1 (compounds of group A) and 2 (compounds of group B). For oligosaccharides of group A the H-1α signal of GlcNAc-**2** resonates at $\delta = 5.205$–5.210 ppm and the NAc protons at $\delta = 2.041$–2.044 ppm, provided that Man-**3** is unsubstituted at C-6 (for numbering of the monosaccharide residues, see Fig. 1) [17]. If Man-**3** is substituted at C-6 and Man-**4′** is unsubstituted at C-3, the H-1α doublet of GlcNAc-**2** is observed at $\delta = 5.212$–5.216 ppm and the NAc singlet at $\delta = 2.054$–2.061 ppm. The GlcNAc-**2** H-1α signal resonates at $\delta = 5.23$–5.25 ppm and the NAc-protons resonate at

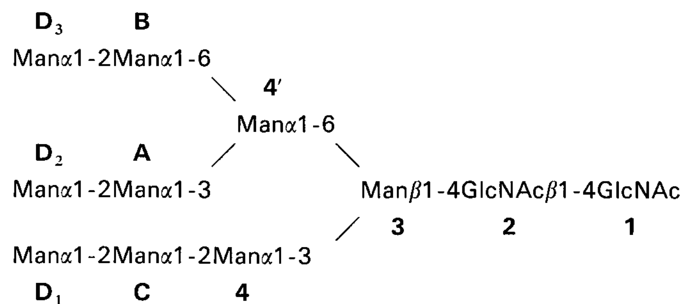

Figure 1. Numbering of the monosaccharide residues in an oligomannose-type oligosaccharide.

Table 1. ^{1}H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides with the Manβ1-4GlcNAc element in common, derived from urine and brain of the α-mannosidosis cat. Chemical shifts, measured at 27°C in ^2H_2O, are given in ppm relative to internal acetone (δ = 2.225 ppm). Compunds are represented by shorthand symbolic notation [17]: ● GlcNAc; ◆, Man. For numbering of the monosaccharide residues, see Fig. 1.

Reporter group	Residue		Chemical shift in									
			U1.1	*B7*	*U1.2*	*U3*	*B9*	*U5*	*B11.1*	*B11.2*	*U7.1*	*U7.2*
H-1	GlcNAc-**2**	α	5.208	5.207	5.215	5.213	5.207	5.212	5.213	5.206	5.212	5.248
		β	n.d.[a]	n.d.	4.72[b]	4.722	n.d.	4.721	n.d.	n.d.	4.72	4.72
	Man-**3**	α[c]	n.d.	n.d.	n.d.	4.791	n.d.	4.781	n.d.	n.d.	4.780	n.d.
		β	n.d.	n.d.	n.d.	4.783	n.d.	4.774	n.d.	n.d.	4.773	n.d.
	Man-**4**		5.111	5.109	–	5.104	5.358	5.352	5.351	5.35	5.345	5.101
	Man-**4′**		–	–	4.918	4.917	–	4.918	4.919	–	4.917	4.873
	Man-**A**	α[c]	–	–	–	–	–	–	–	–	–	5.080
		β	–	–	–	–	–	–	–	–	–	5.101
	Man-**B**		–	–	–	–	–	–	–	–	–	4.914
	Man-**C**		–	–	–	–	5.050	5.051	5.051	5.302	5.303	–
	Man-**D$_1$**		–	–	–	–	–	–	–	5.051	5.043	–
H-2	Man-**3**	α[c]	4.242	4.244	4.09	4.265	4.224	4.246	4.24	4.22	4.244	4.25
		β	4.232	4.233		4.254	4.216	4.238			4.235	
	Man-**4**		4.07	4.073	–	4.070	4.107	4.105	4.104	4.08	4.080	4.08
	Man-**4′**		–	–	3.97	3.973	–	3.977	3.98	–	3.98	4.15
	Man-**A**	α[c]	–	–	–	–	–	–	–	–	–	4.05
		β	–	–	–	–	–	–	–	–	–	4.07
	Man-**B**		–	–	–	–	–	–	–	–	–	3.98
	Man-**C**		–	–	–	–	4.067	4.069	4.07	4.104	4.105	–
	Man-**D$_1$**		–	–	–	–	–	–	–	4.07	4.064	–
NAc	GlcNAc-**2**	α	2.042	2.042	2.061	2.057	2.041	2.057	2.057	2.041	2.057	2.044
		β				2.054		2.054			2.054	

[a] n.d., Not determined.
[b] Some chemical shift values are given with only two decimals because of spectral overlap.
[c] α and β stand for the α- and β-anomers, respectively, of GlcNAc-**2**.

Table 2. ^{1}H-chemical shifts of structural-reporter-group portons of the constituent monosaccharides for the oligosaccharides with the Manβ1-4GlcNAcβ1-4GlcNAc element in common, derived from urine and brain of the α-mannosidosis cat. Chemical shifts, measured at 27°C in $^{2}H_2O$, are given in ppm relative to internal acetone (δ = 2.225 ppm). Compounds are represented by shorthand symbolic notation [17]: ●, GlcNAc; ◆, Man. For numbering of the monosaccharide residues see Fig. 1.

Reporter group	*Residue*		*Chemical shift in*											
			U2.1	*U2.2*	*B8*	*U4*	*U6.1*	*U6.2*	*U6.3*	*U6.4*	*U8*[a]	*U9.1*	*U9.2*	*U9.3*
H-1	**GlcNAc-1**	α	5.190	5.190	5.189	5.188	5.189	5.189	5.189	5.189	5.188	5.188	5.188	5.188
		β	4.696	4.696	4.695	4.696	4.695	4.695	4.695	4.695	4.695	4.695	4.695	4.695
	GlcNAc-2	α[b]	4.614	4.61	4.607	4.612	4.60	4.60	4.60	4.60	4.601	4.60	4.60	4.60
		β	4.606		4.599	4.603					4.592			
	Man-3		4.768	n.d.[c]	n.d.	4.780	n.d.	n.d.	n.d.	n.d.	4.780	4.780	4.780	4.780
	Man-4		–	5.105	5.107	5.101	5.10[d]	5.349	–	–	5.097	5.333	5.345	5.345
	Man-4′		4.916	–	–	4.915	4.894	4.916	4.91	4.870	4.870	4.870	4.870	4.870
	Man-A		–	–	–	–	5.10	–	–	5.073	5.092	5.092	5.403	5.092
	Man-B		–	–	–	–	–	–	5.144	4.910	4.907	4.908	4.908	5.143
	Man-C		–	–	–	–	–	5.051	–	–	–	5.305	5.055	5.055
	Man-D$_1$		–	–	–	–	–	–	–	–	–	5.041	–	–
	Man-D$_2$		–	–	–	–	–	–	–	–	–	–	5.055	–
	Man-D$_3$		–	–	–	–	–	–	5.042	–	–	–	–	5.041
H-2	**Man-3**		4.081	4.231	4.231	4.254	4.24	4.24	4.07	4.07	4.250	4.24	4.24	4.24
	Man-4		–	4.069	4.069	4.067	4.07	4.11	–	–	4.076	4.11	4.13	4.12
	Man-4′		3.969	–	–	3.974	4.12	3.97	4.12	4.13	4.145	4.15	4.15	4.15
	Man-A		–	–	–	–	4.07	–	–	4.07	4.065	4.07	4.10	4.07
	Man-B		–	–	–	–	–	–	4.02	3.98	3.984	n.d.	n.d.	4.02
	Man-C		–	–	–	–	–	4.07	–	–	–	4.11	4.07	4.07
	Man-D$_1$		–	–	–	–	–	–	–	–	–	4.07	–	–
	Man-D$_2$		–	–	–	–	–	–	–	–	–	–	4.07	–
	Man-D$_3$		–	–	–	–	–	–	4.07	–	–	–	–	4.07
NAc	**GlcNAc-1**	α	2.038	2.038	2.038	2.038	2.038	2.038	2.038	2.038	2.038	2.038	2.038	2.038
		β									2.036			
	GlcNAc-2	α[b]	2.080	2.063	2.063	2.078	2.064	2:078	2.064	2.064	2.064	2.064	2.069	2.064
		β									2.063			

[a] Measured at 600 MHz.
[b] α and β stand for the α- and β-anomers, respectively, of GlcNAc-**1**.
[c] n.d., not determined.
[d] Some chemical shift values are given only with two decimals because of spectral overlap.

δ = 2.044–2.050 ppm when Man-**3** is substituted at C-6 and Man-**4′** is substituted at C-3 [17]. A few general comments can also be made regarding the number and attachment positions of the mannose residues at the nonreducing end of the oligosaccharides of both group A and B. By comparing the sets of chemical shift values of the Man H-1 and H-2 atoms, the structure at the nonreducing end of an oligomannose-type oligosaccharide can easily be deduced [17]. For αMan residues the H-1 signals occur in the region δ = 4.86–5.40 ppm, whereas for βMan-**3** the H-1 resonates between δ = 4.76–4.79 ppm. The Man H-2 signals resonate in the region δ = 3.96–4.27 ppm. For a terminal α(1-3)-linked Man-**4** residue the H-1 signal typically is found at δ = 5.10–5.11 ppm, with the H-2 signal at δ = 4.06–4.08 ppm. Similarly, the presence of a terminal Man-**4′** residue can be deduced from the typical combination of Man H-1 and H-2 signals at δ = 4.92 ppm and δ = 3.96–3.98 ppm, respectively. The further presence of Man-**A**, -**B**, -**C**, -$\mathbf{D_1}$, -$\mathbf{D_2}$, and -$\mathbf{D_3}$ can be recognized by characteristic combinations of Man H-1 and H-2 signals [17].

For oligomannose-type carbohydrate chains it has been shown [20] that a linear relationship is obtained when plotting the logarithm of the retention time on a HPLC NH_2-column against the number of mannose residues. In this way the number of mannose residues of a compound having the general formula $Man_iGlcNAc_j$ ($i = 1$–$9, j = 1$–2) can be predicted. Compounds having one or two *N*-acetylglucosamine residues fall on two different lines. This information was used in the present study for those HPLC fractions which contain more than one component.

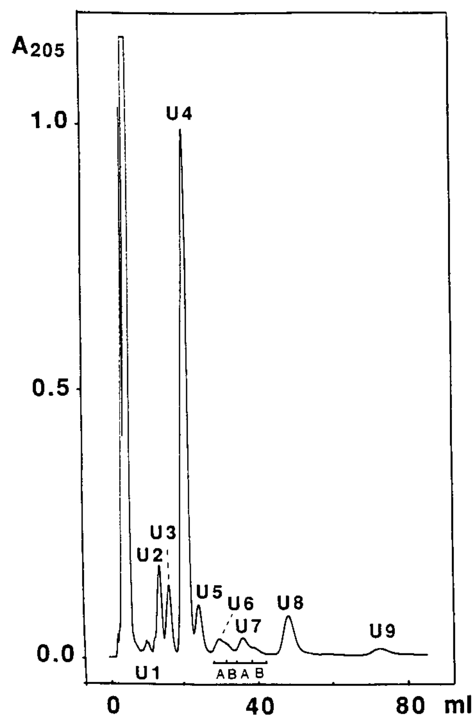

Figure 2. Fractionation pattern at 205 nm of the urinary oligosaccharides on a HPLC Lichrosorb-NH_2 10 µm column (250 × 4.6 mm). The mixture of oligosaccharides was dissolved in 200 µl water. The column was eluted isocratically with water/acetonitrile, 35/65 by vol, at a flow rate of 1.5 ml min^{-1} at room temperature. The injection volume was 5 µl.

Oligosaccharides from urine

Neutral oligosaccharides were isolated from the urine of the affected cat by ion-exchange chromatography on Dowex-type cation- and anion-exchange resins. The carbohydrate-containing fractions were combined and separated by HPLC on Lichrosorb-NH_2. The HPLC analysis revealed the presence of nine carbohydrate positive fractions, denoted U1–U9 (Fig. 2). These fractions were analysed by ^{1}H-NMR spectroscopy.

Fraction U1. The ^{1}H-NMR spectrum of fraction U1 shows the presence of two oligosaccharides in a molar ratio of approximately 2:1 (spectrum not shown). The minor component (U1.1) is identified as the trisaccharide Man α1-3Manβ1-4GlcNAc. The chemical shift values for U1.1 match those obtained previously for the same oligosaccharide structure (cf. compound 5 in [17]) isolated from human α-mannosidosis urine.

The major component (U1.2) in fraction U1 is a positional isomer of oligosaccharide U1.1, namely Manα1-6Manβ1-4GlcNAc. The terminal Man-**4′** is recognized by its H-1 and H-2 signals, and the presence of a terminal GlcNAc-2 residue is inferred from the characteristic H-1α and NAc signals. To support the assignments for U1.1 and U1.2, a 2D-HOHAHA spectrum of U1 was recorded with a 40 ms mixing time (spectrum not shown). This experiment proved the H-1/H-2 connectivities for the terminal mannose residues. The structural-reporter groups of U1.2 fit the ^{1}H-NMR data of the terminal structural element Manα1-6Manβ1-4GlcNAc, present in glycoasparagines isolated from the urine of patients with aspartylglucosaminuria and Gaucher's disease (cf. compound 3 in [17]). Note that the chemical-shift value of GlcNAc-**2** H-1α of compound U1.2 differs from that published by Warren *et al.* for an identical oligosaccharide structure isolated from swainsonine-intoxicated sheep (cf. compound $Man_2GlcNAc$ in [20]).

Fraction U2. The ^{1}H-NMR spectrum of fraction U2 shows one major component (U2.1) constituting about 90% of this fraction (spectrum not shown). The structure of this compound is Manα1-6Manβ1-4GlcNAcβ1-4GlcNAc. The presence of a *N,N′*-diacetylchitobiose unit at the reducing end is deduced from the typical GlcNAc H-1 and NAc signals (see Table 2). The terminal α(1-6)-linked mannose residue is recognized from the typical combination of H-1 and H-2 signals of Man-**4′**, and the NAc signal of GlcNAc-**2** (cf. U2.2 and U4). The structural-reporter groups of U2.1 match those of the terminal structural element Manα1-6Manβ1-4GlcNAc of a closely related glycoasparagine (cf. compound

3 in [17]) and those of the corresponding alditol (cf. compound $Man_2GlcNAc$ in [20]).

The remaining minor (10%) signals in the ^{1}H-NMR spectrum of fraction U2 stem from a positional isomer (U2.2) having a terminal α(1-3)-linked mannose residue. The structure of U2.2 is Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc. This is inferred from the signals at $\delta = 5.105$ ppm (Man-**4** H-1), $\delta = 4.231$ ppm (Man-**3** H-2), $\delta = 4.069$ ppm (Man-**4** H-2), and $\delta = 2.063$ ppm (NAc of GlcNAc-**2**). The relevant chemical-shift values of U2.2 match those published for an α(1-6)-fucosylated analogue isolated from bovine fibrin (cf. Manα1-3Manβ1-4GlcNAcβ1-4(Fucα1-6)GlcNAc, denoted {M2 + F} in [21]).

Fraction U3. The ^{1}H-NMR spectrum of fraction U3 shows one component (U3) having the structure Manα1-3(Manα1-6)Manβ1-4GlcNAc (spectrum not shown). The chemical shift values for the structural-reporter group signals of U3 match those obtained for the same carbohydrate structure prepared by exo- and endo-glycosidase digestions of a Pronase digest of Cohn's fraction IV (cf. compound C in [22]).

Fraction U4. The ^{1}H-NMR spectrum of fraction U4 (Fig. 3) shows one component (U4), being the most abundant (52%) oligosaccharide in the urine. The chemical shift values of the H-1 and H-2 signals of Man-**3**, -**4**, and -**4′** establish the branching pattern and the structure at the non-reducing end of this oligosaccharide to be identical to that in compound U3. However, in contrast to U3 oligosaccharide U4 contains an intact *N*,*N′*-diacetylchitobiose unit at the reducing end, evidenced by the typical H-1 and NAc signals of GlcNAc-**1** and -**2** (see Table 2). Thus the structure of U4 is Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc. The chemical shift values of the structural-reporter-group protons for U4 match completely those previously reported for a similar oligosaccharide prepared by exo- and endo-glycosidase digestions of a Pronase digest of Cohn's fraction IV (cf. compound B in [22]).

Fraction U5. The ^{1}H-NMR spectrum of fraction U5 (spectrum not shown) shows one major (85%) component (U5). The minor (15%) component stems from the closely eluting preceding larger fraction U4. The GlcNAc H-1 and NAc values indicate that compound U5 contains GlcNAc-**2** at the reducing end (see Table 1). The H-1 and H-2 signals of Man-**4′** are characteristic for this residue in terminal position. As compared to U3, the additional α-anomeric signal at $\delta = 5.051$ ppm in U5 is assigned to a terminal α(1-2)-linked Man-**C** residue. This is supported by the lowfield position of Man-**4** H-1 at $\delta = 5.352$ ppm [17], affording the structure Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc for U5. An identical oligosaccharide isolated from swainsonine-intoxicated sheep has previously been analysed as alditol by ^{1}H-NMR spectroscopy (cf. compound $Man_4GlcNAc$ in [20]). Since some of the chemical shift values for the structural-reporter groups of the non-reducing oligomannose element differ in that report compared to those given here, the Man H-1/H-2 resonances were unambiguously assigned for U5 on the basis of a 2D-HOHAHA spectrum recorded with a mixing time of 43 ms.

Fraction U6. Fraction U6 was collected in two parts, denoted U6A and U6B, respectively. The relative elution times on HPLC indicate that these fractions contain compounds having the general formula $Man_4GlcNAc_2$. The ^{1}H-NMR spectra of these fractions indicate a large heterogeneity (spectra not shown). However, in both fractions the same signals are present, the only difference being their relative intensities. For this reason the ^{1}H-NMR spectrum of the fraction containing larger amounts of material (U6A) was used for the structure determination. Because of the heterogeneity of this fraction a 2D HOHAHA spectrum with a 76 ms mixing time was recorded, and all Man H-1/H-2 connectivities were deduced from this spectrum. From the ^{1}H-NMR data of fraction U6 the structure of four isomeric $Man_4GlcNAc_2$ oligosaccharides could be established. The *N*,*N′*-diacetylchitobiose unit at the reducing end is confirmed by the H-1 and NAc signals of GlcNAc-**1** and -**2** (see Table 2). An α-anomeric mannose signal at $\delta = 5.10$ ppm together with the H-2 resonance at $\delta = 4.07$ ppm is typical for terminal α(1-3)-linked mannoses (Man-**4** and Man-**A**). In combination with the characteristic set of signals for the terminal element Manα1-3Manα1-6Manβ (see the H-1 and H-2 signals of Man-**A** and -**4′**, cf. compound 66 in [17], and the NAc signal of GlcNAc-**2** for compound U6.1 in Table 2), and the fact that the Man-**4′** H-1 signal of highest intensity is the one belonging to the mono-3-substituted Man-**4′** residue, the most abundant oligosaccharide (U6.1) in fraction U6 is deduced to be Manα1-3(Manα1-3Man α1-6(Manβ1-4GlcNAcβ1-4GlcNAc.

A signal at $\delta = 5.349$ ppm (Man-**4** H-1) together with a signal at $\delta = 5.051$ ppm (Man-**C** H-1) indicate a compound having the terminal sequence Manα1-2Manα1-3Manβ (cf. compound 66 in [17]). In combination with a signal of comparable intensity at $\delta = 4.916$ ppm (coupled to a H-2 signal at $\delta = 3.97$ ppm), being typical for a terminal α(1-6)-linked Man-**4′**, and a NAc signal at $\delta = 2.078$ ppm, characteristic for GlcNAc-**2** when Man-**4′** is unsubstituted, compound U6.2 in fraction U6 is deduced to be Man α1-2Manα1-3(Manα1-6)Manβ1-4GlcNAcβ1-4GlcNAc.

In the ^{1}H-NMR spectrum of fraction U6 there are also signals of comparable intensity at $\delta = 5.144$ ppm and $\delta = 5.042$ ppm. These signals are assigned to Man-**B** and Man-$\mathbf{D_3}$ in the terminal sequence Manα1-2Manα1-6Manα ($\mathbf{D_3}$-**B**-**4′**-, cf. compound 67 in [17]), affording the following structure: Manα1 - 2Manα1 - 6Manα1 - 6Manβ1 - 4GlcNAc β1-4GlcNAc (U6.3).

Finally, the α-anomeric Man H-1 signal at $\delta = 4.870$ ppm, coupled to a H-2 signal at $\delta = 4.13$ ppm, is typical for a 3,6-disubstituted Man-**4′** residue, yielding the following

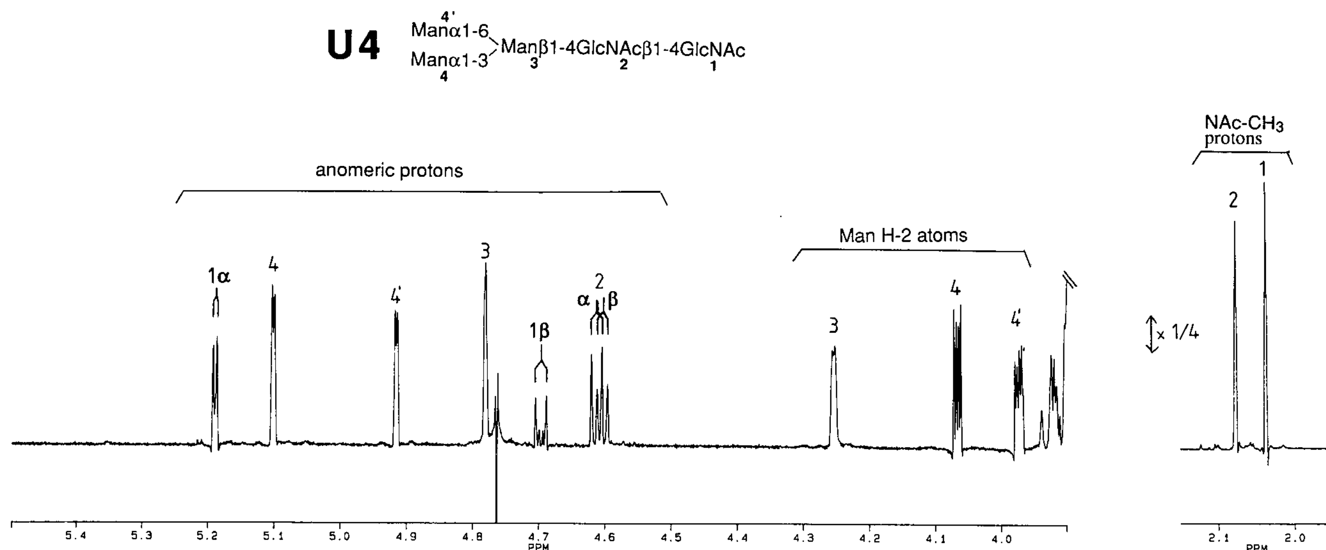

Figure 3. Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of oligosaccharide U4 obtained from cat α-mannosidosis urine.

structure: Manα1 - 3(Manα1 - 6)Manα1 - 6Manβ1 - 4GlcNAc β1-4GlcNAc (U6.4) (cf. the alditol $Man_4GlcNAc_2$-II in [20]).

Fraction U7. Fraction U7 was collected in two parts, denoted U7A and U7B. The relative elution times on HPLC indicate that these fractions contain compounds having the general formula $Man_5GlcNAc$. From the GlcNAc H-1 and NAc signals it is obvious that all carbohydrate chains in fractions U7A and U7B have GlcNAc-**2** at the reducing end (see Table 1). The ^{1}H-NMR spectrum of fraction U7A shows five α-anomeric signals of equal intensity at $\delta =$ 5.212 ppm (αGlcNAc-**2**), $\delta = 5.345$ ppm (Man-**4**, 2-substituted), $\delta = 5.303$ ppm (Man-**C**, 2-substituted), $\delta = 5.043$ ppm (Man-$\mathbf{D_1}$), and $\delta = 4.917$ ppm (Man-**4'**, unsubstituted) (spectrum not shown). Thus the structure of this major compound (U7.1) of fraction U7 is Manα1-2Manα1-2Manα1-3(Man α1-6)Manβ1-4GlcNAc. Oligosaccharide U7.1 accounts for about 90% of the carbohydrate chains in fraction U7A; the remaining 10% is due to overlap with U7B.

The anomeric region of the 500 MHz ^{1}H-NMR spectrum of fraction U7B is shown in Fig. 4. In addition to signals belonging to compound U7.1, an αGlcNAc-**2** H-1 signal is present at $\delta = 5.248$ ppm, indicative of Man-**A** α(1-3)-linked to Man-**4'** [17]. This is supported by the H-1 signals at $\delta = 5.080$ ppm (Man-**A**, when GlcNAc-**2** is α) and $\delta = 5.101$ ppm (Man-**A**, when GlcNAc-**2** is β). The chemical shift value of Man-**4'** ($\delta = 4.873$ ppm) points to a disubstituted Man-**4'** residue, confirmed by the H-1 signal of Man-**B** at $\delta = 4.914$ ppm. A terminal Man-**4** is recognized by the combination of a H-1 signal at $\delta = 5.101$ ppm and a H-2 signal at $\delta = 4.08$ ppm. In conclusion, the proposed structure for the major component in fraction U7B is Man α1-3(Manα1-6)Manα1-6(Manα1-3)Manβ1-4GlcNAc (U7.2).

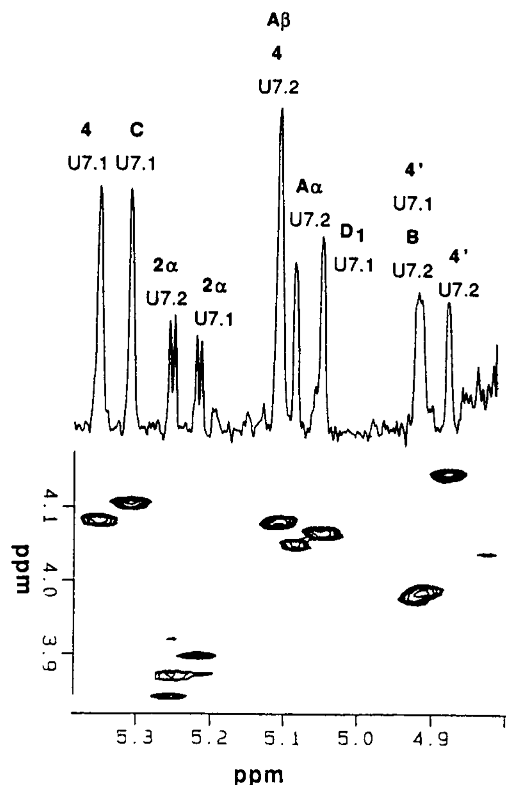

Figure 4. The α-anomeric region of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of the mixture of oligosaccharides U7.1 and U7.2 (fraction U7B), together with the H-1/H-2 cross-peaks from the 2D HOHAHA spectrum, which was recorded with a 40 ms MLEV-17 mixing period.

For fraction U7B a 2D HOHAHA spectrum (see Fig. 4) with a 40 ms mixing time was recorded in order to unambiguously establish the mannose H-1/H-2 connectivities. The pertinent ^{1}H-NMR data of compound U7.2 match those obtained for an identical oligosaccharide structure released by endo-H from recombinant tissue plasminogen activator (cf. compound B1 in [23]).

Fraction U8. The 600-MHz ^{1}H-NMR spectrum of fraction U8 indicates one compound, denoted U8 (spectrum not shown) The ^{1}H-chemical-shift values of this compound match completely those obtained for an identical oligosaccharidestructure isolated from hen ovalbumin (cf. compound {M5} in [21]). The structure of oligosaccharide U8 is Manα1-3(Man α1-6)Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc.

Fraction U9. The elution position on HPLC suggests that fraction U9 contains oligosaccharides with the general formula $Man_7GlcNAc_2$. From the 500 MHz ^{1}H-NMR spectrum of fraction U9 three oligosaccharides can be identified, denoted U9.1, U9.2, and U9.3, respectively, having the following structures: Manα1-3(Manα1-6)Manα1-6(Manα1-2Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (U9.1), Manα1-2Manα1-3(Manα1-6)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (U9.2), and Manα1-2Manα1-6(Manα1-3)Manα1-6(Manα1-2Manα1-3)Manβ1-4GlcNAcβ1-4GlcNAc (U9.3). The presence of the *N,N'*-diacetylchitobiose element at the reducing end is deduced from the characteristic set of GlcNAc-**1** and -**2** H-1 and NAc signals (see Table 2). The mannose structural-reporter-group signals for the mixture of oligosaccharides present in fraction U9 match those previously obtained for a mixture of three $Man_7GlcNAc_2$-glycopeptides isolated from kidney bean glycoprotein II[24].

Brain-derived oligosaccharides

The mixture of oligosaccharides isolated from the brain was separated by FPLC anion-exchange chromatography on Mono Q (not shown). Only the neutral peak turned out to contain carbohydrate material, and was subfractionated by HPLC into thirteen fractions on a Lichrosorb-NH_2 column (Fig. 5). The carbohydrate positive fractions B7–B13 were further characterized by ^{1}H-NMR spectroscopy. However, the amounts of material in B10, B12, and B13 were too low for structure determination by ^{1}H-NMR spectroscopy, but the HPLC retention times suggest the following types of compounds: $Man_3GlcNAc_2$ in B10, $Man_4GlcNAc_2$ in B12, and $Man_5GlcNAc$ in B13.

Fraction B7. Fraction B7 represents the major accumulated oligosaccharide in brain, accounting for 37% of the total amount of oligosaccharides in the brain tissue. The structural-reporter-group regions of the 500-MHz ^{1}H-NMR spectrum of B7 are shown in Fig. 6. Because the ^{1}H-NMR structural reporters of fraction B7 match those of the urinary compound U1.1 (see Table 1), B7 is identified as Manα1-3Manβ1-4GlcNAc.

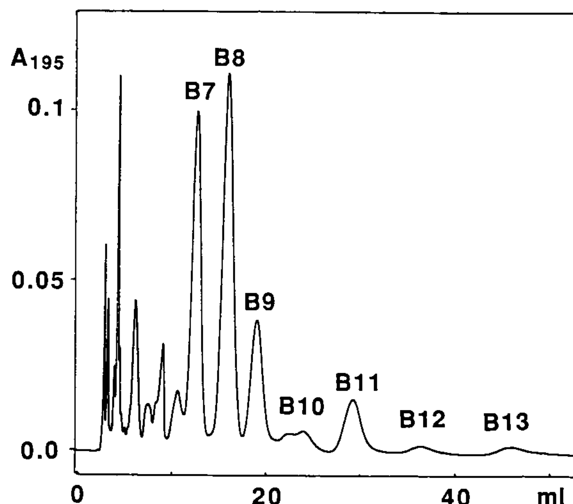

Figure 5. Fractionation pattern at 195 nm of the brain-derived oligosaccharides on a HPLC Lichrosorb-NH_2 10 μm column (250 × 4.6 mm). The mixture of oligosaccharides was dissolved in 200 μl water. The column was eluted isocratically with water/acetonitrile, 25/75 by vol, at a flow rate of 1.0 ml min^{-1} at room temperature. The injection volume was 10 μl.

Fraction B8. The ^{1}H-NMR spectrum (not shown) of fraction B8 shows that this fraction contains a single compound, whose structural-reporter groups match those of the urinary compound U2.2 (see Table 2). Thus, the structure of compound B8 is Manα1-3Manβ1-4GlcNAcβ1-4GlcNAc.

Fraction B9. The 500-MHz ^{1}H-NMR spectrum (not shown) of fraction B9 indicates that it contains one compound, denoted B9. From the GlcNAc-**2** H-1α and NAc signals it can be deduced that B9 contains a reducing GlcNAc-**2**. Two αMan H-1 signals are present at $\delta = 5.358$ ppm (Man-**4**, 2-substituted) and at $\delta = 5.050$ ppm (Man-**C**, terminal), respectively, and therefore the structure of compound B9 is Manα1-2Manα1-3Manβ1-4GlcNAc. The ^{1}H-NMR structural-reporter groups of B9 match those obtained for an identical oligosaccharide structure isolated from human α-mannosidosis urine (cf. compound 64 in [17]).

Fraction B11. From the elution position on HPLC, the components of fraction B11 are predicted to have the general formula $Man_4GlcNAc$. The 500-MHz ^{1}H-NMR spectrum (not shown) indicates the presence in equimolar amounts of two compounds, namely: Manα1-2Manα1-3(Manα1-6)Manβ1-4GlcNAc (B11.1) and Manα1-2Manα1-2Manα1-3Manβ1-4GlcNAc (B11.2). The presence of GlcNAc-**2** at the reducing end is inferred from the GlcNAc-**2** H-1α and NAc signals (Table 1). The structural-reporter groups of compound B11.1 match those of the identical urinary oligosaccharide structure U5. Because many of the signals from compounds B11.1 and B11.2 overlap, a 2D HOHAHA experiment with a 40-ms mixing time was carried out. Compound B11.2 is recognized from the structural

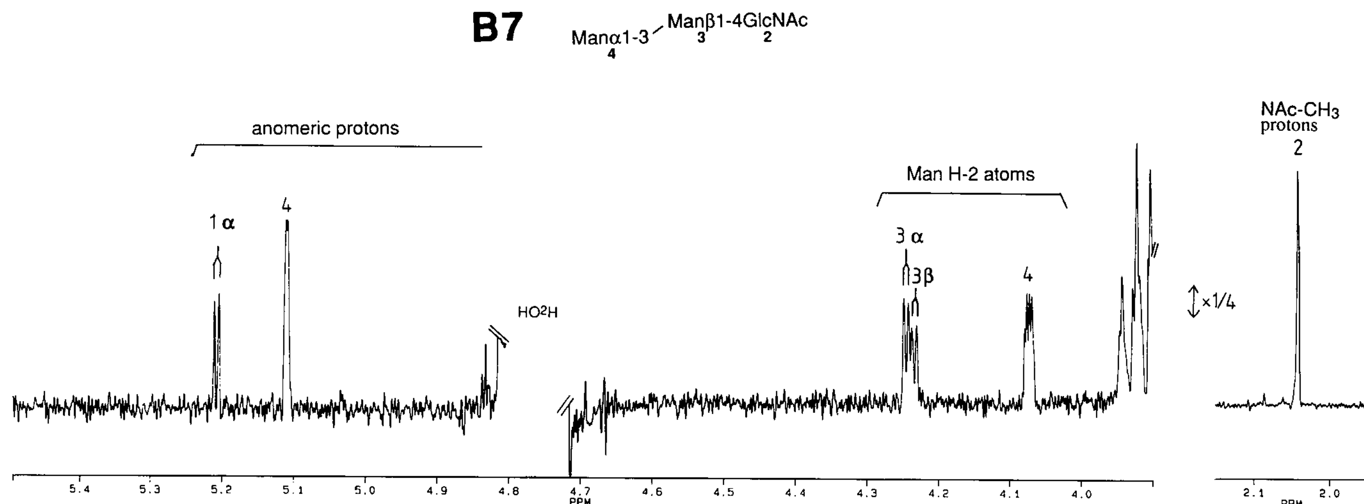

Figure 6. Structural-reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of oligosaccharide B7 obtained from the brain of the α-mannosidosis cat.

reporters of Man-$\mathbf{D}_1$ (H-1, $\delta = 5.051$ ppm; H-2, $\delta = 4.07$ ppm), Man-**C** (H-1, $\delta = 5.302$ ppm; H-2, $\delta = 4.104$ ppm), and Man-**4** (H-1, $\delta = 5.35$ ppm; H-2, $\delta = 4.08$ ppm). The H-1 and H-2 signals from Man-$\mathbf{D}_1$ in B11.2 and Man-**C** in B11.1 coincide, both being terminal α1-2Man-linked residues. However, from the intensity of the anomeric signal at $\delta = 5.05$ ppm it is evident that two protons contribute. Furthermore, the GlcNAc-**2** H-1α signal is sensitive to the presence of Man-**4**′. This is clearly seen in the spectrum of fraction B11, where the signal at $\delta = 5.206$ ppm is attributed to compound B11.2, lacking Man-**4**′, and the signal at $\delta = 5.213$ ppm to B11.1, containing Man-**4**′.

Discussion

This study describes the isolation and structure determination of 17 urinary and five brain-derived oligosaccharides from a Persian cat suffering from α-mannosidosis. In Table 3 the structures of both the urinary and the brain-derived oligosaccharides are compiled, together with their relative amounts. For comparison, the oligosaccharides previously identified in human α-mannosidosis urine [25, 25], urine of swainsonine-intoxicated sheep [20], and feline α-mannosidosis urine and brain [10] are also included. The results obtained in this study for the urinary oligosaccharides are in close agreement with those reported by Abraham *et al.* [12] and by Warren *et al.* [10]. In addition to previously reported structures we have identified an additional structural isomer of $Man_5GlcNAc$ (compound U7.2) which also occurs in low amounts in human α-mannosidosis urine [25, 26], two additional isomers of $Man_4GlcNAc_2$ (compounds U6.2 and U6.3), and a new isomer of $Man_2GlcNAc$, namely Manα1-3Manβ1-4GlcNAc (compound U1.1). The trisaccharide U1.1 has the same structure as the major urinary oligosaccharide in human α-mannosidosis [25–27]. However, in contrast to the chromatographic profiling studies by Warren *et al.* [10], the accumulated oligosaccharides found in brain differ from those found in urine. In general, two main classes of oligosaccharides can be distinguished: one in which the oligosaccharides have a single *N*-acetylglucosamine residue at the reducing end ($Man_nGlcNAc$-series), and another class to which oligosaccharides having an intact *N,N*′-diacetylchitobiose unit at the reducing end belong ($Man_nGlcNAc_2$-series). In urine about 29% of the oligosaccharides belong to the $Man_nGlcNAc$ series and 71% to the Man_nGlcAc_2 series, whereas the corresponding values for the brain-derived oligosaccharides are 77% and 23%, respectively. Another significant difference between the brain-derived and the urinary oligosaccharides is the low amount of α(1-6)-linked mannose residues in brain (5%) as compared to urine (99%). However, the exact cause of the different oligosaccharide patterns in urine and brain is still not clear. The patterns might not only reflect tissue differences in activity and specificity of residual α-mannosidase(s) but also in *N*-glycoprotein glycan structures.

Up to now the major accumulated oligosaccharides in dogs [28], goats [29], and cats [10] suffering from lysosomal diseases have been found to end in Manβ1-4GlcNAcβ1-4GlcNAc, whereas the major accumulated oligosaccharides in man end in Manβ1-4GlcNAc [30]. Therefore, different catabolic routes have been proposed for the breakdown of *N*-glycoprotein glycans in man as compared to dogs, goats, and cats [11, 31, 32]. However, our recent finding of an intact *N,N*′-diacetylchitobiose unit in a sialyloligosaccharide from human galactosialidosis urine [33] and our findings in the present report, suggest the sequential action of an aspartylglucosaminidase (cleaving the GlcNAc-Asn linkage) and an endo-β-*N*-acetylhexosaminidase (hydrolysing the GlcNAcβ1-4GlcNAc linkage) in both man and cat. Li *et al.* have isolated two kinds of endo-β-*N*-acetylhexosaminidases from human kidney [34]. One of the enzymes acts directly on oligomannose-type glycopeptides, while the other enzyme,

Table 3. Comparison of the structures of oligosaccharides isolated from feline α-mannosidosis (α-man) urine and brain, human α-mannosidosis urine, and ovine swainsonine toxicosis (OST) urine. For the shorthand symbolic notation, see Table 1.

Abbreviated formula	*Structure*	*Amount (%)*						
		Feline α-man				*Human α-man*		*OST*
		Urine		*Brain*		*Urine*		*Urine*
		This study	[9][a]	*This study*	[9]	[24]	[25]	[19]
$Man_2GlcNAc$		0.2 (U1.1)	–	37 (B7)	–	67	74	–
		0.3 (U1.2)	–	–	–	–	–	3
$Man_3GlcNAc$		12 (U3)	7	–	9	–	9	3
		–	–	17 (B9)	–	16		–
$Man_4GlcNAc$		12 (U5)	4[b]	5 (B11.1)	8[c]	–	–	4[d]
		–	–	5 (B11.2)	–	3	7	–
		–	–	–	–	2		–
$Man_5GlcNAc$		5 (U7.1)	5[e]	–	4[f]	–	–	5
		(U7.2)	–	–	–	5	3	–
		–	–	–	–			–
$Man_6GlcNAc$		–	–	–	–	4	3	–
		–	–	–	–			–
		–	–	–	–			–
$Man_7GlcNAc$		–	–	–	–	2	2	–
		–	–	–	–			–
		–	–	–	–			–
$Man_8GlcNAc$		–	–	–	–	0.7	1	–
		–	–	–	–			–
		–	–	–	–			–

Continued on page 27

Table 3—*continued.*

Abbreviated formula	*Structure*	*Amount (%)*						
		Feline α-man				*Human α-man*		*OST*
		Urine		*Brain*		*Urine*		*Urine*
		This study	[9][a]	*This study*	[9]	[24]	[25]	[19]
$Man_9GlcNAc$		–	–	–	–	0.4	1	–
$Man_2GlcNAc_2$		6 (U2.1)	5	–	6	–	–	21
		1 (U2.2)	–	23 (B8)	–	–	–	–
$Man_3GlcNAc_2$		52 (U4)	67	–	49	–	–	1
		–		–		–	–	17
		–	–	–	–	–	–	8
$Man_4GlcNAc_2$		2 (U6.1)	4[b]	–	8[c]	–	–	4[d]
		(U6.2)	–	–	–	–	–	–
		(U6.3)	–	–	–	–	–	–
		(U6.4)	–	–	–	–	–	20
$Man_5GlcNAc_2$		8 (U8)	5[e]	–	4[f]	–	–	11
$Man_6GlcNAc_2$	n.d.[g]	–	+	–	+	–	–	–
$Man_7GlcNAc_2$		2 (U9.1)	+	–	–	–	–	–
		(U9.2)		–	+	–	–	–
		(U9.3)		–	–	–	–	–

[a] Values are given for "cat 58".
[b] [f] The values represent half the total amount of the respective fraction, since the actual amount of the individual oligosaccharides has not been determined.
[g] n.d., not determined.

which can hydrolyse both oligomannose- and complex-type oligosaccharides, requires the presence of an aspartylglucosaminidase to remove the Asn moieties of the glycopeptides.

At least two explanations can be given for the difference between the brain-derived and the urinary oligosaccharides in cat α-mannosidosis. Either it may reflect a difference in the activity of an endo-*N*-acetylhexosaminidase in different tissues or it shows that the oligosaccharides of each glycoprotein entering the lysosomes experiences a catabolic pathway which is dependent on the aglycone (polypeptide). The latter possibility resembles the "site-directed processing" proposed for the anabolism of glycoprotein glycans [35], but further work is necessary to see if it also holds for the catabolism.

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