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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 $^1$H-NMR spectra have also been used for determination of relative amounts of oligosaccharides.

*500 and 600 MHz $^1$H-NMR spectroscopy*

Oligosaccharides were repeatedly exchanged in $^2H_2O$ (99.96 atom-% $^2$H, Aldrich, USA) with intermediate lyophilization. Resolution-enhanced $^1$H-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 ($\delta$) 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° $^1$H-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 $^1$H-NMR spectrum of the corresponding oligosaccharide. Relevant $^1$H-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

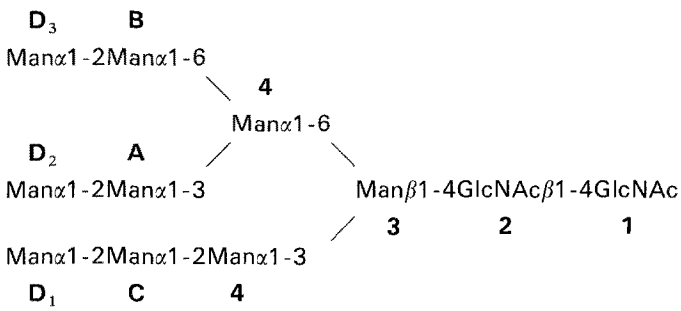


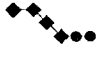

**Figure 1.** Numbering of the monosaccharide residues in an oligomannose-type oligosaccharide.

**Table 1.** $^1$H-chemical shifts of structural-reporter-group protons of the constituent monosaccharides for the oligosaccharides with the Man$\beta$1-4GlcNAc element in common, derived from urine and brain of the $\alpha$-mannosidosis cat. Chemical shifts, measured at 27°C in $^2H_2O$, are given in ppm relative to internal acetone ($\delta$ = 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** | $\alpha$ | 5.208 | 5.207 | 5.215 | 5.213 | 5.207 | 5.212 | 5.213 | 5.206 | 5.212 | 5.248 |
| | | $\beta$ | n.d.[a] | n.d. | 4.72[b] | 4.722 | n.d. | 4.721 | n.d. | n.d. | 4.72 | 4.72 |
| | Man-**3** | $\alpha$[c] | n.d. | n.d. | n.d. | 4.791 | n.d. | 4.781 | n.d. | n.d. | 4.780 | n.d. |
| | | $\beta$ | 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** | $\alpha$[c] | – | – | – | – | – | – | – | – | – | 5.080 |
| | | $\beta$ | – | – | | – | – | – | – | – | – | 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** | $\alpha$[c] | 4.242 | 4.244 | 4.09 | 4.265 | 4.224 | 4.246 | 4.24 | 4.22 | 4.244 | 4.25 |
| | | $\beta$ | 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** | $\alpha$[c] | – | – | – | – | – | – | – | – | – | 4.05 |
| | | $\beta$ | – | – | – | – | – | – | – | – | – | 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** | $\alpha$ | 2.042 | 2.042 | 2.061 | 2.057 | 2.041 | 2.057 | 2.057 | 2.041 | 2.057 | 2.044 |
| | | $\beta$ | | | | 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] $\alpha$ and $\beta$ stand for the $\alpha$- and $\beta$-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 Man$\beta$1-4GlcNAc$\beta$1-4GlcNAc element in common, derived from urine and brain of the $\alpha$-mannosidosis cat. Chemical shifts, measured at 27°C in $^2H_2O$, are given in ppm relative to internal acetone ($\delta$ = 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** | $\alpha$ | 5.190 | 5.190 | 5.189 | 5.188 | 5.189 | 5.189 | 5.189 | 5.189 | 5.188 | 5.188 | 5.188 | 5.188 |
| | | $\beta$ | 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** | $\alpha$[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 |
| | | $\beta$ | 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-$\mathbf{D_1}$ | | – | – | – | – | – | – | – | – | – | 5.041 | – | – |
| | Man-$\mathbf{D_2}$ | | – | – | – | – | – | – | – | – | – | – | 5.055 | – |
| | Man-$\mathbf{D_3}$ | | – | – | – | – | – | – | 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-$\mathbf{D_1}$ | | – | – | – | – | – | – | – | – | – | 4.07 | – | – |
| | Man-$\mathbf{D_2}$ | | – | – | – | – | – | – | – | – | – | – | 4.07 | – |
| | Man-$\mathbf{D_3}$ | | – | – | – | – | – | – | 4.07 | – | – | – | – | 4.07 |
| NAc | GlcNAc-**1** | $\alpha$ | 2.038 | 2.038 | 2.038 | 2.038 | 2.038 | 2.038 | 2.038 | 2.038 | 2.038 | 2.038 | 2.038 | 2.038 |
| | | $\beta$ | | | | | | | | | 2.036 | | | |
| | GlcNAc-**2** | $\alpha$[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 |
| | | $\beta$ | | | | | | | | | 2.063 | | | |

[a] Measured at 600 MHz.
[b] $\alpha$ and $\beta$ stand for the $\alpha$- and $\beta$-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.

$\delta$ = 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 $\delta$ = 4.86–5.40 ppm, whereas for βMan-**3** the H-1 resonates between $\delta$ = 4.76–4.79 ppm. The Man H-2 signals resonate in the region $\delta$ = 3.96–4.27 ppm. For a terminal α(1-3)-linked Man-**4** residue the H-1 signal typically is found at $\delta$ = 5.10–5.11 ppm, with the H-2 signal at $\delta$ = 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 $\delta$ = 4.92 ppm and $\delta$ = 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.

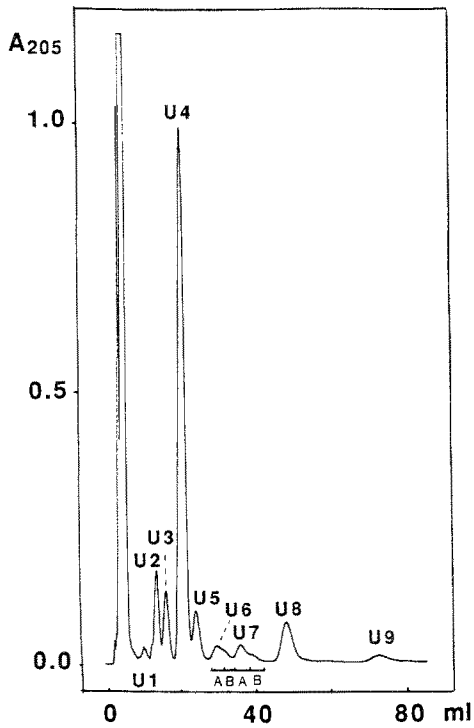



**Figure 2.** Fractionation pattern at 205 nm of the urinary oligosaccharides on a HPLC Lichrosorb-$NH_2$ 10 μm column (250 × 4.6 mm). The mixture of oligosaccharides was dissolved in 200 μl water. The column was eluted isocratically with water/acetonitrile, 35/65 by vol, at a flow rate of 1.5 ml $min^{-1}$ 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 (**$D_3$-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

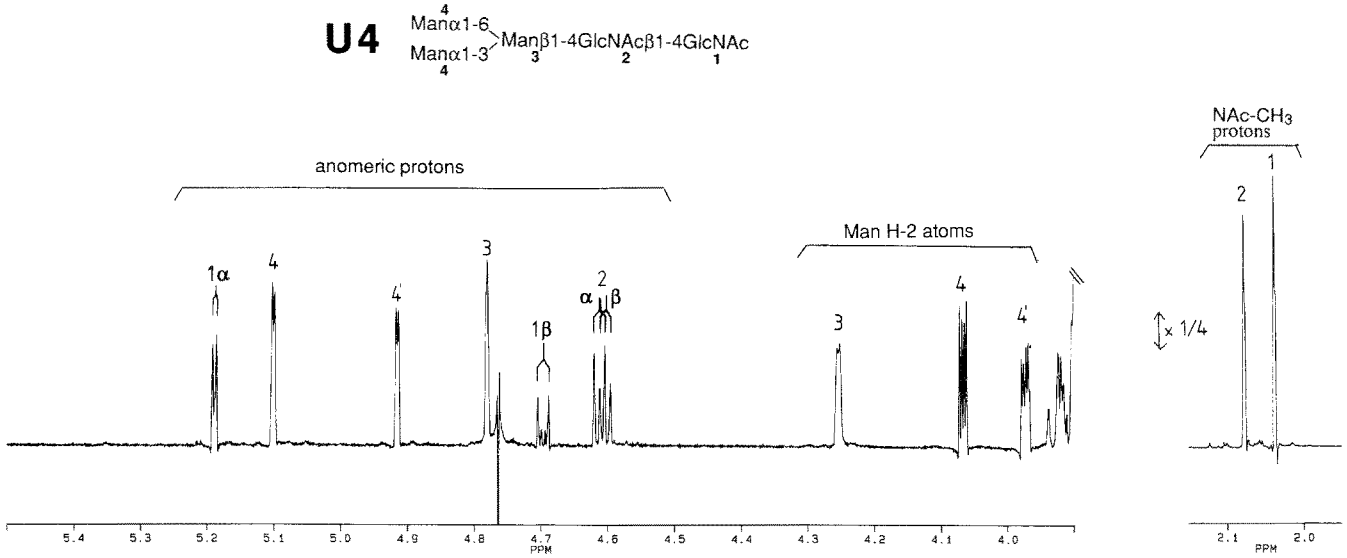



**Figure 3.** Structural-reporter-group regions of the resolution-enhanced 500-MHz $^1$H-NMR spectrum of oligosaccharide U4 obtained from cat α-mannosidosis urine.

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).

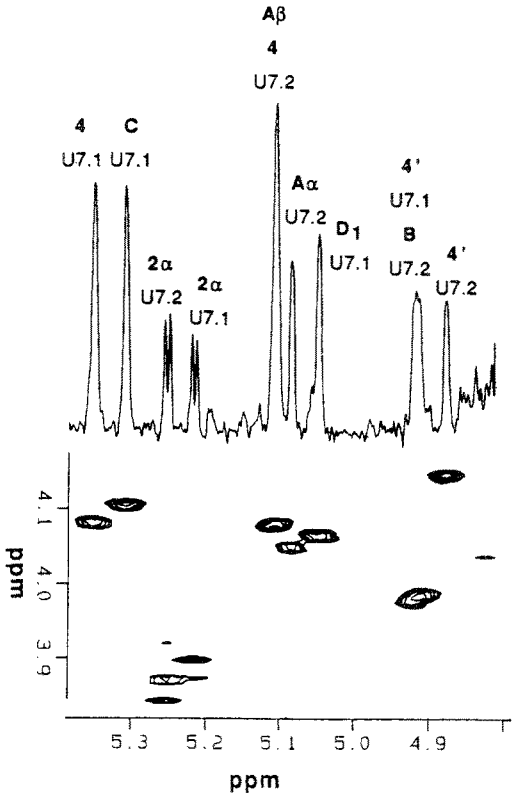



**Figure 4.** The α-anomeric region of the resolution-enhanced 500-MHz $^1$H-NMR spectrum of the mixture of oligosaccharides U7.1 and U7.2 (fraction U7B), together with the H-1/H-2 cross-peaks from the 2D HOHAHA spectrum, which was recorded with a 40 ms MLEV-17 mixing period.

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(Manα1-6)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.

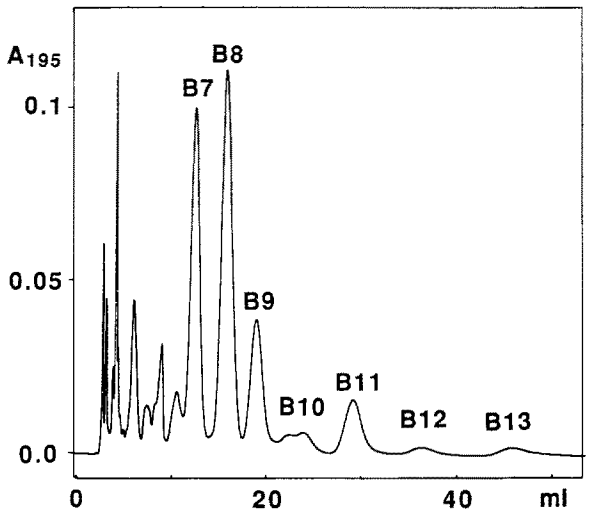



**Figure 5.** Fractionation pattern at 195 nm of the brain-derived oligosaccharides on a HPLC Lichrosorb-$NH_2$ 10 μm column (250 × 4.6 mm). The mixture of oligosaccharides was dissolved in 200 μl water. The column was eluted isocratically with water/acetonitrile, 25/75 by vol, at a flow rate of 1.0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

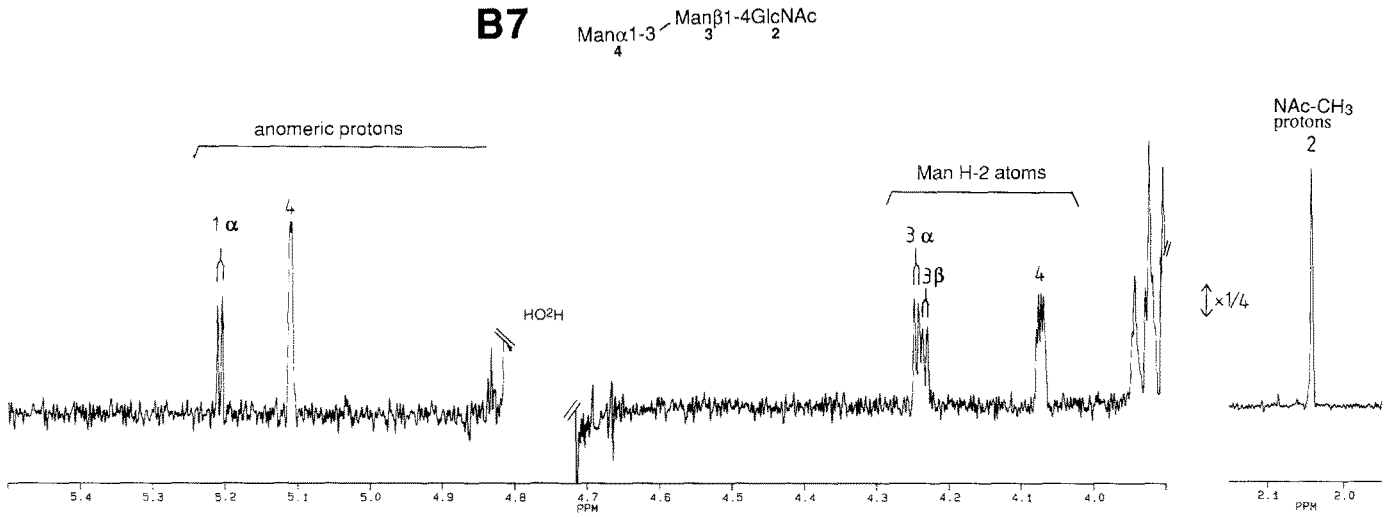



**Figure 6.** Structural-reporter-group regions of the resolution-enhanced 500-MHz $^1$H-NMR spectrum of oligosaccharide B7 obtained from the brain of the α-mannosidosis cat.

reporters of Man-**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-**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 |
| |  | 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 |
| $Man_4GlcNAc_2$ |  | (U6.1) | 4[b] | – | 8[c] | – | – | 4[d] |
| |  | (U6.2) | – | – | – | – | – | – |
| |  | (U6.3) | – | – | – | – | – | – |
| |  | (U6.4) | – | – | – | – | – | 20 |
| | | 2 | | | | | | |
| $Man_5GlcNAc_2$ |  | 8 (U8) | 5[e] | – | 4[f] | – | – | 11 |
| $Man_6GlcNAc_2$ | n.d.[g] | – | + | – | + | – | – | – |
| $Man_7GlcNAc_2$ |  | (U9.1) | | – | – | – | – | – |
| |  | (U9.2) | + | – | + | – | – | – |
| |  | (U9.3) | | – | – | – | – | – |
| | | 2 | | | | | | |

[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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