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Mannosyl-β(1-4)-*N*-Acetylglucosaminyl-β(1-*N*)-Urea, a Compound Isolated from the Urine of Patients with β-Mannosidosis

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A previously unknown substance, mannosyl-β(1-4)-*N*-acetylglucosaminyl-β(1-*N*)-urea, has been isolated from the urine of patients with β-mannosidosis in addition to the main metabolite mannosyl-β(1-4)-*N*-acetylglucosamine. Structural investigation was carried out by fast atom bombardment mass spectrometry and high-resolution ^{1}H-nuclear magnetic resonance spectroscopy at 500 MHz. It was postulated that the occurrence of this carbohydrate-urea conjugate in urine results mainly from urine handling.

The deficiency of the lysosomal enzyme β-mannosidase (EC 3.2.1.25) has recently been discovered in man [1-3]. Patients with this disorder of glycoprotein metabolism excrete large amounts of the disaccharide mannosyl-β(1-4)-*N*-acetylglucosamine in their urine. A rapid diagnosis, based upon the detection of this disaccharide in urine, can be made by one-deminsional TLC of untreated urine [3]. In this paper the characterization of mannosyl-β(1-4)-*N*-acetylglucosaminyl-β(1-*N*)-urea, isolated from the urine of two patients with β-mannosidosis, is described.

Materials and Methods

Twenty-four-hour collections of urine obtained from two patients with β-mannosidosis [3] were stored at -20°C prior to analysis. TLC of urinary oligosaccharides was performed on silica sheets (10 × 10 cm, Alufolien, No 5553, Merck, Darmstadt, W. Germany) in

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system A, *N*-butanol/acetic acid/water, 2/1/1 by vol, and system B, *n*-propanol/acetic acid/water, 85/1/15 by vol [3]. After drying at room temperature, the chromatograms were dipped in a staining reagent (40 mg orcinol dissolved in a mixture of 4 ml concentrated sulfuric acid and 80 ml acetone) and subsequently heated at 105°C for 10 min. Specific staining for the detection of carbamido-containing compounds was done with the Ehrlich reagent, yielding a yellow color [4]. Oligosaccharides were isolated from 350 ml of urine by a sequence of chromatographic techniques. Urine was first desalted by passing it over a cation exchange column (Dowex 50-X8, H^+-form, 100-200 mesh) and an anion exchange column (Dowex 1-X8, acetate-form, 100-200 mesh). Both columns were eluted with water. The eluate was concentrated by rotary evaporation at 38°C and then subjected to gel chromatography in five runs on a Bio-Gel P-2 column (120 × 0.6 cm, Bio-Rad Labs, Richmond, CA, USA) with water elution. Carbohydrate-positive fractions of interest were finally purified by HPLC on a Partisil 10 SAX column (25 cm × 0.4 cm, Whatman 4226.001) which was eluted with acetonitrile/water, 4/1 by vol. The detection wavelength was 205 nm.

Sugar composition analysis was carried out by GLC according to the method of Kamerling *et al.* [5]. Fast atom bombardment mass spectra were recorded on a ZAB-2F VG mass spectrometer using glycerol as matrix.

500 MHz ^{1}H-NMR spectroscopy was performed on a Bruker AM-500 spectrometer, operating in the Fourier transform mode, and equipped with a Bruker Aspect-3000 computer. The spectra were obtained with quadrature phase-detection using a 90° pulse. Prior to ^{1}H-NMR spectroscopic analysis the oligosaccharide fractions were treated three times with ^2H_2O at room temperature with intermediate lyophilization. Finally, the samples were redissolved in 0.45 ml ^2H_2O (99.96 atom % ^{2}H, Aldrich, Milwaukee, WI, USA). Chemical shifts were expressed in ppm relative to internal sodium 4,4-dimethyl-4-silapentanesulfonate (DSS).

Natural-abundance ^{13}C-NMR spectra were obtained in ^2H_2O at 50.76 MHz on a Bruker WP-200 spectrometer.

Results and Discussion

The characteristic disaccharide Manβ1-4GlcNAc occurring in the urine of patients with β-mannosidosis is readily detected by one-dimensional TLC in system A using the orcinol-sulfuric acid stain. However, because of co-elution with lactose, we developed system B for the separation of this disaccharide from lactose [3]. In system B Manβ1-4GlcNAc has a greater mobility than lactose and is found between the positions of lactose and sucrose. When analysing oligosaccharides by TLC in desalted and concentrated urine samples of the two patients with β-mannosidosis, we observed in addition to the band of Manβ1-4GlcNAc an unusual strong band in the position of lactose in system B. This band stained yellow with the Ehrlich reagent, indicating the presence of a carbamido group. Further purification by gel filtration on Bio-Gel P-2 did not give rise to a good separation between Manβ1-4GlcNAc and the unknown compound as could be judged by TLC in system B. The Bio-Gel P-2 fractions of interest were subsequently subjected to HPLC. On the Partisil 10 SAX column the unknown compound was more retarded than Manβ1-4GlcNAc. This purified "Ehrlich positive" fraction was investigated by gas chromatography, NMR spectroscopy and FAB mass spectrometry.

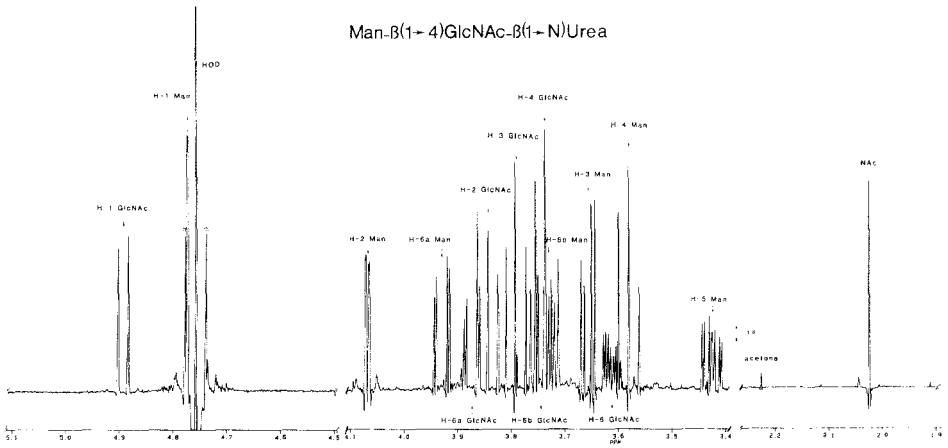

Figure 1. 500 MHz ^{1}H-NMR spectrum of mannosyl-β1-4-*N*-acetylglucosaminyl-β1-*N*-urea in ^2H_2O solution at 300 K; spinning side band.

Table 1. ^{1}H Chemical shifts in ppm for Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-*N*-Urea.

Residue	Proton	Manβ1-4GlcNAc	Manβ1-4GlcNAcβ1-*N*-Urea
α-GlcNAc	H-1	5.211 ($J_{1,2}$ = 3.1Hz)	
	H-2	3.903	
	H-3	3.944	
	H-4	3.745	
	H-5	3.923	
	H-6a/b	~3.80	
	NAc	2.044	
β-GlcNAc	H-1	4.724 ($J_{1,2}$ = 7.8Hz)	4.892 ($J_{1,2}$ = 9.4Hz)
	H-2	3.730	3.843
	H-3	3.722	3.790
	H-4	3.730	3.736
	H-5	3.579	3.611
	H-6a/b	n.d./n.d.[a]	3.873/3.747
	NAc	2.044	2.023
β-Man	H-1	4.769 ($J_{1,2}$ ~ 1Hz)	4.774 ($J_{1,2}$ = 0.8 Hz)
	H-2	4.071/4.061[b]	4.068
	H-3	3.662	3.656
	H-4	3.584	3.580
	H-5	3.438	3.425
	H-6a/b	3.944/n.d.	3.929/3.731

[a] n.d. = not detected.
[b] two signals, due to anomerization [6].

Table 2. ^{13}C Chemical shifts in ppm for *N*-acetylglucosamine, GlcNAcβ1-*N*-Urea, Manβ1-4GlcNAc and Manβ1-4GlcNAcβ1-*N*-Urea. Internal standard: acetone; δCH_3 = 31.55 ppm.

		Chemical shift in			
Residue	Carbon atom	GlcNAc	GlcNAc-Urea	Man-GlcNAc	Man-GlcNAc-Urea
α-GlcNAc	C_1	92.09	—	91.82	—
	C_2	55.34	—	54.99	—
	C_3	72.82	—	71.40	—
	C_4	71.35	—	**80.56**	—
	C_5	71.95	—	70.47	—
	C_6	61.88	—	61.47	—
	CH_3	23.21	—	23.21	—
β-GlcNAc	C_1	96.18	81.64	96.22	81.51
	C_2	57.98	55.57	57.47	55.06
	C_3	75.16	75.54	73.63	74.04
	C_4	71.12	70.94	**80.19**	**79.95**
	C_5	77.20	78.41	75.95	77.15
	C_6	62.03	61.91	61.58	61.44
	CH_3	23.48	23.28	23.50	23.30
β-Man	C_1	—	—	101.40	101.41
	C_2	—	—	71.86	71.84
	C_3	—	—	74.11	74.11
	C_4	—	—	67.93	67.94
	C_5	—	—	77.71	77.75
	C_6	—	—	62.22	62.25

Sugar composition analysis by gas chromatography after methanolysis, re-*N*-acetylation and trimethylsilylation revealed mannose and *N*-acetylglucosamine in a molar ratio of approximately 10:1.

The 500 MHz ^1H-NMR spectrum of the non-derivatized compound is shown in Fig. 1. Its NMR data are summarized in Table 1, together with those of the reference compound Manβ1-4GlcNAc, which has been characterized previously [3].

The sets of chemical shift values of mannose in both compounds are identical. This is indicative of the absence of structural alterations in the mannose part of the molecule. The chemical shift of H-1 of mannose at δ 4.774 ppm together with the small coupling constant $J_{1,2}$ = 0.8 Hz (H-1*ax*, H-2*eq*) point to a β-configuration for the mannose residue [6]. The coupling constant $J_{1,2}$ = 9.4 Hz (H-1*ax*), H-2*ax*) in *N*-acetylglucosamine is characteristic of a β-glycosidic linkage. The relatively large value suggests an *N*-type of glycosidic linkage [6]. The anomeric proton resonates at a rather low field in view of its axial position (δ = 4.893 ppm), which can be ascribed to an electron-withdrawing group attached to C-1. The chemical shift of the singlet of the *N*-acetyl group is typical for *N*-acetylglucosamine linked to nitrogen of an amide group [6].

The (1-4)-linkage between mannose and *N*-acetylglucosamine was unambigiously proven by ^{13}C-NMR spectroscopy. The chemical shift of C-4 of *N*-acetylglucosamine in the disaccharide Manβ1-4GlcNAc moved downfield by approximately 9 ppm when compared to that of C-4 of free *N*-acetylglucosamine (see Table 2). The C-4 atom of *N*-acetylglucosamine in the unknown substance was observed at δ = 79.95 ppm, which is

almost identical with the value for C-4 in Manβ1-4GlcNAc. This indicates the mannose residue in the unknown compound is attached to C-4 of the *N*-acetylglucosamine. Furthermore, the chemical shift of C-1 of *N*-acetylglucosamine at δ 81.51 ppm is indicative of an *N*-type of glycosidic linkage [6].

The FAB mass spectrum showed mass values at 426, 448 and 464, which were interpreted as $[M+H]^+$, $[M+Na]^+$ and $[M+K]^+$, respectively.

Based on the data of the Ehrlich staining reaction, carbohydrate composition analysis, NMR spectroscopy and mass spectrometry the urinary compound was identified as Manβ1-4GlcNAcβ1-*N*-urea. The *N*-glycosidic linkage between *N*-acetylglucosamine and urea is rather stable, as was also observed for GlcNAcβ1-*N*-Asn [7] and therefore it was only partially cleaved under the methanolysis conditions used. This explains why the molar ratio Man:GlcNAc strongly deviates from the theoretical 1:1.

The occurrence in urine of other carbohydrate-urea conjugates *viz* 3,6-anhydro-*N*-acetylhexosamine-urea and *N*-acetylmannosamine-urea has recently been described in sialuria [8]. However, configurations of the *N*-glycosidic linkages were not determined. Wieruszeski *et al.* [8] stated that these urinary components did not originate from treatment of storage of the urine. In contrast with their findings we showed that concentration of the urine samples by rotary evaporation at a pressure of 20 mm Hg and 38°C increased the formation of the Manβ1-4GlcNAc-Urea conjugate with an accompanying decrease of the disaccharide Manβ1-4GlcNAc. In well preserved and carefully treated urine we could barely detect a trace of Manβ1-4GlcNAcβ1-*N*-Urea. This leads to the conclusion that carbohydrate-urea conjugates are mainly the result of urine handling.

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