A 500 MHz ^{1}H NMR STUDY OF URINARY OLIGOSACCHARIDES FROM PATIENTS WITH MANNOSIDOSIS

H. VAN HALBEEK, L. DORLAND, G. A. VELDINK, J. F. G. VLIEGENTHART, G. STRECKER+, J.-C. MICHALSKI+, J. MONTREUIL+ and W. E. HULL*

Department of Bio-Organic Chemistry, University of Utrecht, Croesestraat 79, 3522 AD Utrecht, The Netherlands, +Laboratoire de Chimie Biologique, Université des Sciences et Techniques de Lille I, Villeneuve d'Ascq, France and **Bruker Analytische Messtechnik, GmbH, D-7512 Rheinstetten-Fo., FRG*

Received 22 September 1980

1. Introduction

Mannosidosis is an autosomal, recessively heritable, inborn error of glycoprotein metabolism characterized by a lysosomal deficiency of acidic α-mannosidase leading to the accumulation of mannose-rich oligosaccharides in tissues, fibroblasts and urine [1–7]. The structure elucidation of these oligosaccharides, produced by endo-β-*N*-acetylglucosaminidase digestion of the sugar chains, is of great interest, since they represent possible intermediates in the processing of the biosynthesis of asparagine-linked carbohydrate chains [7–9].

Here, the investigation of several of these oligosaccharides by 500 MHz ^{1}H NMR spectroscopy is described. Some smaller compounds were already chemically identified [3,5]. The NMR parameters of these smaller oligosaccharides in combination with those of three oligomannoside-type glycopeptides isolated from the urine of a patient with Gaucher's disease [10], enable the interpretation of the structural reporter group regions of the 500 MHz ^{1}H NMR spectrum of an oligosaccharide containing 9 mannose residues, in terms of structural assignments.

2. Materials and methods

The isolation of mannose-rich oligosaccharides from urine of patients with mannosidosis has been described [3,5]. The structures of compounds Man_2. GlcNAc (M_2G), Man_3. GlcNAc (M_3G) and Man_4. GlcNAc (M_4G), as determined by chemical methods [3,5], are presented in fig.1.

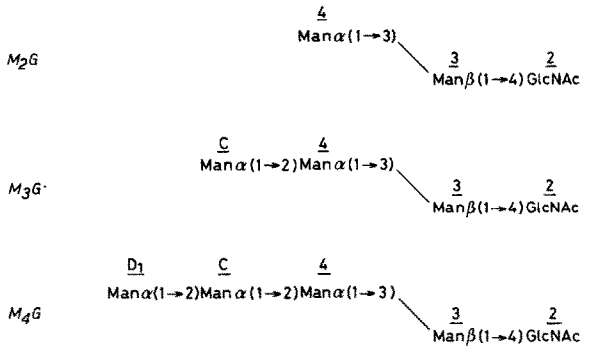


Fig.1. Structures of the urinary mannosidosis oligosaccharides, used as reference compounds.

Solutions of the oligosaccharides were exchanged several times in D_2O with intermediate lyophilization. NMR spectral analysis of ~0.02 M solutions of the compounds in D_2O (99.96 atom % D, Aldrich) was carried out on a Bruker WM-500 spectrometer, operating in the Fourier transform mode at probe temperatures of 300 K or 330 K. Compound M_4G* was analysed by 360 MHz ^{1}H NMR spectroscopy on a Bruker HX-360 spectrometer, operating under the same conditions. Chemical shifts at 300 K are given relative to sodium-2,2-dimethyl-2-silapentane-5-sulphonate (indirectly to acetone in D_2O: δ = 2.225 ppm). Resolution enhancement of the ^{1}H NMR spectra was achieved by Lorentzian to Gaussian transformation according to [11].

3. Results and discussion

Besides the 3 oligosaccharides listed in fig.1, another

series of mannose-rich oligosaccharides was isolated from the urine of mannosidosis patients, varying in mannose-content from 4–9 residues. Compounds M_4G^* and M_9G were obtained in a pure state. Purification of the other oligosaccharide fractions is still in progress.

From the compounds M_2G, M_3G, M_4G as well as from M_4G^* and M_9G, high-resolution ^1H NMR spectra were recorded at 300 K and 330 K.

The 360 MHz ^1H NMR spectral features of the trisaccharide M_2G have appeared [12]. The NMR data of this oligosaccharide, refined by 500 MHz ^1H NMR spectroscopy are compiled in table 1. The NMR spectrum of this oligosaccharide ending on GlcNAc 2 is a

Table 1
^1H chemical shifts of structural reporter groups of constituent monosaccharides for some mannosidosis oligosaccharides[a]

Reporter group	Residue[b]	Compound anomer	Compound and schematic structure[c]				
			M_2G	M_3G	M_4G	M_4G^*[d]	M_9G
H-1 of	2	α	5.209	5.206	5.207	5.250	5.231
		β	4.720	4.718	4.719	4.716	4.714
NAc of	2	α	2.043	2.041	2.043	2.044	2.050
		β	2.041		2.041		2.046
H-1 of	3	α	4.787	4.776	4.774	4.776	4.776
		β	4.783	4.772	4.771		4.772
	4	α,β	5.111	5.356	5.343	5.348	5.337
	4′	α	–	–	–	4.899	4.872
		β					4.869
	A	α	–	–	–	–	5.398
		β					5.407
	B	α,β	–	–	–	–	5.142
	C	α,β	–	5.050	5.296	5.056	5.308
	D_1	α,β	–	–	5.046	–	5.048
	D_2	α	–	–	–	–	5.058
		β					5.063
	D_3	α,β	–	–	–	–	5.040
H-2 of	3	α	4.244	4.224	4.220	4.238	4.239
		β	4.233	4.213	4.209	4.227	4.229
	4	α	4.075	4.108	4.087	4.109	4.089
		β	4.071	4.105	4.082		
	4′	α	–	–	–	<4.0	4.158
		β					4.155
	A	α	–	–	–	–	4.106
		β					4.103
	B	α,β	–	–	–	–	4.025
	C	α,β	–	4.069	4.105	4.068	4.109
	D_1	α,β	–	–	4.066	–	4.069[e]
	D_2	α	–	–	–	–	4.073[e]
		β					
	D_3	α,β	–	–	–	–	4.066[e]

[a] Chemical shifts are given at 300 K; from the spectra recorded at 330 K, no dramatic temperature dependency of the chemical shift of any structural reporter groups has been observed, in contrast to [16]
[b] For coding of monosaccharide residues and complete structures see fig.1–3
[c] (•—) Mannose; (○—) *N*-acetylglucosamine; [d] Measured at 360 MHz; [e] Assignments may be interchanged

superposition of the subspectra of its two anomers containing GlcNAc 2 in the α- and β-pyranose form in a ratio α:β = 2:1, respectively. The effect of anomerization is recognizable in the structural reporter group signals of the reducing end monosaccharide, as well as in those of the other two residues. Obviously, this effect is more pronounced at 500 MHz than at 360 MHz [12]. The relatively large coupling constant $J_{1,2}$ of the β-linked Man 3 (0.95 Hz) is reflected in the presence of a well-resolved doublet for its H-1 in both α- and β-form of M_2G (cf. [10,13]). The α- and the β-linked mannose residues in M_2G can still be recognized on the basis of their H-1 and H-2 resonance patterns [13].

The tetrasaccharide M_3G (see fig.1) can be conceived as an extension of M_2G with an α(1→2) linked mannose residue at the non-reducing end. The 500 MHz ^{1}H NMR spectral parameters of M_3G are summarized in table 1. By selective homonuclear decoupling the relatively sharp lined signals at δ = 5.050 ppm and δ = 4.069 ppm were proved to belong to neighbouring protons; they are assigned to H-1 and H-2 of the α(1→2) linked Man C, respectively. The above set of chemical shift values are characteristic for a terminal α(1→2) linked mannose residue. Upon attachment of Man C to Man 4 at C-2, the H-1 and H-2 signals of Man 4 undergo downfield shifts (Δδ = 0.245 ppm and 0.034 ppm, respectively) and are slightly broadened.

Extension of M_3G with Man D_1, α(1→2)-linked to Man C, leads to the pentasaccharide M_4G (see fig.1). The relevant parts of the 500 MHz ^{1}H NMR spectrum of M_4G are given in fig.2, and its NMR features in table 1. The chemical shifts (see table 1), coupling constants and linewidths of the H-1 and H-2 signals of the terminal Man D_1 closely resemble those of Man C in M_3G.

The doublet at δ = 5.296 ppm is ascribed to H-1 of Man C. Again α(1→2) substitution of a mannose residue (C) by another mannose (D_1), leads to a shift increment of the H-1 of the substituted residue: Δδ = 0.246 ppm, that is comparable with the effect on δH-1 of Man 4 due to attachment of Man C to Man 4 (vide supra). Analogously, H-2 of Man C undergoes a shift increment (Δδ = 0.035 ppm), going from M_3G to M_4G (cf. Δδ for H-2 of Man 4 in the step from M_2G to M_3G). Attachment of the α(1→2)-linked Man D_1 influences also the chemical shifts of H-1 and H-2 of the remote residue Man 4 (Δδ = –0.013 ppm and –0.020 ppm, respectively).

The assignment of all α-mannose H-2 signals was

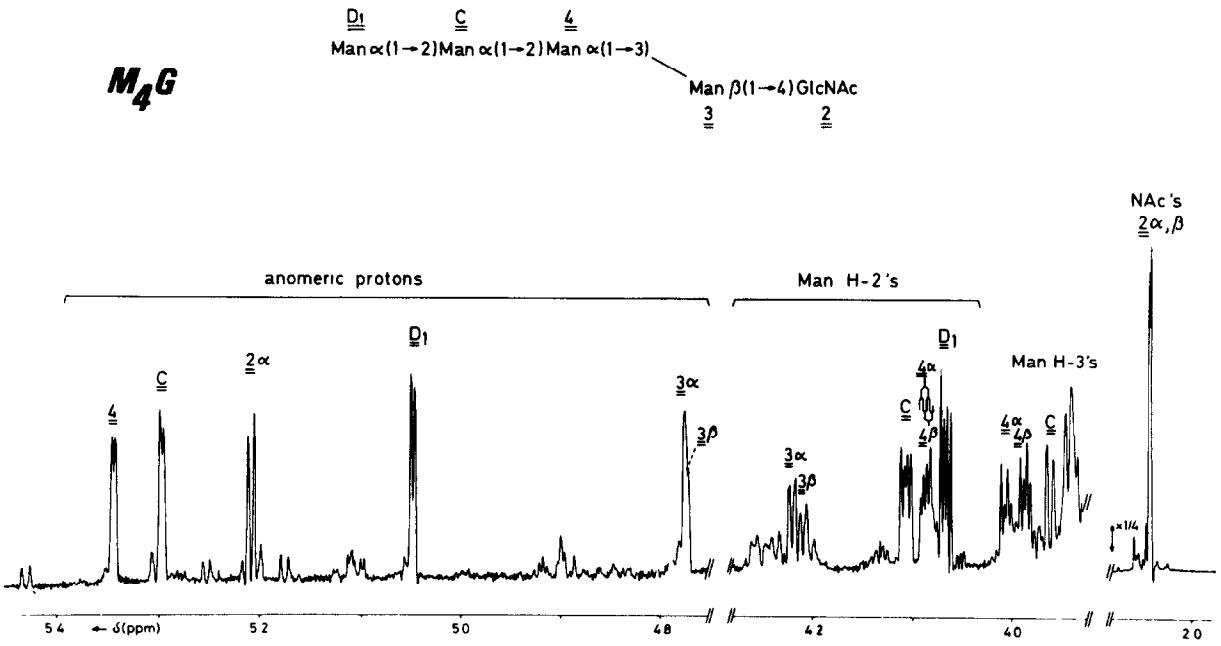
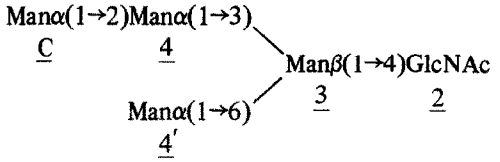


Fig.2. Structural reporter group regions of the resolution-enhanced 500 MHz ^{1}H NMR spectrum of oligosaccharide M_4G in D_2O at 300 K. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

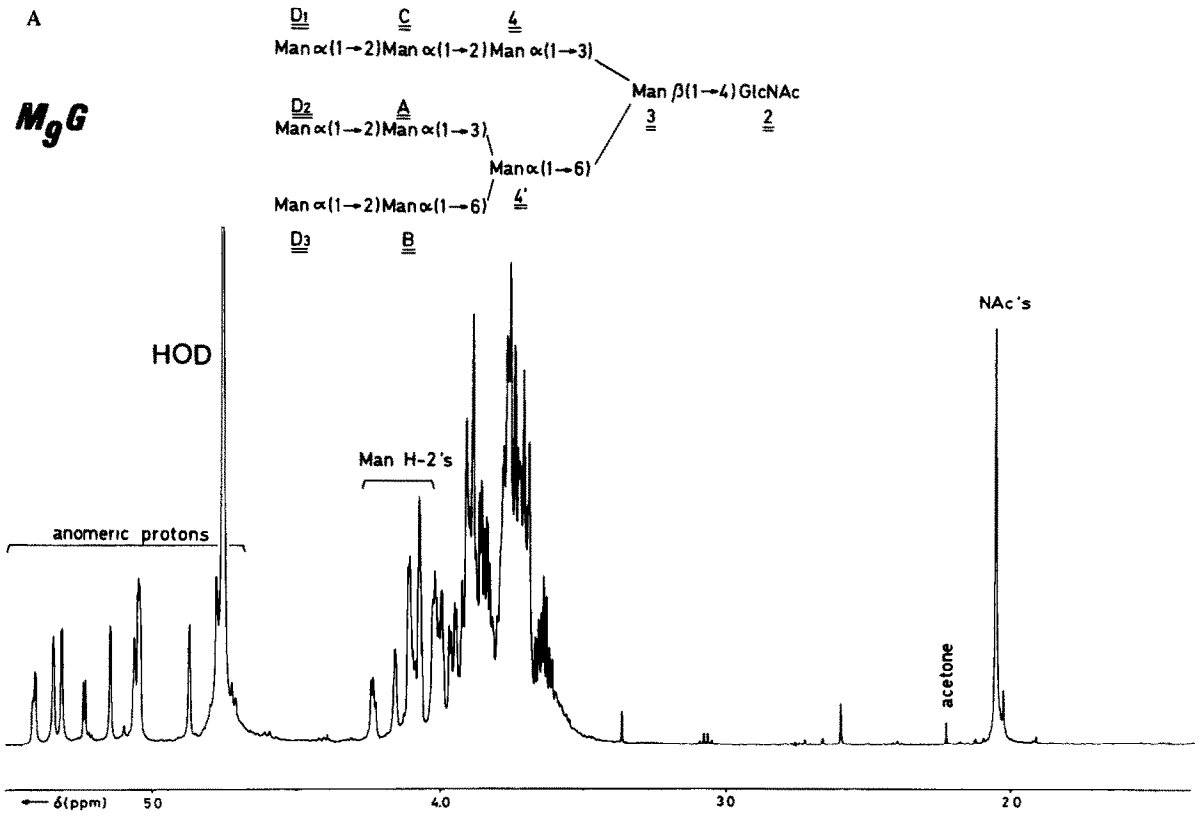
proved by selective irradiations of the α-mannose H-1 resonances. From the spectrum of M_4G (fig.2) it is clear that the linewidths of the α-linked mannoses H-1 doublets decrease going from the internal Man 4 via Man C to the terminal Man D_1. Doubling of signals due to anomerization of M_4G manifests itself only in the signals of GlcNAc 2 and Man 3. The more remote the residue is from the reducing end, the less the effect of the anomeric configuration of GlcNAc 2.

The structure of another pentasaccharide containing 4 mannoses, M_4G^*, could be elucidated with 360 MHz ^{1}H NMR spectroscopy. The spectral parameters of M_4G^* are listed in table 1. Based on the presence of signals at δ = 4.716 ppm (H-1 of 2 β), δ = 2.044 ppm (*N*-acetyl-CH_3 of 2), δ = 4.776 ppm (H-1 of 3) and δ ≃ 4.23 ppm (H-2 of 3), M_4G^* contains the Manβ-(1→4)GlcNAc moiety. The identity of the chemical shift values for H-2 of Man 4 in M_3G and M_4G^* (δ = 4.109 ppm) implicates that Man 4 in M_4G^* bears a terminal α(1→2)-linked Man C (δH-1 = 5.056 ppm, δH-2 = 4.068 ppm). The occurrence of an H-1 doublet at δ = 4.899 ppm indicates that the fourth mannose is α(1→6)-linked [10]. The signal of H-2 of this residue is hidden in the bulk of the skeleton protons (3.5 < δ < 4.0 ppm), which points to the terminal position of this α(1→6)-linked mannose. The occurrence of two terminal residues in M_4G^* identifies this α(1→6)-linked mannose as Man 4′. This is further evidenced by earlier 360 MHz ^{1}H NMR observations on oligosaccharides containing a terminal Man 4′ [14,15]. Therefore, the structure of M_4G^* turns out to be:



The disubstitution of Man 3 is reflected in the small change of the resonance position of its H-2 as compared to M_2G, M_3G and M_4G. The chemical shift of H-1 of GlcNAc 2 in the α-anomer of M_4G^* is significantly different from that of this proton in M_2G, M_3G and M_4G.

The occurrence of oligosaccharide M_4G^* in mannosidosis urine might have been traced before (e.g.



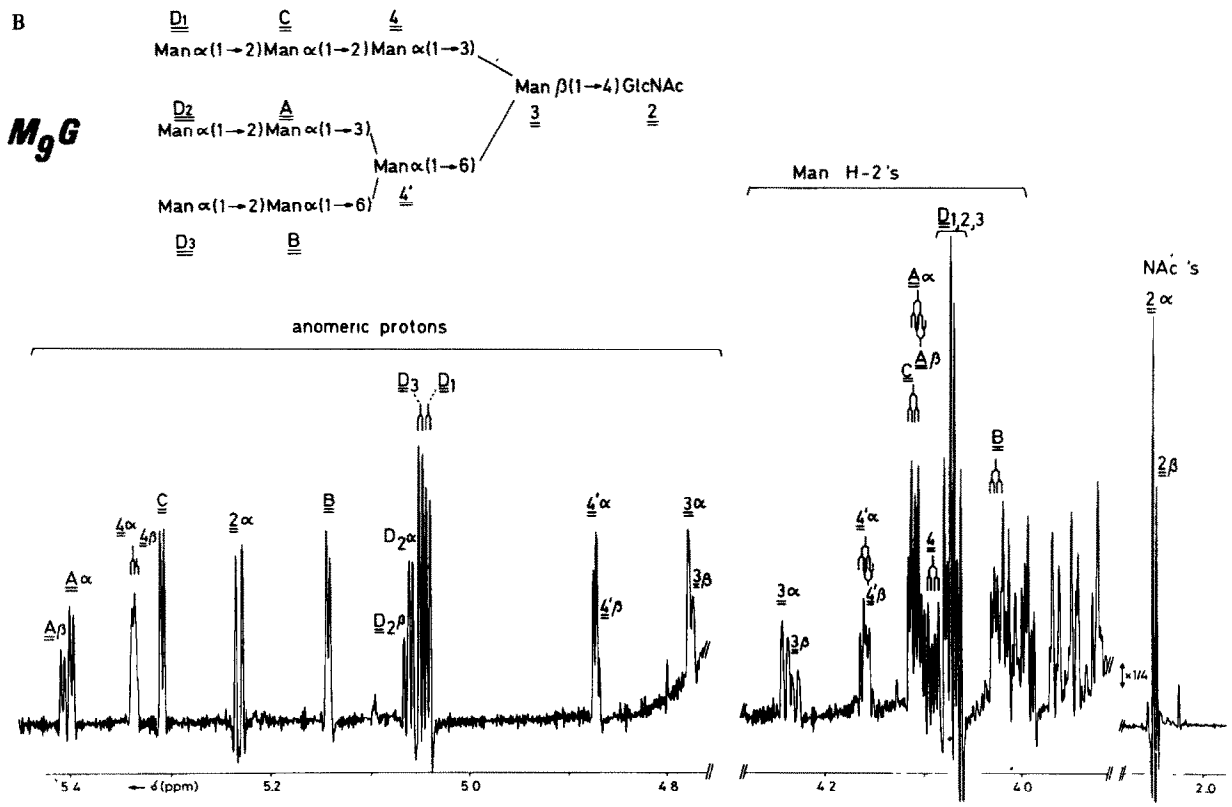


Fig.3 (A) Overall 500 MHz ^{1}H NMR spectrum of oligosaccharide M_9G in D_2O at 300 K, together with its structure. (B) Structural reporter group regions of the resolution-enhanced 500 MHz ^{1}H NMR spectrum of oligosaccharide M_9G in D_2O at 300 K. The numbers and letters in the spectrum refer to the corresponding residues in the structure.

[4]), but was not described for the urine investigated in [7].

The 500 MHz ^{1}H NMR spectrum of the largest mannose-containing oligosaccharide isolated from this mannosidosis urine, M_9G, is depicted in fig.3A. The expanded structural reporter group regions are shown in fig.3B and the NMR spectral features of M_9G are included in table 1. Integration of the anomeric region of the spectrum is consistent with the occurrence of 9 mannose residues and one *N*-acetyl-glucosamine. GlcNAc 2 is the reducing end sugar as is evident from its δH-1 values in the α- and β-anomer of M_9G ($|\Delta\delta_{\alpha-\beta}| > 0.5$ ppm). Man 3 is β(1→4)-linked to GlcNAc 2 (δH-1 ≃ 4.77 ppm). Based upon the presence of 3 anomeric proton signals at δ ≃ 5.05 ppm it can be concluded that M_9G contains 3 terminal α(1→2)-linked mannose residues, pointing to 3 branches in the structure.

Analogously to the data of M_4G (see table 1), the H-1 signals at δ = 5.337 ppm, δ = 5.308 ppm and δ = 5.048 ppm are ascribed to the upper branch mannose residues 4, C and D_1, respectively. The H-2 resonances of these mannoses are found at δ = 4.089 ppm, δ = 4.109 ppm and δ ≃ 4.07 ppm, respectively.

The disubstitution of Man 3 by an α(1→3)- and an α(1→6)-linked mannose is evident from the chemical shifts of its H-2 resonances (δ = 4.239 ppm (α-) and δ = 4.229 ppm (β-anomer of M_9G)) (cf. M_4G*). The H-1 signals of Man 4′ are observed at δ ≃ 4.87 ppm. This assignment is in accordance with that for 2 glycoasparagines of the oligomannoside type isolated from urine of a patient with Gaucher's disease (M_4GP and M_5GP, [10]). The H-2 signals of Man 4′ were identified by selective irradiation of the signals at δ ≃ 4.87 ppm; they are found at δ ≃ 4.16 ppm. The doubling of both the H-1 and H-2 resonances of Man 4′ with relative intensities in the anomeric ratio is more pronounced than for the corresponding signals of the α(1→3)-linked Man 4.

As derived [10], α(1→3) substitution of Man 4′ by

Man A influences mainly the δ-value of H-2 of Man 4′ ($\Delta\delta \simeq 0.18$ ppm); α(1→6) substitution of Man 4′ by Man B has a typical effect on the H-1 chemical shift of Man 4′ ($\Delta\delta \simeq -0.03$ ppm). The effect upon δH-1 of Man 4′ due to attachment of Man A, is negligibly small; the same holds for the influence of Man B on δH-2 of Man 4′, both compared to the above-mentioned $|\Delta\delta|$-values. Therefore, from the chemical shift values of H-1 and H-2 of Man 4′ in M_9G, it is evident that this residue is substituted at C-3 and C-6 by Man A and Man B, respectively. Owing to the presence of the latter two residues, H-1 of GlcNAc 2 (α) undergoes a small upfield shift ($\Delta\delta \simeq -0.02$ ppm) (cf. [10]), as compared to M_4G*.

As discussed before, all chains are terminated with an α(1→2)-linked mannose residue. Combination with the occurrence of 9 mannose residues in compound M_9G leads to the conclusion that Man A and Man B bear directly a terminal α(1→2)-linked mannose. This deduction affords already the primary structure of M_9G, given in fig.3.

The NMR parameters of Man A and B are in accordance with this interpretation. The H-1 doublet at $\delta = 5.142$ ppm and the H-2 signal at $\delta = 4.025$ ppm are assigned to Man B. Compared to the chemical shift values of the structural reporter groups of a terminal Man B (δH-1 = 4.908 ppm, δH-2 = 3.985 ppm, M_5GP [10]), both H-1 and H-2 are shifted downfield (ΔδH-1 = 0.234 ppm; ΔδH-2 = 0.040 ppm). These shift increments are indicative of an α(1→2) substitution of Man B by Man D_3 (vide supra).

Based on the same reasoning, Man A must be substituted by Man D_2 at C-2. Its H-1 and H-2 signals are observed at $\delta \simeq 5.40$ ppm and $\delta \simeq 4.105$ ppm, respectively. The corresponding values for a terminal Man A are: δH-1 = 5.093 ppm and δH-2 = 4.066 ppm (M_5GP, [10]). The shift increment, observed for H-1 of Man A in the step from M_5GP to M_9G is 0.05 ppm larger than that described above as typical for attachment of an α(1→2)-linked mannose.

The doubling of the structural reporter group signals of Man A in the spectrum of M_9G due to anomerization is clearly observable; $|\Delta\delta_{\alpha-\beta}|$ for its H-1 is even larger than that for the corresponding proton of Man 4′. Apparently, anomerization effects upon the H-1 and H-2 signals of Man B are absent. In view of these observations the single H-1 doublet at $\delta = 5.040$ ppm is ascribed to Man D_3, whereas the set of doublets at $\delta = 5.058$ ppm and $\delta = 5.063$ ppm (relative intensity 2:1) are attributed to H-1 of Man D_2 in α- and β-anomer of M_9G, respectively. The H-2 signals of Man D_2 and Man D_3 are found at $\delta \simeq 4.07$ ppm. A tentative assignment is given in table 1.

Comparison of the NMR parameters of the 3 branches of M_9G reveals that the anomeric configuration of GlcNAc 2 exerts its influence far more pronounced on the A–D_2 branch than on the other two, suggesting that this branch occurs in the sphere of influence of the anomeric center of GlcNAc 2, in contrast to the other two. An argument in favour of this proposal is the unexpectedly large downfield shift of H-1 of Man A due to attachment of Man D_2.

The structure of M_9G given in fig.3 is in accordance with that elucidated along chemical routes [7]. It fits the scheme of the biosynthetic pathway for *N*-glycosidically linked carbohydrate chains of glycoproteins [8,9].

4. Concluding remarks

In this study the NMR parameters of Man_2. GlcNAc, Man_3. GlcNAc, Man_4. GlcNAc and Man_4. GlcNAc*, isolated from the urine of mannosidosis patients, were established. The NMR characteristics of terminal and non-terminal α(1→2)-linked mannose residues, as well as the shift effects induced by such linked residues, are now well-defined. Since also the NMR data of the second branching point were available [10], the primary structure of Man_9. GlcNAc could completely be derived from the 500 MHz ^1H NMR spectrum.

In principal, the NMR analysis of reducing oligosaccharides seems to be more complex than that of non-reducing compounds (glycopeptides, oligosaccharide-alditols), because they consist of a mixture of compounds. In fact, the anomerization effect on the chemical shifts of structural reporter groups can be used as an additional tool for interpretation.

Acknowledgements

This investigation was supported by the Netherlands Foundation for Chemical Research (SON) with financial aid from the Netherlands Organization for the Advancement of Pure Research (ZWO), the Netherlands Foundation for Cancer Research (KWF, grant UUKC - OC 79-13), the Centre National de la Recherche Scientifique (Laboratoire Associé no. 217:

Biologie physicochimique et moléculaire des glucides libres et conjugués) and the Institut National de la Santé et de la Recherche Médicale (contract no. 78.1.0523). J.-C. M. is Attaché de Recherche de l'INSERM; G. S. is Maître de Recherche de CNRS.

References

[1] Nordén, N. E., Lundblad, A., Svensson, S., Öckerman, P.-A. and Autio, S. (1973) J. Biol. Chem. 248, 6210–6215.
[2] Nordén, N. E., Lundblad, A., Svensson, S. and Autio, S. (1974) Biochemistry 13, 871–874.
[3] Strecker, G., Fournet, B., Bouquelet, S., Montreuil, J., Dhondt, J. L. and Farriaux, J. P. (1976) Biochimie 58, 579–586.
[4] Tsay, G. C., Dawson, G. and Matalon, R. (1974) J. Pediat. 84, 865–868.
[5] Strecker, G. and Montreuil, J. (1979) Biochimie 61, 1199–1246.
[6] Chester, M. A., Hultberg, B., Nordén, N. E. and Szabó, L. (1980) Biochim. Biophys. Acta 627, 244–249.
[7] Yamashita, K., Tachibana, Y., Mihara, K., Okada, S., Yabuuchi, H. and Kobata, A. (1980) J. Biol. Chem. 255, 5126–5133.
[8] Turco, S. T., Stetron, B. and Robbins, P. W. (1977) Proc. Natl. Acad. Sci. USA 74, 4411–4414.
[9] Tabas, I., Schlesinger, S. and Kornfeld, S. (1978) J. Biol. Chem. 253, 716–722.
[10] Van Halbeek, H., Dorland, L., Veldink, G. A., Vliegenthart, J. F. G., Michalski, J.-C., Montreuil, J., Strecker, G. and Hull, W. E. (1980) FEBS Lett. 121, 121, 65–70.
[11] Ernst, R. R. (1966) in: Advances in Magnetic Resonance (Waugh, J. S. ed) vol. 2, pp. 1–135, Academic Press, London, New York.
[12] Dorland, L., Haverkamp, J., Schut, B. L., Vliegenthart, J. F. G., Spik, G., Strecker, G., Fournet, B. and Montreuil, J. (1977) FEBS Lett. 77, 15–22.
[13] Van Halbeek, H., Dorland, L., Vliegenthart, J. F. G., Schmid, K., Montreuil, J., Fournet, B. and Hull, W. E. (1980) FEBS Lett. 114, 11–16.
[14] Dorland, L., Haverkamp, J., Vliegenthart, J. F. G., Strecker, G., Michalski, J.-C., Fournet, B., Spik, G. and Montreuil, J. (1978) Eur. J. Biochem. 87, 323–329.
[15] Herlant-Peers, M.-C., Montreuil, J., Strecker, G., Dorland, L., Van Halbeek, H., Veldink, G. A. and Vliegenthart, J. F. G. (1980) Eur. J. Biochem. in press.
[16] Grey, A. A., Atkinson, P. H., Ceccarini, C. and Carver, J. P. (1979) abst. Ann. Meet. Soc. Complex Carbohydrates, 15, Toronto, Canada.