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URINARY EXCRETION OF A NOVEL HEXASACCHARIDE AND A GLYCOPEPTIDE ANALOGUE IN FUCOSIDOSIS

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SUMMARY

Repeated Biogel P6 chromatography of the urine from a patient with fucosidosis yielded several fractions containing fucosyloligosaccharides and glycopeptides. Two of these were shown by ^{1}H nuclear magnetic resonance (^{1}H-n.m.r.) spectroscopy and permethylation analysis to have the following structures respectively: (I) αfuc (1→3) [βgal (1→4)] βglcNAc (1→2) αman (1→3/6) βman (1→4) glcNAc and (II) αfuc (1→3) [βgal (1→4)] βglcNAc (1→2) αman (1→3/6) βman (1→4) βglcNAc (1→4) [αfuc (1→3/6)] βglcNAc-Asn.

Fucosidosis is an inherited metabolic disorder, characterized biochemically by an absence of activity of the lysosomal enzyme α-L fucosidase in tissues resulting in an accumulation of fucose-containing glycosphingolipids, glycoproteins, glycopeptides and oligosaccharides in various tissues of patients with this disease (1-6). In the past few years, the isolation and characterization of several oligosaccharides and glycopeptides excreted in the urine of patients with fucosidosis have been described in the literature (4-8). Recently, we had the opportunity to study the excretion of complex carbohydrates in the urine of the first French Canadian patient to be diagnosed with fucosidosis (9). A novel fucosyl hexasaccharide and a glycopeptide analogue were isolated and characterized.

Materials and Methods

Thin layer chromatography (t.l.c.) of 24-hour urine samples was performed on Quantum Silica gel Q5 plate (Pierce Chemical Co., ILL). Twenty µl samples were applied as one cm bands, thoroughly dried and developed for 15 hr in n-butanol : acetic acid : water (2:1:1 by vol.). The carbohydrates were revealed with the orcinol-sulphuric acid reagent (10). Oligosaccharides and glycopeptides were isolated as follows: The 24-hour urine sample was concentrated in vacuo at room temperature and dialysed for three days at 4° against distilled water containing 0.01% sodium azide. The dialysate was concentrated and applied to a Biogel P6 column. Frac-

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tions were monitored by the phenol-sulphuric acid reaction for carbohydrate containing materials. The isolated fractions were further purified by repeated chromatography on the same column. The compositions of the isolated carbohydrate fractions and their sodium borohydride treated products were determined by gas-liquid chromatography (g.l.c.) on a 3% ECNSS-M column after the compounds had been consecutively hydrolyzed, reduced and acetylated (11). Permethylation of the oligosaccharide, glycopeptide and their sodium borohydride treated products were performed essentially by the method of Hakomori (12) and the resulting permethylated compounds acetolyzed, hydrolyzed, reduced and acetylated for g.l.c.-mass spectrometric analyses (13) on an LKB 9000 instrument interfaced with a Varian MAT Model 100-SS Computer. Both 3% ECNSS-M and 3% OV-101 columns were used for the g.l.c. analyses. 220 MHz ^{1}H-n.m.r. spectra were recorded on a Varian Associates HR-220 MHz Spectrometer. The oligosaccharides and glycopeptides were repeatedly exchanged in deuterium oxide (D_2O) and the spectra of the D_2O solutions recorded at 70°. The chemical shifts are in ppm from an external tetramethylsilane standard.

Results

Thin layer chromatographic analyses of the fucosidosis urine (lane 2, Fig. 1) clearly showed excretion of carbohydrate compounds not found in normal urine and also more complex than lactose. The t.l.c. pattern was also markedly different from that of G_{M1}-gangliosidosis Type I urine (lane 3, Fig. 1). Compounds in bands I and II were isolated by Biogel chromatography in yields of 10 and 13 mg/100 ml urine, respectively.

Compositional analyses of Compound I showed that it contained fucose, galactose, N-acetylglucosamine and mannose in approximate molar ratio of 1.1 : 1 : 1.8 : 1.9. There were equal amounts of reducing and non-reducing N-acetylglucosamine respectively. The slightly lower mobility of I in the t.l.c. as compared to that of a pentasaccharide (P) in G_{M1}-gangliosidosis (14) suggested that it was probably a hexasaccharide. Permethylation analyses of both the sodium borohydride treated and intact hexasaccharide I were informative. Both from their respective T-values and mass spectral fragmentations (15), the galactose and fucose residues were deduced to be unsubstituted and thus were at non-reducing termini of the hexasaccharide. There was, however, heterogeneity in the mannosyl linkages; the 2-linked mannose was present in a major proportion to smaller amounts of 3- and 6-linked moieties. No doubly substituted mannosyl residues could be detected, which indicated the presence of a linear dimannosyl core in the

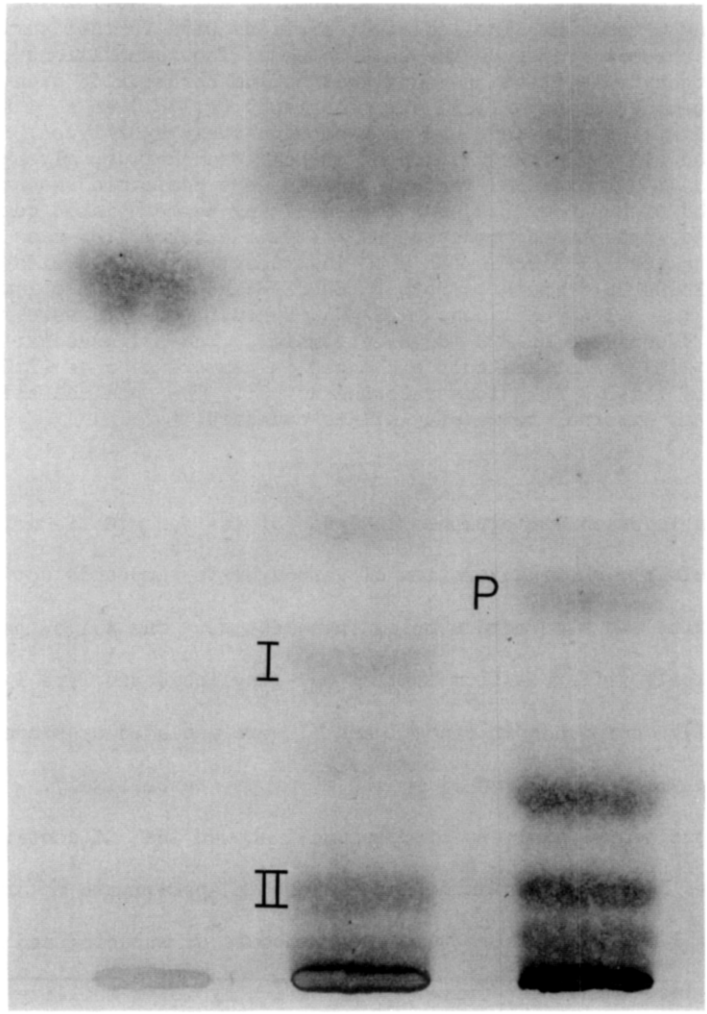

Figure 1. Thin-layer chromatogram of 24-hour urine samples. Lanes 1, 2 and 3 are: control plus lactose, fucosidosis, and G_{M1}-gangliosidosis type I, respectively.

hexasaccharide, as opposed to the branched trimannosyl core structure found in the major oligosaccharides excreted in both G_{M1} and G_{M2}-gangliosidosis (16). Analyses of the N-acetylglucosaminyl residues on the OV-101 column of both the untreated and borohydride reduced hexasaccharide confirmed the presence of both a reducing and non-reducing residue, the former

being 4-linked and the latter 3,4-linked. Thus, in the untreated hexasaccharide, two well separated g.l.c. peaks were eluted at high temperatures. The lesser retained peak gave mass spectral fragmentations typical of a 4-linked N-acetylglucosaminyl residue (17). This N-acetylglucosamine was at the reducing terminus of the hexasaccharide since on borohydride

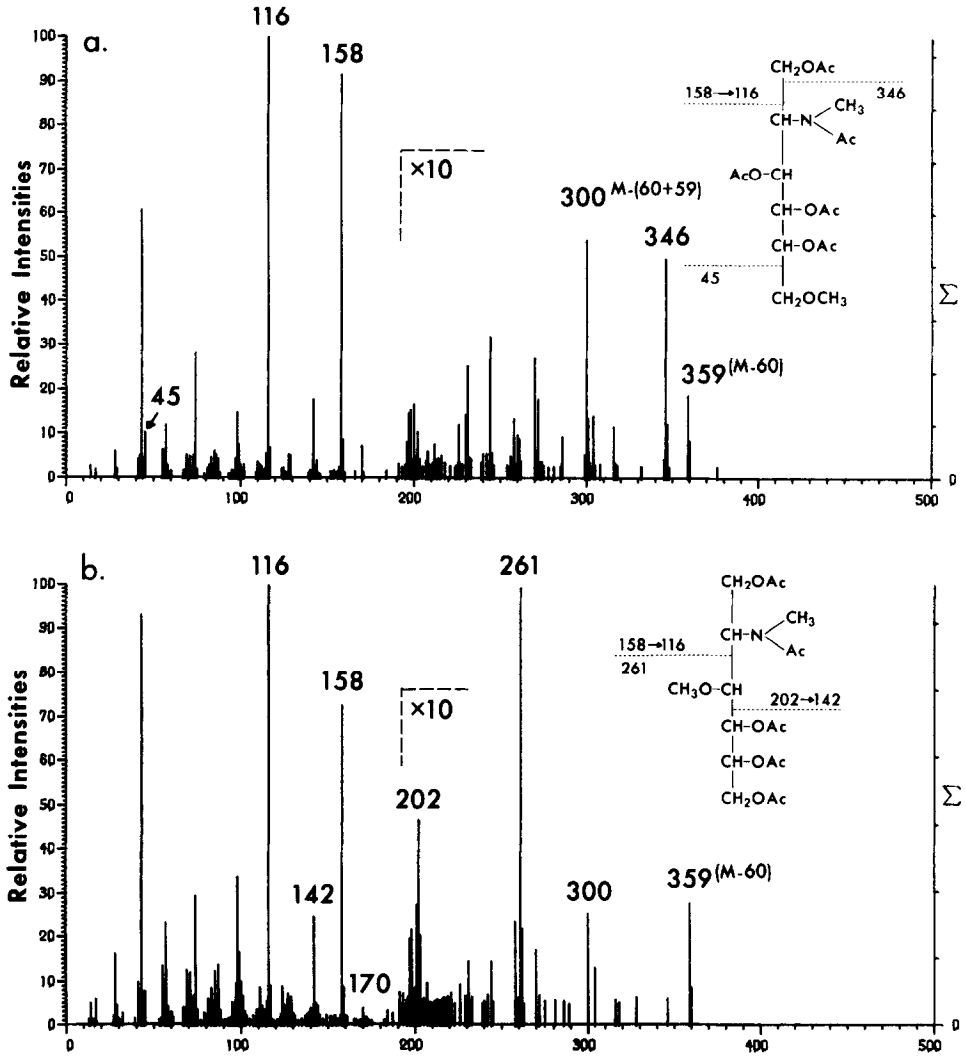

Figure 2. (a) Mass spectrum of 1, 3, 4, 5-tetra-O-acetyl-2-deoxy-2-(N-methyl acetamido)-6-O-methyl-D-glucitol (see also Ref. 17). (b) Mass spectrum of 1, 4, 5, 6-tetra-O-acetyl-2-deoxy-2-(N-methyl acetamido)-3-O-methyl-D-glucitol (see also Ref. 17).

treatment the g.l.c. peak disappeared with concomitant appearance of a compound with characteristic mass spectrum of a reduced 4-linked N-acetyl-glucosamine residue (18). The peak of longer retention time was clearly from a non-reducing compound since it was present in both the untreated and borohydride reduced hexasaccharide. Analysis of its mass spectrum showed that the compound was 1,3,4,5-tetra-O-acetyl-2-deoxy-2-(N-methyl-acetamido)-6-O-methyl-glucitol (Fig. 2a). The more probable fragmentations are shown in the figure.

In the ^{1}H-n.m.r. spectrum of compound I, Figure 3, there were several anomeric absorptions which were attributable to the following residues: at 5.69 ppm, reducing α-N-acetylglucosamine; at 5.60 ppm, α-mannosyl (1→3); at 5.59 ppm, α-fucosyl (1→3); at 5.36 ppm, α-mannosyl (1→6); at 5.28 ppm and shoulder, β-mannosyl (1→4) and reducing β-N-acetylglucosamine respectively; at 5.10 ppm, β-N-acetylglucosaminyl (1→2), and at 4.92 ppm (a well resolved doublet, J 7.7 Hz), β-galactosyl. The presence of fucose was confirmed by the typical absorption at 1.66 ppm attributable to the C-CH_3 of 6-deoxy sugars. The two upfield absorptions of N-acetyl groups at 2.54 and 2.55 ppm reflect the difference in the two N-acetylglucosamine residues of the hexasaccharide. It is of interest to note that the anomeric

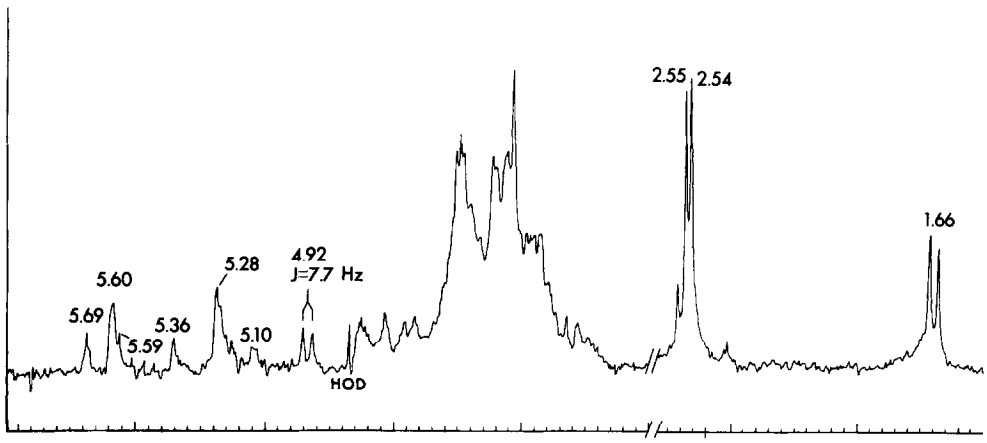

Figure 3. ^{1}H-n.m.r. spectrum of hexasaccharide I in D_2O at 70°. Absorptions in ppm.

I βGal 1→4 βGlcNAc 1→2 αMan 1→3/6 βMan 1→4 GlcNAc
αFuc 1↗3

II βGal 1→4 βGlcNAc 1→2 αMan 1→3/6 βMan 1→4 βGlcNAc 1→4 βGlcNAc-Asn.
αFuc 1↗3 αFuc 1↗3/6

P βGal 1→4 βGlcNAc 1→2 αMan 1→3/6 βMan 1→4 GlcNAc.

Figure 4. Structures of oligosaccharides and glycopeptides.

absorption at 5.10 ppm attributed to the non-reducing N-acetylglucosaminyl residue is more downfield than the 4-linked N-acetylglucosaminyl absorption in the G_{M1}-gangliosidosis pentasaccharide and octasaccharide at 5.06 ppm (14,18). Such deshielding is no doubt the result of additional substitution at the neighboring carbon 3 position of the non-reducing N-acetylglucosaminyl residue in the hexasaccharide I by fucose. From the ^{1}H-n.m.r. spectroscopy and permethylation analyses data, it can be concluded that the hexasaccharide I has the structure shown in Figure 4.

The constituents of the slower moving compound II on the t.l.c. (Fig. 1) were similar to those of the hexasaccharide I, except for the presence of aspartic acid. Its composition was, however, different; fucose, galactose, N-acetylglucosamine and mannose were in the approximate molar ratios of 2.1 : 1.0 : 2.6 : 2.0, respectively. Furthermore, no reducing N-acetylglucosamine was found; thus compound II appeared to be a glycopeptide with a glcNAc-Asn residue. Both the ^{1}H-n.m.r. spectroscopic and permethylation analyses suggest that compound II was an analogue of the hexasaccharide I. The ^{1}H-n.m.r. spectrum of II showed the same anomeric absorptions as those present in the spectrum of I. However, the signal at 5.69 ppm was absent, confirming the absence of a reducing N-acetylglucosamine residue. Around the 2.55 ppm region (N-acetyl group) of the spectrum, the signals were also more complex, suggesting the presence of different N-acetylglucosamine residues. The permethylation analyses of compound II confirmed these observations. Thus, although the g.l.c. pattern obtained from compound

II was similar to that from compound I, there were important differences in the N-acetylglucosaminyl residues at higher retention times on the g.l.c. In addition to the 4-linked and 3,4-linked N-acetylglucosamine residues present in the hexasaccharide I, compound II also contained a 4,6-linked residue, which was identified by its mass spectrum, shown in Fig. 2b. Another significant difference in compound II was that the 4-linked residue was glycosidically linked, whereas it was at the reducing terminus in the hexasaccharide I.

From the ^{1}H-n.m.r. and permethylation analyses of both compounds I and II, it can be concluded that they are structurally related as shown in Figure 4.

Discussion

The absence of the lysosomal enzyme α-L-fucosidase in patients with fucosidosis leads to the accumulation of fuco-sphingolipids in tissues (3). Invariably, fucose was found to be at the non-reducing terminal and linked α1→2 to galactose. Several urinary oligosaccharides and glycopeptides have also been isolated from fucosidosis patients. In all of the reported structures of these compounds, the residues at the non-reducing termini are α-L-fucosyl moieties, which are glycosidically linked to galactosyl and/or to 4-linked N-acetylglucosaminyl-asparagine residues (3-8). In the present study, however, both galactose and fucose were found to be at the non-reducing termini of the compounds. The α-L-fucose was only linked to 4-linked N-acetylglucosaminyl residues: in compound I it is linked α1→3 to N-acetyl-lactosaminyl residue whereas in compound II, an additional fucose is linked α1→3 and 1→6 to N-acetylglucosaminyl asparagine. The excretion of these compounds in the urine of this French Canadian patient afflicted with fucosidosis is most likely the result of an incomplete catabolism of human plasma α_1 acid glycoproteins. These have recently been shown to contain sequences such as αfuc (1→3) [βgal (1→4)] βglcNAc as part of their structures (19). The presence of a fucose residue linked

α1→3 to N-acetylglucosamine of the N-acetyllactosaminyl structure appears to be a factor in the inhibition of the action of β-galactosidase on such compounds (20). Thus, in the compounds excreted in the urine of this patient, both galactosyl and fucosyl residues are present at the non-reducing termini, although only α-L-fucosidase was deficient and the level of β-galactosidase normal. The compounds we have so far identified in this patient possess the linear dimannosyl N-acetylglucosaminyl type structure, similar to the type found in mannosidosis (21), in smaller proportions in G_{M2}-gangliosidosis (22), in G_{M1}-gangliosidosis (14,23), and more recently in sialidosis (24). However, we have been unable to detect any compound analogous to those with a trimannosyl branch core shown to be present in major proportions in G_{M1}-gangliosidosis (18), G_{M2}-gangliosidosis (25), and more recently, in the brain of a fucosidosis patient (5). It is possible that in the present patient an active endo-α-mannosidase (23) is present, but there is no evidence for the existence of such an enzyme.

Addendum

After this manuscript was submitted for publication, the article by Nishigaki et al. (26) describing the structures of several oligosaccharides excreted in the urine of a 2-year old female patient with fucosidosis was brought to our attention. The novel hexasaccharide I, with minor heterogeneities in the mannosyl linkages described in our paper, was also found to be present in the Japanese patient. In addition, the authors noted that more than two-thirds of the fucosyl-containing compounds were in the form of glycopeptides. It is most likely that some of these glycopeptides have structures similar to the isomeric glycopeptides II described in our paper.

The excretion of oligosaccharide I, with N-acetylglucosamine at the reducing terminal, indicates the presence of an active endo-β-N-acetylglucosaminidase. However, the kinetics of this reaction are probably affected by substitution of the chitobiosyl unit by a fucosyl molecule, as is seen by the large ratio of glycopeptide to reducing oligosaccharide excreted by

patients with fucosidosis (7,8,26). Substitution of the 4-linked N-acetylglucosaminyl residue at the neighboring C-3 position appears to exert a more pronounced inhibition by steric and possibly electronic effects, than at the C-6 position. An analogous situation regarding the lack of exo-β-galactosidase activity on C-3 substituted N-acetyllactosaminyl unit in both compounds I and II is discussed above. The glycopeptide αfuc 1→6 glcNAc-Asn which is a major compound excreted by fucosidosis patients (5-8) would support this view. It is probably formed as a result of the action of endo-β-N-acetylglucosaminidase on glycopeptides containing the βglcNAc 1→4 [αfuc (1→6)] βglcNAc-Asn type structures, such as one of the isomers of compound II.

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