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Isolation and ^{1}H-NMR Spectroscopic Identification of the Glucose-containing Lipid-linked Precursor Oligosaccharide of *N*-Linked Carbohydrate Chains

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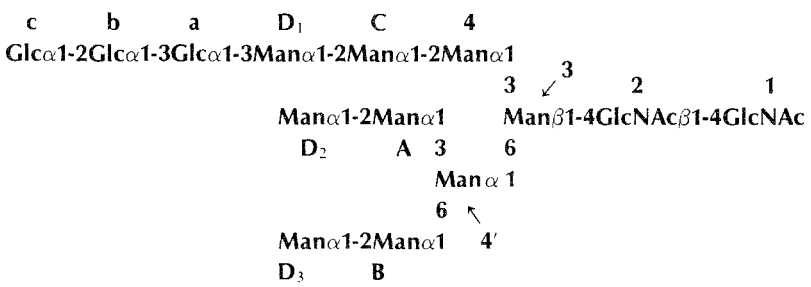
The lipid-linked precursor of *N*-type glycoprotein oligosaccharides was isolated from porcine thyroid microsomes after incubation with UDP[^{3}H]Glucose. The carbohydrate was released from dolichol pyrophosphate by mild acid hydrolysis, purified by gel filtration and characterized by 500-MHz ^{1}H-NMR spectroscopy in combination with enzymatic degradation. The parent oligosaccharide was found to be $Glc_3Man_9GlcNAc_2$. The three glucose residues are present in the linear sequence Glcα1-2Glcα1-3 Glc, the latter being α(1-3)-linked to one of the mannose residues. In order to establish the branch location of the triglucosyl unit, the parent compound was digested with jack-bean α-mannosidase. The oligosaccharide product was purified by gel filtration, and identified by ^{1}H-NMR as $Glc_3Man_5GlcNAc_2$ lacking the mannose residues A, D_2, B and D_3. Therefore, the structure of the precursor oligosaccharide is as follows:



Abbreviations: Man, D-mannose; GlcNAc, *N*-acetyl-D-glucosamine; Glc, D-glucose; NMR, nuclear magnetic resonance.

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The biosynthesis of *N*-linked carbohydrate chains in eukaryotic glycoproteins is initiated by the transfer of an oligosaccharide containing two *N*-acetylglucosamine, nine mannose and three glucose residues, from dolichol pyrophosphate to asparagine residues forming part of the Asn-Xaa-Ser(Thr) signal sequence in the protein [1, 2]. The protein-bound oligosaccharide then undergoes a number of processing steps starting with the removal of glucose residues [3].

The primary structure of the biosynthetically labelled precursor oligosaccharide, $Glc_3Man_9GlcNAc_2$, either bound to lipid [1] or to protein [4] was partly characterized in the late 1970's by chemical methods, *i.e.* by methylation analysis and periodate oxidation, in combination with sequential exoglycosidase treatment. Recently, renewed interest in the $Glc_3Man_9GlcNAc_2$ compound originated from structural studies on the glycoproteins from yeast mutants [5] and from other low-evolutionary organisms e.g. *Asteria rubens* [6]. These glycoproteins may possess unprocessed, Glc_3-containing carbohydrate chains or, alternatively, chains retaining one or two glucose residues, while some mannose-trimming may have already occurred. The structure of these glucose-containing protein-bound oligosaccharide chains has been unambiguously established by modern techniques, especially by 500-MHz ^{1}H-NMR spectroscopy [5, 6]. In those glycoproteins, glucose residues were found to be arranged in a linear sequence, Glcα1-2Glcα1-3Glc, attached in α(1-3)-linkage to the Manα1-2Manα1-2Manα1-3 branch (D_1-C-4). The reason why the yeast and *Asteria* oligosaccharides are not trimmed down to regular, agluco oligomannoside-type structures is still unclear. To investigate if glucose may be arranged in different sequences on lipids and proteins, we decided to isolate the mammalian, lipid-linked precursor oligosaccharide from porcine thyroid tissue, and to reinvestigate its structure by 500-MHz ^{1}H-NMR spectroscopy.

Materials and Methods

Crude microsomes (2.76 g protein) prepared daily from porcine thyroid glands (600 g of tissue) were incubated with 6 μM UDP-[^{3}H]glucose (300 μCi, 3.1 Ci/mmol, Amersham, UK) for 10 min at 37°C, in a total volume of 75 ml, as previously described [7, 8]. The ^{3}H-labelled oligosaccharide-lipids were isolated by sequential delipidation and final extraction with chloroform/methanol/water, 10/10/3 by vol. 21 $\times$ 10^6 Dpm of oligosaccharide-lipid were obtained, corresponding to 0.5 nmol of orcinol/sulfuric acid positive material per gram of tissue.

The lipid extract was further purified on DEAE-cellulose (3 $\times$ 40 cm, acetate form) (Pharmacia, Sweden), equilibrated in chloroform/methanol/water, 10/10/3 by vol. The column was extensively washed with the developing solvent, followed by 1 mM ammonium acetate in this solvent before oligosaccharide-lipids were finally eluted with 75 mM ammonium acetate in the same solvent (79% recovery). The ^{3}H-oligosaccharides were then released from the lipid carrier by 0.02 M HCl in 50% propanol (0.5 ml, 35 $\times$ 10^4 dpm), for 30 min at 100°C (82% recovery). Purification of the glucose-labeled oligosaccharide was accomplished by filtration on Bio-Gel P-6 (200-400 mesh, Bio-Rad, Richmond, CA, USA) 1.5 $\times$ 150 cm column in 0.1 M ammonium bicarbonate (52% recovery). The overall yield of the purification was 33% (7 $\times$ 10^6 dpm), corresponding to 35-40 nmol of oligosaccharide.

After desalting the oligosaccharide on a column (1.5 $\times$ 100 cm) of Bio-Gel P-2 (Bio-Rad, elution with water), it was repeatedly exchanged in ^2H_2O (99.96 atom % ^{2}H, Aldrich,

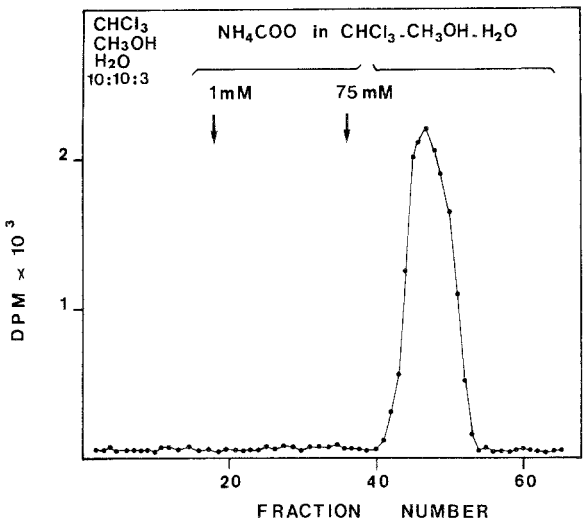


Figure 1. DEAE-cellulose chromatography in chloroform/methanol/water, 10/10/3 by vol, of ^{3}H-labelled oligosaccharide-lipids. Arrows indicate changes in ammonium acetate concentration in the solvent.

Milwaukee WI, USA) with intermediate lyophilization, and then subjected to ^{1}H-NMR spectroscopy.

After recording the ^{1}H-NMR spectrum, the ^{3}H-labelled oligosaccharide was incubated with α-mannosidase (50 mU) from jack bean (Sigma, St. Louis, MO, USA), overnight at 37°C, in a 50 mM sodium acetoacetic acid buffer pH 4.6. The digestion was stopped by partitioning with chloroform/methanol. The digest was passed over a column (2 × 100 cm) of Bio-Gel P-4 (< 400 mesh, Bio-Rad) at 55°C, eluted with water. Fractions of 1.5 ml were collected and monitored for radioactivity. The resulting ^{3}H-labelled oligosaccharide was prepared for ^{1}H-NMR spectroscopy as mentioned above.

^{1}H-NMR Spectroscopy was performed at 500 MHz, on a Bruker WM-500 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen, The Netherlands). The spectra were recorded at 37°C or at 42°C. Chemical shifts (δ) are expressed in ppm downfield from internal 4,4-dimethyl-4-silapentanesulfonate (measured indirectly to internal acetone at δ 2.225)[9].

Results and Discussion

As reported previously [7], the ^{3}H-glucose labelled material extracted by chloroform/methanol/water, 10/10/3 by vol, behaved on DEAE-cellulose as glyco-diphospho-lipids. No [^{3}H-]labelled neutral glycolipids, nor any contamination by Dol-P-[^{3}H-]Glc which would have eluted with 1 mM ammonium acetate in chloroform/methanol/water, 10/10/3 by vol, could be detected (Fig. 1). Bio-Gel P-6 filtration of the mild acid hydrolysate showed that the [^{3}H-]labelled oligosaccharide is of relatively high molecular mass, and essentially homogeneous in size (Fig. 2). The lipid material obtained after acid hydrolysis has been characterized by HPLC as dolichol pyrophosphate (C. Ronin and F. W. Hemming, unpublished results). Also a single spot was obtained when this compound was analyzed by paper chromatography (data not shown).

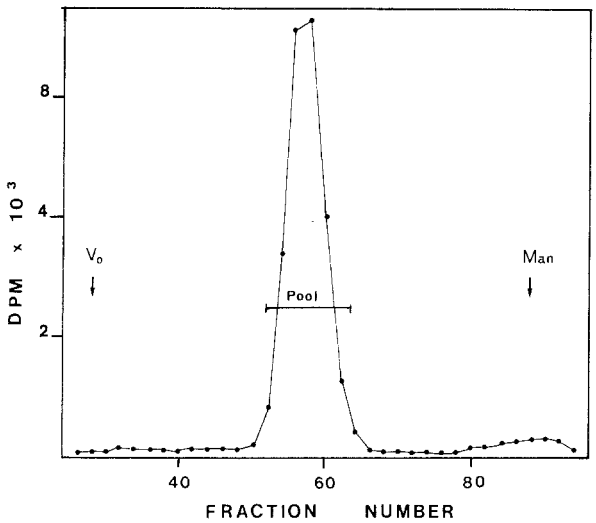


Figure 2. Bio-Gel P-6 filtration of the ^{3}H-labelled oligosaccharide obtained by mild acid hydrolysis of the DEAE-cellulose purified ^{3}H-labelled oligosaccharide lipids.

A 500-MHz ^{1}H-NMR spectrum of the ^{3}H-labelled oligosaccharide was recorded and the anomeric-proton region ($4.7 < \delta < 5.7$) is depicted in Fig. 3A. The chemical shift data (Table 1) showed that an oligomannoside type oligosaccharide is involved. The presence of the reducing *N,N'*-diacetylchitobiose unit is evident from the occurrence of the *N*-acetyl singlets at δ 2.039 (GlcNAc-**1** reducing) and 2.068 (GlcNAc-**2**) *cf.* [10, 11]. The H-1 signals of GlcNAc-**2** and of the β-anomer of GlcNAc-**1** are obscured by the HO^{2}H-line (δ = 4.66 at 37°C). The H-1 signal of the α-anomer of GlcNAc-**1** cannot be observed separately, therefore, it should coincide with one of the α-anomeric signals having $J_{1,2}$ 3.5 Hz, and is most probably found at δ 5.182. This is supported by the finding that in Manβ1-4GlcNAcβ1-4GlcNAc the H-1 of GlcNAc-**1** resonates at δ 5.189 (J. van Pelt, H. van Halbeek, J.F.G. Vliegenthart, O. van Diggelen and M.Z. Jones, unpublished results). The number and intensity of H-1 signals with $J_{1,2} \leqslant 1.5$ Hz (typical of mannose residues [9]) indicated (see Fig. 3A) that nine mannose residues are present in the oligosaccharide in equimolar amounts. Their chemical shifts were found to be very similar to those reported for Man$_9$GlcNAc$_2$Asn [9, 12] (see Table 1). Therefore, it may be concluded that the structures of the oligomannoside portions are identical. The three additional anomeric doublets observed in the $4.7 < \delta < 5.7$ spectral region (see Fig. 3A), namely, at δ 5.182 ($J_{1,2}$ = 3.8 Hz), δ 5.277 ($J_{1,2}$ = 4.1 Hz) and δ 5.533 ($J_{1,2}$ = 3.8 Hz), respectively, are attributed to the glucose residues. The intensity ratio of each of these doublets, relative to e.g. the Man-**4** H-1 signal at δ 5.334, is close to 1:1. The chemical shifts and coupling constants of the glucose H-1 signals indicate that these residues are α-glycosidically linked. The close resemblance of the chemical shifts values of the three glucose H-1 atoms with the corresponding values [13] (*c.f.* Table 1) for the synthetic tetra- (and hexa-) saccharides Glcα1-2Glcα1-3Glcα1-3Man[α1-2Manα1-2Man] strongly suggests the type of linkage in these compounds to be the same.

Comparison of the chemical shifts of the mannose H-1 signals for the porcine thyroid oligosaccharide and Man$_9$GlcNAc$_2$Asn (Table 1) indicates that there are not significant

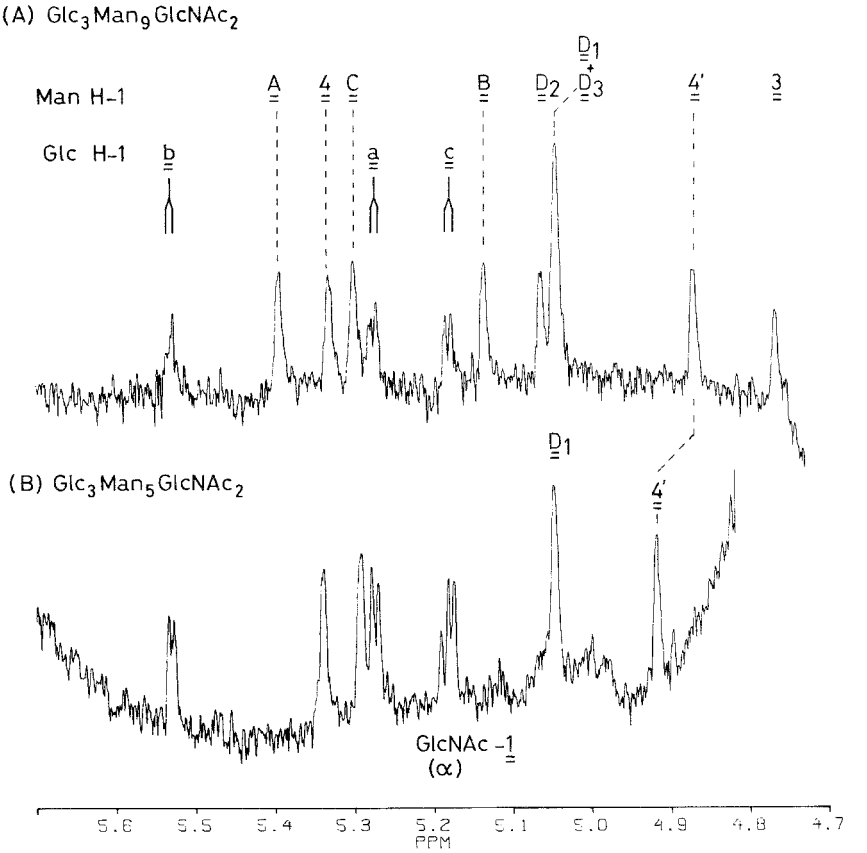


Figure 3. Anomeric proton region of the 500-MHz ^{1}H-NMR spectra of (A) $Glc_3Man_9GlcNAc_2$, the parent precursor oligosaccharide obtained from porcine thyroid gland; (B) $Glc_3Man_5GlcNAc_2$, α-mannosidase digested precursor oligosaccharide from porcine thyroid gland.

shift effects on these reporter groups upon attachment of the glucose residues. Thus, at that point, the branch location of the Glc_3 sequence was the only structural feature that could not be deduced straightforwardly. To identify the mannose residue to which the glucose residues are attached, the parent $Glc_3Man_9GlcNAc_2$ compound was subjected to α-mannosidase digestion. The resulting ^{3}H-labelled oligosaccharide fraction was purified from the incubation mixture by Bio-Gel P-4 filtration. The elution position of the tritium corresponded to that of a dextran-hydrolysate oligomer consisting of 11 units, thereby suggesting the composition of the cleavage product to be $Glc_3Man_{4-5}GlcNAc_2$. The relevant part of the ^{1}H-NMR spectrum of the mannosidase-digested oligosaccharide is depicted in Fig. 3B. From comparison with Fig. 3A it can readily be inferred that the H-1 signals stemming from Man-**A**, -$\mathbf{D}_2$, -**B** and -$\mathbf{D}_3$ have disappeared, whereas those of Man-**4**, -**C** and -$\mathbf{D}_1$ and those of the glucose residues, remained present at essentially unaltered positions. It should be noted that the H-1 signal of the α-anomer of GlcNAc-**1** is clearly visible at δ 5.179 [11] (relative intensity 0.5 proton). The H-1 signal of Man-**4'** has undergone a downfield shift, from δ 4.873 to δ 4.914, indicating that

Table 1. ^{1}H-Chemical shifts of pertinent structural-reporter groups of constituting monosaccharides for the glucose-containing precursor oligosaccharide from porcine thyroid, its α-mannosidase digestion product, and three reference compounds of related structure.

Reporter group	Residue	Chemical shift in[a]				
		$G_3M_9GN_2$	$G_3M_{10}GN$	M_9GN_2Asn	$G_3M_5GN_2$	G_3M_3
H-1	GlcNAc-**1**	4.76(β)[b] 5.182(α)	—	5.092(βN)	4.7 (β)[c] 5.179(α)	—
	GlcNAc-**2**	4.6[c]	5.24(α)	4.610	4.6[c]	—
	Man-**3**	4.769	4.77	4.77	4.764	—
	Man-**4**	5.334	5.33	5.334	5.336	5.38(α)
	Man-**C**	5.302	5.29	5.308	5.289	5.31
	Man-$\mathbf{D_1}$	5.046	5.04	5.049	5.044	5.05
	Man-**4'**	4.873	4.87	4.869	4.914	—
	Man-**A**	5.398	5.09(β) 5.11(α)	5.404	—	—
	Man-$\mathbf{D_2}$	5.065	—	5.061	—	—
	Man-**B**	5.137	5.12	5.143	—	—
	Man-$\mathbf{D_3}$	5.046	5.04	5.042	—	—
	Glc-**a**	5.277	5.27	—	5.270	5.28
	Glc-**b**	5.533	5.52	—	5.527	5.54
	Glc-**c**	5.182	5.17	—	5.174	5.20
NAc	GlcNAc-**1**	2.039(α,β)	—	2.015(βN)[d]	2.039(α,β)	—
	GlcNAc-**2**	2.068	n.d.[e]	2.067	2.076	—

[a] Data were acquired at 500 MHz for ^2H_2O solutions at 37°C for $Glc_3Man_9GlcNAc_2$, 42°C for $Glc_3Man_5GlcNAc_2$. Reference compound $Glc_3Man_{10}GlcNAc$ was investigated at 500 MHz and 40°C [5]. $Man_9GlcNAc_2Asn$ at 500 MHz and 27°C [11], and oligosaccharide Glc_3Man_3 at 400 MHz and 20°C [12]. Chemical shifts are expressed in ppm downfield from internal DSS (sodium 4,4-dimethyl-4-silapentane-1-sulfonate).

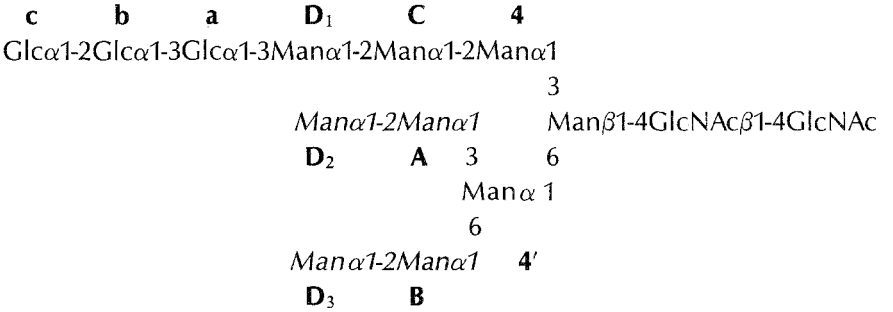
[b] (α) and (β) refer to the pertinent anomer of the reducing oligosaccharide.

[c] Signal obscured by HO^2H resonance at applied probe temperature. In the table heading compounds are denoted by their compositional formula; G=Glc, M=Man, GN=GlcNAc. For complete structures, including numbering system of residues, see text.

[d] (N) refers to the glycopeptide having GlcNAc-**1** β-linked to the side chain NH_2 of Asn.

[e] n.d., not determined.

this residue occupies a terminal position (*c.f.* [9]) in the resulting $Glc_3Man_5GlcNAc_2$ compound. Thus, it was proved that the Glc_3 moiety is attached to the Man-($\mathbf{D_1}$-**C-4**) branch. Since the chemical shifts of the Glc-(**a-b-c**) and Man-(**4-C-**$\mathbf{D_1}$) H-1 signals of the two thyroid oligosaccharides on the one hand, and of the synthetic hexasaccharide Glc_3Man_3 [13] and the yeast compound $Glc_3Man_{10}GlcNAc$ [5] on the other hand (see Table 1) are identical (obviously except for the Man-**4** compared to the reducing mannose in the α-hexasaccharide), we conclude that the structure of the parent and the mannosidase-degraded oligosaccharide from porcine thyroid are as follows:



The residues in italics are sensitive to jack bean α-mannosidase.

The assignment of the glucose anomeric doublets (Table 1) is according to [5, 13].

In conclusion, the lipid linked precursor oligosaccharide from thyroid appears to be identical in its triglucosylated branch with the recently characterized $Glc_3Man_xGlcNAc_2$ moieties of yeast mannan [5] and *Asteria rubens* [6]. It should be noted that determination of the branch location of the triglucosyl sequence in the intact oligosaccharide by means of ^{1}H-NMR spectroscopy was hampered by the lack of significant effects on the mannose structural-reporter groups upon attachment of Glc_3 in α1-3 linkage to a Man-**D** residue. Conclusive evidence could, however, be obtained after α-mannosidase degradation. Therefore, the present strategy based on purification with the help of radiolabeling followed by enzymatic digestion monitored by ^{1}H-NMR spectroscopy may be of potential interest to investigate future carbohydrate structures available in very limited amounts.

Acknowledgements

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