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A Novel Type of Carbohydrate Structure Present in Hen Ovomucoid*

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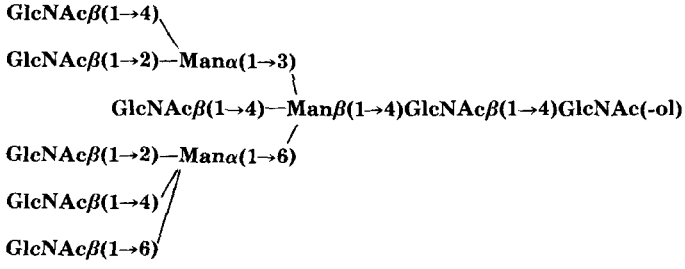
Hen ovomucoid is characterized by a high degree of microheterogeneity of its carbohydrate moieties, as was recently demonstrated by hydrazinolysis in combination with high performance liquid chromatography (Paz Parente, J., Strecker, G., Leroy, Y., Montreuil, J., and Fournet, B. (1982) *J. Chromatogr.*, in press). This approach resulted in 17 oligosaccharide-alditol fractions; the major one could be purified to homogeneity. Primary structural analysis of this fraction was carried out by methylation analysis, partial acid hydrolysis, and 500-MHz ^{1}H NMR spectroscopy. Combination of these techniques enabled the unambiguous determination of a novel type of asparagine-bound carbohydrate chain:



Hydrazinolysis of glycoproteins allows one to obtain mixtures of oligosaccharides which are easily separated by HPLC.[1] In this way, Mellis and Baenziger (1) developed a method for the separation of neutral oligosaccharides obtained by hydrazinolysis from desialylated orosomucoid by high performance liquid chromatography utilizing a Micropak AX-5 ion exchange column. Recently, the hydrazinolysis products of the neutral, β-glycopeptide fraction from hen ovomucoid were subjected to HPLC; this afforded 17 oligosaccharide-alditol fractions (2). The aim of the present study is the elucidation of the primary structure of the major one among these oligosaccharide-alditols by employment of methylation analysis, partial acid hydrolysis, mass spectrometry, and 500-MHz ^{1}H NMR spectroscopy.

EXPERIMENTAL PROCEDURES

Ovomucoid was prepared according to Fredericq and Deutsch (3). The asialoglycopeptide, designated β-glycopeptide, was isolated after pronase digestion of ovomucoid according to Monsigny *et al.* (4) and the oligosaccharides of the β-glycopeptide were released by hydrazinolysis (5). These oligosaccharides were re-*N*-acetylated (6) and then reduced with $NaBH_4$. The resulting oligosaccharide-alditols were fractionated by semipreparative HPLC on Amino AS.5A (5 μm, Touzard et Matignon) (2). The major fraction *11* was further analyzed.

The carbohydrate composition of the oligosaccharide alditol fraction *11* was determined by GLC after methanolysis (0.5 M HCl/methanol; 24 h; 80 °C) and pertrifluoroacetylation (7).

1 mg of oligosaccharide-alditol fraction *11* was methylated according to Finne *et al.* (8). The permethyloligosaccharide-alditol was methanolysed and the products were identified by GLC-MS (9) after peracetylation.

The permethylated oligosaccharide-alditol fraction *11* (1.2 mg) was submitted to partial acid hydrolysis (formic acid 85%; 30 min; 80 °C) followed by reduction with $NaBD_4$ (0.2 M) and ethylation of the free hydroxyl groups (10). The partially methylated, ethylated products were analyzed by GLC-MS on a capillary column (0.4 mm × 60 m) of OV 101 (temperature programmed 5 °C/min from 140 to 320 °C; helium pressure, 0.4 bar).

Prior to ^{1}H NMR spectral analysis, 1 mg of oligosaccharide-alditol fraction *11* was repeatedly exchanged in D_2O (99.96 atom % D, Aldrich) with intermediate lyophilization. ^{1}H NMR spectroscopic analysis was performed on a Bruker WM-500 spectrometer operating at 500 MHz in the Fourier transform mode at a probe temperature of 300 K (11). Chemical shifts are given relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (indirectly to acetone in D_2O:δ = 2.225 ppm).

RESULTS AND DISCUSSION

The separation of the oligosaccharide-alditols from β-glycopeptide of hen ovomucoid by semipreparative HPLC is illustrated in Fig. 1. The major (~15%) oligosaccharide-alditol fraction *11* is composed of galactose, mannose, *N*-acetylglucosamine, and *N*-acetylglucosaminitol with molar ratios 0.19, 3, 6.8, and 1.01, respectively. Mannose has been taken as 3. The GlcNAc+GlcNAc-ol/Man ratio is 2.6. A similar, relatively high value of the ratio total GlcNAc:Man, being 2.6, has been found in glycopeptides derived from turtledove ovomucoid (12).

The results of methylation analysis of fraction *11* (see Table I) reveal the presence of two different mono-*O*-methylmannosides in equal amounts: methyl 2-mono-*O*-methylmannoside and methyl 3-mono-*O*-methylmannoside. These derivatives were identified by GLC-MS (9, 12, 13). Assuming the presence of a mannotriose core structure (14), the identifica-

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[1] The abbreviations used are: HPLC, high performance liquid chromatography; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography combined with mass spectrometry.

tion of the 2-mono-*O*-methyl-3,4,6-tri-*O*-acetylmannoside suggests the occurrence of an intersecting GlcNAc residue, $\beta(1\rightarrow4)$ linked to Man **3**. The 3-mono-*O*-methyl-2,4,6-tri-*O*-acetylmannoside points to a trisubstitution either of Man **4** or of Man **4'**. Discrimination between these two possibilities was achieved by partial acid hydrolysis of the permethylated oligosaccharide-alditol *11*, followed by identification of the resulting partially ethylated, methylated disaccharide-alditols, Man → Man-ol. The mass spectra of the partially ethylated methylated Man-(1→6)-Man-ol and Man-(1→3)-Man-ol are given in Fig. 2. The structure of the disaccharide-alditol 2,4,6-tri-*O*-ethyl-3-mono-*O*-methylmannopyranosido-(1→6)-1,3,4,5-tetra-*O*-ethyl-2-mono-*O*-methylmannitol (Fig. 2*A*) is characterized by the aA fragments: aA_1 *m/z* 261, aA_2 *m/z* 229 and 215, which show the substitution pattern of the nonreducing hexose (mannose) residue, and the alditol fragment, ald *m/z* 292. The presence of an ald J_1 ion, *m/z* 352, is due to the occurrence of a methyl group at C-3 of the nonreducing hexose, corresponding to 3-mono-*O*-methyl-Man. The fragments *m/z* 220 and 407 allow us to confirm the (1→6) linkage between mannose and mannitol. The occurrence of the ion *m/z* 104 indicates that the mannitol residue is substituted by a methyl group at C-2 (*m/z* 104, $C_2H_5O\underset{1}{C}HD—\underset{2}{C}H=\overset{+}{O}CH_3$). The structure of the disaccharide-alditol 2,4-di-*O*-ethyl-3,6-di-*O*-methylmannopyranosido-(1→3)-1,4,5,6-tetra-*O*-ethyl-2-mono-*O*-methylmannitol (Fig. 2*B*) is confirmed by the aA fragments aA_1 *m/z* 247, aA_2, 215 and 201, reflecting the substitution at the nonreducing hexose, and the alditol fragment, ald *m/z* 292. The fragment *m/z* 175, in combination with 129 (elimination of ethanol), suggests a (1→3) glycosidic linkage in this disaccharide-alditol. These results lead to the conclusion that the (1→3)-linked core Man (**4**) bears 2 GlcNAc residues, namely in (1→2)-linkage and in

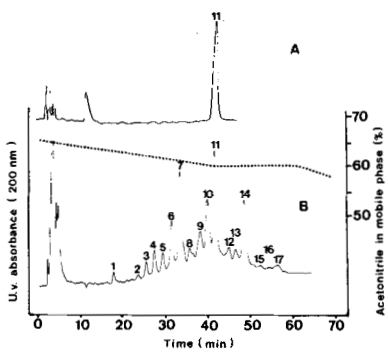


FIG. 1. **Chromatographic analysis of oligosaccharide *11* (*A*) obtained by semipreparative high performance liquid chromatography of oligosaccharides obtained by hydrazinolysis of hen ovomucoid neutral glycopeptide and (*B*) on 5 μM Amino AS-5A (Touzard and Matignon).**

TABLE I

Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated glycopeptides derived from oligosaccharide 11

Methylated monosaccharides	Molar ratio
	mol/mol oligosaccharide
3,6-di-OMe-Man[a]	1 (0.88)
2-mono-OMe-Man	1 (1.2)
3-mono-OMe-Man	1
3,4,6-tri-OMe-Glc-NAcNMe	6 (5.8)
3,6-di-OMe-Glc-NAcNMe	1 (0.90)
1,3,5,6-tetra-OMe-Glc-ol-NAcNMe	1 (0.93)

[a] OMe, *O*-methyl; NAcNMe, acetyl-*N*-methyl.

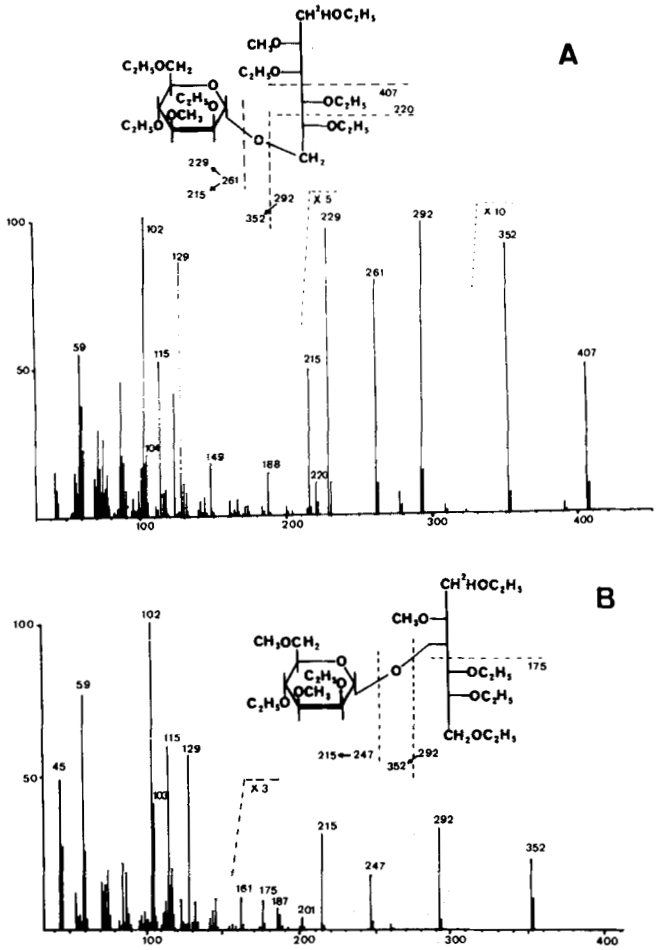


FIG. 2. **Mass spectra of 3-*O*-Me-2,4,6-*O*-Et-Man-(1 → 6)-2-*O*-Me-1,3,4,5-*O*-Et-Man-ol (*A*) and of 3,6-*O*-Me-2,4-*O*-Et-Man-(1 → 3)-2-*O*-Me-1,4,5,6-*O*-Et-Man-ol (*B*).**

(1→4)-linkage (GlcNAc **5** and **7**, respectively), while the (1→6)-linked Man residue (**4'**) is trisubstituted by GlcNAc residues, namely in (1→**2**)-linkage by GlcNAc **5'**, in (1→6)-linkage by GlcNAc **7'**, and in (1→4)-linkage by GlcNAc **10'**.

In order to obtain independent proof of the structure of fraction *11*, the sample was subjected to 500-MHz ^1H NMR spectroscopy. Pertinent regions of the resolution-enhanced spectrum are depicted in Fig. 3. For the interpretation of the spectrum in terms of primary structural assignments, advantage was taken from the spectral data for a glycopeptide from chicken ovotransferrin (15, 16) and from those for another oligosaccharide-alditol fraction (*7*) from hen ovomucoid (2). The chemical shifts of the structural reporter groups (11, 16) of fraction *11* and of the two reference substances are compiled in Table II.

Comparison of the data for the ovotransferrin glycopeptide with those for ovomucoid fraction *7* demonstrates the identical branching pattern of the mannotriose core, *i.e.* substitution by GlcNAc **7**, **5**, **9**, and **5'**, in both structures. Apparently, the spectral features of the peripheral residues are not affected by the transformation of GlcNAc→Asn into GlcNAc-ol (see Table II) thereby making this type of reduced oligosaccharide suitable compounds for ^1H NMR investigation.

The presence of the GlcNAcβ(1→4)GlcNAc-ol structural element in *11* comes to expression in: (i) the occurrence of a GlcNAc-ol H-2 signal at $\delta = 4.255$ ppm in the region of the Man H-2 signals; and (ii) an *N*-acetyl signal of GlcNAc-ol at $2.05 < \delta < 2.07$ ppm, instead of $2.00 < \delta < 2.01$ ppm as usual for GlcNAc **1** β-linked to Asn (16). Furthermore, the chemical

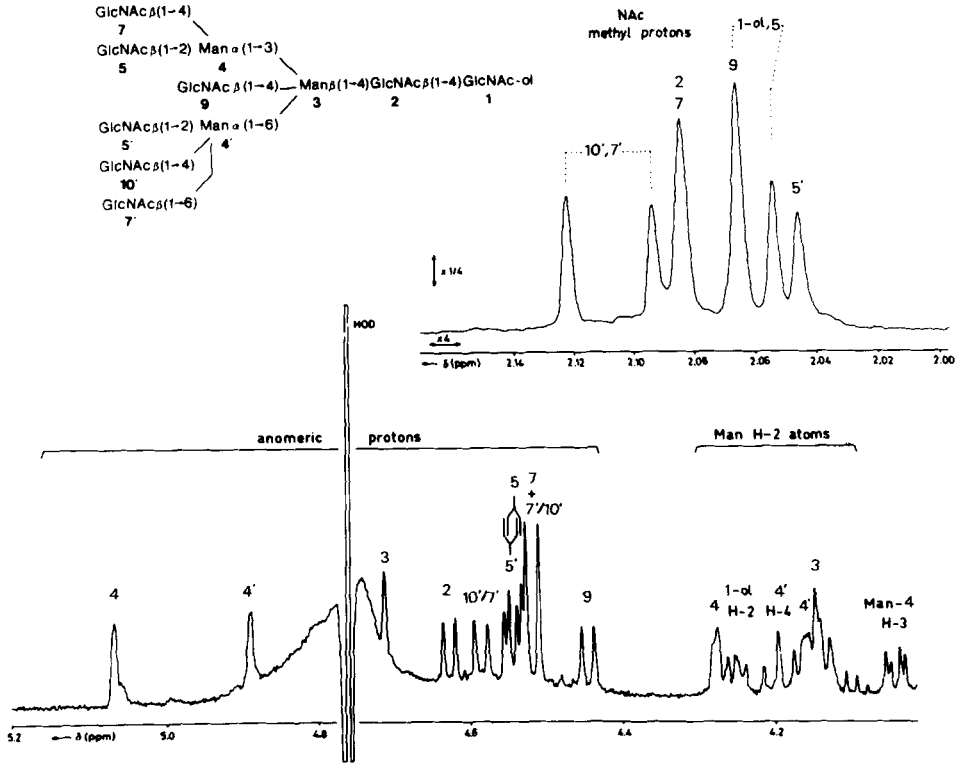


FIG. 3. **Structural reporter group regions of the resolution-enhanced, 500 MHz ^{1}H NMR spectrum of oligosaccharide-alditol *11* derived from hen β-ovomucoid, in D_2O at 300 K.** The *numbers in the spectrum* refer to the corresponding residues in the structure. The relative intensity scale, as well as the chemical shift scale, of the *N*-acetyl proton region (see *inset*) differ from those of the other part of the spectrum, as indicated.

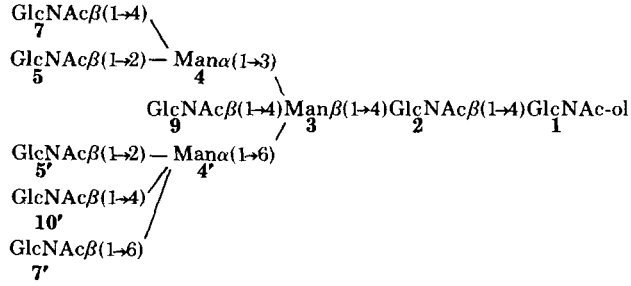
shifts of H-1 and of the *N*-acetyl signal of GlcNAc **2** are slightly but significantly altered as compared to the values for a glycopeptide (see Table II).

The presence of the mannotriose core is clearly deducible from the occurrence of three Man H-1 signals having $4.7 < \delta < 5.1$ ppm and $J_{1,2} < 2$ Hz, and of three corresponding H-2 signals in the typical spectral region $4.0 < \delta < 4.3$ ppm. The core is extended at the peripheral side with six GlcNAc residues in β-linkage, because the spectrum reveals 7 anomeric doublets ($4.4 < \delta < 4.7$ ppm; $J_{1,2} > 8$ Hz), one of which belongs to GlcNAc **2** of the core. This conclusion is corroborated by the presence of 8 *N*-acetyl signals, two of which are attributed to GlcNAc **2** and GlcNAc-ol.

The H-1 signal at rather high field position ($\delta = 4.443$ ppm), together with one of the *N*-acetyl signals at $\delta = 2.065$ ppm, points to the presence of the intersecting GlcNAc **9** (compare with the ovotransferrin glycopeptide, Table II, and with related substances (16, 17)). This conclusion is supported by the chemical shifts of H-1 and H-2 of Man **4** (see Table II, compare with *7*), which further indicate the branching pattern of Man **4** to be as in triantennary structures, that is, with GlcNAc **5** in β(1→2)- linkage and GlcNAc **7** in β(1→4)-linkage. The Man **4** H-3 signal, which is a reporter group particularly for this type of disubstitution of Man **4** (16), is found at $\delta = 4.043$ ppm (see Fig. 3), corroborating the presence of this structural element in the upper branch of *11*.

The predominant differences in chemical shift between the reporter groups of fractions *11* and *7* are observed for H-1 and H-2 of Man **4'**. The set of values for *11* (δH-1 = 4.889 ppm; δH-2 = 4.161 ppm) has not been found, so far, and reflects the trisubstitution of Man **4'**. It is well known (16) that the attachment of GlcNAc in β(1→4)-linkage to an α-Man residue (**4**) does not influence the chemical shift of H-1 of this Man, but only gives rise to a downfield shift for Man H-2 ($\Delta\delta \simeq 0.03$ ppm). Introduction of GlcNAc in β(1→6)-linkage to an α-Man residue (**4'**) results in upfield shifts both for H-1 ($\Delta\delta \simeq -0.07$ ppm) and for H-2 ($\Delta\delta \simeq -0.02$ ppm) of the α-Man (16). In the step from *7* to *11* (see Table II), for H-1 of Man **4'**, an upfield shift is observed ($\Delta\delta = -0.11$ ppm) while H-2 shifts downfield ($\Delta\delta = 0.015$ ppm). The directions of the shift effects on Man **4'** H-1 and H-2 are in line with the 2,4,6-trisubstitution of Man **4'**, revealed by methylation analysis in combination with partial acid hydrolysis. However, due to steric hindrance of the residues **5'**, **7'**, and **10'**, precise values for the chemical shifts of H-1 and H-2 of Man **4'** cannot be deduced by starting at the data of *7*, and simply adding the aforementioned effects of attachment of β(1→4)-linked and β(1→6)-linked GlcNAc to these. Tentative assignments for the H-1 doublets and the *N*-acetyl singlets of GlcNAc residues **5'**, **7'**, and **10'** are indicated in Table II and Fig. 3.

In summary, the 500-MHz ^{1}H NMR spectrum clearly shows the detailed branching pattern of the mannotriose core in fraction *11* from hen β-ovomucoid. Owing to the trisubstitution of Man **4'**, its H-4 signal emerges out of the bulk of the skeleton protons toward $\delta = 4.194$ ppm; this signal can be conceived as a structural reporter group for this particular branching pattern. The differences in the chemical shifts of most of the structural reporter groups of *11*, as compared to *7* (see Table II) suggest a considerable change of spatial structure by the introduction of GlcNAc residues **7'** and **10'**. The structure of oligosaccharide-alditol *11* appears to be as follows:



This structure is a novel type of glycoprotein carbohydrate chain. The simultaneous presence of GlcNAc residues **7**, **5**, **5'**, and **9** in one structure had been described for chicken ovotransferrin (15, 16). The presence of Man **4'**, trisubstituted as

TABLE II

^1H chemical shifts of structural reporter groups of constituent monosaccharides for two oligosaccharide-alditol fractions (7 and 11) from hen β-ovomucoid and for a glycopeptide from chicken ovotransferrin (15,16)

Reporter group	Residue	Chemical shift[a] in: Ovotransferrin	Ovomucoid fraction 7	Ovomucoid fraction 11
			ppm	
H-1 of	**1**	5.056		
	2	4.613	4.632	4.626
	3	4.687	4.696	4.712
	4	5.057	5.057	5.067
	4′	4.998	4.999	4.889
	5	4.537	4.540	4.539
	5′	4.545	4.543	4.545
	7	4.520	4.516	4.517
	7′			4.517[b]
	9	4.463	4.464	4.443
	10′			4.583[b]
H-2 of	**1-ol**		4.246	4.255
	3	4.146	4.146	4.145
	4	4.282	4.284	4.276
	4′	4.141	4.146	4.161
NAc of	**1**	2.008		
	1-ol		2.055	2.065[c]
	2	2.077	2.079	2.084
	5	2.057	2.055	2.054[c]
	5′	2.048	2.048	2.045
	7	2.082	2.083	2.084
	7′			2.093[d]
	9	2.063	2.064	2.065
	10′			2.122[d]

[a] Chemical shifts were acquired at 500 MHz; they are given in parts/million downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate in D_2O at 300 K. Compounds are represented by schematic structures (*cf.* (16)): ○— = GlcNAc; ◇— = Man. For complete structures and numbering of residues, see Fig. 3.

[b,c,d] Assignments are interchangeable.

shown above, once has been suggested for a glycopeptide mixture from turtledove ovomucoid (12). However, by HPLC it turned out to be possible to purify such a penta-antennary structure with an additional intersecting GlcNAc residue to homogeneity, thereby permitting rapid and reliable structural elucidation by combination of permethylation analysis, partial acid hydrolysis, and 500-MHz ^1H NMR spectroscopy.

The first structure of ovomucoid glycan was proposed by Stacey and Wooley (18), in 1940–1942, and was described by the authors as follows: "By glycosidic attachment, seven *N*-acetylglucosamine units radiate from a central core of three D-mannose units. It is considered that this compound does not represent a repeating unit but rather that it depicts the whole molecule as being that of a hen decasaccharide. In regard to the structure of ovomucoid itself, it would appear that the peptide constituents are mainly attached to the *N*-acetyl-D-glucosamine terminal residues." Reading these sentences, it is amazing that they still remain true 40 years later.

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