Primary structure of a novel *N*-glycosidic carbohydrate unit, derived from hen ovomucoid

A 500-MHz ^{1}H-NMR study

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The *N*-glycosidic carbohydrate chains of hen *β*-ovomucoid were released from the protein by hydrazinolysis, and separated by HPLC. Primary structural analysis of 3 major fractions was conducted by applying 500-MHz ^{1}H-NMR spectroscopy in combination with methylation analysis. One of the fractions investigated appeared to consist of an intersected penta-antennary structure extended with one Gal residue. The location of the latter in a certain branch could be established unambiguously by NMR. This structure is a novel member of the family of *N*-glycosidic carbohydrates of glycoproteins.

Ovomucoid *Carbohydrate structure* *(Micro)heterogeneity* *Hydrazinolysis* *HPLC* *^{1}H-NMR*

1. INTRODUCTION

In [1] we have described the separation by HPLC of 17 oligosaccharides which were released from hen ovomucoid by hydrazinolysis. Recently, the primary-structural analysis of oligosaccharide *11*, the most abundant one, was carried out by methylation analysis, partial acid hydrolysis and 500-MHz ^{1}H-NMR spectroscopy [2]. Combination of these techniques enabled to connect unambiguously the primary structure of oligosaccharide *11* to the novel type of pentaantennary *N*-glycosidic glycans discovered in turtle-dove ovomucoid [3,4].

Here, we report on the primary structure of oligosaccharides *1*, *7* and *14*, determined by application of 500-MHz ^{1}H-NMR spectroscopy in combination with methylation analysis.

2. MATERIALS AND METHODS

Hen ovomucoid was prepared as in [5]. After pronase digestion, the asialo glycopeptide designated '*β*-glycopeptide' was isolated as in [6]. Oligosaccharides were released from the *β*-glycopeptide by hydrazinolysis [7]. Subsequently they were re-*N*-acetylated [8] and reduced with $NaBH_4$. The resulting mixture of oligosaccharide alditols was fractionated by semi-preparative HPLC; 17 fractions were obtained [1]. Here, the oligosaccharides *1*, *7* and *14* are further investigated.

Quantitative carbohydrate analysis of the oligosaccharides was carried out by GLC after methanolysis and pertrifluoroacetylation [9].

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Abbreviations: HPLC, high-pressure liquid chromatography; GLC, gas-liquid chromatography; GLC-MS, gas-liquid chromatography combined with mass spectrometer; NMR, nuclear magnetic resonance

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Permethylation of the oligosaccharides was performed according to [10] and the partially methylated monosaccharides were identifed by GLC–MS according to [11].

For ^{1}H-NMR analysis, the oligosaccharides were repeatedly exchanged in D_2O. ^{1}H-NMR spectroscopic analysis was performed on a Bruker WM-500 spectrometer (SON hf-NMR facility, Nijmegen) operating at 500 MHz in the Fourier transform mode at a probe temperature of 300 K [12]. Chemical shifts are given in ppm relative to sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS) (indirectly to acetone in D_2O: δ 2.225).

3. RESULTS

3.1. *Carbohydrate composition; permethylation studies*

The molar carbohydrate compositions of oligosaccharides *1*, *7* and *14* are reported in table 1. The number of Man residues for each oligosaccharide being set to 3, one residue of GlcNAc-ol is found in each of the 3 oligosaccharides; besides 1, 5 and 7, GlcNAc residues occur in oligosaccharides *1*, *7* and *14*, respectively. For the latter oligosaccharide, the same relatively high value for the ratio total GlcNAc:Man (2.6) is found, as for the glycopeptides derived from the turtle-dove ovomucoid [4] and oligosaccharide *11* from hen ovomucoid [2].

The molar ratios of the monosaccharide methyl ethers derived from the permethylated oligosaccharides *1*, *7* and *14* are compiled in table 2. The results for oligosaccharide *1* are in accord with a trimannosyl-di-*N*-acetylchitobiose core structure, as has been described also for quail ovomucoid [13]. For oligosaccharide *7*, methyl-2-mono-*O*-methyl mannoside is found, which suggests the occurrence of an intersecting GlcNAc residue (1⟶4)-linked to Man 3. (For numbering of sugar residues see fig.1–3). The same methyl ethers as for *7* were found previously for glycopeptides obtained from chicken ovotransferrin [14]. Methylation analysis of the oligosaccharide *14*, like that of oligosaccharide *11* [2] reveals the presence of two different mono-*O*-methyl-mannosides in equal amounts: methyl 2- and methyl 3-mono-*O*-methyl mannosides. The presence of the two mono-*O*-methyl ethers of mannose had been demonstrated already by methylation analysis of glycopeptides from turtle-dove ovomucoid [4] and of total hen ovomucoid [3,15]. These results indicate that one residue of mannose is substituted at positions 2 and 4, a second mannose at positions 3, 4 and 6 and the third mannose at positions 2, 4 and 6. The difference between oligosaccharides *11* [2] and *14* is the presence of one Gal residue in terminal non-reducing position in *14*, witness the presence of methyl-2,3,4,6-tetra-*O*-methyl galactoside (1⟶4)-linked to one of the GlcNAc residues. No indications useful for localization of the Gal residue in a certain branch can be derived from sugar and methylation analysis.

3.2. *500-MHz ^{1}H-NMR spectroscopy*

To elucidate the primary structures of the hen ovomucoid oligosaccharides *1*, *7* and *14*, 500-MHz ^{1}H-NMR spectra of the compounds in D_2O were recorded. Relevant NMR parameters for the 3 fractions are listed in table 3; for reference purposes, those for fraction *11* have been included (*cf.* [2]). The structural-reporter-group regions of the

Table 1

Carbohydrate composition of oligosaccharide *1*, *7* and *14* obtained by semi-preparative HPLC of glycans liberated from hen ovomucoid neutral-glycopeptide by hydrazinolysis

Oligosaccharide	Molar ratio[a] of				GlcNAc + GlcNAc-ol ratio
	Gal	Man	GlcNAc	GlcNAc-ol	Man
1	0	3	1.30	0.94	0.74
7	0	3	4.64	0.94	1.86
14	1.07	3	6.80	1.07	2.62

[a] Man taken as 3

Table 2

Molar ratios of monosaccharide methyl ethers present in the methanolysates of the permethylated oligosaccharides *1*, *7* and *14*

Oligosaccharide	Partially methylated monosaccharide (mol/mol) of oligosaccharides)									
	2,3,4,6-tetra-OMe-Gal[a]	2,3,4,6-tetra-OMe-Man	3,4,6 tri-OMe-Man	2,4-di-OMe-Man	3,6-di-OMe-Man	2-Mono-OMe-Man	3-Mono-OMe-Man	3,4,6-tri-OMe-Glc NAcNMe[b]	3,6-di OMe-Glc NAcNMe	1,3,5,6-tetra-OMeGlc-ol NAcNMe
1	–	1.90	–	1	–	–	–	–	0.91	0.95
7	–	–	1.1	–	0.87	1	–	3.88	0.9	0.98
14	0.9	–	–	–	0.92	1.1	1	4.82	1.9	0.91

[a] *O*-Methyl is abbreviated as OMe

[b] *N*-acetyl-*N*-methyl is abbreviated as NAcNMe

500-MHz ^{1}H-NMR spectrum of *14*, as a typical example, are depicted in fig.1.

Comparison of the ^{1}H-NMR data for compounds *1*, *7*, *11* and *14* reveals that all 4 of them are reduced oligosaccharides having in common the GlcNAc $\beta(1\longrightarrow4)$-GlcNAc-ol structural element. The spectral features that are characteristic for the presence of this unit are the H-2 signal of GlcNAc-1-ol at $\delta = 4.25$ (seemingly, a broadlined quartet in the Man H-2 region of the spectrum, $4.0 < \delta < 4.3$, see fig.1), the H-1 doublet of GlcNAc-2 at $\delta \simeq 4.63$ (this chemical shift value, in combination with $J_{1,2}$ being 7.85 Hz, points to the $\beta(1\longrightarrow4)$-linkage of GlcNAc-2 to reduced GlcNAc-1; see also [2]), and two *N*-acetyl methyl singlets at $\delta \simeq 2.055$ and $\simeq 2.08$ for GlcNAc-1-ol and GlcNAc-2, respectively. Furthermore, all 4 compounds contain the mannotriose branching core that is usually found in *N*-glycosidic carbohydrate chains. Evidence for this stems from the occurrence of 3 Man H-1 signals, and also of 3 Man H-2 signals, in each of the spectra (see, for example, fig.1). The characteristic shapes of these signals point to a β-glycosidic linkage for one of the mannoses (designated Man-3) and to α-glycosidic linkages for the other two (designated 4 and 4′) [16].

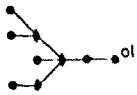
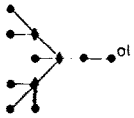
Compound *1* is an incomplete diantennary structure that possesses both Man-4 and Man-4′ in terminal, non-reducing position. This can be concluded from comparison of the H-1 and H-2 chemical shifts of these residues in *1* (see table 3) with those for terminal Man-4 and Man-4′ residues in the oligomannoside-type glyco-asparagines M_2GP and M_5GP [17], or in oligosaccharides like M_2G from mannosidosis urine [18], Ala and Alb from human meconium [19], and *2a* and *2b* from urine of patients with Morquio syndrome type B [20]. Therefore, the structure of compound *1* appears to be Man $\alpha(1\longrightarrow3)$[Man $\alpha(1\longrightarrow6)$]Man $\beta(1\longrightarrow4)$-GlcNAc$\beta(1\longrightarrow4)$GlcNAc-ol.

The resonance positions of the Man-3, -4 and -4′ H-1 and H-2 atoms in the spectrum of compound *7* indicate the branching pattern to be intersected triantennary. This can be readily inferred from comparison of these shift data (see table 3) with those described for a glycopeptide derived from chicken ovotransferrin [14,16]. Moreover, the peripheral part of the structure of the ovotransferrin glycopeptide just mentioned and that of compound *7* from hen ovomucoid are identical. The correctness of this conclusion is proved by the perfect accord between the H-1 and also the NAc signals of the 4 terminal GlcNAc residues for these compounds, both as to their number, as well as to their chemical shifts.

The ^{1}H-NMR spectral features of the intersected pentaantennary structure *11* have been described in detail [2]. It should be mentioned that such a type of branching (i.e., a 2,4-disubstitution of Man-4 and a 2,4,6-trisubstitution of Man-4′ besides the presence of the intersecting GlcNAc-2) gives rise to a unique set of Man H-1 and H-2 chemical shifts. In addition, the Man-4 H-3 and Man-4′ H-4 signals occupy typical positions. Comparison of the corresponding Man H-1, H-2, H-3 and H-4

Table 3

^{1}H chemical shifts of structural-reporter groups of constituent monosaccharides for some major carbohydrates from hen β-ovomucoid[a]

Reporter group	Residue[b]	Compound and schematic structure[c]			
		1	*7*	*11*	*14*
					
H-1 of	2	4.637	4.632	4.626	4.626
	3	~4.78	4.696	4.712	4.712
	4	5.103	5.057	5.067	5.066
	4′	4.915	4.999	4.889	4.890
	5	–	4.540	4.539	4.538
	5′	–	4.543	4.545	4.545
	7	–	4.516	*4.517*	*4.538*
	7′	–	–	4.517[d]	4.518[d]
	8	–	–	–	4.471
	9	–	4.464	4.443	4.443
	10′	–	–	4.583[d]	4.586[d]
H-2 of	1-ol	4.244	4.246	4.255	4.254
	3	4.259	4.146	4.145	4.145
	4	4.067	4.284	4.276	4.277
	4′	3.974	4.146	4.161	4.158
H-3 of	4	n.d.	4.048	4.043	4.044
H-4 of	4′	n.d.	n.d.	4.194	4.194
NAc of	1-ol	2.055	2.055	2.054[e]	2.053[e]
	2	2.076	2.079	2.084	2.084
	5	–	2.055	2.065[e]	2.064[e]
	5′	–	2.048	2.045	2.045
	7	–	2.083	*2.084*	*2.079*
	7′	–	–	2.093[f]	2.093[f]
	9	–	2.064	2.065	2.064
	10′	–	–	2.122[f]	2.121[f]

[a] Chemical shifts are given at 300 K, in ppm downfield from internal DSS in D_2O

[b] For numbering of monosaccharide residues and complete structures, see fig.3

[c] Compounds are represented by schematic structures (*cf.* [17]); (●—) GlcNAc; (◆—) Man; (■—) Gal

[d,e,f] Assignments may have to be interchanged

n.d., value could not be determined

chemical shifts for compounds *7* and *11* (see table 3) reveals that those for Man-4′ deviate considerably, while those for Man-4 are essentially the same for both compounds. This led us to the conclusion [2] that Man-4′ in *11* is the trisubstituted Man residue, the occurrence of which was established by methylation analysis. Apart from the core GlcNAc signals, another seven GlcNAc

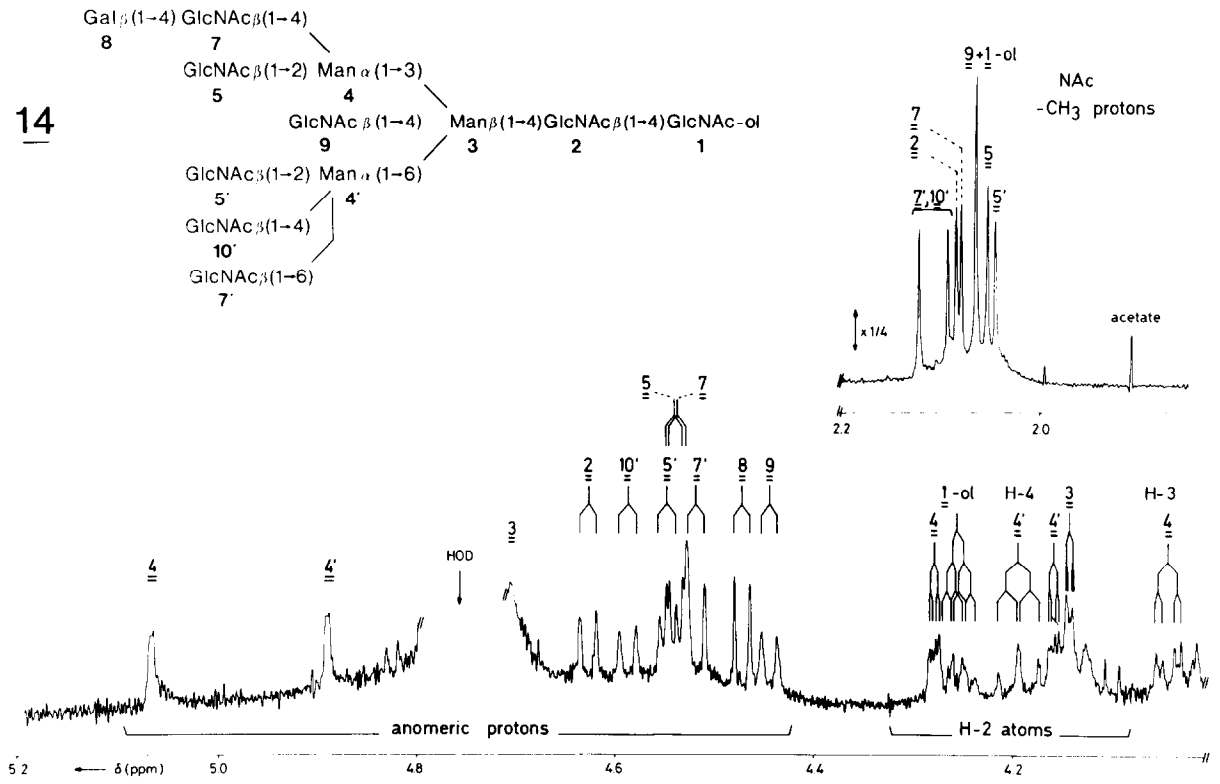


Fig.1. Structural reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectrum of oligosaccharide-alditol *14*, derived from hen ovomucoid, in D_2O at 300 K. The numbers in the spectrum refer to the corresponding residues in the structure. The relative-intensity scale of the *N*-acetyl proton region differs from that of the other part of the spectrum, as indicated.

H-1 doublets and NAc singlets are observed.

Methylation analysis of compound *14* (see table 2) suggested that the latter might differ from structure *11* only in the presence of a terminal Gal residue, (1⟶4)-linked to a GlcNAc. Once this sugestion is verified, the position of Gal in a certain branch remains to be established. In order to facilitate comparison between the 500-MHz ^{1}H-NMR spectra of fractions *11* and *14*, pertinent parts of both are presented in fig.2.

The branching pattern of the mannotriose core in *14* is indeed the same as in *11*, as can be readily inferred from the perfect accordance of the Man H-1 and also the H-2 chemical shifts (see also table 3). The resonance position of Man-4 H-3 (δ 4.044) corroborates the 2,4-disubstitution of this residue, that of Man-4′ H-4 (δ 4.194) confirms the 2,4,6-trisubstitution of the latter residue. From comparison of the β-anomeric region ($4.4 < \delta <$ 4.7) of both spectra (see fig.2), it is obvious that in the spectrum of *14* one additional doublet is present, at δ 4.471 ($J_{1,2}$ = 7.8 Hz). This combination of δ- and *J*-value is known to be characteristic for a terminal Gal residue β(1⟶4)-linked to GlcNAc [10,16]. Thus, compound *14* appears to be an extension of compound *11* with one Gal residue.

The attachment of this Gal residue to structure *11* causes two significant chemical-shift alterations of GlcNAc structural-reporter groups. First, one of the two H-1 doublets at δ 4.517 for *11* is shifted towards δ 4.538 for *14*, while the positions of all other anomeric doublets are unchanged, going from *11* to *14*. Secondly, one of the two *N*-acetyl singlets, coinciding at δ 2.084 for *11*, is shifted towards δ 2.079. The *N*-acetyl signals at δ 2.084 for *11* were unambiguously assigned to GlcNAc-2 and -7 (*cf.* compound *7*, table 3) [2]. The doublets coinciding at δ 4.517 for *11* belong to the H-1 atoms of

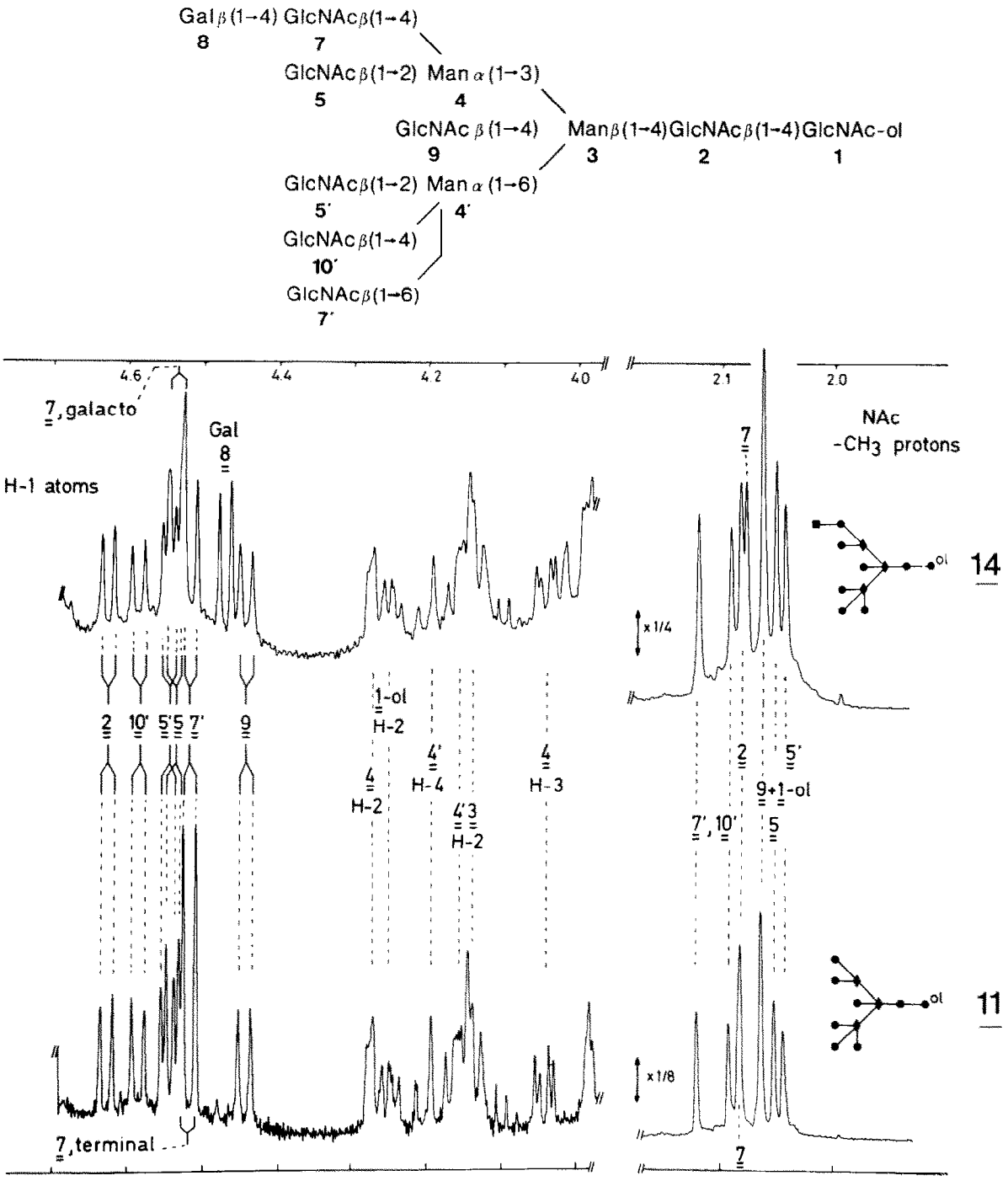


Fig.2. Pertinent structural reporter-group regions of the resolution-enhanced 500-MHz ^{1}H-NMR spectra of oligosaccharide-alditols *11* (lower trace) and *14* (upper trace) from hen ovomucoid. Compounds are represented by schematic structures; (●——) GlcNAc; (◆——) Man; (■——) Gal. The numbers in the spectra refer to corresponding residues in the structures (see fig.1 and 3). The relative-intensity scales of the *N*-acetyl proton regions differ from those of the other parts of the spectra, as indicated.

GlcNAc-7 and either of GlcNAc-7′ or of -10′. The observed shift increments (for H-1, $\Delta\delta \simeq 0.02$ ppm, and for NAc, $\Delta\delta \simeq -0.005$ ppm), if occurring simultaneously, are very typical for the attachment of Gal in $\beta(1\longrightarrow4)$-linkage to GlcNAc [16,21]. Since GlcNAc-2 is already substituted by Man-3 in $\beta(1\longrightarrow4)$-linkage, only GlcNAc-7 can be considered to be the GlcNAc residue that bears Gal in oligosaccharide *14*. Therefore, Gal in fraction *14* has been identified to be Gal-8 (see fig.1).

4. CONCLUSIONS

The structures which can be proposed on the basis of the results of 500-MHz ^{1}H-NMR spectroscopy in conjunction with methylation analysis for the oligosaccharides *1*, *7* and *14* from hen ovomucoid are summarized in fig.3. Oligosaccharide *1* corresponds to the core structure of *N*-glycosidic glycans of glycoprotein; the same primary structure has been isolated from quail

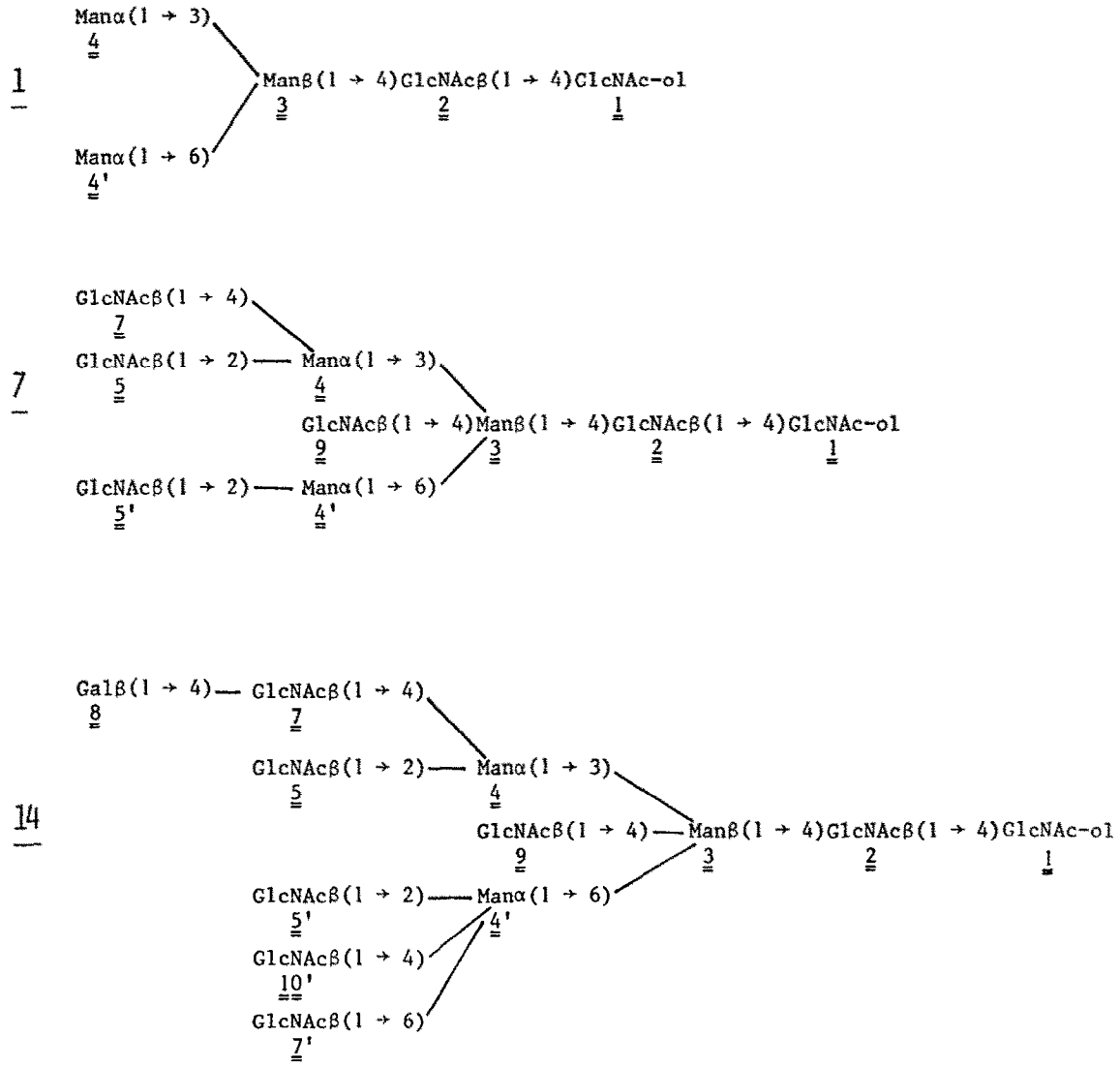


Fig.3. Structures of oligosaccharide-alditols *1*, *7* and *14*, isolated from hen ovomucoid.

ovomucoid [13]. Oligosaccharide *7* is essentially identical to the carbohydrate moiety of chicken ovotransferrin [14]. Oligosaccharide *14* possesses a pentaantennary structure with an intersecting GlcNAc residue and one Gal residue $\beta(1\longrightarrow4)$-linked to GlcNAc-$\underline{\underline{7}}$. It is an extension of oligosaccharide *11*, the NMR parameters of which were described in [2]. The subtle differences between the NMR parameters of the structural-reporter groups of GlcNAc $\underline{\underline{7}}$ for oligosaccharide *14* as compared to *11*, enabled to establish that Gal in *14* is linked to GlcNAc-$\underline{\underline{7}}$. It is remarkable that in hybrid type, *N*-glycosidic oligosaccharides containing a Gal residue, this Gal has been found to occur also in $\beta(1\longrightarrow4)$-linkage to GlcNAc-$\underline{\underline{7}}$ [22]. This novel pentaantennary structure was also demonstrated in turtle-dove ovomucoid [3,4].

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