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The Structure of the Carbohydrate Units of Human Plasma Galactoglycoprotein Determined by 500-megahertz ^{1}H NMR Spectroscopy*

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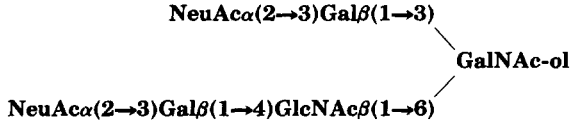
Human plasma galactoglycoprotein (M_r = 81,000) which was recently identified and characterized (Schmid, K., Mao, S. K. Y., Kimura, A., Hayashi, S., and Binette, J. P. (1980) *J. Biol. Chem.* 255, 3221-3226) was found to possess an unusually high carbohydrate content (76%) comprised of NeuAc, Gal, Man, Fuc, GalNAc, and GlcNAc. The aim of the present investigation was to elucidate the primary structure of the oligosaccharide units of this protein.

For the study of the *O*-glycosidic oligosaccharide chains, the protein was subjected to β-elimination and the resulting oligosaccharide preparations were analyzed by 500-MHz ^{1}H NMR spectroscopy. The structure of the predominant glycan, a hexasaccharide:



and that of a tetrasaccharide: NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→6)]GalNAc-ol, were determined. The protein possesses approximately 40 hexasaccharides and 3 tetrasaccharides/mol.

For the isolation of its *N*-glycosidic oligosaccharide chains, the protein was exhaustively digested with proteases followed by chromatography of the desialyzed resulting glycopeptide fraction on concanavalin A-Sepharose. 500-MHz ^{1}H NMR spectroscopy of the obtained preparations revealed the presence of 3 diantennary *N*-glycosidic chains that are extended with a Fuc residue at the Asn-linked GlcNAc.

Many attempts have been made to elucidate the structure and biological function of the carbohydrate moiety of glycoproteins over the past decade (for reviews, see Refs. 1-4). In a study pertinent to this area of investigation, we were recently able to demonstrate a property of the carbohydrate moiety of human plasma α_1-acid glycoprotein that regulates various immune responses (5). In extending our investigation on the structure-function relationship of the carbohydrate moiety of glycoproteins we describe in this paper the structure of the glycans of a recently discovered human plasma glycoprotein, galactoglycoprotein (6). This protein is characterized by its high carbohydrate content (75%) and unusual sugar composition (6, 7).

MATERIALS AND METHODS[1]

RESULTS AND DISCUSSION

The Structure of the O-Glycosidic Oligosaccharides: 500-MHz ^{1}H NMR Spectroscopy—The resolution-enhanced 500-MHz ^{1}H NMR spectrum of fraction E-2 (Fig. 1) indicates the presence of two components whose relative concentrations are approximately 1:10. Evidence in support of this conclusion is the occurrence of two sets of signals differing by a factor of about 10. Compare for example the intensity of the *N*-acetyl singlet of GalNAc-ol at δ 2.042 with that at 2.065.

The chemical shifts of the structural-reporter groups of the main component as well as those of the minor constituent and those of three reference compounds of related structures are listed in Table I (see Miniprint). The higher intensity signals in the spectrum revealed that the major component is a mucin-type oligosaccharide alditol with a 3,6-disubstituted GalNAc-ol residue. The chemical shift of H-2 of GalNAc-ol (δ 4.389) is indicative of the presence of Gal in β(1→3) linkage to GalNAc-ol, whereas the set of H-5 and H-6 chemical shifts (δ 4.265 and δ 3.921, respectively) point to GlcNAc being attached in β(1→6) linkage to GalNAc-ol. In turn, to this GlcNAc residue Gal is attached in β(1→4) linkage because its H-1 doublet ($J_{1,2}$ 8.1 Hz) is found at δ 4.552. Such a value is known to be characteristic for a Galβ(1→4)GlcNAc moiety (see R-1, Table I) (13). Thus, the core of this compound could

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This publication is dedicated to Professor Hans Faillard, University of Saarbrücken, Federal Republic of Germany, on the occasion of his 60th birthday.

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[1] Portions of this paper (including "Materials and Methods," the results on the elucidation of the *N*-glycosidic oligosaccharides, Figs. 1 and 2, and Tables I and II) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 81M-1199, cite the authors, and include a check or money order for $4.40 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

be identified as being identical to the structure R-1 (Table I). Furthermore, the two Gal residues are each terminated by a NeuAc residue in $\alpha(2\rightarrow3)$ linkage. This is evident from the two coinciding NeuAc H-3ax[2] triplets (δ 1.799) and the two H-3eq signals (at δ 2.755 and δ 2.775) of equally high intensity. The chemical shifts of the H-1 ($\delta \simeq 4.54$) and H-3 (δ 4.113) of Gal are consistent with this substitution (15). Therefore, the structure of the main oligosaccharide was identified as shown in Fig. 1. The assignment of the reporter group signals of NeuAc being $\alpha(2\rightarrow3)$ linked to Gal was obtained by comparison of the spectral data (Table I) with those of the reference compounds (R-1, R-2, and R-3). The NeuAc residue that is $\alpha(2\rightarrow3)$-linked to Gal$\beta(1\rightarrow3)$GalNAc-ol (designated NeuAc^{3,3}, Table I) is characterized by δ H-3eq 2.774 and by δ NAc 2.033 (see R-2 and R-3). Therefore, the H-3eq signal at δ 2.755 and the NAc singlet at 2.031 are attributed to NeuAc^{3,4}. These values are in accord with those known for $\alpha(2\rightarrow3)$-sialylated extensions of *N*-acetyllactosamine branches of *N*-glycosidic glycopeptides and oligosaccharides (2, 16). The chemical shifts of H-1 and H-3 of Gal^3 and Gal^4 are characteristic for Gal residues bearing an $\alpha(2\rightarrow3)$-linked NeuAc residue (2, 14). The difference in chemical shift between H-1 of Gal^3 and Gal^4 of the asialo analog being $\Delta\delta = 0.005$ ppm is significantly enlarged ($\Delta\delta = 0.016$ ppm) in the disialo compound. It should be noted that Gal H-1 and GlcNAc H-1 signals could be discerned by the difference in $J_{1,2}$ value (7.85 and 8.1 Hz, respectively) (13), and independently by comparison with the monosialo analog, R-2 (15). The chemical shifts of the H-1 and NAc signals of GlcNAc are also in agreement with those of a *N*-acetyllactosamine unit extended with NeuAc in $\alpha(2\rightarrow3)$ linkage. In particular, the small but significant upfield shift of the NAc singlet ($\Delta\delta$ − 0.004 ppm as compared to R-1) is indicative in this respect (16). The major compound accounts for approximately 90% of the carbohydrate moiety of the protein and agrees with the carbohydrate composition of the native protein (6, 7).

The structure of the minor constituent was identified as follows. The occurrence of the set of NeuAc H-3ax and H-3eq signals of equally low intensity at δ 1.692 and δ 2.724 is highly specific for the NeuAc$\alpha(2\rightarrow6)$[Gal$\beta(1\rightarrow3)$]GalNAc-ol structural element (14). This is corroborated by the presence of GalNAc-ol H-5 at δ 4.245 and H-6′ at δ 3.476 and by the GalNAc-ol NAc singlet at δ 2.042. The position of the Gal^3 H-1 signal, δ 4.541, indicates that another NeuAc residue is $\alpha(2\rightarrow3)$-linked to Gal. Therefore, based on the complete accordance of the data with those described for the same tetrasaccharide (14), the minor component possesses the following structure: NeuAc$\alpha(2\rightarrow3)$Gal$\beta(1\rightarrow3)$[NeuAc$\alpha(2\rightarrow6)$]GalNAc-ol.

It should be noted that the sensitivity of the employed 500-MHz ^{1}H NMR spectroscopy permitted the elucidation of the complete structure of the minor component although it accounts for only approximately 8% of the weight of the analyzed oligosaccharide preparation.

Human plasma GalGP was shown in this study to possess approximately 40 *O*-glycosidic hexasaccharides as well as 3 *O*-glycosidic tetrasaccharides and approximately two *N*-glycosidic diantennary oligosaccharide chains. In contrast, a large number of plasma glycoproteins possess predominantly *N*-glycosidic carbohydrate units (1–3, 16–20). Other plasma proteins such as transferrin (3, 17) and α_1-acid glycoprotein[3] (21) which until recently was the protein that contained the highest carbohydrate content (42%), have only *N*-glycosidic sugar chains (22, 23). Also contrasting the plasma proteins, human glycophorin,[3] a component of the cell membrane of erythrocytes, which has also a very high carbohydrate content (60%), possesses 15 *O*-glycosidic oligosaccharides and only the *N*-glycosidic chain (24). Thus, GalGP, because of its almost exclusive content of *O*-glycosidic sugar chains, appears not to be a typical plasma glycoprotein. Furthermore, judging from some of the above-mentioned observations and especially from the study by Judson *et al.* (25) who isolated a protein from platelets which appears to have a composition similar to GalGP found in serum (6), one might speculate that this protein whose origin is unknown, may be derived from the surface of certain blood cells where it may be associated with various receptor functions.

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[2] The abbreviations used are: H-3ax, H of carbon 3 in axial position; H-3eq, H of carbon 3 in equatorial position; GalGP, galactoglycoprotein; Con A, concanavalin A.

[3] GalGP with approximately 220 amino acid residues possesses about twice as many carbohydrate branches per amino acid as glycophorin which has 130 amino acid residues.

SUPPLEMENTARY MATERIAL TO

"The Structure of the Carbohydrate Units of Human Plasma Galactoglycoprotein Determined by 500-MHz ^{1}H-NMR Spectroscopy" by:

Kazuko Akiyama, Elizabeth R. Simons, Karl Schmid, Herman van Halbeek, Johannes F.G. Vliegenthart, Heinz Haupt, and H. Gerhard Schwick

MATERIALS

Galactoglycoprotein (GalGP) was prepared as described earlier(6,7). However, the following additional step has recently been developed for the final purification of this plasma protein. Small amounts of proteinaceous impurities, mainly α_1-acid glycoprotein, C1-inactivator and albumin were removed by solid phase immunoadsorption. The antiserum utilized for this purpose was obtained from a rabbit which was injected with purified GalGP but which formed antibodies only against the mentioned impurities. The γ-globulin isolated from this serum (chromatography on DEAE-cellulose at pH 7.0, 0.03 M Na-phosphate buffer) was coupled to Sepharose CL-4B (1 g of the γ-globulin fraction per 100 ml of Sepharose CL-4B) using the CNBr-method. Sephadex G-50, G-25 and Con A-Sepharose were from Pharmacia, pronase E (1,000,000 tyrosine units/g) from Kaken Kagaku, Tokyo, Japan, and papain (2 x crystallized, 28 mg protein per ml), α-methyl-D-glucoside and α-methyl-D-mannoside from Sigma.

METHODS

β-Elimination. For the preparation of the O-glycosidic oligosaccharide chains, GalGP (19 mg) was dissolved in 2.5 ml of water, mixed with 2.5 ml of 2 M $NaBH_4$ in 0.2 M NaOH and allowed to stand in the dark at 35°C for 15 h (8). The solution was then neutralized with Dowex-50 W-X2 (H^+) which was removed by pouring it into a small column (0.5 x 5 cm) and washed with water. The resulting solutions were evaporated to dryness, and boric acid was removed subsequently by co-evaporation with methanol. The residue was then dissolved in water and applied to a Sephadex G-50 column (1.2 x 160 cm, 0.4% NH_4HCO_3). The asymmetric peak which eluted between V_o and V_t was separated into the main portion (E-2) and fast (E-1) and slowly (E-3) eluting edges of the peak. It should be noted that the polypeptide chain of the protein was only partially degraded and yielded components with molecular weights ranging from V_o to V_t of the column. Therefore, each fraction (E1,E2 and E3) was next exhaustively digested with papain and pronase and then purified by gel filtration employing Sephadex G-25 (0.9 x 160 cm; 0.4% NH_4CO_3). The recovery of these preparations accounted for 60% of the carbohydrate moiety of the protein.

Papain and Pronase Digestion. For the preparation of the N-glycosidic oligosaccharides, a solution of GalGP (60 mg dissolved in 2.0 ml of activating buffer, pH 7.8) was mixed with 1.0 ml of activated papain solution, which was prepared by mixing 50 µl of papain solution with 1.0 ml of the same buffer, and allowed to stand at 50°C for 1 h. The digestion was carried out at 60°C for 22 h with gentle stirring. The pH was kept constant between 7.8 - 8.0. After 3 and 6 h of incubation 30 µl of papain were again added to the digest. For the pronase digestion 3 mg Ca-acetate were added to the papain digest followed by 1 mg pronase and the reaction was carried out at 40°C for 24 h. Additional enzyme (1 mg each) was added after 3 and 6 h. The proteolytic digest of GalGP was concentrated to approximately 1 ml and applied to a Sephadex G-50 column (2 x 155 cm; 0.4% NH_4HCO_3) (Fig. 2). The absorption was monitored at 230 and 490 nm (9,10). Two major (#3 and #5) and one minor (#2) glycopeptide fractions were obtained. Together with the intermediate fraction (#4) the weight recovery was approximately 85%. Fraction #1 which contained primarily peptides and fraction #4 were too small to be further studied. In order to isolate the N-glycosidic oligosaccharides that are known to account only for a small percentage of the total carbohydrate units of GalGP (6), aliquots of fractions #2,3 and 5 were desialyzed (0.05 N H_2SO_4, 80°C, 1 h). After neutralizing to pH 7 with pyridine, each hydrolysate was desalted by means of a Sephadex G-25 column (1.1 x 17 cm, 0.4% NH_4HCO_3) and lyophilized.

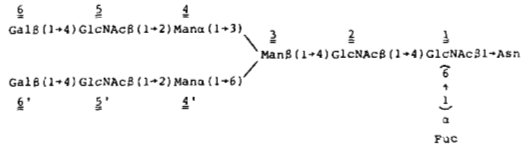
To isolate the N-glycosidic glycopeptide, the asialoglycopeptides described above were chromatographed on a Con A column (1.1 x 10 cm) which had been equilibrated with 20 mM Na acetate buffer, pH 7.0, containing 0.15 M NaCl, 0.1 mM $MnCl_2$ and 0.1 mM $CaCl_2$ (11,12). After eluting the unadsorbed material with this buffer, stepwise elution was carried out, using 0.025 M, 0.05 M and 0.10 M α-methylmannoside in the same buffer. Appropriate fractions were pooled, concentrated and passed through a Sephadex G-25 column (1.1 x 17 cm, 0.4% NH_4HCO_3) followed by lyophilization. Each glycopeptide fraction afforded 3 subfractions.

[4]This buffer contained 6.1 mg NaCN, 43.9 mg cysteine·HCl and 73.1 mg Na_2EDTA per 25 ml. The pH was adjusted to 7.8-8.0.

500 MHz ^{1}H-NMR Spectrometry. A glycopeptide or oligosaccharide preparation was repeatedly exchanged in D_2O (99.96 atom% D, Aldrich) with intermediate lyophilization and analyzed with a Bruker WM-500 spectrometer (SON National hf-NMR-facility, Dept. Biophysics, U. Nijmegen, The Netherlands) operating at 500 MHz in the Fourier transform mode at a probe temperature of 300°K. Chemical shifts are given relative to sodium-4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured indirectly to acetone in D_2O (δ 2.225 ppm) (2).

RESULTS

High-Resolution ^{1}H-NMR Spectroscopy. 500-MHz ^{1}H-NMR spectroscopy revealed that the subfraction E-2 which was eluted with 0.025 M methylglucoside, contained N- as well as O-glycosidic diantennary glycans. A typical carbohydrate composition for fraction #5-E-2 was Fuc, Man, Gal, GalNAc and GlcNAc in the ratio of 0.9 : 3.0 : 3.6 : 0.8 : 3.2 (uncorrected). Glc was present as a contaminant. As is evident from the ^{1}H-NMR data (Table II) the N-glycosidic diantenna has a Fuc residue attached in α(1→6)-linkage to GlcNAc 1. This can be deduced both from the chemical shifts of the Fuc structural reporter group (H-1, H-5 and CH_3) as well as from the characteristic shift effects introduced upon H-1 and NAc of GlcNAc-2 (16). The structure of the N-glycosidic chains of GalGP is as follows:



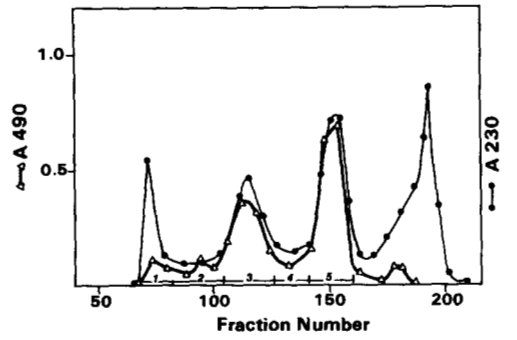


Fig. 2. Gel filtration of the papain-pronase digest of galactoglycoprotein. The flow rate was 6 ml per h, and 2 ml of the effluent were collected per test tube. The weights of fractions 2,3 and 5 were 8.5 16.5 and 20.2 mg, respectively.

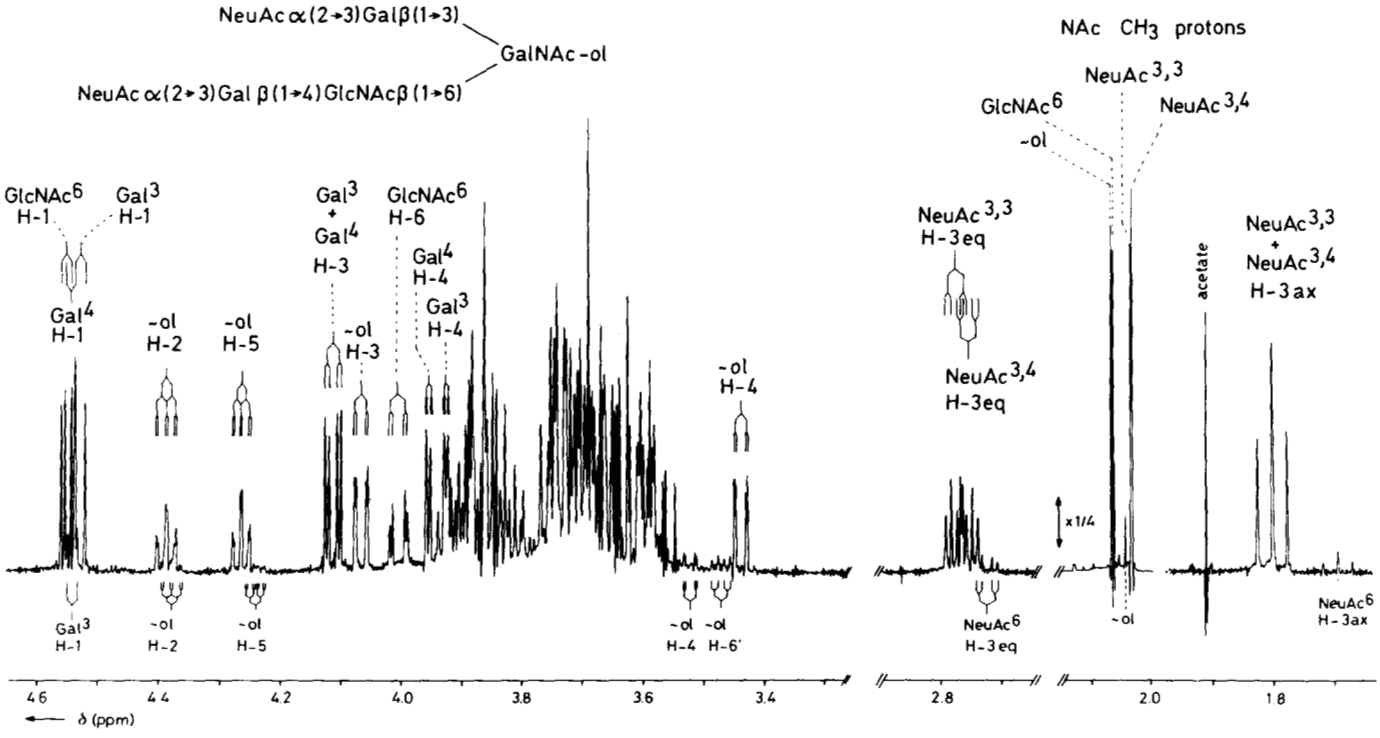


Fig. 1. 500-MHz ^{1}H-NMR spectrum of the oligosaccharide-alditol mixture (E-2) of O-glycosidic carbohydrate chains of GalGP. The assignments belonging to the major compound are indicated above the spectrum and those of the minor compound below. For designation of the monosaccharide residues see footnote to Table I.

Table I

^{1}H-NMR Chemical Shifts of Structural-reporter Groups of Constituent Monosaccharides of GalGP Oligosaccharide alditols and the Reference Compounds R-1, R-2 and R-3

Residue[a]	Reporter group	Chemical shift[b]				
		R-1 (13)	R-2 (15)	GalGP, Major Alditol	R-3 (14)	GalGP, Minor Alditol
						
GalNAc-ol	H-2	4.394	4.390	4.389	4.378	4.380
	H-3	4.060	4.072	4.066	4.067	4.066
	H-4	3.465	3.456	3.439	3.524	3.522
	H-5	4.282	4.272	4.265	4.240	4.245
	H-6	3.931	3.927	3.921	3.84	3.839
	H-6'	3.7	3.7	3.7	3.475	3.476
	NAc	2.067	2.066	2.065	2.042	2.042
Gal^3	H-1	4.465	4.534	4.530	4.541	4.541
	H-3	3.66	4.116	4.113	4.117	4.116
	H-4	3.900	3.922	3.927	3.927	3.927
GlcNAc^6	H-1	4.560	4.559	4.552	-	-
	H-6	3.998	3.993	4.005	-	-
	NAc	2.064	2.066	2.062	-	-
Gal^4	H-1	4.470	4.470	4.546	-	-
	H-3	3.68	3.7	4.113	-	-
	H-4	3.925	3.931	3.956	-	-
NeuAc^{3,3}	H-ax	-	1.801	1.799	1.800	1.799
	H-3eq	-	2.774	2.775	2.774	2.775
	NAc	-	2.033	2.033	2.032	2.031
NeuAc^{3,4}	H-3ax	-	-	1.799	+	-
	H-3eq	-	-	2.755	-	-
	NAc	-	-	2.031	-	-
NeuAc^6	H-3ax	-	-	-	1.692	1.692
	H-3eq	-	-	-	2.723	2.724
	NAc	-	-	-	2.032	2.031

[a] The first superscript at the name of a sugar residue indicates to which position of the adjacent monosaccharide it is glycosidically linked. A second superscript is used to discriminate between identically linked residues, by indicating the type of the next linkage in the sequence.

[b] Chemical shifts are in ppm downfield from internal DSS in D_2O at 27°C, acquired at 500 MHz. In the table-heading, structures are represented by symbolic notation: ◇ = GalNAc-ol; ■ = Gal; ● = GlcNAc; △ = NeuAcα(2→3) and ▲ = NeuAcα(2→6).

Table II

^{1}H-NMR Chemical Shifts of Structural-Reporter-Groups of Constituent Monosaccharides of the Asialo N-Glycosidic Carbohydrate Chain of GalGP and of the Asialo Diantenna of α_1Acid Glycoprotein used as Reference Compound

Reporter-group	Residue[a]	Chemical shift[b]	
		GalGP, monofuco-diantenna	α_1-acid GP, diantenna GP-II-6 (23)
H-1	GlcNAc-1	5.041	5.094
	GlcNAc-2	4.682	4.616
	Man-3	4.77	4.765
	Man-4	5.118	5.121
	Man-4'	4.925	4.928
	GlcNAc-5	4.581	4.582
	GlcNAc-5'	4.581	4.582
	Gal-6	4.467	4.467
	Gal-6'	4.472	4.473
	Fuc	4.87[c]	
H-2	Man-3	4.251	4.246
	Man-4	4.194	4.190
	Man-4'	4.12[d]	4.109
H-5	Fuc	4.12[d]	-
CH_3	Fuc	1.211	-
NAc	GlcNAc-1	2.014	2.009
	GlcNAc-2	2.093	2.079
	GlcNAc-5	2.051	2.050
	GlcNAc-5'	2.047	2.046

(a) For complete structures and the numbering of monosaccharide residues, see text.

(b) Chemical shifts are in ppm downfield from internal DSS in D_2O at 27°C acquired at 500 MHz.

(c) Value could not be determined more accurately due to contamination of the sample with O-glycosidic glycopeptides possessing GalNAc α-linked to Ser/Thr (δ H-1 GalNAc ≈ 4.88).

(d) Value could not be determined more accurately because the H-2 signal of Man-4' and the H-5 signal of Fuc almost coincided (16).