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A TWO-DIMENSIONAL ^{1}H-N.M.R. (500 MHz) AND ^{13}C-N.M.R. (125 MHz) STUDY OF *N*-LINKED GLYCOPEPTIDES DERIVED FROM CALF FETUIN

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ABSTRACT

The oligosaccharide part of an *N*-linked triantennary glycopeptide from calf fetuin with fourteen carbohydrate residues and its smaller derivatives obtained by successive enzymic cleavage of the terminal residues were investigated using 2D ^{1}H-n.m.r. (500 MHz) and ^{13}C-n.m.r. (125 MHz) spectroscopy. Assignments have been made of the resonances of almost all the protons of the constituent carbohydrate residues in these glycopeptides. A comparison of the ^{1}H chemical shifts and coupling constants, as determined from the cross-peaks, has shown the dependence of these parameters on the interactions of spatially related neighbouring carbohydrates. Small conformational changes take place upon elongation of the oligosaccharide side-chains.

INTRODUCTION

The important biological role played by the oligosaccharide side-chains of glycoconjugates[1,2] has led to an increased interest in the elucidation of their primary structures and the determination of their conformations in solution[3–11]. In using n.m.r. spectroscopy to obtain reliable information on these conformations, correct chemical-shift assignment of the proton resonances is an essential prerequisite.

Attention has been paid mainly to *N*-linked glycopeptides and their oligosaccharide chains, and the application of 2D-n.m.r. spectroscopy has simplified the assignment problem[12–20]. Even so, the overlap of ^{1}H-n.m.r. signals observed for relatively large oligosaccharides (2000–3000 daltons) leaves several resonances unassigned. An attractive prospect is the use of ^{13}C,^{1}H heteronuclear-correlated 2D-n.m.r. spectroscopy[21–24], since the large range (50–115 p.p.m.) of chemical shifts of the ^{13}C resonances greatly reduces the overlap of signals relative to that of the proton spectrum, and correlation of the two sets of data may lead to a more complete assignment for both the ^{13}C and the ^{1}H resonances for a given oligosaccharide.

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The assignment of resonances for large oligosaccharides can be assisted by comparison with the data for smaller and simpler related structures. Under certain circumstances, such studies may detect changes in chemical shifts resulting from changes in conformation[6,10,25]. This approach has been applied to an *N*-linked glycopeptide isolated from calf fetuin and having fourteen carbohydrate units, and the results are now reported.

EXPERIMENTAL

The *N*-linked triantennary glycopeptide fraction of calf fetuin was obtained as described[26]. The asialo and asialo-agalacto derivatives were obtained by digestion in sequence with a sialidase and a β-D-galactosidase, each of which was covalently attached to a solid support. The derivatives were purified and desalted by gel-filtration column chromatography (details will be published elsewhere). The digestions were >99% effective as determined from the 500-MHz ^{1}H-n.m.r. spectra of the products.

Spectra were recorded at 30° with a Bruker AM-500 spectrometer (^{1}H at 500, and ^{13}C at 125 MHz). ^{1}H-N.m.r. spectra were recorded with a sweep width of 2200 Hz, 16,384 data points, 90° pulse, and a recycle time of 5.6 s. Chemical shifts were referenced indirectly to sodium 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), using internal acetone (at 2.225 p.p.m.). The ^{13}C-n.m.r. spectra were obtained with a sweep width of 20,000 Hz, 16,384 data points, 80° pulse, and a recycle time of 0.5 s. The composite pulse-decoupling scheme (CPD mode) was used to prevent heating of the sample. Line-broadening of 5 Hz was employed with zero-filling of the data to 32,768 points before processing of the free induction decay (FID). Chemical shifts were referenced to that of 1,4-dioxane at 67.86 p.p.m.

2D Heteronuclear-correlated n.m.r. spectra. — A concentrated aqueous solution (~100 mg/0.5 mL) of the *N*-linked glycopeptide isolated from calf fetuin was used with a pulse sequence which included a proton decoupling in the f_1 domain and a composite 180° carbon inversion pulse. The delays before and after the last 90° (^{13}C) mixing pulse were optimised, with $^1J_{C,H}$ (average) 142 Hz, for observing either –CH– groups or both –CH– and –CH_2– groups. The high-field resonances in both dimensions (methyl groups, C-3 and H-3,3′ of sialic acid) were excluded. Spectral windows of 6945 Hz, with 4096 data points, for ^{13}C and 910 Hz, with 128 data points, for ^{1}H were employed with quadrature detection in each dimension. A total of 128 experiments were acquired each with 1000 transients and a relaxation delay of 1.5 s. The resulting data matrix was zero-filled in each dimension to yield a 8192 × 512 matrix. The f_1 domain was multiplied by a shifted sine-bell function ($\pi/16$) and the f_2 domain by a line-broadening function (5 Hz) prior to processing.

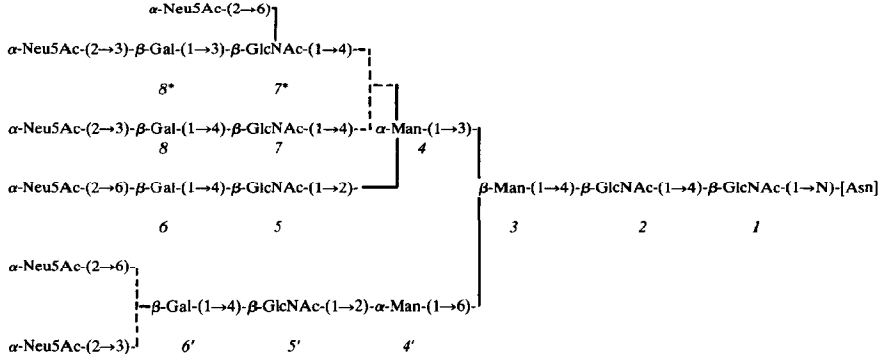
2D Homonuclear-correlated n.m.r. spectra. — Aqueous solutions (~30 mg/0.3 mL) of the *N*-linked glycopeptide and its asialo and asialo-agalacto derivatives were used. Spectra were recorded with spectral widths of 2200 Hz for the homonuclear-correlated (COSY), the magnetisation-transfer COSY (RELAYED-COSY),

and phase-sensitive n.O.e. (NOESY) experiments, and 900 Hz for the phase-sensitive COSY experiments. A 512 × 1024 data matrix was used (512 × 2048 for phase-sensitive COSY) which was zero-filled and multiplied by a shifted sine-bell function ($\pi/32$) in each dimension. Depending on the pulse phase-cycling scheme used, 16–64 scans were accumulated for each experiment with a relaxation delay of 2 s. A coherence transfer delay of 0.278 s was chosen for RELAYED-COSY experiments and a mixing delay of 0.5 s was used in the phase-sensitive NOESY experiments.

The centres of the cross-peaks were taken as the crossing of the chemical shifts, unless an intensity distortion was observed for a given cross-peak, due to strong coupling effects, when the crossing point of the chemical shifts was adjusted accordingly. Coupling constants were determined directly from the expanded plots of the cross-peaks and the relevant cross sections.

RESULTS AND DISCUSSION

The structures of the *N*-linked oligosaccharide side-chains of calf fetuin were only recently fully elucidated by using ^{13}C- and ^{1}H-n.m.r. spectroscopy almost exclusively[26–31]. Several structures were found in the *N*-linked glycopeptide fraction as indicated by dashed arrows below:



Assignments of ^{13}C and ^{1}H resonances of the N*-linked glycopeptide from calf fetuin.* — The glycopeptide fraction was found to be dominated by one major structure[26] (85%), which allowed a clear distinction to be made between the corresponding major and minor resonances, or cross-peaks, in the respective 1D- and 2D-n.m.r. spectra. Chemical shifts of the ^{13}C and ^{1}H resonances obtained from the heteronuclear-correlated spectra and their respective assignments are given in Table I. The experimental conditions were chosen to optimise the cross-peaks resulting from both the –CH– and the –CH_2– groups. However, some important cross-peaks of the type –CH– had disappointingly low intensities under these conditions, so that alternative conditions were used to enhance these cross-peaks. The

TABLE I

^{13}C- AND ^{1}H-N.M.R. CHEMICAL SHIFTS (P.P.M.) OF THE *N*-LINKED TRIANTENNARY GLYCOPEPTIDE[a]

Residue	*Position*					
	1	*2*	*3*	*4*	*5*	*6*
1	79.37	54.95; 54.87	74.06	80.88; 80.69	77.47	61.24†
	5.09	3.85; 3.87	3.76	3.72	3.58	3.87†
2	102.56	56.43*	[b]	79.96	75.96	61.24†
	4.61	3.79	[b]	3.66	3.55	3.87†
3	101.64	71.41	81.63	66.96†	75.71	66.96†
	4.76	4.21	3.77	3.79†	3.58	3.79†
4	100.41	77.37	73.05	79.85	73.80	63.27*
	5.12	4.21	[c]	3.64	3.77†[e]	3.86
4'	98.37	77.62	70.65	68.63	74.06	63.14*
	4.91	4.11	[c]	3.48	3.63	3.86
5	100.78	[d]	[b]	82.04*	75.71*	61.24†
	4.57	3.74	[b]	3.67	3.58*	3.87†
$5'_6$	100.78	[d]	[b]	81.83*	75.71*	61.24†
	4.57	3.74	[b]	3.68	3.58*	3.87†
$5'_3$	100.78	[d]	[b]	79.28	76.02	61.24†
	4.57	3.74	[b]	3.76	3.63	3.87†
7	102.87	55.93	[b]	79.58	76.02	61.24†
	4.54	3.76	[b]	3.74	3.63	3.87†
*7**	102.90†	56.22*	83.30	69.38	75.96*	63.14[e]
	4.54	[c]	3.79	3.65[e]	3.55*	3.86[e]
$6,6'_6$	104.82	72.02	74.16	69.61	74.97	64.62
	4.43	3.53	3.63	3.92	3.81	3.98†
$6'_3$	103.88*	70.65	76.70*	68.76	76.62*	62.29
	4.54	3.57	4.11	3.96	3.71	3.73
8	103.93*	70.65	76.76*[f]	68.76	76.42*[f]	62.29
	4.54	3.57	4.11	3.96	3.71	3.73
*8**	104.87	70.36	76.92	68.57	76.36	62.29
	[c]	3.52	4.08	3.93	3.66	3.73

	Position[g]					
	4	*5*	*6*	*7*	*8*	*9*
Neu5Ac	69.61	52.98	73.71*	69.69*	73.05*	63.95*
(2→3)	3.69	3.84	3.66*	3.55*	3.89	3.87; 3.64
Neu5Ac	69.48	53.17	74.16*	69.76*	72.29*	63.86*
(2→6)	3.65	3.80	3.63*	3.56*	3.89	3.87; 3.64

[a]The top entry is for ^{13}C and the bottom for ^{1}H. Accuracy of the proton chemical shifts is ~0.03 p.p.m. The numbering of residues corresponds to that shown in the text, and subscripts denote Neu5Ac linkage positions on the Gal residues. The ^{13}C signal overlap sometimes combined with weak cross-peaks and yielded only approximate values for the chemical shift (marked †). * Denotes either ^{13}C or ^{1}H assignments that may be interchanged within any column. [b]One-on-one assignments for these positions could not be made. It may be one of the signals: 73.41 with 3.72, 3.85; 73.33, 3.68, 3.89, 3.76, 3.66; and 73.26 with 3.76, 3.89, 3.69 p.p.m. [c]The shift of the proton resonance deviates from its true position and therefore is not given. It results from the fact that the corresponding carbon resonance is heavily overlapped by other resonances which give rise to much stronger cross-peaks. Large differential rates of relaxation for the proton resonances, often observed for large molecules, are responsible for these large

heteronuclear-correlated 2D-n.m.r. spectrum for the anomeric region has been analysed[29] and we made no attempt to optimise experimental conditions for cross-peaks in that region.

The ^{13}C assignments were compared with published data[26]. Correlation of firmly assigned resonances in the ^{1}H-n.m.r. spectrum with corresponding resonances in the ^{13}C-n.m.r. spectrum led to a reversal of a few assignments in the latter spectrum[26]. On the other hand, several unambiguously assigned ^{13}C resonances yielded firm assignments for ^{1}H signals. Thus, the assignments for C-3 and C-5 of Gal-*8* had to be reversed and those for H-5 of Gal-*6* and Gal-*6'*, which are substituted by an α-Neu5Ac-(2→6) residue, reassigned. Most of the assignments were confirmed independently, using phase-sensitive COSY and RELAYED-COSY to trace out the proton connectivities. The resulting ^{1}H assignments were also compared with the data for related oligosaccharides[8,9,16,18–20,29,32–34].

No complete assignments of the ^{1}H resonances of Neu5Ac have been reported for sialyloligosaccharides. For Neu5Ac, the resonances of H-4/9 fall in the range 3.65–3.90 p.p.m. and overlap with resonances belonging to other carbohydrate units. The chemical shifts (~3.67 and 3.64 p.p.m., respectively) for the H-4 and H-6 resonances of the Neu5Ac linked to Gal were found to be somewhat different from the corresponding chemical shifts (3.80 and 3.54 p.p.m., respectively) in free α-Neu5Ac[33], which reflects shielding by the aglycon group. For the same reason, there is also a slight difference in the chemical shift for the ^{1}H resonances of the Neu5Ac, depending on the linkage position [(2→3) *vs.* (2→6)].

Surprisingly, the chemical shifts of ^{1}H resonances of Gal-*8** and GlcNAc-*7** were similar to those of the corresponding resonances of Gal-*8* and GlcNAc-*7*, whereas the corresponding ^{13}C resonances had much larger differences in chemical shifts. Therfore, assignments for these protons were obtained through the heteronuclear-correlated spectrum. For example, the C-3 resonance of GlcNAc-*7** was shifted down-field (10 p.p.m.) relative to that of C-3 of GlcNAc-*7*, but the corresponding ^{1}H resonance was hardly affected, as was evident also from the absence of a RELAYED-COSY cross-peak between H-1 and H-3 of GlcNAc-*7** outside the observed range of chemical shifts for the H-3 resonance of the GlcNAc residues substituted either at C-2 or at C-4.

Comparison of ^{1}H-n.m.r. chemical shifts. — The chemical shift data were obtained principally from the phase-sensitive COSY spectra of the title glyco-

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differences in the intensities of cross-peaks. Subsequent overlap of cross-peaks having low intensity with the tails of the more prominent cross-peaks leads to the observed distortion. For the correct proton chemical shift see Table II. [d]The corresponding resonance may be either 56.13 or 55.86 p.p.m. [e]Tentative assignments. [f]Assignments reversed from that given in ref. 26. [g]For comparison, the ^{1}H-n.m.r. chemical shifts for the resonances of 3- and the 6-linked Neu5Ac are: H-4, 3.69 and 3.66; H-5, 3.85 and 3.81; H-6, 3.63; H-7, 3.56 and 3.56; H-8, 3.88 and 3.88 p.p.m.; as determined by homonuclear 2D-n.m.r. techniques.

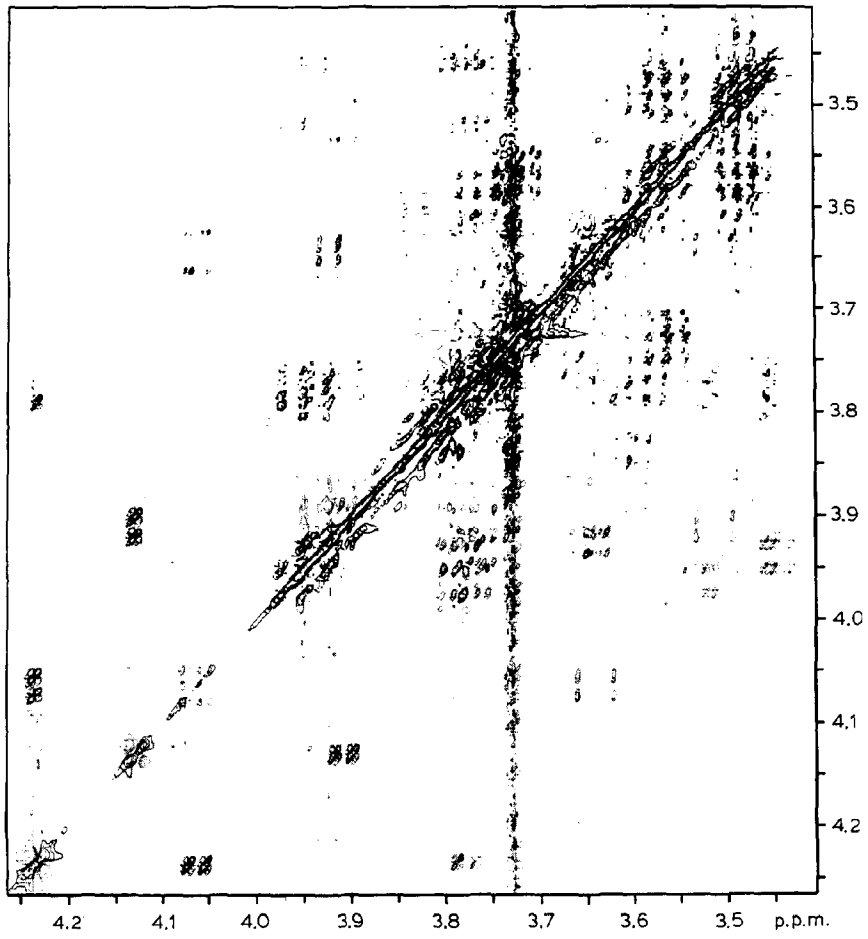
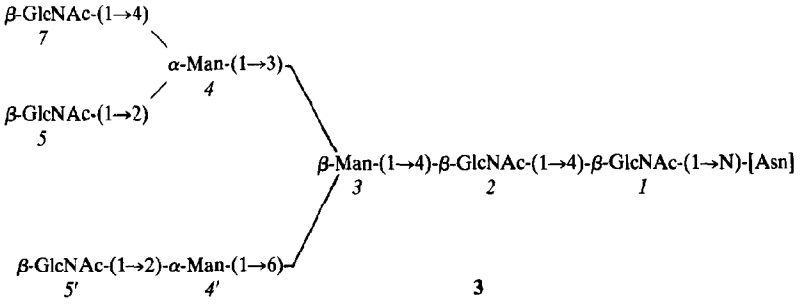


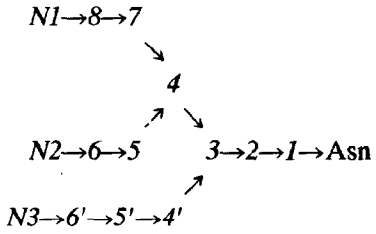
Fig. 1. Part of the phase-sensitive COSY spectrum of glycopeptide **3** (70mM in D_2O, pH 5.5, 30°). Weaker contours represent cross-peak multiplet components with positive (out of the plane of the paper) intensities and *vice versa* for the darker contours.



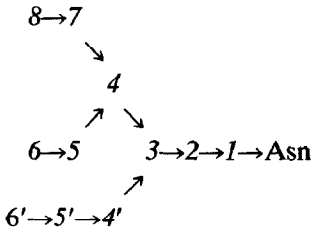
3

peptides. In this relatively new version of COSY, the multiplet components of the cross-peaks appear with alternate phases, to yield a much improved resolution for the pattern of cross-peaks[35]. High digital and spectral resolutions were employed (~0.4 Hz in the f_2 dimension). An example of a phase-sensitive COSY spectrum is shown in Fig. 1.

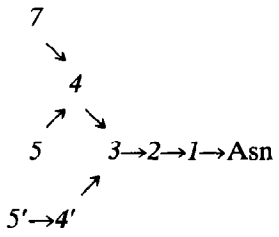
RELAYED-COSY and triple-quantum-filtered COSY spectra were used to aid the assignments of the various cross-peaks. NOESY spectral data were used only to confirm assignments. Thus, observation of a cross-peak in the NOESY spectrum between the chemical shift positions of the H-1 and H-5 resonances of Gal-*6* has served merely as an additional proof of the assignment of the H-5 resonances by other methods. Chemical shift data and assignments obtained for the *N*-linked glycopeptide of fetuin and its fragments are listed in Table II. The chemical shift data and assignments for the pentasaccharide glycopeptide were taken from a recent work[9]. The corresponding structures are shown below:



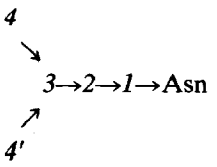
1 (parent glycopeptide)



2 (−Neu5Ac)



3 (−Gal)



4 (−GlcNAc)

Definite assignments for several important ^{1}H resonances could not be made due to severe overlap and second-order coupling effects. These were the assignments for the H-5, H-6, and H-6′ of most of the Gal residues in the structures studied, and for H-4/6′ of each GlcNAc residue in the native *N*-linked glycopeptide. In principle, the missing assignments could be obtained from the heteronuclear-correlated 2D-n.m.r. spectra, but the chemical shifts of the ^{1}H resonances so determined did not allow an accurate evaluation of small changes in the chemical shift under the restrictive experimental conditions.

The substituent chemical shift (SCS) data, given in Table III as $\Delta\delta$ values, were obtained by the subtraction of the chemical shift of a given ^1H resonance from that of the corresponding resonance in the parent molecule. Two types of SCS can be distinguished, originating (*a*) in through-bond electronic interactions, and hence largest for the proton at the glycosylation site (an α-position) and rapidly decreasing with increasing number of intervening bonds; and (*b*) from through-space interactions, mainly from shielding by neighbouring groups. The latter includes the effects of magnetic anisotropy and electric field and shows a strong dependence on distance and orientation and hence reflects conformational features. However, it is generally difficult to extract specific information on the relative orientation of two carbohydrate residues which participate directly in glycosylation. In such struc-

TABLE II

^1H-N.M.R. CHEMICAL SHIFT DATA (P.P.M.) FOR THE *N*-LINKED GLYCOPEPTIDES (**1–4**) DERIVED FROM CALF FETUIN[a]

Proton	**1**	**2**	**3**	**4**[b]	*Proton*	**1**	**2**	**3**	*Proton*	**1**	**2**
3					*5*				6_6		
1	4.77	4.74	4.75	4.78	1	4.57	4.56	4.54	1	4.44	4.49
2	4.21	4.20	4.21	4.25	2	3.75	3.74	3.70	2	3.52	3.54[e]
3	3.78	3.75	3.75	3.76	3	3.72	3.69	3.54	3	3.66	3.67[e]
4	3.78	3.80	3.80	3.75	4		3.72	3.45	4	3.92	3.92
5		3.54[d]	3.61	3.64	5		3.56	3.42	5		*f*
6	3.78	3.77	3.77	3.79	6	3.99	3.97	3.91	6	3.82[d]	*f*
6′	3.95	3.94	3.96	3.86	6′	3.86	3.83[e]	3.74	6′	3.99[d]	*f*
4					*5′*				$6'_3$		
1	5.12	5.11	5.11	5.10	1	4.59	4.57	4.55	1	4.54	4.46
2	4.22	4.21	4.21	4.06	2	3.76	3.75	3.70	2	3.56	3.53[e]
3	4.09	4.04	4.03	3.88	3	3.69	3.68	3.54	3	4.12	3.66[e]
4	3.61	3.62	3.61	3.62	4		3.72	3.47	4	3.96	3.92
5	3.77	3.76	3.76	3.64[c]	5		3.56	3.42	*8*		
6	3.88	3.87	3.87	3.93	6		3.97	3.91	1	4.55	4.46
6′	3.74	3.74	3.75	3.73	6′		3.84[e]	3.76	2	3.57	3.53
4′					*7*				3	4.12	3.66
1	4.91	4.91	4.91	4.91	1	4.54	4.54	4.52	4	3.96	3.93
2	4.11	4.10	4.10	3.97	2	3.79	3.79	3.74	*8**		
3	3.89	3.87	3.89	3.90	3	3.69	3.72	3.56	1	4.50	4.43
4	3.48	3.48	3.49	3.62	4		3.72	3.46	2	3.51	3.51
5	3.62	3.61	3.61	3.64[c]	5		3.63[d]	3.50	3	4.09	3.64
6		3.89	3.89	3.93	6		4.00	3.94	4	3.93	3.91
6′		3.62	3.62	3.73[c]	6′		3.83	3.75			
					*7**						
					1	4.57	4.56				
					2	3.90	3.89				
					3		3.79				

[a]Recorded for solutions in D_2O (~70mM) at 30° and pH 5.5. Chemical shifts are given to the nearest 0.01 p.p.m., though accuracy of measurement is ±0.002 p.p.m. [b]Data taken from Paulsen *et al.*[43]. [c]Homans *et al.*[17] gave 3.79 p.p.m. for H-5 of Man-*4*, 3.75 p.p.m. for H-5 of Man-*4′*, and 3.70 p.p.m. for H-6′ of Man-*4′*. [d]Tentative assignment only. [e]Assignments may be interchanged down any column. [f]H-5 and H-6,6′ are located at 3.72 and 3.74 p.p.m., as determined from the triple-quantum-filtered COSY spectrum.

TABLE III

^{1}H-N.M.R. SUBSTITUENT CHEMICAL SHIFT (SCS) VALUES OF THE *N*-LINKED GLYCOPEPTIDE DERIVATIVES (**2–4**) FROM CALF FETUIN[a]

Proton	**2**	**3**	**4**[b]	*Proton*	**2**	**3**	*Proton*	**2**
Man-*3*				GlcNAc-*5*			GlcNAc-*7**	
1	**0.03**	−0.01	**−0.03**	1	0.01	0.02	1	0.01
2	0.01	−0.01	**−0.04**	2	0.01	*0.04*	2	0.01
3	**0.03**	0.00	−0.01	3	**0.03**	*0.15*	3	
4	−0.02	0.00	**0.05**	4		*0.27*	4	
5		−0.07[c]	**−0.03**	5		*0.14*	*Gal-6*$_6$	
6	0.01	0.00	−0.02	6	0.02	*0.06*	1	**−0.05**
6′	0.01	−0.02	**0.10**	6′	**0.03**	*0.09*	2	−0.02
Man-*4*[d]				GlcNAc-*5′*			3	−0.01
1	0.01	0.00	*0.01*	1	0.02	0.02	4	0.00
2	0.01	0.00	*0.15*	2	0.01	*0.05*	Gal-*6′*$_3$	
3	**0.05**	0.01	*0.15*	3	0.01	*0.14*	1	*0.08*
4	−0.01	0.01	*−0.01*	4		*0.25*	2	*−0.02*
5	0.01	0.00	*0.12*	5		*0.14*	3	*0.46*
6	0.01	0.00	*−0.06*	6	0.00	*0.06*	4	*0.04*
6′	0.00	−0.01	*0.02*	6′	0.00	*0.08*	Gal-*8**	
Man-*4′*				GlcNAc-*7*			1	*0.07*
1	0.00	0.00	*0.00*	1	0.00	0.02	2	*0.00*
2	0.01	0.00	*0.13*	2	0.00	*0.05*	3	*0.45*
3	0.02	−0.02	*−0.01*	3	**−0.03**	*0.16*	4	*0.02*
4	0.00	−0.01	*−0.13*	4		*0.26*		
5	0.01	0.00	*−0.03*	5		*0.13*		
6		0.00	*−0.04*	6		*0.06*		
6′		0.00	*−0.11*	6′		*0.08*		

[a]See footnote *a* of Table II. An average standard deviation of 0.002 p.p.m. (63 entries) was observed for all SCS values ≤0.02 p.p.m. Italicised entries refer to SCS values of glycosylated residues. Long range SCS values with $\Delta\delta > 0.02$ p.p.m. are indicated in bold type. [b]These values were derived in part from published data[43], for which the reference signal (acetone) was set at 2.22 p.p.m. from DSS, compared with 2.225 p.p.m. used in the present work; therefore, the accuracy of the glycosylation shifts was taken as ±0.02 p.p.m. only. [c]This value was not used because of the uncertainty of the assignment. [d]The italicised values list the combined effect of the 2,4-disubstitution of this Man residue.

tures, shielding occurs at a short distance for any possible conformation of the residues so that information on shielding is non-specific. On the other hand, long-range SCS effects, observed for carbohydrate rings that are widely separated, unmistakably point to a conformation bringing these two rings into close proximity[10,36]. The second type of SCS was also useful for the analysis of the primary structures of oligosaccharides[4]. Unless influenced by either or both of these types of SCS, the chemical shifts of all the other resonances are expected to remain constant within experimental error. Indeed, the chemical shifts of most of the ^{1}H resonances of the glycopeptides studied remained essentially constant. In the analysis of the SCS values, only differences in the chemical shifts of ≥0.03 p.p.m. are considered to be unambiguous, even though the accuracy of the n.m.r. measurements was nearly an order of magnitude better.

The entries italicised in Table III refer to SCS values for glycosylated residues, and most of the values listed can be rationalised as follows. The glycosylation shifts observed for GlcNAc-*5*, -*5'*, and -*7* confirmed the previously reported regularity in which the SCS values are largest for the protons at the glycosylation site (α-SCS) and the two vicinal positions (β-SCS)[36]. They are clearly a result of both through-bond and short-distance through-space interactions. The β-SCS values for sialylation are small, possibly because of mutual cancellation of the through-bond and the through-space contributions. The same argument applies to the β-SCS values of the 2-substituted Man-*4'* residue. A seemingly unexpected, large, negative, up-field γ-SCS value was observed for Man-*4'*, which results from a shielding of H-4 of Man by the axially oriented GlcNAc-*5'*. Indeed, in the minimum energy conformations calculated for several oligosaccharides carrying the same disaccharide fragment[9], H-4 is located within the shielding cone of the acetyl group of GlcNAc-*5'*. Correspondingly, the H-6 and H-6' of Man-*4'*, which enter the acetyl shielding cone during the rotation of the hydroxymethyl group, have their resonances shifted up-field. The changed orientation of the electron pairs on O-2 of Man-*4'* may also contribute to the observed γ-SCS value. The approximate additivity of the calculated SCS values is best demonstrated for the 2,4-diglycosylated Man-*4*, for which the chemical shift of the H-4 resonance remains practically unchanged (−0.01 p.p.m.) because of equally strong α-deshielding and γ-shielding effects. The SCS values for the other ring protons are expected to follow similar additivity rules. For further verification, the calculated SCS values for the presently unavailable 4-substituted Man are included as the last entry in Table IV, where some of the relevant SCS values are grouped together. They are classified by the position of the observed proton relative to the substitution site.

TABLE IV

^{1}H-N.M.R. GLYCOSYLATION SHIFTS FOR SOME CARBOHYDRATE SUBSTITUTIONS IN OLIGOSACCHARIDE STRUCTURES[a]

Residue	*Substitution by*	*SCS (p.p.m.) for protons in position*		
		α	β	γ
β-Gal-(1→4)	α-Neu5Ac-(2→3)	0.45	~0.02	0.05
β-GlcNAc-(1→2)	β-Gal-(1→4)	0.26	0.14	0.05
β-GlcNAc-(1→4)	β-Gal-(1→4)	0.26	0.14	0.06
α-Man-(1→6)	β-GlcNAc-(1→2)	0.13	−0.01	−0.13
α-Man-(1→3)[b]	β-GlcNAc-(1→4)	0.12	0.12	~0.02

[a]The SCS values are obtained by subtraction of the chemical shift of the resonance of a given proton in the unsubstituted carbohydrate (column 1) from that of the corresponding proton after substitution by another carbohydrate (column 2). The actual SCS values were calculated from Table III, taking averages where reasonable. [b]The SCS values for a 4-substituted Man were calculated by using the experimental data given for the 2,4-diglycosylated Man-*4* and the 2-glycosylated Man-*4'* residues, assuming additivity for SCS values.

Appreciable long-range SCS values for most of the protons belonging to Man-*3* in the inner core occur on substitution by three GlcNAc residues (set in bold type in Table III). On the basis of this observation, it is proposed that the relative orientations of Man-*4* and Man-*4'*, in the inner core pentasaccharide, must be somewhat altered upon substitution by three GlcNAc residues.

Glycosylation of the Gal residues by Neu5Ac did produce some long-range SCS values (H-1 and H-3 of Man-*3*, H-3 of Man-*4*, H-3 and H-6' of GlcNAc-*5*, and H-3 of GlcNAc-*7*) as shown in Table III. Some of these shifts were noted previously[4] and more recently it was suggested that the Neu5Ac is folded back toward the inner residues of the antenna[6]. This is probably true for the Gal-*6*–GlcNAc-*5*–Man-*4*– antenna which is exclusively substituted by an α-Neu5Ac-(2→6) residue[28]. In order to help to clarify this point, attempts were made to observe inter-residue n.O.e. peaks for the protons of Neu5Ac. A n.O.e. between the axial H-3 of Neu5Ac and H-3 of the penultimate Gal residue was observed for C-3 substitution but not for C-6 substitution. Only a few of the Neu5Ac protons could be monitored for the inter-residue n.O.e. because of serious overlap of signals. Other inter- and intra-residue n.O.e. cross-peaks, connecting protons other than the Neu5Ac protons, were observed in the NOESY spectrum, which corresponded to the already well-characterised n.O.e. connectivities, previously published, for related oligosaccharides.

No generalised empirical set of SCS data can be derived for carbohydrate substitutions and their value is limited. They can be used to distinguish between two different types of substitution on the same residue, but cannot be used to predict the ^{1}H-n.m.r. spectrum of a given oligosaccharide. Each type of substitution involves both the nature of the substituting carbohydrate residue, the position of substitution, the anomeric configuration, and changes in the molecular conformation.

Analysis of the coupling constants. — The magnitudes of the observed $J_{H,H}$ values are known to be correlated with the electronegativity of the nearby substituents and with the conformation of the molecule. When there is molecular flexibility, the *J* values are often an average of several values associated with two or more conformers in equilibrium. The combination of high resolution and the phase-sensitive mode in acquiring the spectra has made it possible to determine the coupling constants for several cross-peaks.

The vicinal $J_{3,4}$ values of Man-*4* and Man-*4'* differ by 0.5 ±0.11 Hz (9.5 and 10 Hz, respectively), which is most likely a result of the introduction of GlcNAc-*7* at C-4 of Man-*4* relative to Man-*4'*. It was not possible to account for contributions to the *J* values arising from a possible flattening of the pyranoside ring of Man-*4* as a result of the 2,4-disubstitution by GlcNAc. The geminal $J_{6,6'}$ values for Man-*3* and Man-*4* also differ by 2.1 ±0.13 Hz (10.9 and 13.0 Hz, respectively). Geminal coupling constants depend on the relative orientation of vicinal electronegative substituents[37,38] and differences can be attributed largely to different relative rotamer populations of the substituted hydroxymethyl of Man-*3* compared with the free hydroxymethyl group of Man-*4*.

Unexpected differences were observed for the vicinal $^3J_{5,6'}$ and the geminal $^2J_{6,6'}$ values of GlcNAc-*1* and GlcNAc-*2* (7.5 *vs.* 4.7 Hz and 11.5 *vs.* 12.6 Hz, respectively). They are large enough to be observed by direct inspection of the two cross-peaks (Fig. 2). These differences in coupling constants (2.8 ±0.14 Hz and 1.1 ±0.21 Hz, respectively) reflect different rotamer populations for the two hydroxymethyl groups which can arise only from different interactions for the, otherwise unperturbed, hydroxymethyl groups of the inner core GlcNAc-*1* and -*2*. We have found that for GlcNAc-*2*, rather than for GlcNAc-*1*, these *J* values deviate from the accepted values observed for GlcNAc-*5*, -*5'*, -*7* and other model compounds[39]. It was suggested previously that the α-D-Man-(1→6) arm of biantennary structures adopts partially a conformation in which that arm is tilted toward the inner core residues (Man-*3*–GlcNAc-*1*–GlcNAc-*2*)[40]. In such a conformation, it is possible that the hydroxymethyl group of GlcNAc-*2* will be affected by the presence of the folded-back arm more than will the hydroxymethyl group of GlcNAc-*1*. A recent study pointed to the possible existence of some non-bonded interactions between GlcNAc-*5'* and GlcNAc-*2* in related biantennary structures[10]. The respective chemical shift differences for these H-6 resonances are only 0.02 and 0.06 p.p.m., *i.e.*, less than the typical down-field shift of >0.1 p.p.m. frequently encountered as a result of non-bonded interactions of an oxygen and a given proton[41,42]. Nevertheless, the change in the coupling constants clearly indicates the existence of such interactions. However, it cannot be ruled out that the observed dependency of some SCS values on the molecular conformation, and of the changes in the magnitudes of the coupling constants, is a result of deviations from the chair conforma-

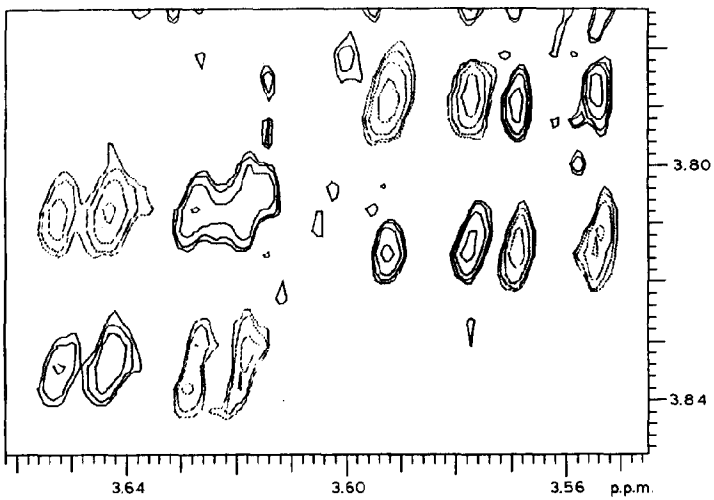


Fig. 2. Contour plot of a section of the 500-MHz 2D phase-sensitive COSY spectrum _g the two H-6,6' cross-peaks of GlcNAc-*1* and GlcNAc-*2*. Differences in the magnitudes of their $J_{5,6}$ values (4.7 Hz for GlcNAc-*1* and 7.5 Hz for GlcNAc-*2*) lead to the observed different appearances of the cross-peaks. Dashed contours represent components with positive intensity.

tion. Thus, for Man-*3*, the smallest deviation from the corresponding, expected, monosaccharide chair conformation, undoubtedly, will affect the relative orientation of the antennae.

The flexibility of the α-D-Man-(1→6) arm is retained to a large degree in the triantennary structure as was shown by the analysis of the coupling constants in the relevant cross-peaks. As the size of the oligosaccharide structures increases from five to fourteen residues, definite changes in the relative orientations of the various antennae occur. Therefore, it is predicted that a somewhat different set of interresidue n.O.e. factors will be derived for each of the related structures.

Calculations of the conformations of oligosaccharides are based mainly on studies of n.O.e. Thus, it is imperative that n.O.e.-based assignments will not be used as a sole source of assignment, especially when the same n.O.e. data are used later in a semi-iterative manner in the calculation of the conformation. Sometimes, wrong assignment of a single proton resonance can be critical for the proposed conformation of an oligosaccharide as demonstrated by the conformation proposed[11,30] for the α-D-Man-(1→6) antenna, which was apparently contested[17] because of disagreement over a single proton assignment (H-5 of Man-*4*). Therefore, we have restricted our assignments to data derived from sources other than n.O.e. experiments in order to provide unbiased data.

It is important to study the conformation of glycopeptides, in preference to the corresponding oligosaccharide side-chains, because they are more likely to possess the conformations present in the parent glycoproteins. We have provided spectral assignments for a triantennary glycopeptide even though the sample was a mixture of closely related structures. These assignments form a basis for using n.O.e. methods in the elucidation of the solution conformations adopted by *N*-linked triantennary side-chains of glycoproteins.

Finally, it has been demonstrated that the high-resolution phase-sensitive COSY technique provides, in addition to the more accurate chemical shift values, a useful way to extract information on coupling constant for overlapping resonances. Subsequently, these coupling constants and additional n.O.e. data can be used as constraint parameters in calculations of the minimum energy conformations of complex oligosaccharides, in much the same way as is done for the elucidation of the 3D structures of proteins.

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