500 MHz ^1H n.m.r. of oligosaccharides of *N*-acetyl-lactosamine-type released from human erythrocyte glycopeptides by endo- β -galactosidase

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500 MHz ^1H n.m.r. spectroscopy has been used in structural studies of three linear and five branched oligosaccharides of *N*-acetyl-lactosamine-type that were released from desialylated blood group O erythrocyte glycopeptides by treatment with the endo- β -galactosidase of *Bacteroides fragilis* followed by reduction. The following oligosaccharide alditols were characterized:

INTRODUCTION

Oligosaccharides of the poly-*N*-acetyl-lactosamine series are widely distributed on human and other mammalian cells as deduced from immunochemical studies with the monoclonal autoantibodies, anti-i and anti-I, directed at the linear and branched sequences, respectively (Childs *et al.*, 1980; Thorpe & Feizi, 1984). Structural studies of oligosaccharides of this type have been greatly helped by the availability of endo- β -galactosidases which fragment the long-chain sequences into shorter oligosaccharides (Hounsell *et al.*, 1981; Fukuda *et al.*, 1979, 1984a,b; Scudder *et al.*, 1986). The endo- β -galactosidase of *Bacteroides fragilis* is one such enzyme which cleaves β -galactosidic linkages on linear

domains of oligosaccharides and glycolipids with poly-*N*-acetyl-lactosamine backbones but not those adjacent to branch points (Scudder *et al.*, 1984). In order to assess the specificity of *B. fragilis* endo- β -galactosidase towards branched poly-*N*-acetyl-lactosamine structures on membrane glycopeptides, the oligosaccharides released by this enzyme from desialylated human erythrocyte glycopeptides were isolated and partially characterized by fast atom bombardment-mass spectrometry (f.a.b.-m.s.) of permethylated derivatives and g.l.c.-m.s. of partially methylated alditol acetates (Scudder *et al.*, 1987). In the present investigation the complete structure of the six major oligosaccharides (di- to hepta-saccharide) have been determined using 500 MHz ^1H n.m.r. spectroscopy and information obtained on two

Abbreviations used: f.a.b.-m.s., fast atom bombardment-mass spectrometry; h.p.t.l.c., high performance thin layer chromatography; DSS, sodium 4,4-dimethyl-4-silapentane 1-sulphonate.

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Table 1. The ^1H n.m.r. chemical shifts (p.p.m. from DSS) of oligosaccharide alditols obtained from desialylated human erythrocyte (blood group O) glycopeptides by endo- β -galactosidase digestion and of a chemically synthesized pentasaccharide alditol standard

Symbols used: $\bigcirc$, Gal β 1-4; ϕ , Gal-ol; $\square$, GlcNAc β 1-3 (to Gal-ol); $\square$, GlcNAc β 1-3 (to Gal); $\blacksquare$, GlcNAc β 1-6; $\triangle$, Fuc α 1-2. N.d., not determined. Signals in the spectrum of fraction VI which could not be assigned unambiguously to either isomer are indicated by /.

Alditol	Proton	Human erythrocyte oligosaccharides						Standard
		II	III	IV	Vla	Vlb	VIIa	Penta-ol
Gal _{ext} ³	H-1		4.451	4.520	4.448		4.465	4.451
	H-2		3.532	N.d.	3.561/7		3.538	3.533
	H-4		3.922	N.d.	N.d.		3.923	3.923
Gal _{ext} ⁶	H-1					4.472	4.479	4.477
	H-2					3.567/1	3.559	3.541
	H-4					—	3.923	3.923
Gal _{int}	H-1				4.431	4.431	4.430	
	H-4				4.148	4.148	4.151	
GlcNAc to Gal-ol	H-1	4.591	4.614	4.591	4.618/4.614		4.612	4.606
	NAc	2.072	2.069	2.077	2.077	2.077	2.076	2.070
GlcNAc (1-3)	H-1				4.685/4.667		4.689	
	NAc				2.032	2.032	2.030	
GlcNAc (1-6)	H-1				4.589/4.585		4.624	4.573
	NAc				2.063/2.057		2.058	2.051
Fuc	H-1			5.312				
	H-5			4.224				
Gal-ol	—CH ₃			1.233				
	H-2	3.997	3.993	3.998			N.d.	3.985
	H-3	3.874	3.873	N.d.	N.d.	N.d.	N.d.	N.d.
	H-4	3.795	3.795	N.d.	N.d.	N.d.	N.d.	N.d.
	H-5	4.135	4.131	4.133	4.127	4.127	4.123	4.251

Table 2. The chemical shifts (p.p.m.) and coupling constants (Hz) for protons of the *N*-acetylglucosamine residue of alditol II GlcNAc β 1-3Gal-ol and the coupling constants of related oligosaccharides GlcNAc β 1-3Gal and GlcNAc β 1-3GalNAc-ol

	Chemical shifts (p.p.m.)		Coupling constants (Hz)		
	GlcNAc β (1-3Gal-ol)		GlcNAc β (1-3Gal-ol)	GlcNAc β (1-3Gal)*	GlcNAc β (1-3GalNAc-ol)†
H-1	4.591	$J_{1,2}$	8.5	8.5	8.1
H-2	3.746	$J_{2,3}$	10.4	10.2	10.2
H-3	3.573	$J_{3,4}$	8.5	9.0	8.4
H-4	3.439	$J_{4,5}$	9.5	9.6	9.8
H-5	3.471	$J_{5,6}$	2.3	2.2	2.0
H-6	3.954	$J_{5,6'}$	6.1	6.0	6.3
H-6'	3.746	$J_{6,6'}$	12.1	11.2	12.0

* GlcNAc β 1-3Gal obtained from keratan sulphate proteoglycan (Hounsell *et al.*, 1986).

† GlcNAc β 1-3GalNAc-ol obtained from meconium glycopeptides (Hounsell *et al.*, 1985).

minor fucosylated oligosaccharides (hepta- and octasaccharide).

MATERIALS AND METHODS

Erythrocyte oligosaccharides

The oligosaccharides were obtained from desialylated erythrocyte glycopeptides by digestion with endo- β -galactosidase of *B. fragilis* and purified by Bio-Gel P4 chromatography after reduction (Scudder *et al.*, 1987). Oligosaccharide alditol fractions designated II-IX were

obtained and two additional fractions, X and XI, containing oligosaccharide alditols and glycopeptides. Of these fractions II-VI and VIII-IX appeared homogeneous by h.p.t.l.c. (Scudder *et al.*, 1987): fraction VII showed a major slow-migrating band and a minor faster-migrating band designated VIIa and VIIb, respectively, and oligosaccharide fraction VI is shown in the present study to contain two hexasaccharides, VIa and VIb. Due to the low yield of fractions V and IX and the polydispersity of fractions X and XI these were not analysed by n.m.r.

Standard oligosaccharide alditol

The chemically synthesized oligosaccharide standard (analysed as its alditol):

was a gift of Dr. A. Veyrières, Université Paris-Sud Orsay, France (Augé *et al.*, 1979).

N.m.r. spectroscopy

The oligosaccharides (10–200 nmol) were lyophilized four times with deuterated water (D4501, Sigma Chemical Co., Poole, Dorset, U.K.) taken up in 400 μ l deuterated water (D9764, Sigma) and transferred to n.m.r. tubes (Wilmad Glass Co., Buena, NJ, U.S.A.) with 1 μ l of acetone as internal standard. The 500 MHz ¹H n.m.r. spectra were obtained at 22 °C with a Bruker AM 500 Spectrometer operating in the Fourier transform mode and equipped with an Aspect 3000 computer. Chemical shifts were measured from acetone which resonates at 2.225 p.p.m. from sodium 4,4-dimethyl-4-silapentane 1-sulphonate (DSS). Coupling constants were obtained from a first-order analysis of the spectra.

Spectra were obtained using quadrature detection with a spectral width of 6400 Hz and the results were collected using 32000 data points (giving 0.4 Hz/point digital resolution). Usually 300–500 transients were accumulated. Before Fourier transformation, the free-induction decay was multiplied by an exponential function giving a line broadening of 0.5 Hz to improve the signal-to-noise ratio. Resolution-enhanced spectra were obtained by a Gaussian multiplication technique (Ferrege & Lindon, 1979).

RESULTS AND DISCUSSION

Table 1 shows the 500 MHz ¹H n.m.r. chemical shifts for the major oligosaccharides released from desialylated erythrocyte glycopeptides by *B. fragilis* endo- β -galactosidase (oligosaccharides in fractions II–IV and VI, and oligosaccharide VIIa) and the standard pentasaccharide alditol. The proton assignments are discussed below.

Straight-chain oligosaccharide alditols II, III and IV

Complete assignment of the signals in oligosaccharide II could be made by using spin-decoupling techniques to connect multiplets from coupled nuclei and from considerations of the measured coupling constants. The chemical shifts were similar to those previously reported (Donald & Feeney, 1986) for the proposed disaccharide alditol GlcNAc β 1–3Gal-ol (Tables 1 and 2). For comparison the chemical shifts of the GlcNAc residue are shown in Table 2, together with the coupling constants. This emphasizes their similarity to those found in the non-reduced oligosaccharide, GlcNAc β 1–3Gal (obtained from bovine corneal keratan sulphate; Hounsell *et al.*, 1986) and those of the related oligosaccharide GlcNAc β 1–3GalNAc-ol (Hounsell *et al.*, 1985), thus confirming the presence of GlcNAc β 1.

It could be deduced that the linkage of GlcNAc to Gal-ol in oligosaccharides II–IV is 1–3 rather than 1–6 by comparison of the chemical shifts of H-5 of the Gal-ol moieties (4.135, 4.131 and 4.133 p.p.m., respectively;

Table 1) with the H-5 of GalNAc-ol in oligosaccharides having GlcNAc linked to the 3- or 6-position e.g. Gal β 1–4GlcNAc β 1–3GalNAc-ol (Van Halbeek *et al.*, 1982) and Gal β 1–4GlcNAc β 1–6GalNAc-ol (Feeney *et al.*, 1986). The H-5 GalNAc-ol chemical shifts are 4.143 and 4.027 p.p.m., respectively.

In the trisaccharide III, the presence of a Gal residue linked β 1–4 to GlcNAc could be deduced by the similarity in chemical shifts of H-1, H-2 and H-4 of the non-reducing-end Gal in the Gal β 1–4GlcNAc β 1–3 sequence (Gal_{ext}³) of this alditol (4.451, 3.532 and 3.922 p.p.m., respectively; Table 1) with those of the H-1, H-2 and H-4 protons of the standard pentasaccharide alditol (4.451, 3.533 and 3.923 p.p.m., respectively; Table 1) and the similarity of the H-1 and NAc chemical shifts of –4GlcNAc β 1–3 in these two alditols (4.614, 2.069, 4.606 and 2.070 p.p.m., respectively; Table 1). The assignments agree with those previously reported for this trisaccharide alditol obtained from Tamm–Horsfall glycoprotein (Donald & Feeney, 1986).

In the tetrasaccharide IV, the presence of Fuc in an α 1–2-linkage to the non-reducing-end Gal β 1–4 of oligosaccharide III was evident from a comparison of the chemical shifts of the H-1, H-5 and –CH₃ of this Fuc (i.e. 5.312, 4.224 and 1.233 p.p.m., respectively; Table 1), with the chemical shifts of these protons in fucose residues of the following related oligosaccharide sequences: 5.306, 4.228 and 1.231 p.p.m., respectively, for Fuc α 1–2Gal β 1–4GlcNAc β 1–6 (Rao *et al.*, 1985); 5.189, 4.293 and 1.233 p.p.m., respectively, for Fuc α 1–2Gal β 1–3GlcNAc β 1–3 (Hounsell *et al.*, 1987); 5.028, 4.882 and 1.180 p.p.m., respectively, for Fuc α 1–4(Gal β 1–3)GlcNAc β 1–3Gal (Hounsell *et al.*, 1987); and, 5.127, 4.830 and 1.174 p.p.m., respectively, for Fuc α 1–3(Gal β 1–4)GlcNAc β 1–3Gal (Hounsell, 1987).

It was thus established that oligosaccharide alditols II, III and IV have the following structures:

Branched oligosaccharide alditols of fractions VI and VII

By h.p.t.l.c., f.a.b.-m.s. and g.c.-m.s. analysis, oligosaccharide fraction VI appeared to be homogeneous (Scudder *et al.*, 1987). However, the ¹H n.m.r. spectrum featured a series of overlapping multiplets indicative of an approximate 50:50 mixture of two isomeric hexasaccharides, designated VIa and VIb (Table 1). Oligosaccharide fraction VII, which had been shown by h.p.t.l.c. to have a major and minor component, gave a well-resolved ¹H n.m.r. spectrum. From a comparison of the intensities of the α - and β -anomeric signals and CH₃ group signals of fucose, it could be deduced that there was an approximate 4:1 mixture of an unfucosylated and fucosylated oligosaccharide, designated VIIa (Table 1) and VIIb, respectively (see the following section).

The assignment of the signals for the unfucosylated oligosaccharides, VIa, VIb and VIIa, in the spectra of fractions VI and VII were made by comparison of the chemical shift results with those of the linear oligosaccharide alditols II–IV and the standard pentasaccharide alditol (Table 1). For example, the β -anomeric

Table 3. Structures of the oligosaccharides of desialylated blood-group O erythrocyte glycopeptides released by *B. fragilis* endo- β -galactosidase and characterized in reduced form by 500 MHz ^1H n.m.r.

Oligosaccharides VIIb and VIII could not be characterized solely from their n.m.r. spectra due to low resolution caused by small sample sizes (< 10 nmol). The information obtained was combined with f.a.b.-m.s. and g.l.c.-m.s. data giving the molecular mass, sequence, and positions of linkages (Scudder *et al.*, 1987).

Structure	Oligosaccharide
GlcNAc β 1-3Gal-ol	II
Gal β 1-4GlcNAc β 1-3Gal-ol	III
Fuc α 1-2Gal β 1-4GlcNAc β 1-3Gal-ol	IV
$\begin{array}{c} \text{GlcNAc}\beta 1 \text{---} 6 \\ \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1 \text{---} 3 \end{array} \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1\text{---}3\text{Gal-ol}$	VIa
$\begin{array}{c} \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1 \text{---} 6 \\ \text{GlcNAc}\beta 1 \text{---} 3 \end{array} \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1\text{---}3\text{Gal-ol}$	VIb
$\begin{array}{c} \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1 \text{---} 6 \\ \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1 \text{---} 3 \end{array} \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1\text{---}3\text{Gal-ol}$	VIIa
$\begin{array}{c} \text{Fuc}\alpha 1\text{---}2\text{Gal}\beta 1\text{---}4 \text{---} \text{GlcNAc}\beta 1 \text{---} 6 \\ \text{GlcNAc}\beta 1 \text{---} 3 \end{array} \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1\text{---}3\text{Gal-ol}$	VIIb
$\begin{array}{c} \text{Fuc}\alpha 1\text{---}2 \text{---} \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1 \text{---} 6 \\ \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1 \text{---} 3 \end{array} \text{Gal}\beta 1\text{---}4\text{GlcNAc}\beta 1\text{---}3\text{Gal-ol}$	VIII

chemical shift of 4.451 p.p.m. for H-1 of Gal linked β 1-4 to GlcNAc β 1-3 (Gal_{ext}³) in oligosaccharide III was similar to that assigned to this proton in oligosaccharide VIa and VIIa (4.448 and 4.465 p.p.m., respectively). The chemical shifts of 4.472 and 4.479 p.p.m. for the β -anomeric protons of a Gal residue of VIb and VIIa, respectively, could then be assigned to the Gal linked β 1-4 to GlcNAc β 1-6 (Gal_{ext}⁶). Additional signals for the β -anomeric protons of Gal at 4.431 p.p.m. (fraction VI) and 4.430 p.p.m. (fraction VII) and the downfield H-4 signals at 4.148 and 4.151 p.p.m., respectively, are characteristic of a Gal residue branched at C-6 and C-3 (Dua *et al.*, 1985; Hounsell, 1987). The presence of unbranched Gal-ol linked at C-3 only by a GlcNAc β 1-residue could be deduced from the characteristic multiplets for H-5 of Gal-ol at 4.127 p.p.m. (fraction VI) and 4.123 p.p.m. (fraction VII) compared with those of similar shifts of 4.140, 4.131 and 4.133 p.p.m. found for oligosaccharide alditols II, III and IV, respectively.

A similar approach to that described above for assigning the signals for the Gal/Gal-ol protons was used to assign signals for the H-1 and NAc protons of the GlcNAc residues of fractions VI and VII. The chemical shifts of H-1 (4.618, 4.614 and 4.612 p.p.m.) and NAc (2.077 and 2.076 p.p.m.) for oligosaccharides VIa, VIb and VIIa were similar to those for the GlcNAc residue linked to Gal-ol in oligosaccharide III (4.614 and 2.069 p.p.m., respectively; Table 1). The assignment of the proton signal of the GlcNAc residue linked β 1-3 to internal branched Gal in oligosaccharide VIIa was made from the similarity of the H-1 and NAc chemical shifts (4.689 and 2.030 p.p.m., respectively) to those of GlcNAc in the sequence Gal β 1-4GlcNAc β 1-3Gal of several

oligosaccharides for which ^1H n.m.r. data has been reported in the literature, eg. 4.688 and 2.042 p.p.m. for Gal β 1-4GlcNAc β 1-3Gal β 1-3GalNAc-ol (Van Halbeek *et al.*, 1982) and 4.708 and 2.034 p.p.m. for Gal β 1-4GlcNAc β 1-3Gal β 1-4Glc-ol (Hounsell, 1987). The signals for the H-1 β -anomeric protons of GlcNAc in the spectrum of fraction VI at 4.685, 4.667, 4.589 and 4.585 p.p.m. could not be assigned unambiguously.

From the results obtained, it could be established that the major branched oligosaccharides released from the erythrocyte glycopeptides have the following structures:

Branched fucosylated oligosaccharide alditols VIIb and VIII

Because of the small amounts (approx. 10 nmol) of oligosaccharide VIIb (in fraction VII) and oligosaccharide VIII (the major component of fraction VIII) available, little accurate chemical-shift data could be obtained from ^1H n.m.r. analysis. However, in the spectra of fractions VII and VIII the H-1, H-5 and $-\text{CH}_3$ resonances characteristic of a Fuc residue in the

sequence Fuc α 1-2Gal β 1-4GlcNAc were identified at 5.314, 4.227 and 1.227 p.p.m., respectively (compared, for example, with chemical shifts of the equivalent protons in the Fuc residue of oligosaccharide IV at 5.312, 4.224 and 1.233 p.p.m, respectively; Table 1 and discussed above).

The similarity of the chemical-shift data for the H-1, H-5 and —CH₃ of the Fuc residue in oligosaccharides IV, VIIb and VIII, and those reported in the literature (Rao *et al.*, 1985), with the Fuc residue in the same Fuc α 1-2Gal β 1-4GlcNAc sequence linked β 1-6 to GalNAc-ol (5.310, 4.226 and 1.233 p.p.m., respectively), suggest that ¹H n.m.r. analysis based on such data cannot be used to distinguish between Fuc residues linked to the C-3 or C-6 branches of Gal. The n.m.r. data could therefore only be used to confirm the presence of this sequence on either branch of the heptasaccharide and octasaccharide alditol structures deduced for VIIb and VIII, respectively (Table 3), by f.a.b.-m.s. and g.l.c.-m.s. (Scudder *et al.*, 1987). In addition, the n.m.r. data could be used to deduce the β -anomeric configuration for all linkages besides fucose and the absence of any branched galactitol (see below).

Comparison of the n.m.r. data of the oligosaccharides from erythrocyte glycopeptides with those from the standard pentasaccharide alditol

The pentasaccharide alditol was analysed in reduced form in order to determine whether the ¹H n.m.r. chemical shifts for straight-chain -3Gal-ol and branched -3/6Gal-ol could be used to distinguish between the two alternatives. As can be seen from Table 1 the chemical shifts for Gal-ol in oligosaccharides II-IV, VIa, VIb and VIIa were significantly different from those of the standard pentasaccharide alditol. This provided additional proof to that obtained from f.a.b.-m.s. and g.l.c.-m.s. (Scudder *et al.*, 1987) that the major oligosaccharides released from the erythrocyte glycopeptides by *B. fragilis* endo- β -galactosidase did not contain a branched reduced-end galactose residue (the oligosaccharides shown in Table 3 make up more than 85% of the oligosaccharides released from desialylated human blood-group-O erythrocyte glycoproteins by the *B. fragilis* enzyme).

Together, these observations indicated that, as with erythrocyte glycolipids described previously (Scudder *et al.*, 1984), the endo- β -galactosidase of *B. fragilis* cannot hydrolyse branch-point β -galactosidic linkages on erythrocyte membrane glycopeptides. This enzyme therefore has strict specificity for linear domains on poly-*N*-acetyl-lactosamine backbones. This knowledge is important for the interpretation of studies using endo- β -galactosidase to deduce structural and functional information (e.g. see Loomes *et al.*, 1984; Rastan *et al.*, 1985) where the released products are available in amounts too small for physico-chemical characterization.

The ¹H n.m.r. assignments reported in the present study are amongst the first to be obtained from oligosaccharides of cell-membrane glycoproteins and will be valuable in recognizing these types of poly-*N*-

acetyl-lactosamine sequences in other studies of cell membrane and secreted glycoproteins or glycolipids. As has been discussed previously (Hounsell *et al.*, 1984), the ¹H n.m.r. data of the same oligosaccharide sequence of three to five monosaccharides are usually comparable in different oligosaccharides and therefore the new data reported here, for internal branched galactose residues linked to *N*-acetylglucosamine, will be of additional value in interpreting the spectra of oligosaccharides of branched and straight-chain poly-*N*-acetyl-lactosamine-type with diverse substituents.

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