Oligosaccharides obtained from a blood-group-Sd(a+) Tamm–Horsfall glycoprotein

An n.m.r. study

Alastair S. R. DONALD* and James FEENEY†

*Division of Immunochemical Genetics, M.R.C. Clinical Research Centre, Watford Road, Harrow, Middx HA1 3UJ, U.K., and

†Biomedical Nuclear Magnetic Resonance Centre, National Institute for Medical Research, Mill Hill, London NW7 1AA, U.K.

Treatment of Tamm–Horsfall urinary glycoprotein with *Bacteroides fragilis* endo- β -galactosidase over a range of enzyme concentrations, pH and temperature resulted in the release of a small but constant proportion of the terminal sugars, which indicates the presence in the glycoprotein of relatively few enzyme-susceptible –GlcNAc β 1–3Gal β 1–4GlcNAc– units. Three oligosaccharides were isolated from the enzyme digest and characterized as Gal β 1–4GlcNAc β 1–3Gal (I), NeuAca2–3Gal β 1–4GlcNAc β 1–3Gal (II) and GalNAc β 1–4(NeuAca2–3)Gal β 1–4GlcNAc β 1–3Gal (III) by methylation analysis and exo-glycosidase digestion. The alditols of these oligosaccharides and related structures were examined by 500 MHz ^1H -n.m.r. spectroscopy aided by spin–spin decoupling and two-dimensional correlated spectroscopy. An almost complete assignment of proton shifts was possible, and significant differences between the signals of some of the protons in the blood-group-Sd^a-active oligosaccharide III and literature values for the corresponding signals in the structurally related Cad-blood-group determinant are noted.

INTRODUCTION

The Sd^a-blood-group determinant is carried on the erythrocytes of about 92% of individuals and is the sole antigen of the Sid blood-group system (Macvie *et al.*, 1967; Renton *et al.*, 1967). The urine of Sd(a+) persons also shows Sd^a activity (Morton & Terry, 1970), and the major carrier of the urinary Sd^a determinant has been shown (Soh *et al.*, 1979) to be the T–H glycoprotein (Tamm & Horsfall, 1950), of which it is an integral part. Another carrier of this blood-group activity is a mucin-type glycoprotein that occurs in trace amounts in urine (Cartron & Blanchard, 1982).

The reactivity of Sd(a+) erythrocytes with certain lectins (see Race & Sanger, 1975), notably *Dolichos biflorus* lectin, that bind to structures with a terminal *N*-acetylgalactosamine residue suggested that this sugar formed part of the determinant. Such a view was reinforced by the observation that T–H glycoprotein from Sd(a+) individuals contained 1–2% of *N*-acetylgalactosamine compared with less than 0.2% in the glycoprotein from Sd(a–) persons (Soh *et al.*, 1980). *N*-Acetylgalactosamine was shown to occur in terminal GalNAc β 1–4Gal units when this disaccharide with a ^3H label in *N*-acetylgalactosamine was obtained by hydrazinolysis/acid hydrolysis of a T–H glycoprotein labelled in terminal galactose and *N*-acetylgalactosamine residues (Donald *et al.*, 1982). Although the disaccharide reacted with the *Dolichos* lectin, it was inactive when tested against human anti-Sd^a sera and thus does not comprise the complete Sd^a determinant.

The pentasaccharide GalNAc β 1–4(NeuAca2–3)-Gal β 1–4GlcNAc β 1–3Gal has been isolated (Donald

et al., 1983) from an endo- β -galactosidase digest of T–H glycoprotein, and this oligosaccharide was active against human anti-Sd^a sera. An n.m.r. study (Williams *et al.*, 1984) of glycopeptides derived from T–H glycoprotein has confirmed the presence of the branched terminal trisaccharide unit that occurs in the pentasaccharide. Derivatives of the pentasaccharide lacking either *N*-acetylgalactosamine or sialic acid were inactive human anti-Sd^a sera (Donald *et al.*, 1983, 1984a), indicating that both these sugars are essential constituents of the Sd^a determinant. We now report additional evidence supporting the structure proposed for the pentasaccharide, describe two other oligosaccharides isolated from the endo- β -galactosidase digests of T–H glycoprotein, and give 500 MHz ^1H -n.m.r. data for all three compounds together with those of related structures.

EXPERIMENTAL

Materials

The T–H glycoprotein was a gift from Professor W. T. J. Morgan (M.R.C. Clinical Research Centre) and was ^3H -labelled in terminal galactose and *N*-acetylgalactosamine units as described previously (Donald *et al.*, 1982). The *Bacteroides fragilis* endo- β -galactosidase was prepared by the procedure of Scudder *et al.* (1983). Jack-bean β -galactosidase and β -hexosaminidase, bovine kidney β -hexosaminidase and *Clostridium perfringens* neuraminidase were obtained from Sigma Chemical Co. *Vibrio cholerae* neuraminidase was obtained from Koch–Light Laboratories. The ion-exchange resins AG1 X4 (200–400 mesh) and AG50W X8 (200–400 mesh) were

Abbreviation used: T–H glycoprotein, Tamm–Horsfall glycoprotein.

* To whom correspondence should be addressed.

purchased from Bio-Rad Laboratories, and the $^3\text{H}_2\text{O}$ (99.96%) was from Aldrich Chemical Co. The Fractogel TSK HW-40(S) and Zerolit 225 X4 (200–400 mesh) were from BDH Chemicals. The NaB^3H_4 was obtained from Amersham International. The CPK molecular models were purchased from Harvard Apparatus.

General methods

Rotary evaporations were conducted with a water-bath temperature of less than 40 °C. Reduction with NaB^3H_4 (2 mCi) was performed in NaBH_4 (1%) with a 4-fold excess of reducing agent to oligosaccharide. The pyridine/acetate buffers were prepared from 0.4 M-acetic acid by the addition of pyridine to pH 5.4 and dilution to the appropriate molarity.

Descending paper chromatograms were run on Whatman no. 40 paper, and sugars were located with alkaline AgNO_3 stain (Trevelyan *et al.*, 1950). The solvents used were: solvent A, ethyl acetate/pyridine/water (2:1:1, by vol.); solvent B, ethyl acetate/pyridine/acetic acid/water (5:5:1:3, by vol.). The silica gel 60 high-performance t.l.c. plates were from BDH Chemicals, and were run in the following solvents: solvent C, butan-1-ol/acetone/water (6:5:4, by vol.); solvent D, ethanol/butan-1-ol/pyridine/water/acetic acid (100:10:10:30:3, by vol.); solvent E, propan-1-ol/water (7:3, v/v). The plates were dipped in 0.5% (w/v) orcinol in 5% (v/v) H_2SO_4 in ethanol and heated at 100 °C to locate the sugars. Cellulose t.l.c. plates from BDH Chemicals were run in butan-1-ol/propan-1-ol/0.1 M-HCl (1:2:1, by vol.) (solvent F) and stained with alkaline AgNO_3 . Chromatography on a column (160 cm $\times$ 1 cm) of Fractogel was carried out by elution with 50 mM-pyridine/acetate buffer, pH 5.4. The Zerolit 225 (K^+ form) column (160 cm $\times$ 1 cm) was run in water. The eluates from the columns were monitored with a differential refractometer.

Analytical methods

Hexosamines and hexosaminitols were determined on an LKB 4400 amino acid analyser after hydrolysis with 6 M-HCl for 2 h at 100 °C. The column (27.5 cm $\times$ 0.4 cm) of Ultrapak 8 resin was operated at 85 °C with buffer A (buffer A, 0.35 M- Na^+ /0.15 M-citrate adjusted to pH 4.85 with HCl) for hexosamine analysis and buffer B [as buffer A but with the addition of H_3BO_3 to 0.3 M and ethanol to 25% (v/v) and adjusted to pH 5.09 with HCl] for hexosamine/hexosaminitol determination. γ -Amino-n-butyric acid was used as internal standard in both assays. *N*-Acetylneuraminic acid was assayed by either (i) the thiobarbituric acid procedure (Warren, 1959) after hydrolysis in 0.05 M- H_2SO_4 for 1 h at 80 °C, or (ii) as methoxynneuraminic acid after release of this derivative with 0.5 M-HCl in methanol for 18 h at 80 °C and determination on the amino acid analyser with buffer B above. The trisaccharides NeuAca2-3Gal β 1-4Glc and NeuAca2-6Gal β 1-4Glc were used to provide standards for sialic acid in method (ii). For the determination of neutral sugars, samples were hydrolysed with 3 M-trifluoroacetic acid at 100 °C for 2 h, and the hydrolysates were passed through columns of AG50W (H^+ form) and AG1 (acetate form). The sugars were then converted into their trimethylsilyl ethers (Bhatti & Clamp, 1968) and assayed by g.l.c. on a 38 m SE 30-support-coated open tubular column with a temperature programme of 150 °C (5 min), 150–250 °C at 8°/min, then 250 °C (2 min).

Methylation analysis

Reduced oligosaccharides were methylated with methyl iodide in dimethylformamide, with $\text{BaO}/\text{Ba}(\text{OH})_2$ as catalyst, as described previously (Donald, 1981).

Sequential exo-glycosidase digestion

These experiments were performed at 37 °C in the buffers recommended by the suppliers of the enzymes. The anomeric specificities of the jack-bean β -galactosidase and β -hexosaminidase were confirmed in tests on appropriate α - and β -linked oligosaccharides. The batch of bovine kidney β -hexosaminidase used was found to have some capacity to cleave *N*-acetylgalactosamine from the tetrasaccharide GalNAca1-3(Fuca1-2)Gal β 1-4Glc (P. Greenwell, personal communication), indicating some α -activity. The hexosaminidases were used at a ratio of 20–40 units/ μmol of oligosaccharide and the other enzymes at a ratio of 2–5 units/ μmol . The products of digestion of NaB^3H_4 -reduced oligosaccharides with the β -galactosidase and β -hexosaminidases were separated by paper chromatography in solvent B and oligosaccharides were located with a radiochromatogram scanner. Released hexoses and *N*-acetylhexosamines were recovered from the paper and assayed as described under 'Analytical methods' above. The neuraminidase digestion products were passed through a column (2 cm $\times$ 0.4 cm) of AG1 (acetate form) and non-absorbed oligosaccharides were purified by paper chromatography. Sialic acid was recovered from the resin with 0.4 M-pyridine/acetate buffer, pH 5.4, and identified by t.l.c. as described by Schauer (1978) with solvents E and F.

Small-scale digestion with endo- β -galactosidase

To ^3H -labelled T-H glycoprotein (500 μg) in water (10 μl) was added 1 M-sodium acetate buffer, pH 5.9 (5 μl), endo- β -galactosidase (0.02 to 16 munits) and water (to 30 μl). The mixture was incubated overnight at 37 °C and then separated by paper chromatography in solvent A for 48 h. Areas of the paper corresponding to regions A and B of Fig. 1 were cut out and their radioactivities counted in a scintillation counter.

Large-scale endo- β -galactosidase digestion

To T-H glycoprotein (1.8 g) in water (40 ml) was added 1 M-sodium acetate buffer, pH 5.9 (400 μl), the endo- β -galactosidase (0.4 unit) and a drop of toluene. The mixture was incubated for 48 h at 37 °C and then dialysed from 20/32 Visking tubing against water (6 $\times$ 500 ml). The diffusible products were concentrated and streaked on to three sheets of chromatography paper, which were eluted with solvent A for 48 h. The paper was divided into regions A–D as indicated in Fig. 1.

^1H -n.m.r. studies

Samples for ^1H -n.m.r. spectroscopy were repeatedly evaporated from $^3\text{H}_2\text{O}$ at room temperature and dissolved in $^3\text{H}_2\text{O}$ (400 μl). The 500 MHz ^1H -n.m.r. spectroscopy was carried out on a Bruker AM 500 spectrometer operating in the Fourier-transform mode with quadrature detection and utilizing a Bruker Aspect 3000 computer. The spectral width was 6000 Hz and the data were collected for several hundred transients with 32 k data points. Two-dimensional correlated spectroscopy experiments were performed with a digital resolution in the F2 dimension of 1.17 Hz/point (COSY) and 2.34 Hz/point (RELAYED COSY).

The chemical shifts were measured with respect to internal acetone but are expressed in p.p.m. from 4,4-dimethyl-4-silapentane-1-sulphonate (taken as 2.225 p.p.m. from the acetone signal). All experiments were performed with a probe temperature of 295 K.

RESULTS AND DISCUSSION

When ^3H -labelled T-H glycoprotein was incubated with *B. fragilis* endo- β -galactosidase and the products were separated by paper chromatography the radiochromatogram shown in Fig. 1 was obtained, and, as previously reported (Donald *et al.*, 1983), the major radioactive component of fraction B was the Sd^a-active pentasaccharide. Experiments aimed at increasing the yield of this oligosaccharide have now been conducted. It was found that the amount of radioactivity in peak B was a constant 6–7% of that remaining at the origin of the chromatogram when between 0.2 munit and 16 munits of the enzyme per mg of glycoprotein were used for the incubation. At enzyme concentrations below 0.2 munit/mg of glycoprotein no breakdown products at all were visible. Subsequent experiments performed with 0.4 munit/mg of glycoprotein established that neither increase of temperature to 47 °C or 55 °C nor variation of pH over the range 5–6 nor prior partial removal of sialic acid from the glycoprotein by exhaustive neuraminidase treatment resulted in a higher proportion of the radioactivity occurring in peak B. As Sd(a+) T-H glycoproteins contain 1–2% *N*-acetylgalactosamine compared with less than 0.2% found in Sd(a–) glycoproteins, virtually all of this sugar in the former is presumably present as part of the Sd^a determinant. Analysis of endo- β -galactosidase digests of several Sd(a+) T-H glycoproteins has now shown that only 5–10% of the *N*-acetylgalactosamine present was diffusible. Thus only a small proportion of the carbohydrate chains carrying the Sd^a determinant in T-H glycoprotein can contain endo- β -galactosidase-susceptible –GlcNAc β 1–3Gal β 1–4GlcNAc– sequences.

Large-scale digestion of ^3H -labelled T-H glycoprotein

Fig. 1. Paper chromatography of the endo- β -galactosidase digest of an Sd(a+) T-H glycoprotein

The trace shown is that of a ^3H -labelled glycoprotein (500 μg) digested with 0.4 munit of *B. fragilis* endo- β -galactosidase at pH 5.9, chromatographed on paper for 48 h in solvent A and scanned for radioactivity. The arrows indicate the positions of the milk oligosaccharides LNT (Gal β 1–3GlcNAc β 1–3Gal β 1–4Glc) and LNFP I (Fuc α 1–2Gal β 1–3GlcNAc β 1–3Gal β 1–4Glc). The paper was divided into regions A–D as indicated by the bars.

Table 1. Compositions and molar proportions of constituent monosaccharide residues of the reduced oligosaccharides

Molar proportions are expressed relative to galactitol (Gal-ol).

Oligosaccharide	Composition (molar proportions)				
	GalNAc	NeuAc	Gal	GlcNAc	Gal-ol
I	0	0	1.03	1.07	1.00
II	0	1.02*	1.09	1.07	1.00
III	1.00	1.06†	1.09	1.00	1.00

* Assayed by the thiobarbituric acid method.

† Determined on an amino acid analyser as described in the Experimental section.

(1.8 g) with the endo- β -galactosidase was followed by dialysis and paper chromatography of the diffusible products. The material recovered from fraction D (see Fig. 1) was further purified by paper chromatography and on columns of Zerolit 225 (K⁺ form) and Fractogel, and a ^3H -labelled trisaccharide (oligosaccharide I) was obtained in a yield of 2.1 mg. Fraction C on examination by t.l.c. (solvent D) contained predominantly a single component, which was present to a lesser extent in fraction B, in which a compound with mobility identical with that of the Sd^a-active pentasaccharide described previously (Donald *et al.*, 1983) was evident. Fractions B and C were pooled and separated by paper chromatography for 98 h in solvent B into two components, which were eluted from paper and desalted on a column of Fractogel. A non-radioactive tetrasaccharide (oligosaccharide II, 4.2 mg) and a ^3H -labelled pentasaccharide (oligosaccharide III, 4.4 mg) isolated previously (Donald *et al.*, 1983) were obtained in pure form.

Oligosaccharides I–III were reduced with NaB³H₄ for structural studies. Analytical values for these reduced compounds are given in Table 1. Methylation of the reduced oligosaccharides was followed by hydrolysis, and all three compounds yielded 1,2,4,5,6-penta-*O*-methylgalactitol and 3,6-di-*O*-methylglucosamine. In addition, oligosaccharide I yielded 2,3,4,6-tetra-*O*-methylgalactose, oligosaccharide II gave 2,4,6-tri-*O*-methylgalactose, and oligosaccharide III gave 2,6-di-*O*-methylgalactose and 3,4,6-tri-*O*-methylgalactosamine. No attempt was made to identify the methyl ethers of the neuraminic acid.

The Sd^a-active pentasaccharide III was reported (Donald *et al.*, 1983) to be resistant to the action of *V. cholerae* and *C. perfringens* neuraminidases and to jack-bean β -hexosaminidase. Sequential glycosidase digestion therefore had to be carried out after removal of sialic acid by acid hydrolysis, a procedure that also eliminates *O*-acetyl groups and prevents full characterization of the sialic acid. The pentasaccharide has been reported to be susceptible to bovine kidney β -hexosaminidase (Donald *et al.*, 1984b), which removed [^3H]GalNAc to leave a product chromatographically identical with tetrasaccharide II. The sialic acid was readily removed from both the digestion product and tetrasaccharide II with *C. perfringens* neuraminidase and identified as *N*-acetylneuraminic acid by t.l.c. with

solvents E and F, which separate the naturally occurring sialic acids (Schauer, 1978). The neutral neuraminidase digestion products had chromatographic mobilities identical with those of trisaccharide I, and on sequential degradation with jack-bean β -galactosidase and β -hexosaminidase both compounds released first galactose and then a mixture of *N*-acetylglucosamine and [^3H]galactitol.

The results of the methylation studies and of the sequential exo-glycosidase digestion indicate that the structures of the oligosaccharides are:

The alditols of oligosaccharides I–III and the disaccharide GlcNAc β 1-3Gal-ol, obtained from oligosaccharide I by β -galactosidase treatment, form a series in which the carbohydrate chains are elongated by one monosaccharide residue at a time in the order that probably occurs during biosynthesis. The 50 MHz ^1H -n.m.r. spectra of this series of oligosaccharides and several related structures have been examined. The ^1H chemical shifts for these compounds are given in Table 2. The signals were assigned by using spin-spin-decoupling experiments and by comparing the ^1H chemical shifts of the members of this closely related series. For the pentasaccharide III the assignment was aided by two-dimensional COSY and RELAYED-COSY experiments. By using these methods an almost complete assignment of the proton signals was possible, and this not only has provided valuable confirmatory evidence for

Table 2. ^1H chemical shifts of the constituent monosaccharides for the reduced oligosaccharides and related structures

The values given for pentasaccharide III refer to the compound obtained on reduction with NaB^2H_4 .

		Chemical shift (p.p.m.)						
		β -MeGalNAc	GalNAc β 1-4Gal	GlcNAc β 1-3Gal-ol	Tri-saccharide I	Tetra-saccharide II	Sd ^a -active pentasaccharide III	
Gal-ol	H ₍₁₎			3.661	3.659	3.653	3.665	
	H ₍₁₎			3.661	3.659	3.652	3.655	
	H ₍₂₎			3.993	3.990	3.993	3.989	
	H ₍₃₎			3.868	3.879	3.874	3.871	
	H ₍₄₎			3.791	3.800	3.795	3.791	
	H ₍₅₎			4.132	4.134	4.146	4.133	
	H ₍₆₎			3.661	3.671	3.67	3.665	
	H _(6')			3.661	3.659	3.67	3.665	
GlcNAc	H ₍₁₎			4.587	4.616	4.607	4.599	
	H ₍₂₎			3.741	3.75	3.783	3.781	
	H ₍₃₎			3.586	3.72-3.78	—	3.726	
	H ₍₄₎			3.442	3.72	—	3.66	
	H ₍₅₎			3.487	3.628	3.612	3.62	
	H ₍₆₎			3.950	4.023	4.034	4.020	
	H _(6')			3.741	3.828	3.824	3.844	
	N-Ac			2.072	2.069	2.069	2.068	
Gal	H ₍₁₎		4.562 (β)		4.455	4.531	4.527	
			5.223 (α)					
	H ₍₂₎		3.361 (β)		3.536	3.566	3.335	
	H ₍₃₎		3.740 (β)		3.666	4.127	4.151	
			3.953 (α)					
H ₍₄₎			4.074 (β)		3.925	3.957	4.109	
			4.134 (α)					
	NeuAc	H _{(3)eq.}					2.754	2.664
		H _{(3)ax.}					1.826	1.927
		H ₍₄₎					—	3.831
H ₍₅₎						—	3.765	
H ₍₆₎						—	3.538	
H ₍₇₎						—	3.593	
N-Ac						2.031	2.031	
GalNAc	H ₍₁₎	4.383	4.627 (β)				4.719	
			4.614 (α)					
	H ₍₂₎	3.891	3.891 (β)				3.913	
			3.914 (α)					
	H ₍₃₎	3.711	3.725 (α, β)				3.886	
	H ₍₄₎	3.935	3.911 (α, β)				3.912	
	H ₍₅₎	3.683	3.653 (α, β)				—	
	N-Ac	2.035	2.067 (β)				2.013	
		2.059 (α)

Fig. 2. Resolution-enhanced 500 MHz ^1H -n.m.r. spectra of GlcNAc β 1-3Gal-ol (a) and reduced oligosaccharide I (b)

Spectra were recorded on samples in $^2\text{H}_2\text{O}$ at 295 K.

these structures but has also firmly established the chemical-shift values of some novel structural reporter protons.

The disaccharide GlcNAc β 1-3Gal-ol gave a well-resolved spectrum (Fig. 2a) with few overlapping signals. Two multiplets at $\delta = 4.132$ and 3.993 p.p.m. are characteristic of the galactitol residue, and these and other signals from this reduced sugar are virtually unchanged by the subsequent addition of galactose, *N*-acetylneuraminic acid and *N*-acetylgalactosamine residues to form higher members of the series. The galactitol multiplet at $\delta = 3.993$ p.p.m. could be assigned unequivocally to $\text{H}_{(2)}$ following the observation that reduction of the pentasaccharide with NaB^2H_4 instead of NaBH_4 causes this multiplet to simplify. The *N*-acetylglucosamine signals in the disaccharide show slight but significant differences from any of those so far reported for sequences involving GlcNAc-Gal, GlcNAc-

Man and GlcNAc-GalNAc-ol. The $\text{H}_{(1)}$ signal for the *N*-acetylglucosamine is found at $\delta = 4.587$ p.p.m. ($J_{1,2} = 8.2$ Hz). In the next member of the series, trisaccharide I (see Fig. 2b), the additional galactose residue has an $\text{H}_{(1)}$ signal at $\delta = 4.455$ p.p.m. ($J_{1,2} = 7.8$ Hz), indicating that it is also attached via a β -linkage, and the signals from the other protons are in accord with the sugar being in a terminal position (see, e.g., Hounsell *et al.*, 1984). Substitution at the *N*-acetylglucosamine residue with this galactose residue results in a relatively high value for *N*-acetylglucosamine $\text{H}_{(6)}$ ($\delta = 4.023$), which implies that the linkage between the sugars is 1-4 rather than 1-3 (Van Halbeek *et al.*, 1982). Further elongation of the chain to form either tetrasaccharide II or pentasaccharide III caused little variation in the shifts of the *N*-acetylglucosamine protons compared with those observed for trisaccharide I.

In the spectrum of tetrasaccharide II (Fig. 3a) the

Fig. 3. Resolution-enhanced 500 MHz ^1H -n.m.r. spectra of reduced oligosaccharides II (a) and III (b)

Oligosaccharide III was reduced with NaBH_4 , thus introducing deuterium at C-1 of the galactitol residue. Spectra were recorded of samples in $^2\text{H}_2\text{O}$ at 295 K.

Fig. 4. Absolute-value RELAYED-COSY spectrum of the Sd^a-active pentasaccharide III

The RELAYED-COSY spectrum (Eich *et al.*, 1982) of the NaB³H₄-reduced oligosaccharide is presented as a two-dimensional contour plot. The F_2 frequencies are labelled iH_j , where i is the residue number (see structure) and j is the position of the proton in the residue. Cross-peaks are observed between scalar coupled nuclei and also between those nuclei that are not coupled to each other but that are both coupled to a third nucleus. Thus for the *N*-acetylglucosamine residue the $H_{(3)}$ (2H3) and $H_{(4)}$ (2H4) signals can be assigned from the cross-peaks with 2H1 and 2H6 respectively. The 2H2 and 2H5 assignments had previously been determined in a COSY experiment in which only the directly coupled nuclei are assigned. The *N*-acetylneuraminic acid assignments ($H_{(3)eq.}$, $H_{(3)ax.}$, $H_{(4)}$, $H_{(5)}$, $H_{(6)}$ and $H_{(7)}$) were made in a similar manner.

highly characteristic shifts that occur on attachment of *N*-acetylneuraminic acid via an α 2-3-linkage to galactose (see Vliegthart *et al.*, 1983) are observed. Compared with the trisaccharide, there is a downfield movement of the galactose $H_{(1)}$ ($\Delta\delta = +0.076$ p.p.m.) to $\delta = 4.531$ p.p.m., a very marked change in galactose $H_{(3)}$ ($\Delta\delta = +0.461$ p.p.m.) to 4.127 p.p.m. and lesser downfield movements in galactose $H_{(2)}$ ($\Delta\delta = +0.030$ p.p.m.) and $H_{(4)}$ ($\Delta\delta = +0.032$ p.p.m.). The shifts for *N*-acetyl-

neuraminic acid $H_{(3)}$ axial ($\delta = 1.826$ p.p.m.) and equatorial ($\delta = 2.754$ p.p.m.) also have values consistent with a NeuAc α 2-3Gal linkage. The *N*-acetyl proton signals for the sialic acid residue were found at 2.031 p.p.m.

The addition of an *N*-acetylgalactosamine residue to the 4-position of the galactose residue of tetrasaccharide II to form the Sd^a-active pentasaccharide III produces a set of characteristic shifts for protons of the *N*-

acetylgalactosamine, *N*-acetylneuraminic acid and galactose residues. Values for shifts close to those observed for the Cad-blood-group determinant [GalNAc β 1-4(NeuAc α 2-3)Gal β 1-3(NeuAc α 2-6)GalNAc-ol] and other structures bearing the same branched terminal trisaccharide unit (Blanchard *et al.*, 1983; Herkt *et al.*, 1985) were recorded for *N*-acetylgalactosamine H₍₁₎ (δ = 4.719), galactose H₍₃₎ (δ = 4.151), galactose H₍₄₎ (δ = 4.109) and *N*-acetylneuraminic acid H_{(3)ax} (δ = 1.927) and H_{(3)eq} (δ = 2.664).

However, some differences are apparent between the shifts given in the references cited above for the Cad determinant and those reported here for the Sd^a-active pentasaccharide III. The galactose H₍₂₎ of the pentasaccharide III is found at an extremely upfield position (δ = 3.335 p.p.m.) whereas the signal from the same proton in the Cad determinant is located in the skeletal region. This difference is not surprising, as the galactose residue is linked β 1-3 to *N*-acetylgalactosaminitol in the Cad determinant and β 1-4 to *N*-acetylglucosamine in the Sd^a determinant. More difficult to explain is that the Sd^a determinant has its *N*-acetylgalactosamine H₍₂₎ at δ = 3.913 p.p.m., a value not very different from those found for this proton in β -MeGalNAc and GalNAc β 1-4Gal, whereas Blanchard *et al.* (1983) report values in the range 3.63–3.42 p.p.m. for this signal in the Cad determinant and its derivatives lacking *N*-acetylneuraminic acid on the *N*-acetylgalactosaminitol residue.

The position of the *N*-acetylneuraminic acid H₍₄₎–H₍₇₎ signals for the Cad determinant have not been reported, but values for these signals for the Sd^a-active pentasaccharide could be assigned as the result of a RELAYED-COSY experiment (see Fig. 4). The *N*-acetylneuraminic acid H₍₆₎ signal (δ = 3.538 p.p.m.) occupies a position well clear of the bulk of the skeletal proton signals, whereas that of *N*-acetylneuraminic acid H₍₇₎ (δ = 3.593) is found at the edge of the skeletal region and both protons can be categorized as structural reporter protons for this oligosaccharide. A signal probably due to *N*-acetylneuraminic acid H₍₇₎ also occurs at δ = 3.594 p.p.m. in the spectrum of the tetrasaccharide II, but as this was not unequivocally characterized its value is not given in Table 2. Although the *N*-acetylneuraminic acid H₍₆₎ signal in tetrasaccharide II was not located, inspection of the spectrum of this compound compared with that of pentasaccharide III reveals that the signal of this H₍₆₎ proton in the latter must have moved upfield by at least 0.05 p.p.m. as a result of increased shielding. An examination of a CPK molecular model of the pentasaccharide indicates that it is not possible for the *N*-acetylgalactosamine to interact directly with the *N*-acetylneuraminic acid H₍₆₎. Thus it appears likely that the observed shielding difference between oligosaccharides II and III results from a conformational change in the *N*-acetylneuraminic acid residue induced by the introduction of the *N*-acetylgalactosamine residue (the two sugar residues are spatially close together). The shielding differences observed for the *N*-acetylneuraminic acid H_{(3)eq} ($\Delta\delta$ = +0.09 p.p.m.) and H_{(3)ax} ($\Delta\delta$ = -0.101 p.p.m.) in oligosaccharides II and III can be explained in a similar manner.

It is possible that the differences noted between certain

of the chemical shifts of the Cad and Sd^a blood-group determinants may indicate conformational differences between the branched terminal trisaccharide units common to both structures. Confirmation of this must await more complete assignment of the protons of the Cad determinant, and nuclear-Overhauser-effect experiments on both structures may lead to a better understanding of the spatial arrangement of the monosaccharide residues in the two blood-group determinants.

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