Glycoconjugate J (1989) 6:319-332

Enzymic Synthesis, Chemical Characterisation and Sd^a Activity of GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAc and GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc

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Received February 28/July 2, 1989.

Key words: carbohydrate antigens, Sd^a-active oligosaccharides, β-4-N-acetylgalactosaminyltransferase, ^{1}H-NMR spectroscopy

The tetrasaccharides GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc and GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAc were synthesised by enzymic transfer of GalNAc from UDP-GalNAc to 3'-sialyllactose (NeuAcα2-3Galβ1-4Glc) and 3'-sialyl-*N*-acetyllactosamine (NeuAcα2-3Galβ1-4GlcNAc). The structures of the products were established by methylation and ^{1}H-500 MHz NMR spectroscopy. In Sd^a serological tests the product formed with 3'-sialyl-*N*-acetyllactosamine was highly active whereas that formed with 3'-sialyllactose had only weak activity.

The human blood group Sid system is defined by antibodies which occur naturally in individuals who lack the antigen Sd^a. These antibodies show considerable variation in their capacity to agglutinate red cell specimens from different individuals; they react strongly with a few cell samples, designated Sd(a++), weakly with most cell samples, Sd(a+), and negatively with 8-9% of cell samples, Sd(a-). Even for the strong positive reactors usually only a proportion of the red cells are agglutinated by human anti-Sd^a reagents [1]. The factors controlling this variability of expression among the red cell population from a single individual and the strength of expression in different individuals are at present unknown.

The lectin *Dolichos biflorus* reacts with human group A red cells [2], and was considered to be specific for blood group A until Cazal *et al.* [3] reported a Mauritian family, called "Cad", in which the red cells of certain non-A family members were strongly agglutinated by the lectin. A link between these observations and the Sid system was forged when Sanger *et al.* [4] demonstrated that the *Dolichos* reactive cells in the Cad family were extremely strong reactors with human Sd^a antibodies and that other Sd(a++) red cells were also

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agglutinated by *Dolichos biflorus* preparations. Another property recorded for the original Cad cells was their polyagglutinability [3] and Sanger *et al.* [4] suggested that the antigen on these cells represented very strongly expressed Sd^a which is detectable by low levels of anti-Sd^a present in the serum of the great majority of individuals. Further studies showed that the reaction of Sd(a++) cells with *Dolichos biflorus* can be inhibited by *N*-acetylgalactosamine [4, 5]. Although this sugar did not inhibit the agglutination of Sd(a+) or Sd(a++) cells by human anti-Sd^a serum the inhibition result pointed to the involvement of carbohydrate in Sd^a specificity and a specific role for *N*-acetylgalactosamine in the antigenic determinant.

Both human and guinea pig urine are potent sources of Sd^a-active material [6]. In human urine the major carrier of this specificity is the Tamm-Horsfall (T-H) glycoprotein [7] although Sd^a activity has also been found on a mucin-type glycoprotein [8]. The presence of *N*-acetylgalactosamine in Sd^a-active T-H glycoproteins and its virtual absence from Sd^a-negative T-H preparations confirmed the earlier inference that this sugar was important for Sd^a specificity [7]. The subsequent isolation of a disaccharide characterised as GalNAcβ1-4Gal from Sd^a-active T-H glycoprotein [9] revealed that the terminal *N*-acetylgalactosamine residue in the Sd^a determinant is β-linked. This was a surprising observation because β-linked *N*-acetylgalactosamine residues had not previously been described in mammalian glycoproteins and *Dolichos biflorus* has a known preference for α-linked *N*-acetylgalactosamine [10]. The disaccharide GalNAcβ1-4Gal inhibited the reaction between Sd(a++) cells and *Dolichos biflorus* lectin but was not inhibitory in the human Sd^a-anti-Sd^a system, thus indicating that a structure larger than the disaccharide is required for competitive binding with the human reagent [9]. Subsequently Donald *et al.* [11] isolated from the *endo*-β-galactosidase digestion products of Sd^a-active T-H glycoprotein a pentasaccharide fragment with the structure :

GalNAcβ1
4
Galβ1-4GlcNAcβ1-3Gal
3
NeuAcα2

that was strongly inhibitory with human anti-Sd^a serum and thus confirmed the role of β-linked *N*-acetylgalactosamine in Sd^a specificity. Tests with related oligosaccharides showed that sialic acid α(2-3)-linked to the sub-terminal β-galactosyl residue was also essential for Sd^a serological activity.

Independently, Cartron and Blanchard [12] studied Cad-positive red cells and suggested that Cad specificity is defined by *N*-acetylgalactosamine residues carried by sialoglycoproteins. A reduced pentasaccharide with the structure :

NeuAcα2
6
GalNAcβ1-4Galβ1-3GalNAc-ol
3
NeuAcα2

was subsequently isolated and characterised [13] from the alkaline borohydride degradation products of a glycophorin A preparation obtained from Cad erythrocytes. This fragment was reported to inhibit both *Dolichos biflorus* lectin and human anti-Sd^a serum [14]; a result suggesting that the terminal trisaccharide common to the "Sd^a pentasaccharide" and the "Cad pentasaccharide" constitutes the determinant structure with which the human anti-Sd^a reagent combines. However, although this same trisaccharide occurs as a terminal non-reducing structure in the glycosphingolipid G_{M2} (GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc-Ceramide) liposomes formed with this compound were not inhibitory in the human Sd^a-anti-Sd^a system [11].

Serafini-Cessi and Dall'Olio [15] described an enzyme in microsomal preparations from guinea pig kidney that transferred ^{14}C-labelled *N*-acetylgalactosamine in β-anomeric linkage to glycopeptides from Tamm-Horsfall glycoprotein and was therefore considered to be involved in the biosynthesis of Sd^a determinant structures. Subsequently it was shown that this enzyme preparation also transferred labelled *N*-acetylgalactosamine to the low molecular weight acceptor 3'-sialyllactose [16]. In order to obtain a clearer idea of the precise structure required for binding with the human Sd^a antibody a number of oligosaccharides related to the Sd^a and Cad determinants are being synthesised on a larger scale with the transferase from guinea pig kidney as the enzyme source. The preparation, chemical characterisation and Sd^a serological activity of two of these compounds, GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc and GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAc, are reported in this paper.

Materials and Methods

UDP-*N*-Acetyl-D-[1-^{14}C]galactosamine (2.3 GBq/mmol) was obtained from Amersham International, UK. Unlabelled UDP-*N*-acetyl-D-galactosamine was prepared according to the method of Carlson *et al.* [17]. HPTLC silica gel 60 plates were purchased from BDH Ltd., Poole, U.K. AG 1-X4 and AG 50W-X8 were obtained from Bio-Rad Labs, Watford, UK.

T-H Glycoproteins were isolated from the urine of Sd^a-positive and Sd^a- negative individuals by the procedure of Tamm and Horsfall [18]. The disaccharide GalNAcβ1-4Gal [9], the penta- and tetrasaccharides GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAcβ1-3Gal and GalNAcβ1-4Galβ1-4GlcNAcβ1-3Gal [11], the tri- and tetrasaccharides Galβ1-4GlcNAcβ1-3Gal and NeuAcα2-3Galβ1-4GlcNAcβ1-3Gal [19] were isolated from the degradation products of Sd^a-active Tamm-Horsfall glycoprotein as described. The glycolipid G_{M2} was a gift from Dr K. Sandhoff, University of Bonn, FRG.

Preparation of Acceptor Substrates

3'-Sialyllactose (NeuAcα2-3Galβ1-4Glc), 3'-sialyl-*N*-acetyllactosamine (NeuAcα2-3Galβ1-4GlcNAc), 6'-sialyllactose (NeuAcα2-6Galβ1-4Glc) and 6'-sialyl-*N*-acetyllactosamine (NeuAcα2-6Galβ1-4GlcNAc) were isolated from human urine as described by Parkkinen and Finne [20] except that the final separation of the monosialyloligosaccharides was achieved by ion-exchange chromatography on a column (160 cm x 1.0 cm) of AG 1-X4 (200-400 mesh, acetate form) eluted with 50 mM pyridine acetate pH 5.4. Aliquots of the eluted

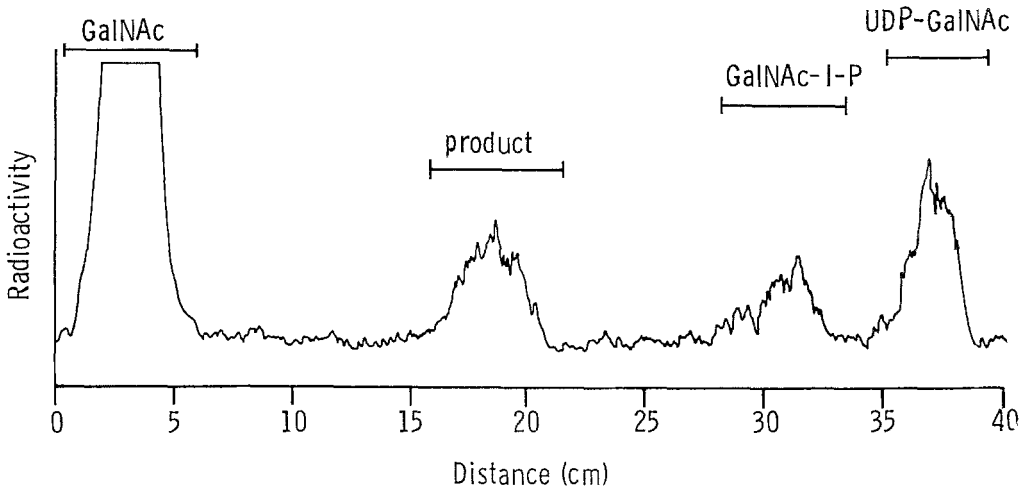

Figure 1. Electrophoretic separation of the reaction products formed with 3'-sialyl-*N*-acetyllactosamine as the acceptor substrate and UDP-*N*-acetyl-[^{14}C]galactosamine as the donor substrate. A picrate marker had the same mobility as *N*-acetylgalactosamine-1-P.

fractions were chromatographed on HPTLC silica gel 60 plates developed in ethanol/butan-1-ol/pyridine/H_2O/acetic acid, 100/10/10/30/3 by vol (Solvent 1), and the oligosaccharides were visualised by dipping the plates in 0.5% orcinol dissolved in ethanol containing 5% H_2SO_4 followed by heating at 100°C. The purity of the isolated 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine was ascertained by examination of their ^{1}H-NMR spectra.

Preparation of β-N-Acetylgalactosaminyltransferase

Freshly excised guinea pig kidneys (5 g) were homogenised in 10 ml 0.15 M KCl containing 0.1% mercaptoethanol for 10-20 seconds in an Ultra-Turrax homogeniser. The homogenate was spun at 14,000 x *g* for 10 min at 4°C in a Sorvall RC-5B centrifuge, the supernatant was then centrifuged in a Beckman L2-65B Ultracentrifuge at 100,000 x *g* for 60 min at 4°C. The supernatant was discarded and the microsomal pellet was resuspended in 4 ml 0.15 M KCl-mercaptoethanol in an all-glass homogeniser. This suspension was used as the enzyme source.

Enzyme Incubation Mixtures

The conditions used for the biosynthesis of the oligosaccharides with respect to pH, cation concentration and presence of detergent were those established as optimal for the guinea pig kidney β-*N*-acetylgalactosaminyltransferase by Serafini-Cessi *et al.* [15, 16]. The incubation mixtures contained in a final volume of 6 ml:- 0.16 M Tris-HCl pH 7.5; 20 mM $MnCl_2$;

2.5 mM ATP; 0.5% Triton X-100; 1.25 mM UDP-*N*-acetylgalactosamine plus 12 µM UDP-*N*-acetyl-[^{14}C]galactosamine (100,000 cpm/nmol); 2.5 mM 3'-sialyllactose or 3'-sialyl-*N*-acetyllactosamine and the microsomal suspension at a final concentration of 4 mg protein/ml. The reaction mixtures were incubated at 37°C for 18 h.

Separation of Enzyme Reaction Products

The incubation mixtures were electrophoresed on Whatman 3MM paper in 40 mM pyridine acetate buffer pH 5.4 at 80 V/cm for 1.5 h on a Shandon Southern High Voltage Model L24 Electrophoresis apparatus. The papers were dried and scanned for radioactivity on a Packard Radiochromatogram Scanner. The areas of the paper containing the radioactive tetrasaccharide products (Fig. 1) were eluted with 100 mM pyridine acetate buffer pH 5.4. The eluate was evaporated to dryness, washed twice with distilled water to free the products from pyridine acetate, reconstituted in approximately 1.5 ml distilled water and loaded on a column (160 cm x 1.0 cm) of Dowex AG 1-X4, 200-400 mesh (acetate form) which had been equilibrated with 5 mM pyridine acetate buffer pH 5.4. The column was eluted with 50 mM pyridine acetate buffer pH 5.4 at 15 ml/h and 5 ml fractions were collected. Aliquots of each fraction were monitored for radioactivity and tested for the presence of sialyloligosaccharides by HPTLC as described for the acceptor substrates. Fractions from the AG 1 column containing radioactivity were pooled and concentrated.

Characterisation of Products of β-N-Acetylgalactosaminyl Transfer

Reduction: Oligosaccharides were converted to the alditols by reduction with a 10-fold excess of sodium borohydride. The excess borohydride was destroyed with acetic acid, Na^+ ions were adsorbed on AG 50W-X8 H^+ resin and boric acid was removed by repeated evaporation with methanol.

500 MHz ^{1}H-NMR spectroscopy: Samples for ^{1}H-NMR spectroscopy were repeatedly evaporated from ^2H_2O at room temperature to replace all exchangeable hydrogens. The spectra were recorded on a Bruker AM 500 spectrometer on samples dissolved in ^2H_2O with a probe temperature of 295 K as described previously [19]. Chemical shifts were measured from an acetone internal standard and then expressed as ppm from sodium 4,4-dimethyl-4-silapentane-1-sulphonate.

Methylation: For this procedure all evaporations were carried out in a stream of nitrogen at room temperature. The dried alditols of the oligosaccharides (100 µg) were methylated with methyl iodide (100 µl) in dimethylformamide (100 µl) with BaO/$Ba(OH)_2$ (100 mg of a freshly ground 1:1 mixture) as a catalyst [21] for 3 h at 45°C. The mixture was diluted with chloroform (500 µl) and the Ba salts were filtered and washed with chloroform (500 µl). The chloroform solution was washed with water (4 x 1 ml) and the aqueous layers were each back-washed with 1 ml chloroform. The chloroform solutions were combined and evaporated. The methylated compound was hydrolysed with 2 M trifluoroacetic acid (50 µl) at 100°C for 3 h, the solution was evaporated, 50% ethanol (50 µl) was added and the mixture was again evaporated. The resultant methyl ethers were reduced with NaB^2H_4 (1 mg in 50 µl of water) for 3 h, the solution was taken to dryness and borate was removed by repeated evaporation with methanol (30 µl). The alditols were converted to alditol acetates by heating

in 50 µl of pyridine/acetic anhydride, 1/1 by vol, for 1 h at 80°C. Toluene (100 µl) was added, the solution was evaporated and the residue was shaken with a mixture of chloroform (100 µl) and water (100 µl). The chloroform layer containing the methyl ethers was evaporated and the residue dissolved in ethyl acetate for GLC analysis.

The alditol acetates were identified by GLC on support coated open tubular capillary columns of SE-30 and SP 1000 (both 38 m in length) operated with a temperature programme of 160°-220°C at 2°C/min. The alditol acetate methyl ethers were identified by their co-migration with authentic standards and their identity was confirmed by GC/MS carried out by Dr A. Lawson, Clinical Research Centre, Harrow, UK.

Serological Analysis

The serological activities of the biosynthesised tetrasaccharides and related compounds were measured in haemagglutination inhibition tests as described previously [22]. Human anti-Sd^a serum was supplied by Miss Joyce Poole, Blood Group Reference Laboratory, Oxford, UK and freeze-dried partially purified *Dolichos biflorus* seed extract was the gift of Dr. G.W.G. Bird, Birmingham Blood Transfusion Centre, UK. Sd(a++) red blood cells were supplied by Dr. R. O'Charoen, National Blood Centre, Bangkok, Thailand; these cells gave strong mixed field agglutination with both the human anti-Sd^a (titre 512) and the *Dolichos* extract (titre 1024) but were not polyagglutinable.

Liposomes containing G_{M2} were prepared by dissolving 1 mg each of lecithin and cholesterol in chloroform/methanol, 2/1 by vol, and adding the solution to 1 mg of the glycolipid. The mixture was shaken well and then evaporated to dryness under a stream of nitrogen. The residue was taken up in 100 µl of 0.15 M NaCl and sonicated for 5 min to give an even suspension which was used in haemagglutination inhibition tests.

Results

Biosynthesis of GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc and GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAc

Preliminary experiments with radioactively labelled UDP-[^{14}C]GalNAc confirmed the observations of Serafini-Cessi *et al.* [16], that 3'-sialyllactose is an acceptor substrate for the guinea pig kidney β-*N*-acetylgalactosaminyltransferase and further showed that 3'-sialyl-*N*-acetyllactosamine functions as an acceptor for this enzyme. Neither 6'-sialyllactose nor 6'-sialyl-*N*-acetyllactosamine gave radioactive products.

Larger scale incubation mixtures containing a mixture of unlabelled and radioactively labelled UDP-GalNAc were used for the biosynthesis of the tetrasaccharides as described in the Methods section. The products formed with both 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine migrated on paper electrophoresis (Fig. 1) with mobilities of 0.6 relative to picrate and the compounds eluted from the electrophoresis papers each gave single peaks on chromatography on AG 1 columns eluted with pyridine acetate buffer pH 5.4. The pooled, concentrated eluate from the incubation mixture containing 3'-sialyllactose gave a

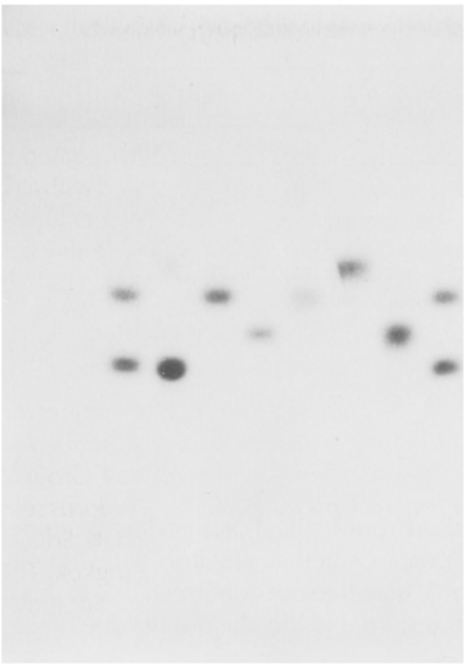

Figure 2. Thin layer chromatogram of products synthesised with UDP-*N*-acetyl-[^{14}C]galactosamine as the donor substrate and 3'-sialyllactose or 3'-sialyl-*N*-acetyllactosamine as the acceptor substrates. Lanes 1 and 8, 3'- and 6'-sialyllactose; lane 2, 6'-sialyllactose; lane 3, 3'-sialyllactose; lane 4, product formed with 3'-sialyllactose; lane 5, product formed with 3'-sialyl-*N*-acetyllactosamine; lane 6, 3'-sialyl-*N*-acetyllactosamine; lane 7, 6'-sialyl-*N*-acetyllactosamine.

single spot on TLC in Solvent 1 with an R_F of 0.56 and a mobility relative to 3'-sialyllactose of 0.91. The product from the incubation mixture containing 3'-sialyl-*N*-acetyllactosamine had an R_F of 0.61 and a mobility relative to 3'-sialyllactose of 0.98 in the same solvent (Fig. 2). The yields for typical incubation mixtures as described in the Methods section, calculated from the incorporated radioactive *N*-acetylgalactosamine, were 662 nmol of the 3'-sialyllactose product and 536 nmol of the 3'-sialyl-*N*-acetyllactosamine product. The experiments were repeated in order to obtain sufficient material for chemical and serological analyses.

Characterisation of Biosynthetic Products

Methylation analysis: The two biosynthetic compounds were reduced with sodium borohydride and subjected to methylation analysis (Table 1). In each of the acceptor trisaccharides the galactosyl residue was substituted at the O-3 position with *N*-acetylneuraminic acid. Hence the presence of 2,6-di-*O*-methyl galactose in the hydrolysis products of the methylated compounds showed that the *N*-acetylgalactosamine residues had been transferred to the O-4 positions of these sugars. The *N*-acetylglucosaminitol methyl ethers arising

Table 1. Methylation analysis of the alditols of the products formed by transfer of *N*-acetylgalactosamine to 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine

Acceptors	Methyl ethers[a] of:		
	GalNAc	Gal	Glc-ol
3'-Sialyllactose	3,4,6	2,6	1,2,3,5,6
3'-Sialyl-*N*-acetyllactosamine	3,4,6	2,6	-

[a] GlcNAc-ol methyl ethers were not identified.

from the compound synthesised from 3'-sialyl-*N*-acetyllactosamine were not identified, but the presence of 1,2,3,5,6-penta-*O*-methyl glucitol in the methylated products of the compound synthesised from 3'-sialyllactose confirmed that in this product the *N*-acetylgalactosamine had not been transferred to the glucose residue.

500 MHz ^{1}H-NMR spectra: The alditols of the purified biosynthetic products were subjected to 500 MHz ^{1}H-NMR analysis in parallel with the alditols of the acceptor substrates 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine. Signals were assigned by comparison with the ^{1}H chemical shifts of reference compounds and were confirmed in most cases by spin-spin decoupling experiments. The spectra for the alditols of 3'-sialyllactose and the product formed by transfer of *N*-acetylgalactosamine to this trisaccharide are given in Fig. 3. The chemical shifts of the products formed with both trisaccharides are shown in Table 2 together with those previously established for the alditol of the Sd^a-active pentasaccharide [19], the alditol of the Cad pentasaccharide [13] and the unreduced oligosaccharide GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc derived from G_{M2} [13]. The spectra for the alditols of 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine, and for the alditols of the products of *N*-acetylgalactosaminyl transfer to these trisaccharides, have not been recorded previously.

The signals for the *N*-acetylgalactosamine, sialic acid and galactose residues of the biosynthetic compounds together form a pattern which is highly characteristic of the branched, terminal trisaccharide unit GalNAcβ1-4[NeuAcα2-3]Gal- that occurs in the Sd^a and Cad pentasaccharides. On the addition of *N*-acetylgalactosamine to the acceptor trisaccharides the chemical shifts for the H-2, H-3 and H-4 protons of galactose are changed to values close to those found in the Sd^a and Cad pentasaccharides. Moreover the signal changes observed for these protons (mean values for the two products; H-2, $\Delta\delta = -0.200$; H-3, $\Delta\delta = +0.022$; H-4, $\Delta\delta = +0.050$) that occur on the addition of this sugar are closely similar to the differences recorded [19] between the shifts of the same galactose protons (H-2, $\Delta\delta = -0.231$; H-3, $\Delta\delta = +0.024$, H-4, $\Delta\delta = +0.052$) in the Sd^a pentasaccharide and the precursor tetrasaccharide lacking the *N*-acetylgalactosamine residue, namely NeuAcα2-3Galβ1-4GlcNAcβ1-3Gal. The GalNAc H-1 and NAc signals of the biosynthetic products were found in similar positions to those recorded for the Sd^a and Cad pentasaccharides and the

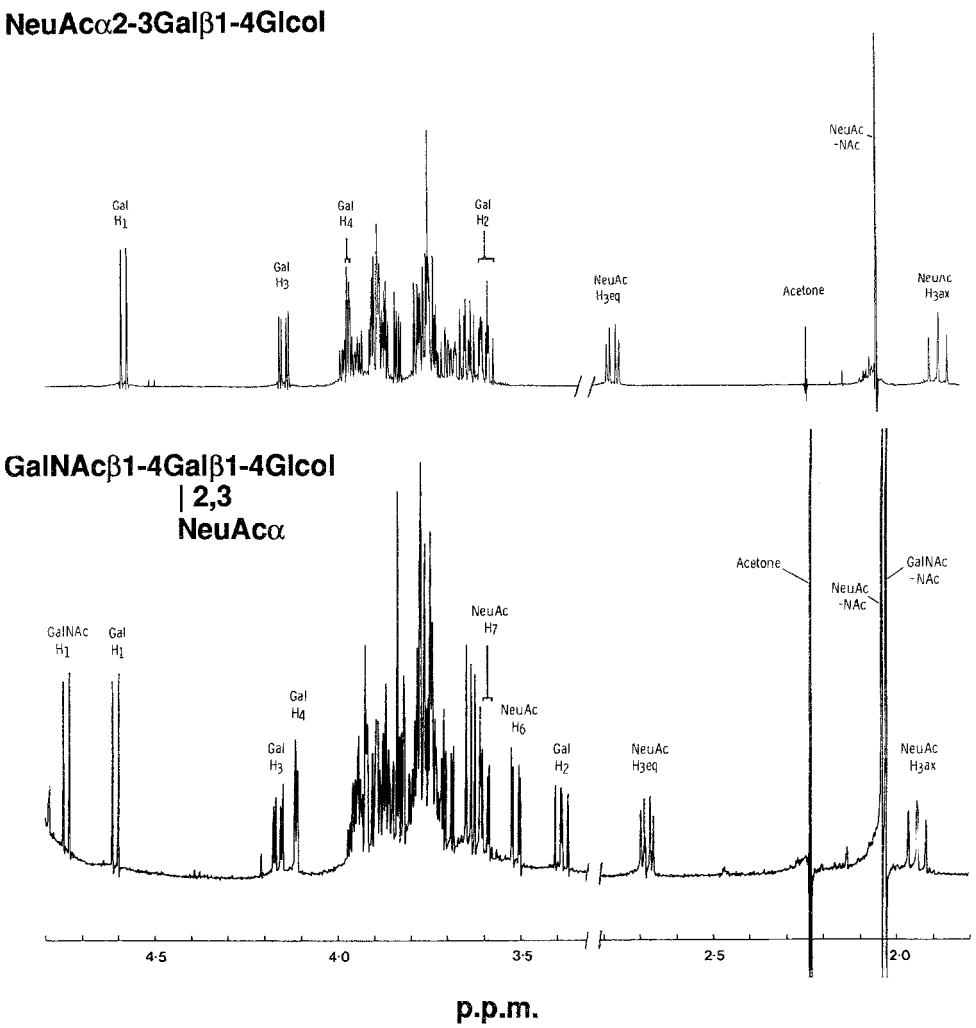

Figure 3. Resolution enhanced 500 MHz ^{1}H-NMR spectra of the alditols of 3'-sialyllactose and the product formed by transfer of *N*-acetylgalactosamine to 3'-sialyllactose.

values of the H-1 coupling constants ($J_{1,2}$ = 8.4 and 8.8 Hz for the 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine products, respectively) confirmed that the *N*-acetylgalactosamine had been transferred in β-anomeric linkage.

The chemical shifts of the NeuAc H-3 axial and equatorial are close to the values observed for the Sd^a and Cad oligosaccharides. The NeuAc H-6 and H-7 signals which were buried in the skeletal proton region of NeuAcα2-3Galβ1-4GlcNAcβ1-3Gal-ol were observed to move markedly upfield in the spectrum of the Sd^a pentasaccharide [18]. A similar shift upfield was observed for the NeuAc H-6 and H-7 protons when *N*-acetylgalactosamine was

Table 2. ^{1}H-Chemical shifts of the constituent monosaccharides of the alditols of the tetrasaccharides synthesised by enzymic transfer of *N*-acetylgalactosamine to 3'-sialyllactose (3'-SL) and 3'-sialyl-*N*-acetyllactosamine (3'-SLN) together with those of the alditols of 3'-SL, 3'-SLN, the Sd[a] and Cad pentasaccharides and the unreduced oligosaccharide from G_{M2}.

Residue	Reporter group	Chemical shift (p.p.m.)						
		Product from 3'-SL	Product from 3'-SLN	3'-SL	3'-SLN	Sd[a] pentasaccharide[a]	Cad pentasaccharide[b,c]	Oligosaccharide from G_{M2}[b,d]
GalNAc	H-1	4.740	4.746	-	-	4.719	4.714	4.744
	NAc	2.025	2.015	-	-	2.013	2.025	2.015
Gal	H-1	4.607	4.612	4.581	4.576	4.527	4.561	4.528
	H-2	3.385	3.396	3.580	3.600	3.335	-	-
	H-3	4.161	4.158	4.140	4.135	4.151	4.162	4.147
	H-4	4.111	4.105	3.963	3.953	4.109	4.091	4.116
NeuAc	H-3eq.	2.677	2.677	2.758	2.762	2.664	2.681	2.664
(α2-3)	H-3ax.	1.939	1.937	1.864	1.846	1.927	1.933	1.923
	H-6	3.510	3.483	-	-	3.538	-	-
	H-7	3.594	3.589	-	-	3.593	-	-
	NAc	2.037	2.031	2.033	2.031	2.031	2.032	2.032
GlcNAc-ol	H-2	-	4.164	-	4.284[e]	-	-	-
	NAc	-	2.050	-	2.050	-	-	-

[a] Data from [18].
[b] Data from [13].
[c] The shifts of the NeuAcα2-6 are not given.
[d] The values shown refer to the β-form of the oligosaccharide.
[e] Signal identified by comparison of spectra of samples reduced with $NaBH_4$ and NaB^3H_4.

transferred to 3'-sialyllactose or 3'-sialyl-*N*-acetyllactosamine. Most of the signals of the Glc-ol and GlcNAc-ol residues of the biosynthetic products are located in the skeletal region and were not identified except for that of the H-2 of GlcNAc-ol which occurs at 4.164 ppm in the product biosynthesised from 3'-sialyl-*N*-acetyllactosamine, somewhat upfield of the value observed for this proton in the alditol of the parent compound (4.284 ppm). The NMR and methylation data eliminate the possibility of 3,4 disubstitution of the Glc-ol and GlcNAc-ol residues and makes other disubstitution patterns appear improbable.

Thus the methylation and NMR data confirm that the *N*-acetylgalactosamine residue has been transferred in β-anomeric configuration to the O-4 position of the β-galactosyl residue in both 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine.

Table 3. Serological activity with human anti-Sd^a serum and *Dolichos biflorus* lectin of the fragments isolated from Tamm-Horsfall glycoprotein and the enzymically synthesised tetrasaccharides.

Compound	Minimum amount giving complete inhibition of Sd(a++) cells (μg/10 μl)	
	Human anti-Sd^a	*Dolichos biflorus*
Tamm-Horsfall (T-H) glycoprotein		
Sd^a-positive	0.02	3
Sd^a-negative	>200	>200
Degradation products of Sd^a-positive T-H glycoprotein		
GalNAcβ1-4Gal	>200	50
GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAcβ1-3Gal	3	12.5
GalNAcβ1-4Galβ1-4GlcNAcβ1-3Gal	>200	n.t.
NeuAcα2-3Galβ1-4GlcNAcβ1-3Gal	>200	>200
Galβ1-4GlcNAcβ1-3Gal	>200	>200
Biosynthetic tetrasaccharides		
GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc	50	12.5
GalNAcβ1-4[NeuAcα2-3]Galβ1-4GlcNAc	6	12.5
Glycolipid G_{M2}		
GalNAcβ1-4[NeuAcα2-3]Galβ1-4Glc-Ceramide	>100	n.t.
Monosaccharides		
GalNAc	>200	50
Gal	>200	>200
GlcNAc	>200	>200

n.t. = not tested

Serological Activity of the Biosynthetic Tetrasaccharides

The tetrasaccharide products were examined in haemagglutination inhibition tests in parallel with specimens of T-H glycoproteins obtained from Sd^a-positive and Sd^a-negative donors, oligosaccharide fragments isolated from Sd^a-positive T-H glycoprotein and liposomes prepared with the glycolipid G_{M2} (Table 3). Strongly reacting Sd(a++) red cells were used as the indicator cells and human anti-Sd^a serum or *Dolichos biflorus* lectin as the agglutinating reagents. The compound formed by addition of GalNAc in β(1-4)-linkage to 3-sialyl-*N*-acetyllactosamine strongly inhibited the human anti-Sd^a serum and was only slightly less active than the Sd^a pentasaccharide isolated from T-H glycoprotein [11]. The tetrasaccharide synthesised from 3'-sialyllactose had much less inhibitory activity and, in agreement with earlier observations, the liposomal preparation of the glycolipid G_{M2}, which carries the same tetrasaccharide, was inactive. In contrast the two biosynthetic tetrasaccharides were equally active as inhibitors of the agglutination of Sd(a++) cells by *Dolichos biflorus* lectin and the degree of inhibition was comparable with that given by the Sd^a-active pentasaccharide fragment.

Discussion

In a search for a source of the enzyme synthesising Sd^a determinants Serafini-Cessi and Dall'Olio [15] examined homogenates of guinea pig kidney and found that microsomal preparations contained a β-*N*-acetylgalactosaminyltransferase that acted preferentially on Tamm-Horsfall glycoprotein from Sd(a-) donors and utilised 3'-sialyllactose, and not 6'-sialyllactose, as an acceptor [16]. The present study has confirmed the acceptor specificity with regard to the 3' and 6' isomers of sialyllactose and has shown that the guinea pig kidney β-*N*-acetylgalactosaminyltransferase also transfers *N*-acetylgalactosamine to 3'-sialyl-*N*-acetyllactosamine but not to 6'-sialyl-*N*-acetyllactosamine. Analysis by methylation and 500 MHz ^{1}H-NMR spectroscopy of the tetrasaccharides formed on a preparative scale with 3'-sialyllactose and 3'-sialyl-*N*-acetyllactosamine confirmed that the enzyme had transferred *N*-acetylgalactosamine from UDP-*N*-acetylgalactosamine in β-anomeric configuration to the O-4 position of the sub-terminal β-galactosyl residue in each acceptor. The product with 3'-sialyllactose, thus has the structure :

GalNAcβ1
4
Galβ1-4Glc
3
NeuAcα2

and corresponds to the oligosaccharide moiety of G_{M2}. The product with 3'-sialyl-*N*-acetyllactosamine has the structure :

GalNAcβ1
4
Galβ1-4GlcNAc
3
NeuAcα2

and is identical with the first four sugars in the Sd^a-active pentasaccharide isolated from Tamm-Horsfall urinary glycoprotein [11].

Earlier tests had shown that liposomes prepared from G_{M2} failed to inhibit the reaction between Sd^a-positive red cells and human anti-Sd^a serum [11] despite the fact that the terminal trisaccharide structure was identical with that of the Sd^a-active pentasaccharide. Since the Cad determinant described by Blanchard *et al.* [13] shares the same terminal trisaccharide, and has Sd^a activity, it seemed possible that the failure of G_{M2} to combine with Sd^a antibodies could be explained by assuming that the proximity of the ceramide moiety was in some way influencing the basic conformation or availability of the oligosaccharide structure. However, the tetrasaccharide synthesised from 3'-sialyllactose had very low inhibitory activity in the Sd^a-anti-Sd^a system (Table 3). In contrast the tetrasaccharide synthesised from 3'-sialyl-*N*-acetyllactosamine was almost as active as the pentasaccharide isolated from Sd^a-positive Tamm-Horsfall glycoprotein. The *N*-acetylamino group on the fourth sugar (GlcNAc) therefore appears to be important either directly for binding to the antibody or indirectly for influencing the conformation of the biosynthetic tetrasaccharide

in such a way that combination with the antibody can take place. With the *Dolichos biflorus* lectin and Sd(a++) indicator cells the two tetrasaccharides were equally inhibitory and thus in this system the nature of the fourth sugar does not play the same role in influencing the binding capacity of the oligosaccharide.

A direct comparison between the Sd^a activity of the determinant structures isolated from Tamm-Horsfall glycoprotein [11] and from Cad-positive erythrocytes [13] has not yet been made. Although the two pentasaccharides share the same terminal trisaccharide the present studies have shown that this is not sufficient to ensure Sd^a activity. The fourth sugars in the two pentasaccharides are different in that the *N*-acetylglucosamine residue in the Sd^a-active T-H structure [11] is replaced by *N*-acetylgalactosaminitol in the Cad structure [13] and moreover in this compound a sialic acid residue is substituted on the O-6 position of this acetyl amino-sugar. Further studies are therefore required to establish whether, despite their overall dissimilarities, the conformations of the two structures are such that they can fulfil equally the requirements for combination with the human Sd^a antibodies.

Acknowledgements

We thank Dr. K. Sandhoff for the gift of G_{M2}, Miss Joyce Poole for supplying the anti-Sd^a serum, Dr. G.W.G. Bird for donating the *Dolichos biflorus* extract, Dr. R. O'Chareon for supplying the Sd(a++) blood and Dr. A. Lawson for carrying out the GC/MS analyses.

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