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Carbohydrate Research 328 (2000) 321–330

CARBOHYDRATE RESEARCH

NMR assignments for glucosylated and galactosylated *N*-acetylhexosaminitols: oligosaccharide alditols related to O-linked glycans from the protozoan parasite *Trypanosoma cruzi*

Christopher Jones [a,*], José O. Previato [b], L. Mendonça-Previato [b]

[a] *Laboratory for Molecular Structure, National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts EN6 3QG, UK*

[b] *Instituto de Microbiologia, Universidade Federal do Rio de Janeiro, Cidade Universitária, 21944-970 Rio de Janeiro, RJ, Brazil*

Received 9 February 2000; accepted 11 April 2000

Abstract

We report full ^{1}H and ^{13}C NMR assignments for 13 gluco- or galacto-pyranosylated derivatives of GlcNAc-ol, GalNAc-ol or ManNAc-ol, many of which have been prepared by enzymatic methods. These spectra are reference data to aid the structural analysis by NMR spectroscopy of glycosylated alditols derived from the mucin of the protozoan parasite *Trypanosoma cruzi*. A series of structural reporter groups for the derivatives from this unusual series of *O*-glycans are described. © 2000 Elsevier Science Ltd. All rights reserved.

Keywords: Parasite mucins; *O*-Glycans; *N*-Acetylhexosaminitols; Enzymatic synthesis; *Trypanosoma cruzi*

1. Introduction

The O-linked glycan chains of the GPI-anchored surface mucin from the protozoan parasite *Trypanosoma cruzi* have unusual structures ([1–4]; Jones et al., unpublished data). They differ from other O-linked glycoprotein glycans in three respects. Firstly, the glycan is linked to Thr residues in the peptide backbone predominantly through α-linked *N*-acetylglucosamine [4]. Secondly, the HexNAc-ol in oligosaccharides isolated by reductive cleavage can be substituted with β-Gal*f* at C-4, β-Gal*p* at C-4 or C-4 and C-6, or β-glucosylated [1–3]. The oligosaccharide alditols isolated from the more complex parasite glycans contain 4,6-disubstituted HexNAc-ol (usually GlcNAc-ol), rather than 3,6-disubstituted GalNAc-ol-containing compounds commonly isolated from mammalian systems. Thirdly, further elaboration of the chain is by galactopyranosylation of the six-arm in a strain-dependent manner, and galacto-pyranosylation or -furanosylation of the β-Gal*f*(1→4)- substituent on HexNAc C-4 [1–4]. In our work on the *O*-glycans from *T.*

Abbreviations: 1D, one-dimensional; 2D, two-dimensional; COSY, correlation spectroscopy; CPS, capsular polysaccharide; GPI, glycophosphoinositol; HexNAc-ol, *N*-acetylhexosaminitol; HSQC, heteronuclear single quantum coherence; PGC, porous graphitised carbon; PNP, *p*-nitrophenyl; TFA, trifluoroacetic acid; TOCSY, total correlation spectroscopy.

* Corresponding author. Tel.: +44-1707-654753 ext. 211; fax: +44-1707-646730.

E-mail address: cjones@nibsc.ac.uk (C. Jones).

0008-6215/00/$ - see front matter
PII: S0008-6215(00)00107-5

cruzi, after release of the glycan with alkaline borohydride, oligosaccharide alditols are fractionated by gel permeation chromatography or HPLC on porous graphitised carbon (PGC) [5,6], and analysed by one- (1D) and two-dimensional (2D) NMR, methylation analysis and, when appropriate, fast atom bombardment mass spectrometry. The isolated oligosaccharides are heterogeneous from the presence of different HexNAc-ol residues. At times only enough material for a 1D ^{1}H NMR spectrum was obtained, or components could not be completely resolved. These fractions can be identified from the 1D ^{1}H NMR spectrum if appropriate authentic model compounds are available, but no systematic study of the ^{1}H NMR spectra of these compounds has been reported.

A series of authentic reference compounds — mono-galactosylated and -glucosylated derivatives of *N*-acetylhexosaminitols — have been prepared from PNP derivatives of β-Glc and β-Gal by the method of Yoon and Ajisaka [7] using enzymic transglycosylation in an aqueous organic solvent mix, and their ^{1}H and ^{13}C NMR spectra fully assigned. These compounds also provide reference samples for methylation analysis.

2. Results

Oligosaccharide preparation.—Parent compounds for five of the desired alditols (β-Gal*p*-(1→3)-GlcNAc, β-Gal*p*-(1→3)-GalNAc, β-Gal*p*-(1→4)-GlcNAc, β-Gal*p*-(1→4)-ManNAc, and β-Gal*p*-(1→6)-GlcNAc) are available commercially. They were reduced with sodium borohydride and purified by HPLC on a PGC column. β-Glc-(1→3)-GlcNAc was prepared by acid hydrolysis of the capsular polysaccharide (CPS) from *Streptococcus pneumoniae* Type 20, as described [8], except that the hydrolysis was performed at 80 °C for 90 min with 0.1 M TFA. The hydrolysis mixture was reduced, desalted and β-Glc-(1→3)-GlcNAc-ol isolated by HPLC.

Other oligosaccharides were prepared according to Yoon and Ajisaka [7], using appropriate glycosidases in an aqueous–triethylphosphate solvent mix with PNP derivatives as donors and *N*-acetylhexosaminitols as acceptors. Initially, the enzymatic transglycosylation reaction mixture was reduced directly and a monosaccharide–alditol fraction isolated directly by HPLC on PGC. In later experiments, the reaction mixture was fractionated, a disaccharide fraction isolated, reduced and desalted. In both cases, the isolated monosaccharide alditols were purified by HPLC on a 20 cm PGC column eluted with a shallow acetonitrile gradient. If the final product remained impure — the most common impurity was the relevant β-Hex-(1→6)-Hexitol — a further chromatographic step on an aminopropyl column was carried out. This method yielded most of the required oligosaccharide alditols, except that formation of the β-Hex-(1→3)-HexNAc derivatives did not occur and yields of β-Hex-(1→4)-HexNAc-ol were variable. Two disaccharide alditols were isolated from the transglycosidation reactions, β-Gal*p*-(1→6)-β-Gal*p*-(1→6)-GalNAc-ol and β-Gal*p*-(1→6)-β-Gal*p*-(1→6)-ManNAc-ol, consistent with sequential addition of Gal residues to the favoured hydroxymethyl groups.

Glucosylation reactions.—Whilst glucosylation of GlcNAc and ManNAc only gave only 6-substituted derivatives, two monosaccharide alditol fractions were isolated from the GalNAc reaction in an approximate ratio of 4:1. The minor, earlier-eluting β-Glc*p*-(1→4)-GalNAc-ol fraction was contaminated with β-Glc-(1→6)-Glc-ol and required re-chromatography on an aminopropyl column.

Galactosylation reactions.—Galactosylation of GalNAc with recombinant *E. coli* enzyme yielded only β-Gal*p*-(1→6)-GalNAc-ol, whilst use of the enzyme from *Aspergillus oryzae* gave a mixture of which the major component was the β-Gal*p*-(1→6)-GalNAc-ol, and minor components were β-Gal*p*-(1→4)-GalNAc-ol and the disaccharide alditol β-Gal*p*-(1→6)-β-Gal*p*-(1→6)-GalNAc-ol. Galactosylation of ManNAc gave the β-Gal*p*-(1→6)-ManNAc-ol and β-Gal*p*-(1→4)-ManNAc-ol as major and minor products respectively. The NMR spectrum of the latter was identical with that of authentic material. Small amounts of β-Gal*p*-(1→6)-β-Gal*p*-(1→6)-ManNAc-ol were also obtained.

Epimerisation of GlcNAc-containing oligosaccharides.—Attempts were made to prepare ManNAc-ol derivatives by controlled alkaline epimerisation of the appropriate GlcNAc-containing disaccharide. Epimerisation of GlcNAc in dilute alkali has been studied [9], and used as a cheap source of GlcNAc/ManNAc mixtures in the enzymatic production of *N*-acetylneuraminic acid [10,11], and is a side reaction in heparin degradation [12]. Epimerisation of the aminosugar was a potential source of additional model compounds: available GlcNAc-containing disaccharides were treated with base, trapped by reduction, and the products analysed by HPLC and NMR spectroscopy. The equilibrium proportions of GlcNAc:ManNAc after dilute alkali equilibriation have been reported to be 4:1, but NaOH treatment of β-Gal*p*-(1→4)-GlcNAc with subsequent borohydride reduction gave a mixture containing β-Gal*p*-(1→4)-GlcNAc-ol and β-Gal*p*-(1→4)-ManNAc-ol in an approximate ratio of 2:1. The NMR spectrum could be interpreted as a mixture of the two compounds, both components of which were already available in a pure form. Attempts to use this approach to prepare β-Gal*p*-(1→3)-ManNAc-ol from the readily available β-Gal*p*-(1→3)-GlcNAc failed, as reaction in either dilute sodium hydroxide or dilute aqueous ammonia gave rise to a complex mixture of degradation products.

Sufficient material was obtained for 13 of the 18 possible derivatives to allow full NMR assignment, and partial assignments could be obtained for one other, which was a component of a mixture. Partial NMR data has previously been reported for two of the compounds studied here. β-Gal*p*-(1→3)-GalNAc-ol is the product of reductive cleavage of the Tn antigen from glycoproteins and several assignment have been reported [13–15]. β-Glc*p*-(1→3)-GlcNAc-ol, identified by methylation analysis, was isolated from the hydrolysate of the *S. pneumoniae* Type 20 CPS, but only the chemical shifts of the Glc H-1 and GlcNAc-ol NAc methyl resonances were reported [8].

The NMR spectra of the monosaccharide alditols were assigned at 500 MHz and 30 °C using conventional methods — TOCSY and COSY to assign the ^{1}H NMR spectrum and heteronuclear correlations to assign the carbon spectrum. These spectra were used for linkage determination of some monosaccharide-alditols made by enzymatic methods. These data are reported in Tables 1–3. Similar assignments were made for two disaccharide alditols isolated from the enzymatic transglycosidations.

Separation of the monosaccharide alditols on the PGC column.—The use of PGC for the separation of oligosaccharide alditols was pioneered in Hounsell's laboratory [5,6]. PGC is robust and the separation depends on hydrophobic patches that interact with planar π-systems in the graphite: components are eluted with an acetonitrile gradient. The oligosaccharide alditols liberated from parasite mucins with alkaline borohydride are well resolved (Jones et al., unpublished work). It was of interest to us to establish general correlations between structure and elution time on these columns as a further aid to characterisation. In the present work, HPLC on a 10 cm PGC column proved valuable to isolate low yields of monosaccharide alditols or disaccharides without preliminary work-up, as they were resolved from excess HexNAc, triethylphosphate, PNP-glycosides and PNP.

Structural dependence of the HPLC elution time.—A knowledge of the relative elution times of structural variants is an aid to structure determination. Three sets of chromatograms were obtained to assess the importance of three factors, the presence of a Glc or Gal substituent, the identity of the HexNAc-ol and the importance of the linkage position. During HPLC of the six β-Hex*p*-(1→6)-HexNAc-ols, the glucosylated compounds eluted later than the galactosylated analogues. This was also true when comparing β-Glc-(1→3)-GlcNAc-ol with β-Gal*p*-(1→3)-GlcNAc-ol. Secondly, in both series of compounds, β-Gal*p*-(1→6)-HexNAc-ol and β-Glc*p*-(1→6)-HexNAc-ol, the order of elution was GalNAc-ol before ManNAc-ol before GlcNAc-ol. The separation of the galactosylated series is shown in Fig. 1(a): essentially the same separation was observed for β-Glc*p*-(1→6)-HexNAc-ol (data not shown). However, β-Gal-(1→4)-GlcNAc-ol

elutes before β-Gal-(1→4)-ManNAc-ol, whilst β-Gal-(1→3)-GalNAc-ol elutes before β-Gal-(1→3)-GlcNAc-ol. Within the series β-Gal*p*-(1→X)-GlcNAc-ol, where X = 3, 4 or 6, the elution order is β-Gal*p*-(1→3)-GlcNAc-ol before β-Gal*p*-(1→4)-GlcNAc-ol, which eluted before β-Gal*p*-(1→6)-GlcNAc-ol. This separation is shown in Fig. 1(b). The same order was observed for the β-Gal*p*-(1→X)-GalNAc-ol series (data not shown), and in the ManNAc-ol series, where compounds are available. The separation of galactosylated GlcNAc-ol derivatives β-Gal*p*-(1→X)-GlcNAc-ol and β-Gal*f*-(1→X)-GlcNAc-ol (X = 3, 4 or 6) by high-performance anion-exchange chromatography has recently been reported [16]. The resolution obtained by HPLC on PGC is comparable with that obtained by anion exchange, and the use of a volatile solvent system simplifies sample recovery for spectroscopic analysis.

Analysis of the partially methylated alditol acetates (PMAAs) from the *N*-acetylhexosaminitols by GC and GC–MS on a DB-1 column showed the order in which the derivatives eluted. Elution times are compared to the peak from permethyl Gal α-methyl glycoside, present in all samples, at 1.000. The order observed was →4)-GlcNAc-ol (2.184), →4)-ManNAc-ol (2.228), →3)-GalNAc-ol (2.238), →6)-GlcNAc-ol (2.260), →3)-GlcNAc-ol (2.261), →6)-GalNAc-ol (2.377) and →6)-ManNAc-ol (2.391). No derivatives containing →3)-ManNAc-ol or →4)-GalNAc-ol were available.

3. Discussion

Synthesis of the components.—Using a combination of approaches the majority of the required monosaccharide alditols were prepared for NMR analysis. The use of glycosidases in organic solvents proved an efficient route for the preparation of the 6-substituted derivatives in the small quantities required for NMR analysis, whilst production of the 4-substituted derivatives was more problematic and no 3-substituted alditols were obtained. Whilst epimerisation of the more readily avail-

Table 1
NMR assignments for GalNAc-ol-containing species

Residue	H-1 C-1	H-1′	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-6′	NAc
GalNAc-ol	3.740 62.88	3.688	4.250 52.86	3.852 69.85	3.396 70.82	3.939 71.18	3.654 64.49	3.683	2.057 23.18
β-Glc*p*-(1→3)-GalNAc-ol	Compound not available								
β-Gal*p*-(1→3)-	4.470 105.14		3.559 72.31	3.667 73.71	3.896 69.67	3.718 76.20	3.773 62.20	3.773	
→3)-GalNAc-ol	3.789 61.73	3.728	4.383 52.56	4.056 77.60	3.503 70.29	4.183 70.61	3.669 64.07	3.621	2.043 23.00
β-Glc*p*-(1→4)-	4.521 103.50		3.315 74.49	3.498 76.91	3.398 70.93	3.350 76.93	3.912 61.90	3.730	
→4GalNAc-ol	3.778 63.54	3.680	4.315 52.03	4.022 68.87	3.720 78.05	4.009 70.83	3.705 62.76	3.606	2.071 23.15
β-Gal*p*-(1→4)-	4.465 104.04		3.533 72.15	3.647 73.86	3.901 69.66	3.712 76.20	3.78 62.04	3.78	
→4)-GalNAc-ol	3.702 62.82	3.66	4.329 51.93	4.030 68.88	3.730 77.91	4.009 70.75	3.81 63.28	3.681	2.071 23.15
β-Glc*p*-(1→6)-	4.513 103.81		3.318 74.28	3.512 76.74	3.392 70.76	3.476 76.99	3.928 61.83	3.725	
→6)-GalNAc-ol	3.744 62.70	3.678	4.266 52.61	3.871 69.55	3.434 70.70	4.125 69.47	3.996 72.87	3.793	2.065 23.00
β-Gal*p*-(1→6)-	4.449 104.44		3.559 72.09	3.670 73.80	3.933 69.75	3.712 76.29	3.772 62.12	3.772	
→6)-GalNAc-ol	3.747 62.75	3.692	4.269 52.64	3.876 69.60	3.449 70.84	4.129 69.29	4.006 73.02	3.796	2.066 23.03

Table 2
NMR assignments for the GlcNAc-ol-containing species

Residue	H-1 C-1	H-1′	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-6′	NAc
GlcNAc-ol	3.761 62.10	3.635	4.071 54.94	3.957 69.59	3.596 72.37	3.755 72.22	3.817 64.01	3.648	2.044 23.35
β-Glc*p*-(1→3)-	4.576 103.93		3.362 74.22	3.524 76.71	3.409 70.95	3.479 76.71	3.944 61.93	3.724	
→3)-GlcNAc-ol	3.780 61.62	3.70	4.258 54.46	4.173 76.71	3.579 71.89	3.878 71.89	3.857 63.80	3.650	2.048 23.22
β-Gal*p*-(1→3)-	4.509 104.58		3.551 71.91	3.676 73.69	3.904 69.73	3.715 76.11	3.776 62.26	3.761	
→3)-GlcNAc-ol	3.768 61.64	3.681	4.244 54.49	4.166 76.73	3.559 71.88	3.903 71.73	3.866 64.11	3.633	2.031 23.22
β-Glc*p*-(1→4)-GlcNAc-ol	Compound not available								
β-Gal*p*-(1→4)-	4.512 104.12		3.579 72.23	3.665 73.73	3.927 69.82	3.697 76.19	3.806 62.22	3.761	
→4)-GlcNAc-ol	3.789 62.05	3.659	4.304 53.79	3.947 69.43 [a]	3.872 79.79	3.946 72.28 [a]	3.878 63.13	3.754	2.063 23.21
β-Glc*p*-(1→6)-	4.505 103.96		3.337 74.41	3.516 76.74	3.402 70.83	3.479 77.05	3.934 61.90	3.739	
→6)-GlcNAc-ol	3.7 62.01	3.639	4.089 54.80	3.980 69.40	3.681 71.96	3.916 71.08	4.146 72.42	3.819	2.055 23.22
β-Gal*p*-(1→6)-	4.440 104.52		3.575 72.01	3.670 73.72	3.935 69.68	3.716 76.21	3.78 62.05	3.78	
→6)-GlcNAc-ol	3.741 61.90	3.640	4.091 54.74	3.980 69.37	3.690 71.86	3.916 71.08	4.148 72.32	3.836	2.055 23.16

[a] Assignment uncertain.

Table 3
NMR assignments for the ManNAc-ol-containing species

Residue	H-1 C-1	H-1′	H-2 C-2	H-3 C-3	H-4 C-4	H-5 C-5	H-6 C-6	H-6′	NAc C-Me
ManNAc-ol	3.851 61.94	3.768	4.053 53.08	3.880 69.09	3.527 70.41	3.746 71.58	3.824 64.12	3.645	2.041
β-Glc*p*-(1→3)-ManNAc-ol	Compound not available								
β-Gal*p*-(1→3)-ManNAc-ol	Compound not available								
β-Glc*p*-(1→4)-ManNAc-ol	Insufficient material for full assignment [a]								
β-Gal*p*-(1→4)-	4.507 104.02		3.568 72.39	3.661 73.79	3.933 69.74	3.703 76.12	3.782 62.27	3.782	
→4)-ManNAc-ol	3.890 63.42	3.750	4.116 54.42	4.073 68.81	3.878 78.45	3.912 72.39	3.839 61.65	3.839	2.031 23.34
β-Glc*p*-(1→6)-	4.503 104.12		3.330 74.41	3.513 76.74	3.396 70.83	3.467 77.05	3.930 61.81	3.738	
→6)-ManNAc-ol	3.858 62.12	3.760	4.058 53.25	3.888 69.12	3.604 70.36	3.893 70.67	4.159 72.85	3.819	2.047 23.18
β-Gal*p*-(1→6)-	4.437 104.70		3.572 72.03	3.680 73.74	3.937 69.85	3.716 76.23	3.776 62.07	3.776	
→6)-ManNAc-ol	3.859 62.07	3.776	4.061 53.20	3.894 69.07	3.619 70.37	3.892 70.63	4.161 72.81	3.849	2.048 23.18

[a] Only sufficient β-Glc-(1→4)-ManNAc-ol was obtained for a 1D ^{1}H NMR spectrum.

able GlcNAc derivatives was effective for the preparation of 4-substituted ManNAc-ol compounds [12], β-Gal*p*-(1→3)-GlcNAc was rapidly degraded under these conditions and the required β-Gal*p*-(1→3)-ManNAc-ol could not be isolated. No experiments were performed in the 6-substituted GlcNAc series, as the relevant ManNAc-ol derivatives were already available.

NMR assignments.—Full assignment of the ^{1}H and ^{13}C NMR spectra of these compounds was achieved by conventional methods and without difficulty. The 1D ^{1}H NMR spectra are key reference data for our work. The distinction between glucosylated or galactosylated materials is clear from the presence or absence of highfield resonances between 3.3 and 3.6 ppm, but the determination of the identity and substitution pattern of the HexNAc-ol is more subtle. A number of 'structural reporter groups' could be defined. Firstly, the peak shape of the HexNAc-ol H-2 resonance appears to be characteristic of the stereochemistry of the HexNAc-ol residue, and, secondly, the pattern of chemical shifts of the HexNAc-ol H-2, H-3, H-4, H-5, H-6a, H-6b′ and NAc are diagnostic of the substitution pattern. Many of these resonances are resolved.

Peak shape of the HexNAc-ol H-2.—The peak shape of this resonance was characteristically different in the three HexNAc-ols, due to the different values of the $^{3}J_{H2,H3}$ coupling constant. In GalNAc-ol and GlcNAc-ol, the two $^{3}J_{H1a,H2}$ and $^{3}J_{H1b',H2}$ couplings are approximately equal (5 Hz), and so, in GalNAc-ol, where $^{3}J_{H2,H3}$ is small the H-2 resonance appears as a triplet, with barely resolvable splitting for each peak. In GlcNAc-ol-containing compounds, $^{3}J_{H2,H3}$ is comparable in size to $^{3}J_{H1a,H2}$ and $^{3}J_{H1b',H2}$ and the H-2 resonance appears as a quartet. In the ManNAc-ol family, the values of the two $^{3}J_{H1a,H2}$ and $^{3}J_{H1b',H2}$ coupling constants are different, and both dif-

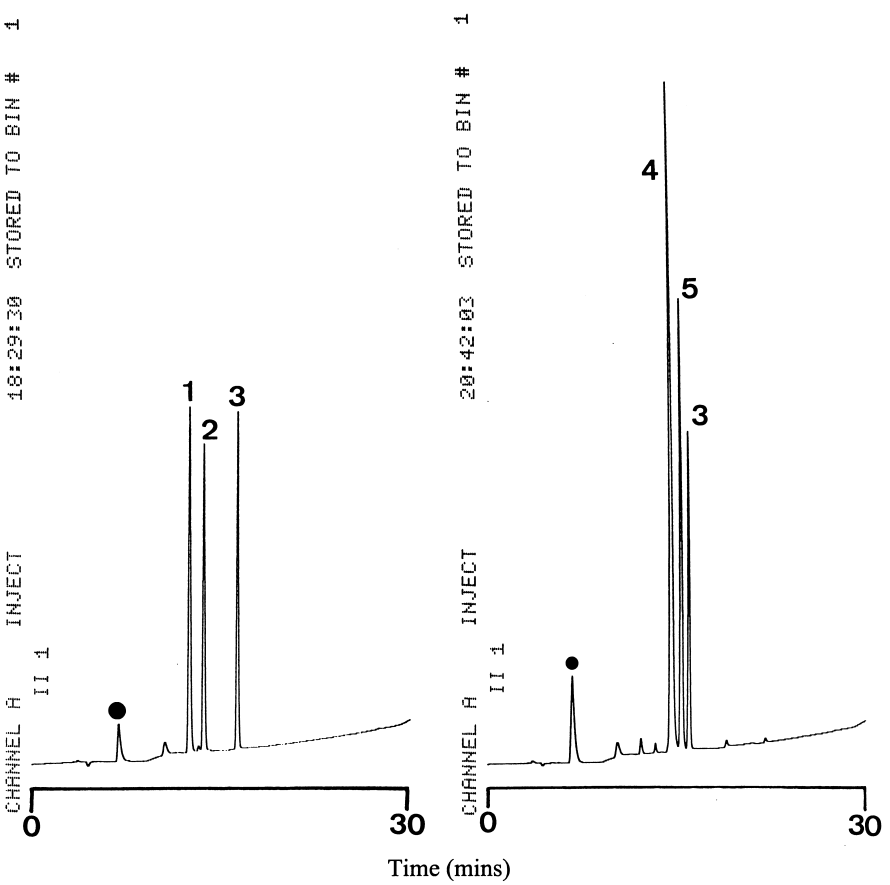

Fig. 1. HPLC separation on a 20 cm 5 μm HyperCarb column of (a) β-Gal-(1→3)-GlcNAc-ol (**1**), β-Gal-(1→4)-GlcNAc-ol (**2**) and β-Gal-(1→6)-GlcNAc-ol (**3**), and (b) of β-Gal-(1→6)-GlcNAc-ol (**3**), β-Gal-(1→6)-GalNAc-ol (**4**) and β-Gal-(1→6)-ManNAc-ol (**5**). The peak indicated by ● arises from TSP-d_4 present in samples recovered from NMR spectroscopy.

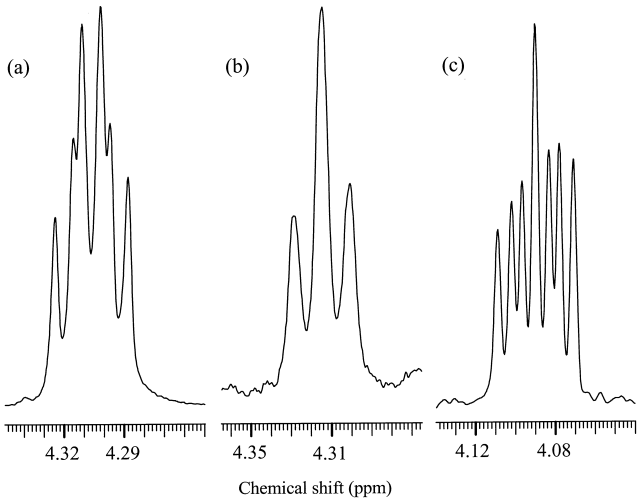

Fig. 2. Peak shapes of the HexNAc-ol H-2 resonance in the 500 MHz NMR spectra of (a) β-Gal*p*-(1→4)-GlcNAc-ol, (b) β-Glc*p*-(1→4)-GalNAc-ol, and (c) β-Gal*p*-(1→6)-ManNAc-ol. In (c), the ManNAc-ol H-2 peak shape is distorted by second-order coupling to the H-3: this is more severe in the spectrum of β-Gal*p*-(1→4)-ManNAc-ol, where the ManNAc-ol H-2 and H-3 are separated by 0.07 ppm.

fer from $^{3}J_{H2,H3}$, so the H-2 resonance has seven lines, with the central line double the intensity of the flanking lines. This is illustrated in Fig. 2.

Structural reporter groups for these components.—The chemical shifts of the H-2, H-3, H-4, H-6a, H-6b′ and NAc appear to be characteristic both of the identity and the substitution pattern of the HexNAc-ol, independent of whether the substituent is β-Glc*p* or β-Gal*p*. These data are summarised in Table 4. For example, all the ManNAc-ol derivatives are characterised by highfield H-2 resonance when 4-substituted, whilst both GlcNAc-ol and GalNAc-ol have lowfield H-2s. The H-5 of both 4- and 6-substituted GalNAc-ol is lowfield, in contrast to GlcNAc-ol- and ManNAc-ol-containing oligosaccharides. It seems these rules can be extrapolated to 4,6-disubstituted systems, when both substituents are in the pyranose form, but different chemical shifts for reporter groups apply when the 4-substituent is a β-Gal*f* residue ([1] and unpublished data).

NMR data reported for related compounds.—Full or partial ^{1}H NMR data have been reported for a number of related structure, particularly those related to the oligosaccharide alditols derived from the various GalNAc-containing core structures from mammalian glycoproteins. These include, for example, β-Gal*p*-(1→3)-GalNAc-ol [13–15], β-GlcNAc-(1→3)-GalNAc-ol, β-GalNAc-(1→3)-GalNAc-ol, β-GlcNAc-(1→6)-GalNAc-ol and β-L-Fuc-(1→6)-GalNAc-ol [13]. The anomeric proton chemical shift was reported for β-Gal*f*-(1→4)-GlcNAc-ol [17], and full assignments for β-Gal*f*-(1→4)[β-Gal*p*-(1→6)]GlcNAc-ol [1] and these were confirmed by Gallo-Rodriguez et al. [18] with synthetic material.

ManNAc-ol in the T. cruzi *mucin.*—Analysis of the oligosaccharide alditols obtained from the GPI-anchored mucin of the protozoan parasite *T. cruzi* clearly indicated that, although the major *N*-acetylhexosaminitol was GlcNAc-ol, a second HexNAc-ol was also present, in amounts ranging from 10 to 30% between samples. Comparison with model compounds now indicate that this is ManNAc-ol rather than GalNAc-ol. It is not clear whether this arises from ManNAc in the original mucin or from rapid epimerisation during cleavage of the oligosaccharides under basic condition: this is the subject of ongoing study and results will be reported elsewhere. The availability of suitable model compounds and the identification of structural reporter groups also demonstrate that the minor component isolated from *T. cruzi* Y-strain, originally identified [2] as a component of a mixture as β-Gal*p*-(1→3)-GlcNAc-ol, is in reality β-Gal*p*-(1→4)-ManNAc-ol. Higher homologues also present therefore are also in this family.

4. Experimental

Laboratory chemicals were obtained from Sigma, Aldrich or Fluka, and HPLC grade MeCN from Fisher Scientific (Loughborough, UK). β-Glucosidase from almonds, β-galactosidase from *E. coli* and β-galactosidase from *A. oryzae* were obtained from Sigma. Authentic samples of β-Gal*p*-(1→3)-GalNAc, β-Gal*p*-(1→3)-GlcNAc, β-Gal*p*-(1→4)-GlcNAc and β-Gal*p*-(1→4)-ManNAc were obtained from Dextra Laboratories (Reading) and β-Gal*p*-(1→6)-GlcNAc from Sigma Chemical Co. (Poole, UK). Disaccharides (1–5 mg) were reduced with aq $NaBH_4$ (20 mg in 0.5 mL) at r.t. for 6 h, excess $NaBH_4$ destroyed

Table 4
Structural reporter groups in mono-substituted β-D-Hex-(1→X)-D-HexNAc-ols and di-substituted D-Gal*p* or β-D-Gal*f*-(1→4)-(β-D-Hex*p*-(1→6)-D-HexNAc-ols [a]

Hexose	HexNAc-ol	HexNAc-ol H-2	HexNAc-ol H-3	HexNAc-ol H-4	HexNAc-ol H-5	HexNAc-ol H-6	HexNAc-ol H-6′	HexNAc-ol NAc
β-Glc*p*-(1→3)- or β-Gal*p*-(1→3)-	GalNAc	**4.38**	**4.06**	**3.50**	**4.18**	3.67	3.62	**2.043**
	GlcNAc [b]	**4.26**	**4.18**	3.57	3.85–3.92	3.88	3.65	**2.043**
	ManNAc	No compounds available						
β-Glc*p*-(1→4)- or β-Gal*p*-(1→4)-	GalNAc	**4.32–4.33**	**4.02–4.03**	3.72–3.73	**4.01**	3.71–3.81	3.61–3.68	**2.071**
	GlcNAc [c]	**4.30**	3.95	3.87	3.95	3.88	3.75	**2.063**
	ManNAc	**4.12**	**4.07**	3.88	3.84	3.84	3.84	**2.036**
β-Glc*p*-(1→6)- or β-Gal*p*-(1→6)-	GalNAc [d]	**4.27**	3.87–3.88	**3.43–3.45**	**4.12–4.13**	**4.00–4.01**	3.79–3.80	**2.066**
	GlcNAc	**4.09**	3.98	3.68–3.69	3.92	**4.15**	3.82–3.84	**2.055**
	ManNAc [e]	**4.06**	3.88–3.89	3.60–3.62	3.89	**4.16**	3.82–3.85	**2.047–2.048**
β-Gal*f*-(1→4)- [f]	GlcNAc	**4.15**	3.93	3.77	3.92	3.82	3.69	**2.056**
β-Gal*f*-(1→4)- [f]	ManNAc	3.92	3.80	3.78	**4.06**	3.98	3.79	**2.033**
β-Gal*p*-(1→4)- [f], β-Gal*p*-(1→6)- or β-Glc*p*-(1→6)-	GlcNAc	**4.33**	3.95		**4.07**	**4.20**	3.95	**2.063**
β-Gal*p*-(1→4)- [f], β-Gal*p*-(1→6)- or β-Glc*p*-(1→6)-	ManNAc	**4.12**	**4.12**	n.d.	**4.03**	**4.22**	3.94	**2.032**
β-Gal*f*-(1→4)-; β-Gal*p*-(1→6)- [f]	GlcNAc	**4.17**	3.93	3.83	**4.04**	**4.15**	3.84	**2.057**
β-Gal*f*-(1→4)-; β-Gal*p*-(1→6)- [f]	ManNAc	Compound not available						

[a] Figures in bold are for those resonances which are likely to be resolved.
[b] Include data obtained for PO_3→6)-α-Glc-(1→6)-β-Glc-(1→3)-β-Gal*f*-(1→3)-β-Glc-(1→3)-GlcNAc-ol isolated from the pneumococcal Type 20 CPS [8].
[c] Includes data obtained for α-NeuNAc-(2→3)-β-Gal*p*-(1→4)-GlcNAc-ol and α-NeuNAc-(2→3)-β-Gal*p*-(1→4)-GlcNAc-ol.
[d] Includes data for β-Gal*p*-(1→6)-β-Gal*p*-(1→6)-GalNAc-ol.
[e] Includes data for β-Gal*p*-(1→6)-β-Gal*p*-(1→6)-ManNAc-ol.
[f] Data from unpublished work on biological samples, or Refs. [1] or [2].

with 20% aq HOAc, and the monosaccharide alditols desalted by HPLC on PGC using a gradient of MeCN between 2 and 15% over 20 min, essentially as described below.

Preparation of the glucosylated and galactosylated monosaccharide alditols.—The derivatives were prepared by incubating either GalNAc, GlcNAc or ManNAc (20 mg) with either Glcβ1-OPNP or Galβ1-OPNP (10 mg) in 100 μL of 40% 50 mM Na acetate buffer pH 4.5/60% $(EtO)_3PO$ in the presence of either β-glucosidase (from almonds) or β-galactosidase (from *E. coli* or *A. oryzae*) at 30 °C. After 2–5 h the reaction was stopped by freezing and aliquots injected onto a PGC column ('HyperCarb', 4.6 mm × 10 cm, Life Science International, Basingstoke,). The column was eluted with a gradient of MeCN (2–100%) in water, containing 0.05% TFA over a period of 40 min, and elution monitored at 206 nm. Disaccharide fractions were collected and dried, resuspended in water and reduced with $NaBH_4$ (ca. 5 mg). Excess $NaBH_4$ was destroyed by the addition of 20% HOAc until evolution of H_2 ceased, and the reaction mixture evaporated to dryness. Monosaccharide alditols were isolated by chromatography on a PGC column (4.6 mm × 20 cm), eluted with a gradient of 2–25% MeCN containing 0.05% TFA, over a period of 30 min. Fractions still impure were further purified by chromatography on a 3 μm Phenomosphere NH_2 aminopropyl column (4.6 × 150 mm, Phenomonex, Macclesfield, UK), using a gradient of 15–40% 3 mM KH_2PO_4 in MeCN [19], with elution monitored at 206 nm. UV-absorbing fractions were collected, dried in vacuo and desalted by chromatography on HyperCarb.

Epimerisation of GlcNAc-containing disaccharides.—β-Gal*p*-(1→3)-GlcNAc or β-Gal*p*-(1→4)-GlcNAc (5 mg, Dextra, Reading UK) were treated with NaOH (50 mM, 50 μL) for 18 h at r.t. [10], reduced with $NaBH_4$, desalted on BioRad AG50x8 100–200 mesh H^+ form, and purified as a mixture by HPLC on PGC. Products were analysed by NMR and HPLC on PGC using a 2–15% MeCN gradient. Additional experiments using 1 or 0.25% aq NH_3 were carried out with β-Gal*p*-(1→3)-GlcNAc.

NMR spectroscopy.—NMR spectra were obtained on a Varian Unity 500 NMR spectrometer equipped with a 5 mm triple resonance PFG probe, running VNMR version 5.3 or 6.1B. Samples were deuterium exchanged twice before redissolution in D_2O (ca. 250 μL) and introduction into the NMR tube. Low volume susceptibility-matched tubes (Shigemi, Tokyo) were used, and spectra were obtained at an indicated probe temperature of 30 °C. Standard pulse sequences as supplied by Varian were used except for the introduction of an echo sequence into the TOCSY sequence, and the use of the Wider and Wüthrich implementation of the HSQC sequence [20]. Chemical shifts are referenced against internal TSP-d_4 acid at 0 ppm (^{1}H) or −1.80 ppm (^{13}C) [21].

Gas chromatography, GC–MS and methylation analysis.—β-Gal*p*-(1→X)-HexNAc-ol in Me_2SO was methylated by the method of Ciucanu and Kerek [22] for 40 min with sonication, the reaction diluted with aq $Na_2S_2O_3$ and permethylated material extracted with $CHCl_3$. The product was methanolysed (0.5 M HCl in MeOH, 18 h, 80 °C), solvent evaporated and the material acetylated with HOAc/C_5H_5N for 24 h at 18 °C. The resulting mixture of permethylated Gal methyl glycosides and partially methylated, partially acetylated N-acetylated *N*-methylhexosaminitol was analysed by GC on DB-1 column (25 m × 0.2 mm i.d.) using a linear temperature gradient from 120 to 180 °C (2 °C min^{-1}, then hold). Peaks were identified by GC–MS as previously described [1].

Acknowledgements

We thank Merck and Co. Inc. (West Point, PA) for the sample of the pneumococcal Type 20 CPS. This work was supported by grants from PRONEX, FINEP, CNPq and PADCT. We are grateful for support for our collaborative work from the Sir Halley Stewart Trust. L.M.-P. is a Howard Hughes International scholar.

References

[1] J.O. Previato, C. Jones, L.P.B. Gonçalves, R. Wait, L.R. Travassos, L. Mendonça-Previato, *Biochem. J.*, 301 (1994) 151–159.

[2] J.O. Previato, C. Jones, M.T. Xavier, R. Wait, L.R. Travassos, A.J. Parodi, L. Mendonça-Previato, *J. Biol. Chem.*, 270 (1995) 7241–7250.

[3] C. Jones, J.O. Previato, A.R. Todeschini, R. Wait, L. Mendonça-Previato, *Abstracts of Papers*, EuroCarb 9, Utrecht, July 1997, B36.

[4] J.O. Previato, M. Sola-Penna, O.A. Agrellos, C. Jones, T. Oeltmann, L.R. Travassos, L. Mendonça-Previato, *J. Biol. Chem.*, 273 (1998) 14982–14988.

[5] M.J. Davies, E.F. Hounsell, *J. Chromatogr. A*, 720 (1996) 227–233.

[6] M.J. Davies, K.D. Smith, R.A. Carruthers, W. Chai, A.M. Lawson, E.F. Hounsell, *J. Chromatogr.*, 646 (1993) 317–326.

[7] J.-H. Yoon, K. Ajisaka, *Carbohydr. Res.*, 292 (1996) 153–163.

[8] J.C. Richards, M.B. Perry, D.J. Carlo, *Can. J. Biochem. Cell Biol.*, 61 (1983) 178–190.

[9] M.A.E. Sallam, *Carbohydr. Res.*, 63 (1978) 127–130.

[10] M. Mahmoudian, D. Noble, C.S. Drake, R.F. Middleton, D.S. Montgomery, J.E.. Piercey, D. Ramlakhan, M. Todd, M.J. Dawson, *Enzyme Microb. Technol.*, 20 (1997) 393–400.

[11] T. Sugai, A. Kuboki, S. Hiramasu, H. Okazaki, H. Ohta, *Bull. Chem. Soc. Jpn.*, 68 (1995) 3581–3589.

[12] T. Toida, I.R. Vlahov, A.E. Smith, R.E. Hileman, R.J. Lindhart, *J. Carbohydr. Chem.*, 15 (1996) 351–360.

[13] S. Martensson, S.B. Levery, T.T. Fang, B. Bendiak, *Eur. J. Biochem.*, 258 (1998) 603–622.

[14] H. van Halbeek, L. Dorland, J. Haverkamp, G.A. Veldink, J.F.G. Vliegenthart, B. Fournet, G. Ricart, J. Montreuil, W.D. Gathmann, D. Aminoff, *Eur. J. Biochem.*, 118 (1981) 487–495.

[15] A.S. Shashkov, N.P. Arbatsky, V.A. Derevitskaya, N.K. Kochetkov, *Carbohydr. Res.*, 72 (1979) 218–221.

[16] M.L. Salto, C. Gallo-Rodriguez, C. Lima, R.M. de Lederkremer, *Anal. Biochem.*, 279 (2000) 79–84.

[17] A.A. Serrano, S. Schenkman, N. Yoshida, A. Mehlert, J.M. Richardson, M.A.J. Ferguson, *J. Biol. Chem.*, 270 (1995) 27244–27253.

[18] C. Gallo-Rodriguez, O. Varela, R.M. de Lederkremer, *J. Org. Chem.*, 61 (1996) 1886–1889.

[19] W. Morelle, G. Strecker, *Biochem. J.*, 330 (1998) 469–478.

[20] G. Wider, K. Wüthrich, *J. Magn. Reson. Ser. B*, 120 (1993) 239–241.

[21] D. Wishart, C.G. Bigam, J. Yao, F. Abildgaard, H.J. Dyson, E. Oldfield, J.L. Markley, B.D. Sykes, *J. Biomol. NMR*, 6 (1995) 135–140.

[22] I. Ciucanu, F. Kerek, *Carbohydr. Res.*, 131 (1984) 209–217.