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^{1}H NMR analysis of novel sialylated and fucosylated lactose-based oligosaccharides having linear GlcNAc(β1–6)Gal and Neu5Ac(α2–6)GlcNAc sequences [1]

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Abstract

Three novel oligosaccharides of human infant faeces have been fully characterised by methylation analysis and 500/600 MHz ^{1}H NMR spectroscopy including DQF-COSY, TQF-COSY, TOCSY and ROESY experiments. The oligosaccharides were shown to be lactose-based structures two of which were substituted at C-6 of Gal with either the Le^x trisaccharide, Gal(β1-4)[Fuc(α1-3)]GlcNAc(β1-, or Neu5Ac(α2-6)Gal(β1-4)GlcNAc-(β1-. They differ from other free oligosaccharides previously isolated from the human by having the (1 → 6) linkage to Gal in the absence of a (1 → 3) branch. The third oligosaccharide has Neu5Ac(α2–6) linked to GlcNAc of the trisaccharide GlcNAc(β1–3)Gal(β1–4)Glc. This is a linear fragment of the disialylated tetrasaccharide sequence Neu5Ac(α2–3)Gal(β1–3)[Neu5Ac(α2–6)]GlcNAc(β1- found in the milk oligosaccharide disialyl LNT (the GlcNAc residue of the tetrasaccharide linked to lactose) and also of N-linked chains (GlcNAc linked to Man). © 1997 Elsevier Science Ltd.

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1. Introduction

The major non-protein component of milk is the disaccharide lactose with minor additional oligosaccharides being found having a lactose core. These can be further glycosylated by glycosyltransferases in the gastrointestinal tract of breast fed infants and similar oligosaccharides have been found in urine. In humans many neutral, sialylated and differently fucosylated oligosaccharides have been isolated from these sources. The oligosaccharides have been essential

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[1] Dedicated to Professor Hans Paulsen on the occasion of his 75th birthday.

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reagents for the characterisation of cell differentiation and tumour antigens [1,2]. After lactose, the most abundant are the tetrasaccharides LNT, Gal(β1–3)GlcNAc(β1–3)Gal(β1–4)Glc, and LNNT, Gal(β1–4)GlcNAc(β1–3)Gal(β1–4)Glc, and their various fucosylated derivatives. Fuc is found at C-2 of non-reducing terminal Gal (blood group H), at C-3 of Glc and at C-3 or C-4 of GlcNAc (Le^x and Le^a, respectively, in the absence of the blood group H Fuc and Le^y and Le^b, respectively, in the presence of the blood group H Fuc). These oligosaccharides can have the A/B blood group antigenic structures attached in the absence or presence of the Le^y or Le^b Fuc, e.g. GalNAc/Gal(α1–3)[Fuc(α1–2)]Gal(β1–4)[Fuc(α1–3)]Glc (A/B Le^y) and on the trisaccharides having Fuc(α1–3)Glc and Fuc(α1–2)Gal of lactose. In addition blood group A/B Le^b-like sequences have been found based on a Gal(β1–3)Glc rather than the lactose core, i.e. GalNAc/Gal(α1–3)[Fuc(α1–2)]Gal(β1–3)[Fuc(α1–4)]Glc [3].

Larger oligosaccharides are classically built up by addition of type I, Gal(β1–3)GlcNAc, and type II, Gal(β1–4)GlcNAc, disaccharide sequences either in a linear fashion (GlcNAc linked to C-3 of Gal) or with branching (GlcNAc linked to C-6 of Gal already linked at C-3) giving, for example LNH (lacto-*N*-hexaose), Gal(β1–4)GlcNAc(β1–6)[Gal(β1–3)GlcNAc(β1–3)]Gal(β1–4)Glc, and iso-lacto-*N*-octaose [4,5], Gal(β1–3)GlcNAc(β1–3)Gal-(β1–4)GlcNAc(β1–6)[Gal(β1–3)GlcNAc(β1–3)]-Gal(β1–4)Glc. These larger oligosaccharides are variously fucosylated, e.g. linear di-Le^x or Le^a–Le^x extensions of LNNT [6,7], Le^b–Le^x [8] and also Le^b–Le^x–Le^x [5]. The (1 → 6) branch of LNH can be extended and fucosylated giving for example, non-reducing terminal H type I and internal Le^x [7], Le^b–Le^x [4], or extended by another branch giving Gal(β1–4)GlcNAc(β1–6)[Gal(β1–3)GlcNAc-(β1–3)]Gal(β1–4)GlcNAc(β1–6)[Gal(β1–3)GlcNAc(β1–3)]Gal(β1–4)Glc [6]. Both arms of LNH can be fucosylated, e.g. giving Le^a or Le^b on the (1 → 3) arm and Le^x on the (1 → 6) arm [7,9–11]. Iso-lacto-*N*-octaose was identified as having either Le^a–Le^x on the (1 → 6) arm or internal Le^x on the (1 → 6) arm and blood group H type I Fuc on the (1 → 3) arm [4] and has also been found with either H-type I and Le^x or Le^b–Le^x on the (1 → 6) arm together with Le^b on the (1 → 3) arm [5]. The Le^b structure has been found additionally in the presence of the blood group A GalNAc, i.e. giving ALe^b [10]. These oligosaccharides were obtained from the faeces of breast fed infants of blood group A mothers [11]. This serves as a good source for novel oligosaccharides which we have analysed in the present study.

In all these free oligosaccharides from human sources described so far the GlcNAc(β1–6)Gal sequence is only found in the presence of GlcNAc(β1–3)Gal, i.e. in the presence of a branch. However, in goats milk, lactosamine and 3-fucosyllactosamine linked (1 → 6) to lactose have been described [12] and the linear GlcNAc(β1–6)Gal linkage has been found in human mucin oligosaccharide chains [13,14]. In the present study we report extensive NMR chemical shift data for oligosaccharides containing the Gal(β1–4)GlcNAc(β1–6)Gal sequence either fucosylated at GlcNAc or sialylated at Gal. Such oligosaccharides may well have arisen from degradation in the gastrointestinal tract rather than specific biosynthesis, but this may not be the case as they would be difficult to manufacture artificially from branched oligosaccharides because the (1 → 6) linkage is the most labile, e.g. is specifically cleaved by acetolysis. However, for the similar situation i.e. (1 → 6) linkages in the absence of (1 → 3) documented in mucin oligosaccharides of meconium, it was argued that specific biosynthesis may have occurred [13]. We have in the present study a third example of a linear motif (italicised) from a previously found branched oligosaccharide DSLNT [15,16], Neu5Ac(α2–3)Gal(β1–3)*[Neu5Ac(α2–6)]GlcNAc(β1–3)Gal(β1–4)Glc*, which can also be found on larger and fucosylated reducing oligosaccharides [17–19]. The non-reducing end tetrasaccharide sequence (absence of lactose in DSLNT) has also been found linked to Man in N-linked glycoprotein glycans [20]. Strecker et al. [15] have documented three monosialylated LNT oligosaccharides having linear Neu5Ac(α2–6)Gal or Neu5Ac(α2–3)Gal and branched Neu5Ac(α2–6)[Gal(β1–3)]GlcNAc. Larger oligosaccharides are variously sialylated, e.g. sialylation of LNH most commonly at C-3 of Gal in the (1 → 3) arm and at C-6 of Gal in the (1 → 6) arm [21–23], but also at C-6 of Gal in the (1 → 3) arm [24,25]. These and larger sialylated oligosaccharides are variously glycosylated with blood group and Le (or sialyl Le) sequences, e.g. sialyl Le^a on the (1 → 3) arm and blood group H-Le^x on the (1 → 6) arm [23], Neu5Ac(α2–3)Gal on the (1 → 3) arm with Le^y or Le^x [11] or Le^b–Le^a [22], on the (1 → 6) arm, Neu5Ac(α2–6)[Gal(β1–3)]GlcNAc(β1–3) and Le^x on the (1 → 6) arm [22], etc. As these motifs may be important recognition signals, it is of considerable interest to have conformational information on these complex oligosaccha-

rides. Detailed NMR data on the linear Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6)Gal, Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6)Gal and Neu5Ac(α2–6)GlcNAc(β1–3)Gal sequences in the present study will help in this analysis.

2. Experimental

Oligosaccharides isolated from the faeces of a breast-fed human infant O-secretor were a kind gift of the late A.S.R. Donald. Briefly these were obtained by the recycling low pressure chromatography [26] pioneered by A.S.R.D as follows: Nappies were extracted with boiling water. On cooling, the solution was filtered and 0.1 vol charcoal (BDH, Poole, UK) and 2.6 mg/mL boric acid were added to the filtrate and shaken for 18 h. This was filtered and washed with water and then 1:1 2-propanol–water. The combined washings were evaporated and loaded via a celite filter onto a Fractogel column (100 × 4.8 cm) and eluted with water. After the first 500 mL, fractions of 12.5 mL were collected and fractions 33–55 containing tetra- to octa-saccharides were further fractionated on an AG1 column (acetate form; 7.5 × 2.5 cm). The water eluate containing the neutral oligosaccharides was further fractionated on two consecutive columns (80 × 5 cm) of Zerolit 225 × 4 (K^+ form). The fraction corresponding to pentasaccharides was further purified on AG1 × 4 (200–400 mesh) in 0.125 M borate adjusted to pH 8.1 to obtain oligosaccharide **1**. Sialylated oligosaccharides **2** and **3** were recovered from the AG1 (acetate) column by elution with 1 M pyridine acetate. The eluate was evaporated and re-evaporated several times from water and the residue taken up in 5 mM pyridine acetate and loaded onto an AG1 × 4 (200–400 mesh) column in the acetate form eluted with 16.6 mM pyridine acetate. The tetra- to hexa-saccharide fraction was purified by recycling from a Fractogel column (95 × 2.5 cm) run in 100 mM NH_4HCO_3 and the main hexose-containing peaks evaporated and rechromatographed to give single components on an AG1 × 4 (200–400 mesh) column eluted in 50 mM borate buffer.

Methylation analysis.—Oligosaccharides (30–40 μg) were permethylated with methyl iodide in a suspension of solid NaOH in Me_2SO [27], extracted into $CHCl_3$ and then treated with anhydrous acidic MeOH (0.5 M; Supelco, Poole, UK) at 80 °C for 18 h and acetylated with 1:1 pyridine–acetic anhydride at room temperature, overnight [28]. The partially *O*-methylated *O*-acetylated methyl glycosides obtained were characterised by GC–MS using a Hewlett Packard 5890 series II GC and a HP5972A MSD operated in the EI mode, with a Hewlett Packard Ultra-2 capillary column (25 m × 0.2 mm), a column temperature from 60 to 265 °C with a gradient of 5 °C/min and a helium pressure of 10 psi with cool on-column injection.

NMR spectroscopy.—The oligosaccharides were lyophilised and redissolved twice in 99.9 atom % D (Sigma, Poole, UK) before final dissolution in 99.96 atom % D and addition of acetone (~ 2 μM) as an internal standard. Final concentrations were greater than 5 mM of oligosaccharide. 500 and 600 MHz spectrometers were run under the software supplied by the manufacturer, Varian (VNMR v4.3). Solvent suppression was carried out using a 2 s presaturation pulse in both the 1D and 2D experiments. The chemical shifts were referenced to the methyl resonance of acetone at 2.225 ppm for spectra recorded at 295 K. Homonuclear ^1H–^1H spectra phase sensitive DQF-

Table 1
Molar ratios of the partially *O*-methylated *O*-acetylated methyl glycosides from oligosaccharides **1**, **2** and **3** identified and quantified by GC–MS

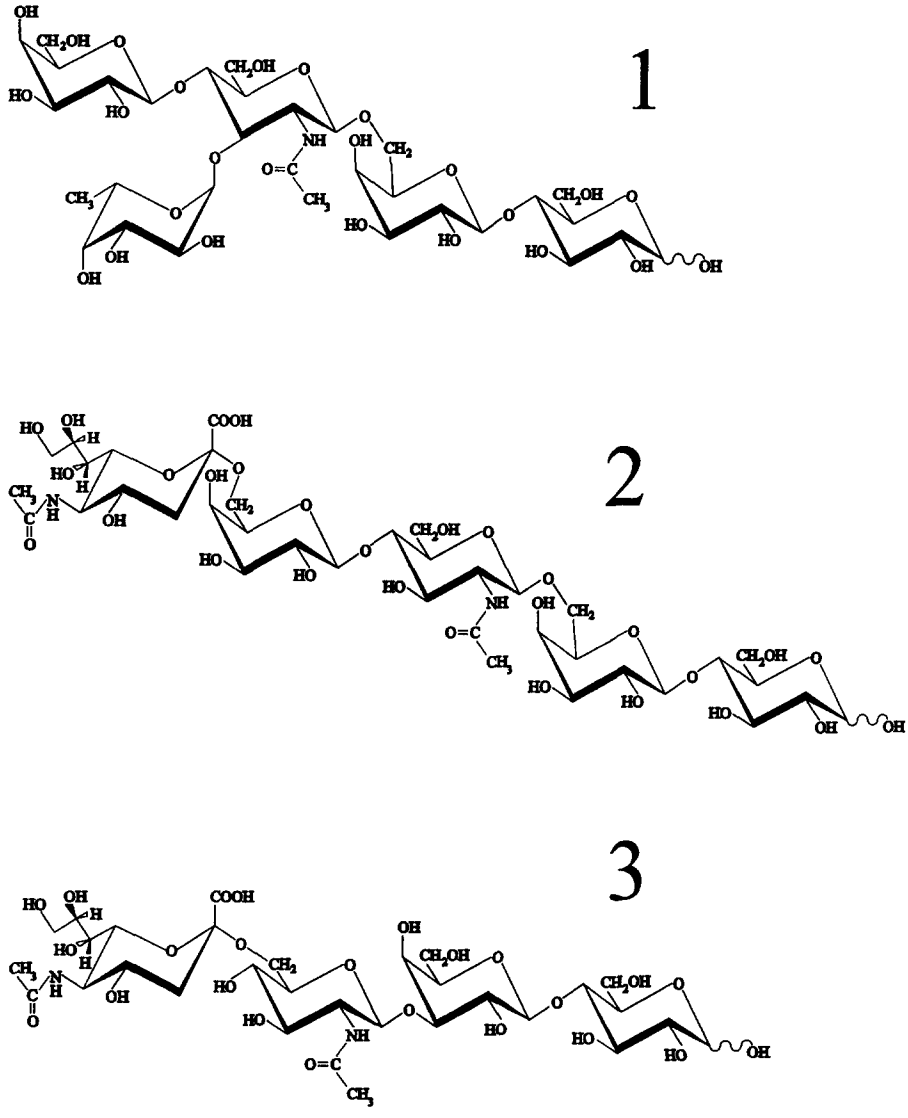
O-methyl derivative	Deduced linkage	Oligosaccharide		
		1	**2**	**3**
2,3,5-tri-*O*-Me-Fuc	Fuc(1-	0.7	–	–
2,3,4,6-tetra-*O*-Me-Gal	Gal(1-	0.8	0.1	–
2,3,4-tri-*O*-Me-Gal	-6)Gal(1-	1.0	2.0	–
2,4,6-tri-*O*-Me-Gal	-3)Gal(1-	–	–	1.0
2,3,6-tri-*O*-Me-Glc	-4)Glc(1-	0.9	1.1	1.0
3,6-di-*O*-Me-GlcN(Me)Ac	-4)GlcNAc(1-	–	0.8	–
3,4-di-*O*-Me-GlcN(Me)Ac	-6)GlcNAc(1-	–	–	0.8
6-mono-*O*-Me-GlcN(Me)Ac	-3,4)GlcNAc(1-	0.8	–	–
4,7,8,9-tetra-*O*-Me-Neu5Ac	Neu5Ac(2-	–	0.7	0.8

COSY, TQF-COSY and TOCSY (with MLEV-17 pulse sequence and 100 ms mixing times) [29–32] measurements were acquired typically with 2D data collections of 4096 complex points in t_2 with between 256 and 512 increments (using 16/32 scans per increment) in t_1 and a spectral width of 5000 Hz. ^{1}H–^{1}H ROESY experiments [33,34] were carried out with 300 ms mixing times and examined for spin diffusion by comparison with the TOCSY.

3. Results

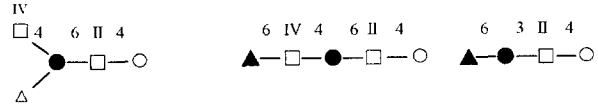
Table 1 gives the results of the methylation analysis of oligosaccharides **1, 2** and **3** (Scheme 1) which shows the presence of 6-linked Gal in oligosaccharides **1** and **2** and 6-linked GlcNAc in oligosaccharide **3**. Chemical shift assignments for the three oligosaccharides were achieved by analysis of 1D ^{1}H and 2D ^{1}H–^{1}H NMR spectra (Table 2) as follows:

Oligosaccharide **1**.—Fig. 1 shows the 1D ^{1}H NMR spectrum and 2D ROESY experiment of oligosaccharide **1.** The shaded area is expanded in Fig. 2 from which it was possible to obtain a near complete assignment of the bulk of the TOCSY and ROESY



Scheme 1. The structures of the oligosaccharides **1, 2** and **3**.

Table 2
^{1}H NMR chemical shifts (δ, ppm) for oligosaccharides **1, 2** and **3** in D_2O at 22°C (○, Glc; ●, GlcNAc; □, Gal; △, Fuc; ▲, Neu5Ac) where [ ] are β anomeric chemical shifts



cross-peaks in the spectral width 3.2 → 4.2 ppm. The TQF-COSY experiment gave an additional assignment for H-5 of Gal^{II}, but it was still not possible from this spectrum to deduce the chemical shifts of H-6a, H6b in the predicted novel linear linkage GlcNAc(β1–6)Gal. The chemical shifts for the Le^x sequence, Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1-, (Table 2) are similar to those previously published [35–40]. Comparing the present data with those for lacto-*N*-fucopentaose III (LNFP III), Gal^{IV}(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)Gal^{II}(β1–4)Glcα/β [37,39], shows that the glycosidic linkage between GlcNAc and Gal^{II} is not (1 → 3) since, although the α-Glc, β-Glc, Gal^{IV} and Fuc chemical shift patterns are very similar, those for the Gal^{II} and GlcNAc residues are different. In our study the Gal^{II} protons H-1 to H-4 are shifted to lower fields, H-4 showing the biggest difference, $\Delta\delta$ H-1 = 0.007, $\Delta\delta$ H-2 = 0.040, $\Delta\delta$ H-3 = 0.066, $\Delta\delta$ H-4 = 0.253. Comparison of the data in Table 2 with those for the octasaccharide difuco-lacto-*N*-neohexaose (DFLNNH), Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–6){Gal(β1–4)-[Fuc(α1–3)]GlcNAc(β1–3)}Gal(β1 → 4)Glcα/β [5], showed that the GlcNAc residue in **1** has chemi-

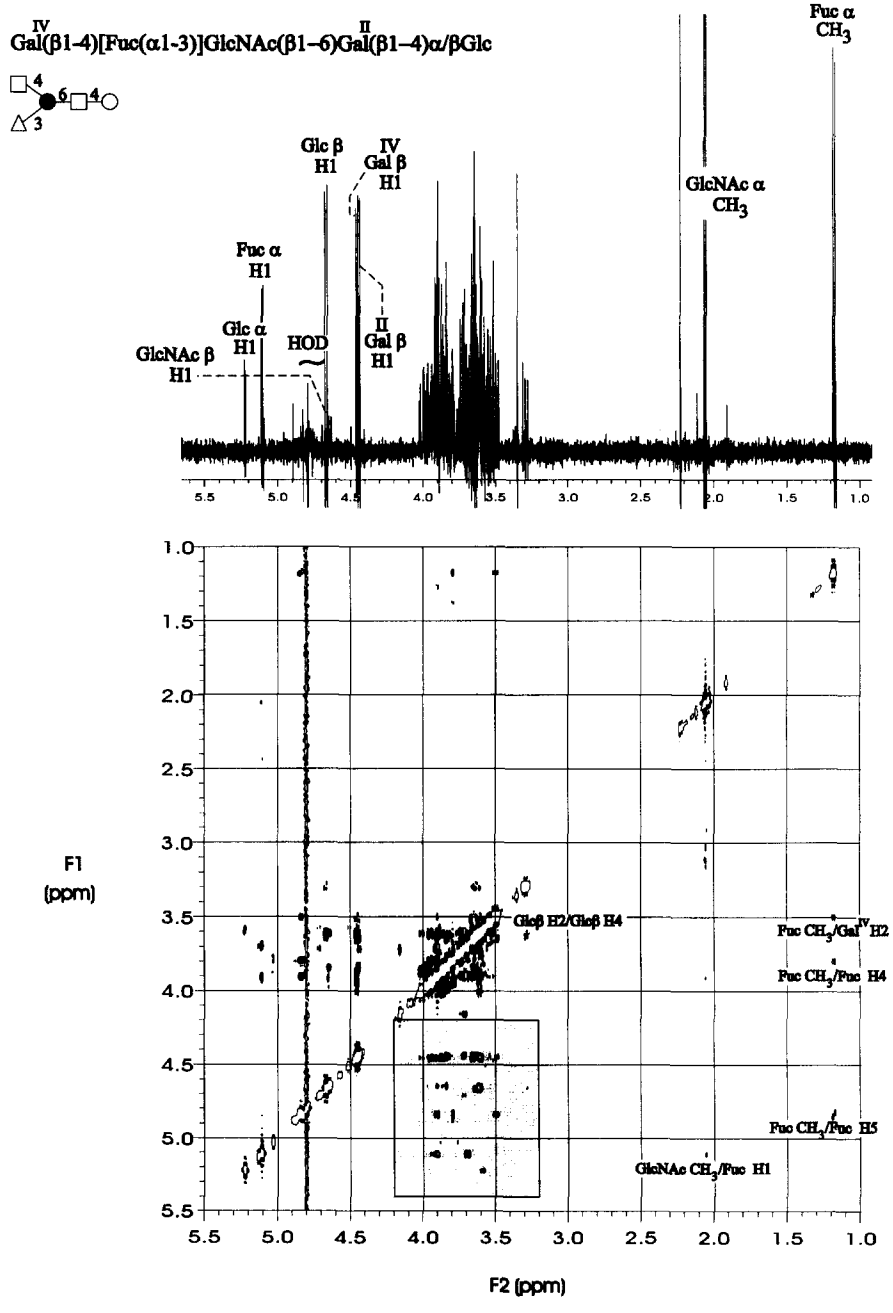


Fig. 1. 1D ^{1}H resolution-enhanced spectrum of **1** and the 2D ^{1}H–^{1}H ROESY spectrum at 295 K.

cal shifts similar to the GlcNAc residue in the Le^x structure on the (1 → 6) arm, i.e. (DFLNNH chemical shifts in italics): H-1, 4.640/*4.642*; H-2, 3.91/*3.896*; H-3, 3.87/*3.824*; H-4, 3.93/*3.934*; H-5, 3.610/*3.608*; H-6b, 4.007/*4.01*; $-COCH_3$, 2.050/*2.051*. 1D NMR spectroscopy on the equivalent goat oligosaccharide [12] gives fewer chemical shifts and compares poorly to our data and the previous literature since the spectra were taken at 77 °C. Comparison with the data for the non-fucosylated GlcNAc(β1–6)Gal(β1–4) sequence reported by Hanisch et al. [14] shows similar shifts for the Gal residue but these do appear to be affected by the proximity of Fuc in oligosaccharide **1** as they are more similar to the chemical shift of this residue in oligosaccharide **2** (see below).

In the present study additional evidence for the presence of the internal, linear GlcNAc(β1–6)Gal linkage in oligosaccharide **1** was obtained by the ROESY experiment (Fig. 2, Table 3) which gives an ROE cross-peak from GlcNAc H-1 at 4.640 ppm to Gal^{II} H-4 at 3.903 ppm, but not H-3 (Fig. 2), whereas both are found in LNFP III [37]. Additional cross-peaks from GlcNAc H-1 (4.640 ppm) and Gal^{II} H-1 (4.443 ppm) to 3.84 ppm are putatively assigned as ROEs from GlcNAc H-1 to Gal^{II} H-5 and Gal^{II} H-1 to Gal^{II} H-5. The signal at 3.84 ppm is a broad signal in the 1D spectrum, signifying H-5 rather than H-6, which is shifted downfield compared to that of H-5 of Gal linked at C-3 (see discussion of oligosaccharide **3** below). In the present study ROEs are found from GlcNAc CH_3 to Fuc H-1 (Fig. 1, Table 3) and Gal^{IV} H-1 to GlcNAc H-6a H-6b (Fig. 2) which were not detected in LNFP III by NOESY [39] or ROESY experiments [37]. This is consistent with a different conformation for the Le^x trisaccharide dependent on the adjacent GlcNAc(β1–6)Gal linkage.

Oligosaccharide **2**.—Although methylation analysis of **2** (Table 1) showed the presence of five monosaccharide residues, the 1D ^1H and 2D ^1H–^1H TOCSY spectra (Fig. 3) show only four signals corresponding to the 'reporter' groups. However, the 1D resolution-enhanced spectrum shows the presence of two Gal H-2 signals at 3.536 and 3.532 ppm, and peak volume analysis of the 'single' H-1 doublet in the 2D ^1H–^1H TOCSY spectrum gave an intensity equivalent of two protons. This is consistent with two Gal residues having a similar chemical environment, i.e. -6)Gal(β1–4)Glc and -6)Gal(β1–4)GlcNAc. In fact the H-2, H-3 and H-4 cross-peaks on the H-1 track for Gal^{II} and Gal^{IV} were indistinguishable. A further 1D resolution-enhanced spectrum at 303 K showed no separation of the H-1 reporter signals and a 2D TOCSY experiment at 303 K showed no chemi-

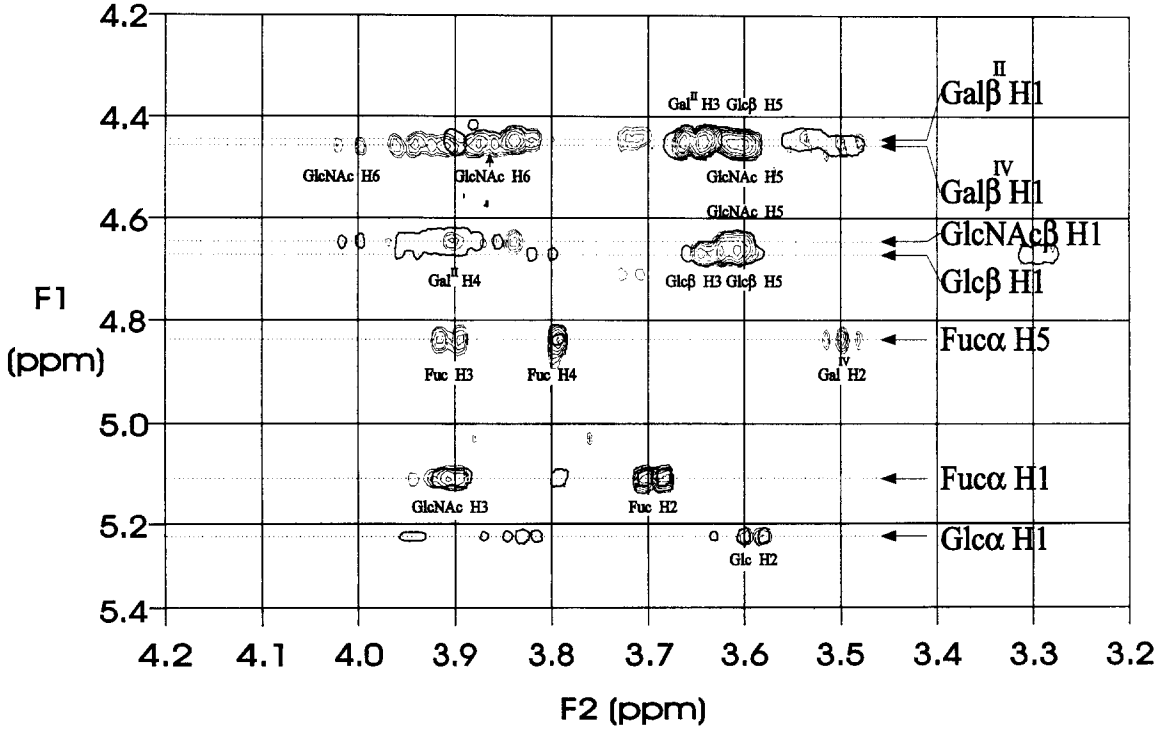


Fig. 2. The 2D ^1H–^1H TOCSY spectrum of oligosaccharide **1** (thick single contour lines) superposed on the 2D ^1H–^1H ROESY spectrum (thin multiple contour lines) shown in Fig. 1.

cal shift difference in any of the cross-peaks. In addition, the small difference in the chemical shifts between the β-Glc and β-GlcNAc ($\Delta\delta = 0.010$) in oligosaccharide **2** (Fig. 3) also makes chemical shift and ROE assignment of these residues difficult. One inter-residue interaction was shown by the ROESY experiment from Gal H-1 to GlcNAc H-6 (presumed to be that of Gal^{IV} because of spatial considerations; Scheme 1 and Table 3). The absence of strong ROEs from Neu5Ac to Gal^{IV} and from GlcNAc to Gal^{II} suggested that neither of these sequences have the (1 → 3) linkage. The chemical shifts for H-3 and H-4 of -6(Galβ1-) were $\Delta\delta$ 0.01 and 0.002 compared to the mucin oligosaccharide reported by Hanisch et al. [14] confirming the linear β-(1 → 6) linkage in our oligosaccharide.

The non-reducing terminal Neu5Ac residue of **2** shows chemical shifts more similar to those of sialyl(α2–6)lactose, than sialyl(α2–3)lactose (Platzer et al. [41] data in italics): Neu5Ac(α2–6): H-3ax, 1.715/*1.743*; H-3eq, 2.666/*2.710*; H-4, 3.558/*3.64*; H-5, 3.864/*3.86*. Neu5Acα2–3: H-3ax, *1.800*; H-3eq, *2.757*, H-4, *3.68*; H-5, *3.84*. The chemical shift of Gal H-3 at 3.651 ppm in our study, is also more similar to that in the literature [41] for the sialyl(α2–6)lactose (*3.66*) than sialyl(α2–3)lactose (*4.115*). The proposed non-reducing trisaccharide sequence of oligosaccharide **2** is represented in branched oligosaccharides [22,42]. The most similar chemical shifts for Neu5Ac in oligosaccharide **2** are those given for di-(2–6)sialyllacto-*N*-hexaose [42], i.e. H-3ax , *1.713*; H-3eq, *2.669*; H-4, *3.55*; H-5, *3.79*; H-6, *3.70*; H-7, *3.65*). In general there is some discrepancy between all the reported chemical shifts for Neu5Ac H-5 of Neu5Ac(α2–6)Gal). Comparison with the NMR data for the Neu5Ac(α2–6)Gal(β1–4)GlcNAc(β1–6) arm of the monosialylated human milk octasaccharide characterised by Grönberg et al. [22], i.e. Gal(β1–4)[Fuc(α1–3)]GlcNAc(β1–3)-[Neu5Ac(α2–6)Gal^{IV}(β1–4)GlcNAc(β1–6)]Gal^{II}-(β1–4)Glcα/β, shows similar shifts for the Gal^{IV} residue, i.e. (Grönberg et al. [22] data in italics): H-1, 4.447/*4.444*; H-2, 3.532/*3.54*; H-3, 3.651/*3.67*; H-4, 3.917/*3.92*; and the GlcNAc(β1–6) residue: H-1, 4.669/*4.67*; H-5, 3.642/*3.63*; $-COCH_3$, 2.083/*2.086*. In the pentasaccharide of the present study Gal^{II} and Gal^{IV} have similar chemical shifts (Table 2), but Gal^{II} has different shifts to those of a branched Gal such as Gal^{II} in the monosialylated octasaccharide shown above (Grönberg et al. [22] data in italics): H-1, 4.447/*4.431*; H-2, 3.536/*3.58–3.59*; H-3, 3.663/*3.71*; H-4, 3.917/*4.150*. The Gal^{II} residue could also be assigned by comparison with the -6)Gal(β1–4)Glcα/β sequence of oligosaccharide **1**.

Oligosaccharide **3**.—Fig. 4 shows a resolution-enhanced 1D ^1H NMR spectrum of oligosaccharide **3** and the 2D ^1H–^1H DQF-COSY experiment. The

Table 3
ROESY cross-peaks detected for oligosaccharides **1** and **2**

Residue	Oligosaccharide **1**		Oligosaccharide **2**	
	Inter-residue	Intra-residue	Inter-residue	Intra-residue
α-Glc		H-1 ↔ H-2		H-1 ↔ H-2
β-Glc	H-5 ↔ Gal^{II} H-1	H-1 ↔ H-3, H-5 H-2 ↔ H-4		H-2 ↔ H-4
β-Gal^{II}	H-1 ↔ Glcβ H-5	H-1 ↔ H-3		H-1 ↔ H-2
β-GlcNAc	H-1 ↔ Gal^{II} H-4, H-5 H-6b ↔ Gal^{IV} H-1 CH_3 ↔ Fuc H-1	H-1 ↔ H-6a		H-1 ↔ H-5 H-1 ↔ H-5 H-6a ↔ H-6b
β-Gal^{IV}	H-1 ↔ GlcNAc H-4, H-5, H-6	H-1 ↔ H-3	H-1 ↔ GlcNAc H-6b	H-1 ↔ H-2
α-Fuc	H-1 ↔ GlcNAc CH_3 H-5 ↔ Gal^{IV} H-2 CH_3 ↔ Gal^{IV} H-2	H-1 ↔ H-2 H-3 ↔ H-5 H-4 ↔ H-5 H-4 ↔ CH_3 H-5 ↔ CH_3		
α-Neu5Ac				H-3ax ↔ H-3eq H-3ax ↔ H-4, H-5 H-3eq ↔ H-4

shaded area is shown expanded in Fig. 5. Of the four reporter groups, those of the reducing α/β-Glc residue have chemical shifts similar to their counterparts in oligosaccharides **1** and **2** (Table 2). The single Gal residue has different chemical shifts to those of the other two oligosaccharides studied. The β-(1 → 6) linkages to Gal in **1** and **2** result in the Gal H-2, H-3 and H-4 signals being shifted to lower field. The chemical shifts of the Neu5Ac(α2–6) residue are also different to that of the Neu5Ac in oligosaccharide **2**, but most similar in the published literature to those of the pentasaccharide Gal(β1–3)-[Neu5Ac(α2–6)]GlcNAc(β1–3)Gal(β1–4)Glcα/β [15], with the latter data in italics for Neu5Ac: H-3ax, 1.690/*1.685*; H-3eq, 2.751/*2.741*; H-4, 3.690/*3.68*; H-5, 3.824/*3.816*, and also for Gal linked to Glc: H-1, 4.438/*4.436*; H-2, 3.583/*3.585*; H-3, 3.715/*3.720*; H-4, 4.172/*4.171*. These data are consistent with a β-(1 → 3) linkage from GlcNAc to Gal and a α-(2 → 6) linkage for Neu5Ac to GlcNAc.

As the Neu5Ac(α2–6) residue is present on a small tetrasaccharide here, particularly one that is

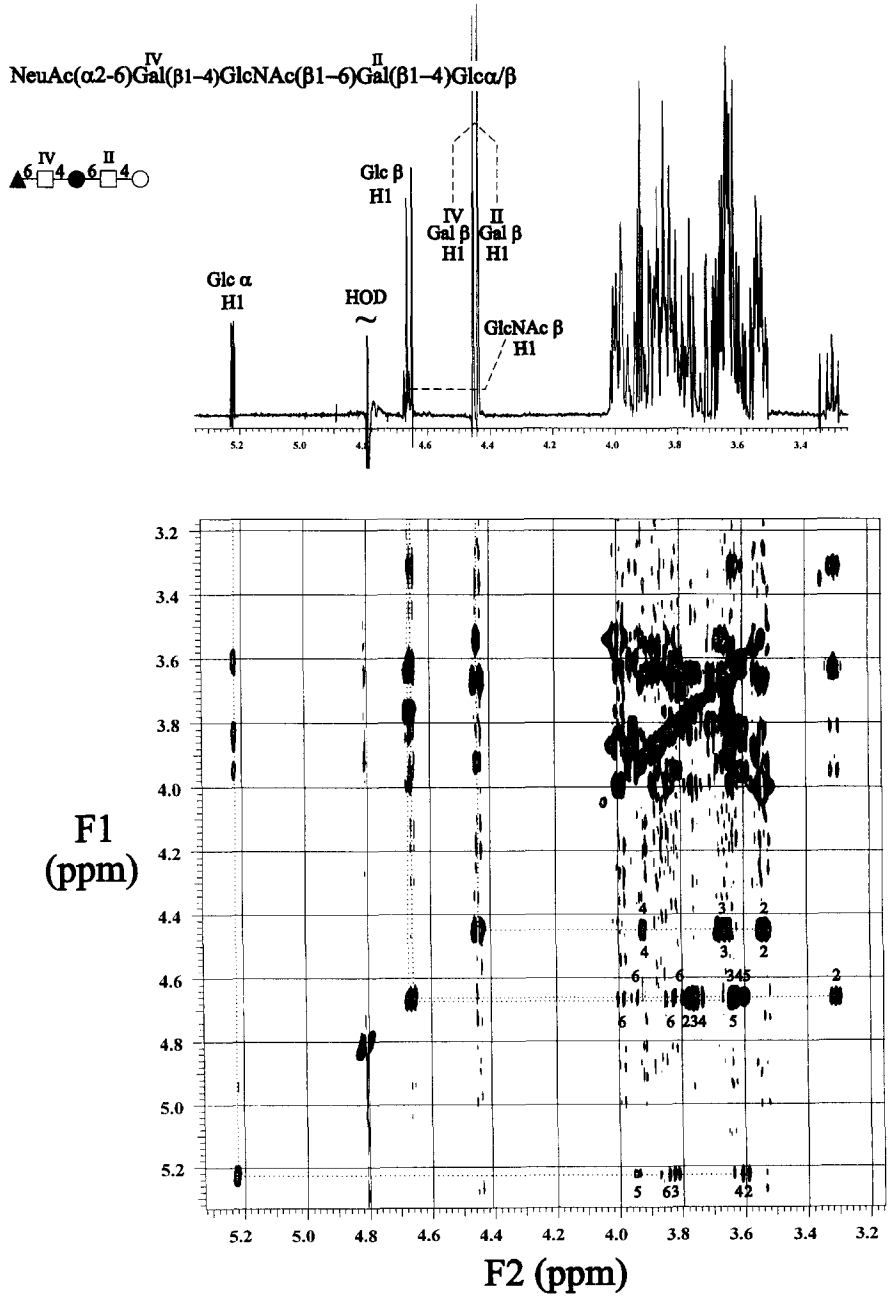


Fig. 3. 1D ^{1}H resolution-enhanced spectrum of **2** and the 2D ^{1}H–^{1}H TOCSY spectrum at 295 K.

monogalactosylated as compared to oligosaccharide **2** with many overlapping Gal signals, it is now possible with oligosaccharide **3** to provide comprehensive chemical shift assignments of the Neu5Ac(α2–6)GlcNAc(β1–3)Gal-motif (Table 2). Previously data for linear Neu5Ac(α2–6)GlcNAc were available only from a non-natural oligosaccharide having the GlcNAc linked directly to an aliphatic spacer arm $(CH_2)_8CO_2CH_3$ [16]. Compared to oligosaccharide **1**, the ROESY spectrum of oligosaccharide **3** (data not shown) confirms the presence of the (1 → 3) linkage to Gal by showing, in the presence of the ROE from Gal^{II} H-1 to Gal^{II} H-5, the absence of the ROE from GlcNAc H-1 to Gal^{II} H-5.

4. Discussion

The results reported here give comprehensive NMR data for three oligosaccharides with novel sequences

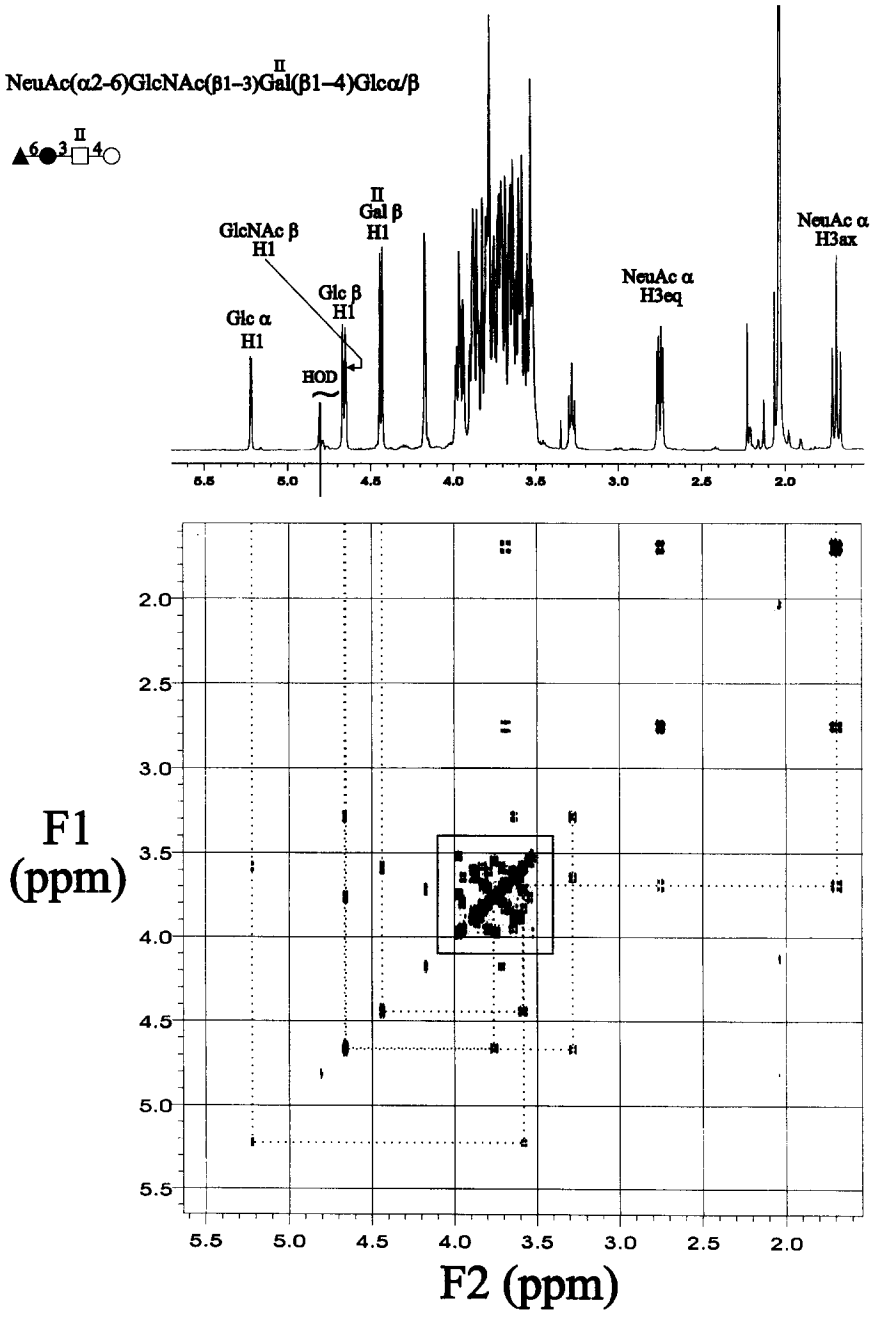


Fig. 4. 1D ^1H resolution-enhanced spectrum of **3** and the 2D ^1H–^1H DQF-COSY spectrum at 295 K. The shaded area is enlarged in Fig. 5.

having substituents at C-6. The first of these has the motif Le^x linked to C-6 of Gal of lactose. Previously this has been extensively characterised as part of the oligosaccharide LNFP III, on branched oligosaccharides, on glycoprotein O-linked cores and on the antennae of N-linked glycoprotein chains. Essentially the same inter-residue contacts for Le^x were described in LNFP III [37] and the allyl β-glycoside of Le^x [43] leading to the conclusion that the lactose part of the molecule has no significant effect on the conformation of the Le^x motif. Sialylated Le^x structures have also been investigated [38,40,43] and show no significant difference in the conformation of the Le^x motif alone, the only extra contact being between Neu5Ac H-3ax and Gal H-3. The majority of the inter-residue ROEs occur between the three residues of the Le^x motif as with the data shown here for oligosaccharide **1**. For GlcNAc and Gal^{II} of LNFPIII, ROEs were also found between GlcNAc H-1 and H-3 and H-4 of Gal^{II} [37]. In the present study the distinguishing feature of the 6-linked Le^x motif compared to the 3-linked version (LNFPIII) was that in **1** no GlcNAc H-1/Gal^{II} H-3 ROE was seen but a clear GlcNAc H-1 to Gal^{II} H-4 ROE and probably to H-5 were detected. For oligosaccharide **2** the unique feature from the NMR point of view was the remarkable similarity of chemical shifts for the two Gal residues although one was substituted by Neu5Ac and the other by GlcNAc. By comparison, oligosaccharide **3** showed the typical chemical shifts for a Gal residue linked at C-3, i.e. the H-4 signal was found outside of the bulk region of signals making it possible to trace around the glycosidic ring to assign the H-5 and H-6 signals. This task was made easier by having only one Gal residue in the oligosaccharide. The complete assignment of this oligosaccharide having a unique linear Neu5Ac(α2–6)GlcNAc sequence was therefore possible. This has previously been found as part of a disialylated tetrasaccharide in milk oligosaccharides (see above) in N-linked chains [20] and also as a novel disialylated pentasaccharide having the Sd^a antigenic motif [44].

Acknowledgements

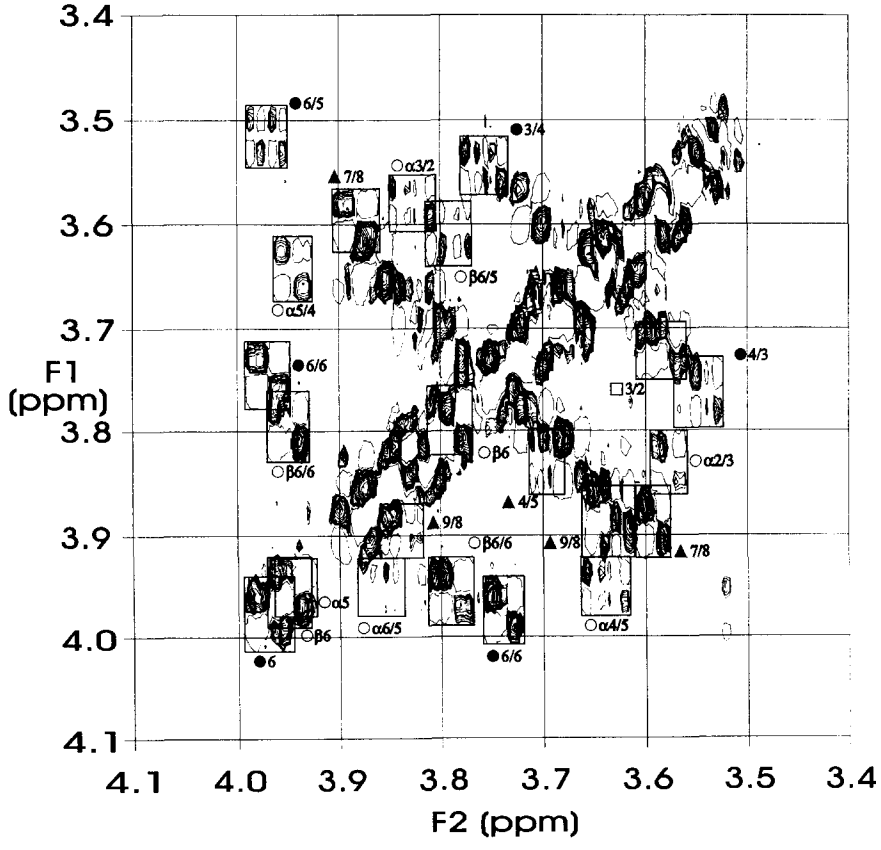


Fig. 5. The enlarged shaded area of the 2D ^1H–^1H DQF-COSY shown in Fig. 4. Single contour lines represent negative peaks.

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Residue	Atom	1	2	3
Glcα[β]	H-1	5.222 [4.667]	5.224 [4.659]	5.220 [4.664]
	H-2	3.589 [3.294]	3.597 [3.308]	3.582 [3.282]
	H-3	3.829 [3.64]	3.825 [3.64]	3.830 [3.639]
	H-4	3.62 [3.61]	3.62 [3.62]	3.637 [3.63]
	H-5	3.943 [3.602]	3.943 [3.597]	3.948 [3.602]
	H-6a	3.86 [3.808]	3.86 [3.808]	3.87 [3.793]
	H-6b	[3.948]	[3.948]	[3.951]
Gal^{II}	H-1	4.443	4.447	4.438
	H-2	3.538	3.536	3.583
	H-3	3.646	3.663	3.715
	H-4	3.903	3.917	4.172
	H-5	3.84		3.791
	H-6a			3.77
	H-6b			3.79
GlcNAc	H-1	4.640	4.669	4.659
	H-2	3.91	3.73–3.78	3.752
	H-3	3.87	3.73–3.78	3.756
	H-4	3.93	3.73–3.78	3.540
	H-5	3.610	3.642	3.505
	H-6a	3.866	3.836	3.740
	H-6b	4.007	3.992	3.973
	NAc	2.050	2.083	2.035
Gal^{IV}	H-1	4.453	4.447	
	H-2	3.496	3.532	
	H-3	3.652	3.651	
	H-4	3.901	3.917	
Fuc	H-1	5.106		
	H-2	3.692		
	H-3	3.903		
	H-4	3.791		
	H-5	4.834		
	CH_3	1.173		
Neu5Ac	H-3ax		1.715	1.690
	H-3eq		2.666	2.751
	H-4		3.558	3.690
	H-5		3.804	3.824
	H-6		3.698	3.594
	H-7		3.650	3.588
	H-8			3.881
	H-9a			3.632
	H-9b			3.833
	NAc		2.028	2.032

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