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DETERMINATION OF THE STRUCTURE OF THREE OLIGOSACCHARIDES FROM NORMAL HUMAN URINE BY USING 60-MHz, CARBON-13 NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

ELISHA BERMAN

Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143 (U.S.A.)

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ABSTRACT

The detailed structures of three trisaccharides obtained from normal, human urine were determined by ^{13}C-nuclear magnetic resonance spectroscopy. These compounds are α-NeuAc-(2→3)-β-D-Gal-(1→4)-D-Glc, α-NeuAc-(2→6)-β-D-Gal-(1→4)-D-GlcNAc (**2**), and α-NeuAc-(2→3)-β-D-Gal-(1→4)-D-GlcNAc (**3**). Trisaccharide **3** has not previously been reported in normal, human urine. The elucidation of these structures by using ^{13}C-n.m.r. spectroscopy is discussed in detail. Some unusual, chemical-shift values found for structure **2** are related to intramolecular interactions between the NeuAc and GlcNAc residues, and may indicate that **2** has a unique conformation. The potential use of these trisaccharides as model compounds in ^{13}C-n.m.r.-spectral studies of intact glycoproteins is critically evaluated. Like ^{1}H-n.m.r. spectroscopy, this technique demonstrates elucidation of the structures of mixtures of oligosaccharides or of glycopeptides that have very closely related structures.

INTRODUCTION

Normal, human urine contains a large number of different, carbohydrate-derived compounds[1]. The presence of these compounds in the urine is linked to the biosynthesis and degradation of the body glycoproteins, glycolipids, and glycans. Under certain pathological conditions, the total urinary carbohydrate-material is increased dramatically, and the number and ratios of the components present are also increased. Many of these pathological disorders are the result of a deficiency in a specific exoglycosidase. During the past few years, the structures of some of the oligosaccharides and glycopeptides excreted in the urine of normal and oxyglycosidase-deficient patients have been elucidated by using conventional methods[1–9]. Recently, high-field, ^{1}H-n.m.r. spectroscopy was employed in the structural elucidation of urine oligosaccharides and glycopeptides[10–12]. Some of the oligosaccharides and glycopeptides found in the urine are structurally related to the asparagine-linked, carbohydrate side-chains of glycoproteins.

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The use of ^{13}C-n.m.r. spectroscopy, like that of ^{1}H-n.m.r. spectrosopy, for structural determination of oligosaccharides and glycopeptides is appealing, owing to the nondestructive nature of the method. Recently, ^{13}C-n.m.r. spectroscopy has been found to be useful in studies of intact glycoproteins[13,14], in particular, for elucidation of the carbohydrate side-chain structures[15]. However, the full potential of such ^{13}C-n.m.r.-spectral studies is strongly dependent on the establishment of a comprehensive, ^{13}C-chemical-shift data-base for small oligosaccharides of known structures, that are to be used as model compounds for the purpose of chemical-shift assignments, and the biological link between oligosaccharides found in urine and glycoproteins in the body makes them ideal, model compounds for use in such studies.

Herein, we discuss the isolation of three trisaccharides from normal, human urine. Their structures were inferred from n.m.r. studies, and their potential use as model oligosaccharides in future ^{13}C-n.m.r.-spectral studies is critically evaluated.

EXPERIMENTAL

Separation of the oligosaccharides. — Normal, human urine (4 L) was concentrated to ~400 mL, and the concentrate was centrifuged. The supernatant liquor was dialyzed (cut-off in mol. wt., 6000–8000) against doubly distilled water. The dialyzate was then concentrated *in vacuo* to ~8 mL, and any precipitate was removed by centrifugation.

The concentrated dialyzate was applied in 1-mL portions to a column (2 × 180 cm) of Bio-Gel P4-400 and eluted with water at 9 mL/h, the elution being monitored at 540 nm by using the orcinol–sulfuric acid test[16]. All of the hexose-positive fractions, extending from the void volume to the major, orcinol-positive fraction, were pooled, lyophilized, and rechromatographed on the same column under the same conditions. The fractions were collected, and lyophilized as shown in Fig. 1.

Fractions 1 and 2 (see Fig. 1) were then chromatographed on a column (1.6×

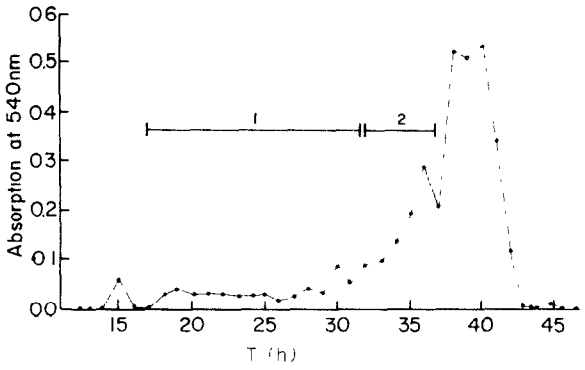

Fig. 1 Bio-Gel P4-400 rechromatography of oligosaccharides obtained from concentrated, normal human urine. [For conditions of chromatography, see Experimental section The bars indicate fractions collected. Void volume was estimated to be ~20 mL.]

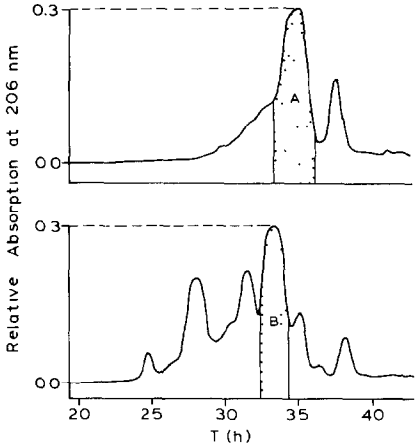

Fig. 2. Bio-Gel P4-400 chromatography of fraction 2 (upper) and fraction 1 (lower), using 0.05M ammonium hydrogencarbonate. [For conditions of chromatography, see Experimental section.]

40 cm) of Dowex-50 X-2-400 ion-exchange resin with mM sodium acetate buffer, pH 2.6, at 5 mL/h, in order to remove oligopeptides and colored impurities. For each case, a number of hexose-positive fractions were eluted, but only the first hexose-positive fraction to be eluted from the column was collected and lyophilized. These fractions were then desalted on the P4 column, and the major fraction in each case was pooled and lyophilized. Fractions thus obtained were analyzed by ^{13}C-n.m.r. spectrosopy for their purity and types of sugars present.

These fractions were further purified on the P4 column by using 0.05mM ammonium hydrogencarbonate buffer, pH 8.0, as the eluant. The elutions were monitored at 206 nm, and the profiles are shown in Fig. 2. The ammonium hydrogencarbonate salt was removed by repeated lyophilization. By ^{13}C-n.m.r. spectroscopy, fractions A and B were subsequently shown to be the major fractions. Fraction B (~12 mg) was found to be a mixture of two isomers of sialyllactosamine, and fraction A (20 mg) to be sialyllactose, as determined by analysis of their respective, ^{13}C-n.m.r. spectra. The purity of the fractions was estimated to be >90% from their respective ^{13}C-n.m.r. spectra.

Digestion with neuraminidase (EC 3.2.1.18). — To a solution of fraction B (~12 mg) in 0.5 mL of 0.1M potassium acetate, pH 4.7, was added one unit of the enzyme (from *Clostridium perfringens*), and the mixture was incubated for 22 h at 37°, diluted to ~1.6 mL with D_2O, and its ^{13}C-n.m.r. spectrum recorded. Based on analysis of this spectrum, all of the NeuAc bound in the (2→3) isomer was removed, and ~60% of the NeuAc was released from the (2→6) isomer. The mixture was separated on the P4 column as already described, and the elution (monitored at 206 nm) yielded three peaks, corresponding roughly with the elution profiles for tri-, di-, and mono-saccharides.

^{13}C-Nuclear magnetic resonance spectroscopy. — The ^{13}C-n.m.r. spectra were recorded at 60.5 MHz, using 10-mm n.m.r.-tubes. The spectra were acquired

with 85° pulses, a spectral window of ~12 kHz, 16 K memory-data points, and a repetition time of 1.0 s. Line broadening of 2 Hz was used in the processing of the spectra. The total numbers of scans, the solvent, the concentration, the temperature, and the pH are given in the Figure legends. Chemical shifts are referenced indirectly to tetramethylsilane, using 1,4-dioxane as the internal standard (67.86 p.p.m.). The deuterium-induced, isotope chemical-shifts were measured from spectra obtained for solutions in H_2O and D_2O, respectively.

RESULTS AND DISCUSSION

Table I lists all of the chemical shifts obtained for the isolated urine oligosaccharides, as well as those for model compounds previously reported. The fourth column in Table I lists the chemical shifts obtained for the major saccharide components resulting from the neuraminidase digestion of fraction B. Fig. 3 shows the ^{13}C-n.m.r. spectrum of fraction B, excluding the methyl-resonance region. The resonances in Fig. 3 are numbered consecutively from left to right, in order to facilitate easier disussion of individual resonances. This numbering is employed in Table

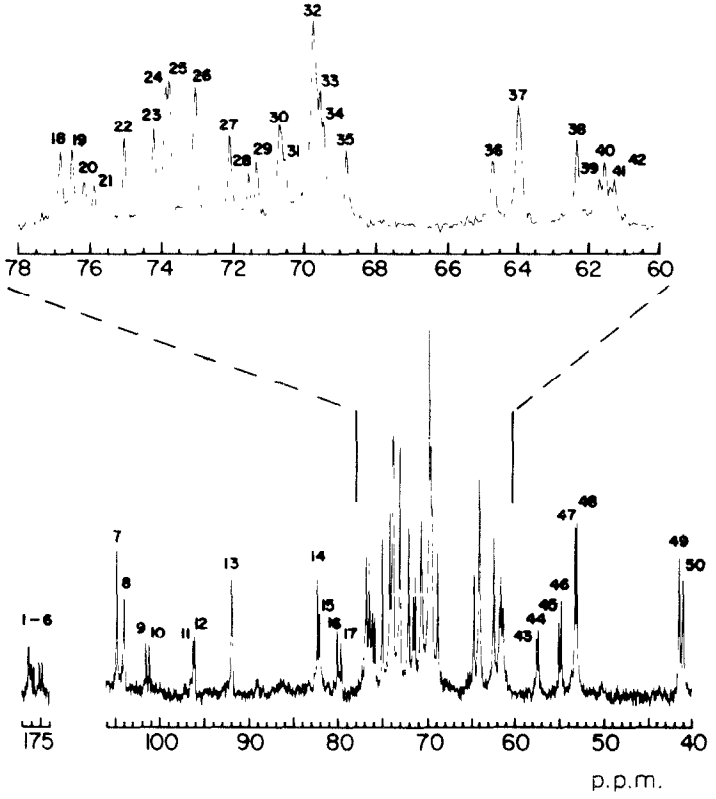

Fig. 3. Proton-decoupled, natural-abundance, ^{13}C-n m.r. spectrum of fraction B (6mM, D_2O, pD 7.0; 118,000 accumulations; 30°). [The chemical shifts of the omitted resonances of the methyls (peaks 51 to 54) are given in Table I. For other spectral conditions, see Experimental section.]

TABLE I

CARBON-13 CHEMICAL-SHIFT ASSIGNMENTS[a]

Carbon atom	*NeuAc(β)*[b]	*β-D-Gal-(1→4)-D-GlcNAc*[c]	*Digestion products*[d]	*α-NeuAc-(2→3)-β-D-Gal-(1→4)-D-Glc*[e]	*α-NeuAc-(2→3)-β-D-Gal-(1→4)-D-GlcNAc*[f]	*α-NeuAc-(2→6)-β-D-Gal-(1→4)-D-GlcNAc*[f]
NeuAc						
1	175.98		175.72	175.08	175.13(5)	175.73(4)
2	97.61		97.70	101.09	101.12(10)	101.49(9)
3	40.63		40.70	40.93	40.95(50)	41.39(49)
4	68.51		68.62	69.59	69.71(32)	69.71(32)
5	53.50		53.60	52.98	53.00(48)	53.19(47)
6	71.45		71.55[g]	74.15	74.18(23)	73.85(24)
7	69.82		69.90	69.41[g]	69.43(34)	69.71(32)
8	71.59		71.67[g]	73.04	73.04(26)	73.04(26)
9	64.55		64.63	63.89	63.97(37)	63.97(37)
10	177.87		177.87	176.29	176.24(2)	176.32(1)
11	23.24		23.40	23.32	23.33(53)	23.33(53)
Gal (β)						
1		104.1	104.21	103.92	103.92(8)	104.78(7)
2		72.3	72.31	70.64	70.67(30)	72.06(27)
3		73.9	73.87	76.43[g]	76.47(19)	73.76(25)
4		69.9	69.90	68.77[g]	68.78(35)	69.53(33)
5		76.6	76.65	76.78[g]	76.80(18)	75.00(22)
6		62.3	62.33	62.29	62.27(38)	64.67(36)
GlcNAc						
1α		91.6	91.86	93.10	91.88(13)	91.88(13)
1β		96.2	96.18	97.05	96.21(11)	96.03(12)
2α		55.1	55.03	72.46	55.02(45)	54.76(46)
2β		57.6	57.56	75.10	57.51(43)	57.32(44)
3α		70.6	70.61	72.65	70.53(31)	70.67(30)
3β		74.9	75.00	75.61	73.76(25)	73.76(25)
4α		80.1	80.18	79.66	80.02(16)	82.22(14)
4β		79.7	79.77	79.53	79.59(17)	81.98(15)
5α		71.5	71.55	71.37	71.54(28)	71.32(29)
5β		76.1	76.14	76.07	76.15(20)	75.87(21)
6α		61.3	61.34	61.25	61.27(42)	61.54(40)
6β		61.3	61.45	61.39	61.39(41)	61.70(39)
COα		176.0	176.1		176.02(3)	174.81(6)
COβ		176.0	176.1		176.02(3)	174.81(6)
$CH_3\alpha$		23.3	23.23		23.19(54)	23.33(53)
$CH_3\beta$		23.5	23.40		23.46(52)	23.59(51)

[a]Chemical shifts are given in p.p.m. downfield from tetramethylsilane as the external reference, for solutions in D_2O (see Experimental section). [b]Taken from ref. 24. [c]Taken from ref. 21. [d]Chemical shifts are taken from the spectrum shown in Fig. 4. [e]Data were taken from a spectrum (not shown) obtained for 20mM solutions, pD 7.2; 20,000 scans. Assignments are based on unpublished data[20]. [f]Assignments given herein. The most likely assignments are listed in Table I. However, there may be interchanges within the following groups of resonances: 1,2; 3–6; 18,19; 24,25; 30,31; 32–35; and 51–53 (see Fig. 3 for peak numbering). Chemical shifts were taken by using the same n.m.r sample, but with 1,4-dioxane added. [g]Assignments not confirmed, and may have to be interchanged.

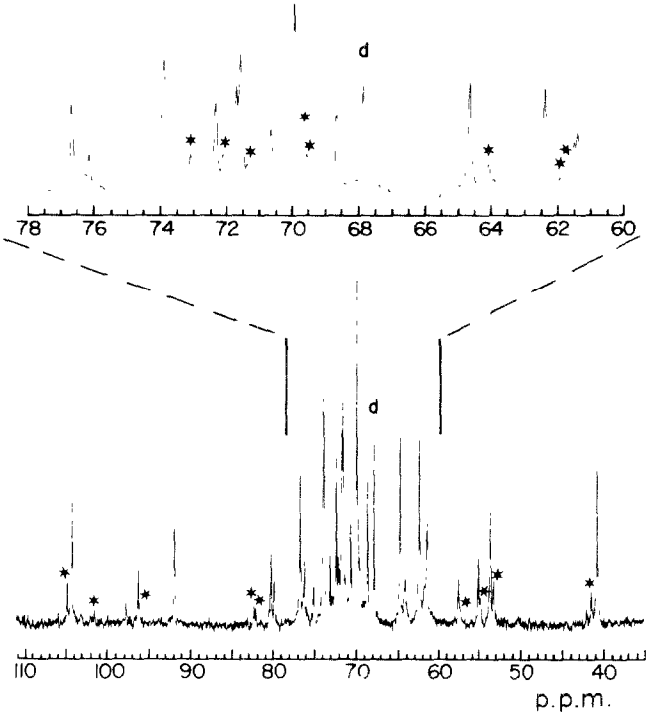

Fig. 4. Proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of the neuraminidase-treated fraction B (sample concentration as for Fig. 3; 30mM potassium acetate in 70% D_2O, pD 4.7; 85,000 accumulations; 31°). [The chemical shifts of the omitted resonances of the carbonyls and methyls are given in Table I. Peaks marked with an asterisk are discussed in the text. The 1,4-dioxane peak is marked by *d*. For other spectral conditions, see Experimental section.]

I for the same reason. Fig. 4 corresponds to fraction B after digestion by neuraminidase, excluding both the carboxyl and the methyl regions.

The ^{13}C-n.m.r.-spectral analysis of these fractions had two principal aims. The first was determination of the structures involved, and the second, provision of a ^{13}C-chemical-shift data-base for future use of these oligosaccharides as model compounds. Although the first aim does not, in general, require complete resonance-assignments, the potential use of the data base mandates as complete a chemical-shift assignment as possible.

Carbon-signal count, the number and integration values of the resonances appearing in the sugar anomeric region (90–110 p.p.m.), together with inspection of other reporter regions in the spectrum (*e.g.*, carbonyl, 172–178; methyl, 15–25; and the sugar C-amino, 53–58 p.p.m.) can provide useful information on the number and types of carbohydrates present in the oligosaccharide structure. Comparison with chemical-shift data previously reported, together with additional data obtained from off-resonance decoupling, coupling constants, deuterium-induced differential isotope-shifts[17], spin–lattice relaxation[14], and pH-dependence[18] experiments, usually suffices to afford a tentative, if not firm, structure elucidation for the

compound under study. However, recourse to complementary analytical methods may be desirable, in order to provide additional confirmation of the structure. In the more-favorable cases, it may not be necessary to perform all of the experiments just listed. Based on the methodology outlined, the results for individual oligosaccharides under study are discussed in subsequent sections.

Structure of A. — The ^{13}C-n.m.r. spectrum of fraction A (not shown) was identical in appearance to that reported[19] for α-NeuAc-(2→3)-β-D-Gal-(1→4)-D-Glc, but the chemical shifts and assignments were slightly modified from the published values. The present results are based on ^{13}C-n.m.r. data obtained from the pH-dependence of the chemical shifts, spin–lattice relaxation measurements, and deuterium chemical-shift effects, collected for the same compound isolated from bovine colostrum[20]. The chemical shifts and assignments are shown in Table I, and were used in the structure elucidation for fraction B (see next section).

Structure of B. — From the total number of peaks (54) and the differential intensities observed in the spectrum (see Fig. 3), it is clear that it is either that of an oligosaccharide of relatively high molecular weight, or of a mixture of smaller oligosaccharides. As will now be shown, it is, in fact, a mixture of two trisaccharides present in the ratio of ~3:2, as determined by peak integrations. Some details regarding structure elucidation will first be dealt with, and then the way in which specific resonance-assignments (see Table I) were made will be discussed.

Direct comparison between the ^{13}C-n.m.r. spectrum of this fraction (see Fig. 3) and that of fraction A (not shown) indicated the presence of the α-NeuAc-(2→3)-β-D-Gal-(1→4)-X unit in the material. Thus, peaks 8 and 10 respectively arise from C-1 of β-Gal and C-2 of α-NeuAc. The intensity difference between the two resonances is attributed to the relatively long, spin–lattice relaxation-time (~5–6 s) for the nonprotonated C-2 of NeuAc in relation to the experimental, sampling repetition-time (1 s). Similarly, such additional resonances as peaks 18, 19, 23, 26, 35, 38, 48, and 50 could be identified. The complete comparison is presented in Table I under the appropriate columns. The pairs of resonances, 9 and 10, 26 and 37 (two resonances each), 47 and 48, and 49 and 50, in chemical-shift positions unique for a terminal α-NeuAc group[19] indicate the presence of a second α-NeuAc group in addition to that already discussed. Thus, the spectrum probably shows two terminal α-NeuAc groups.

From the chemical-shift values and differential intensities observed for peaks 11–13 in the anomeric region, and the four low intensity peaks, 43–46, in the *C*-amino region, it may be concluded that two reducing, amino sugar residues are present; this is based on the observation that, in solution, reducing sugars are typically found at equilibrium between the α- and β-anomeric configurations. Each of these configurations gives rise to a different set of chemical shifts for its ring-carbon atoms[21]. In the present instance, the α and β configurations were found to have about equal populations, which accounts for the greatly diminished intensities observed for the carbon resonances of the reducing sugars. Scanning of the spectrum

for other, low-intensity peaks (*e.g.*, 14–17, 20, 21, 28, 29, and 39–42) indicated, on the basis of chemical-shift considerations[21], that the reducing sugars must be GlcNAc residues, and these two must either have slightly different magnetic environments or be substituted at different positions. The chemical-shift values of the respective α and β anomers for these two GlcNAc residues essentially agree only with reducing GlcNAc residues[21] substituted at O-4. Both residues are unlikely to be substituted at O-3 or O-6, as such substitutions would give rise to a chemical-shift pattern completely different from the one observed[22]. However, the chemical shifts of peaks 14 (82.22) and 15 (81.98 p.p.m.), which should correspond to the substituted carbon atom of at least one of the GlcNAc residues, are too far removed from the position expected (~80 p.p.m.) for O-4 substitution. As will be conclusively demonstrated later in the discussion, it was concluded that these two reducing GlcNAc residues are, indeed, substituted at O-4 by a β-D-Gal residue.

In the anomeric region, one resonance (peak 7) is left unaccounted for. If the intensity of peak 8 is considered as being contributed by one residue, and the intensity of peak 13 likewise (as it corresponds to contributions from the two half-carbon resonances of the α anomers of the reducing GlcNAc residues), peak 7 must correspond approximately to one residue. From chemical-shift considerations of the remaining peaks unaccounted for in the spectrum (22, one component of 25, 27, 33, and 36), it was concluded that the additional residue must be a β-D-Gal substituted at O-6, as evidenced by the lack of peak intensity in the C-6 region (61–63 p.p.m.) for unsubstituted hexose and the position[23] of peak 36. Some of these chemical shifts, and those corresponding to the second α-NeuAc group, agree with those reported[19] for the NeuAc group and Gal residue in α-NeuAc-(2→6)-β-D-Gal-(1→4)-D-Glc.

From total-intensity considerations and the data so far presented, we conclude that there are probably two terminal α-NeuAc groups, two reducing D-GlcNAc, and two β-D-Gal residues. From the unequal intensities observed for the corresponding two reducing residues, the possibility of one overall structure was excluded. As six residues were counted, and as these structures chromatographed as one band (see Fig. 2), it was concluded that there are two trisaccharide units; each structure has a terminal α-NeuAc group, and β-D-Gal and reducing GlcNAc residues. Because it was suspected that both D-GlcNAc residues are substituted at O-4, and one β-D-Gal residue is substituted at O-3 and the other at O-6, these isomeric trisaccharide structures were tentatively established as

α-NeuAc-(2→3)-β-D-Gal-(1→4)-D-GlcNAc (B1) and
α-NeuAc-(2→6)-β-D-Gal-(1→4)-D-GlcNAc (B2).

If these two isomeric structures are present in fraction B, the chemical-shift nonequivalence for the carbon atoms corresponding to the two GlcNAc residues must arise from the different linkage-positions for the terminal α-NeuAc groups,

(2→3) *vs.* (2→6). That includes, in particular, unexpected chemical-shifts for C-4 of one of the GlcNAc residues (peaks 14 and 15). Other noticeable chemical-shift differences were observed for C-1 of the β-D-Gal residues (peaks 7 and 8) and C-5 of the α-NeuAc groups (peaks 47 and 48). In order to prove this point, and to exclude other linkages or different structures, a simple experiment was conducted.

If both structures are isomeric, as proposed, removal of the teminal α-NeuAc group should yield a mixture of free NeuAc and β-D-Gal-(1→4)-D-GlcNAc (lactosamine). The chemical shifts for both components, known from previous studies, are listed in Table I. Therefore, fraction B was treated with neuraminidase (see Experimental section); the ^{13}C-n.m.r. spectrum of the digested mixture is shown in Fig. 4. Relative simplification of the resultant spectrum was observed, with clear indication as to the presence of resonances corresponding to the (more prominent) β anomer (92%) of free NeuAc[24] and lactosamine[21]. From the presence in the spectrum of small peaks that retained their original positions relative to the undigested material (see Fig. 3), it was concluded that ~25% of the original substrate had remained undigested, presumably due to an insufficiently long digestion-time, as peaks 14 and 15 were diminished to ~40% of their original intensity, whereas peaks 18 and 17 had clearly disappeared; this was also evident in other parts of the spectrum. No other resonances were observed in the spectrum.

The presence of the substrate and two products as the sole components was confirmed by column chromatography of the digestion mixture (see Experimental section). The incomplete digestion had some advantages, as the residual peaks (marked by an asterisk in Fig. 4) were used in the assignment procedure next described. As it had now been determined that neuraminidase digestion yields only two known components, the proposed trisaccharide structures B1 and B2 are indeed present in fraction B. It may be noted that, in the ^{13}C-n.m.r. spectrum of the pentasaccharide IV^{6}NeuAcLcOse$_4$, which contains a nonreducing trisaccharide group identical to structure B2, essentially the same set of signals as those assigned to structure B2 (with the β-D-GlcNAc anomer) had been observed[25].

The peak assignments for the two trisaccharide isomers are shown in Table I. These are based on chemical-shift considerations, deuterium-isotope effects, and the discernible peaks, belonging to the undigested substrate (see Fig. 4), in the spectrum of the digestion mixture. The chemical-shift considerations have already been discussed. The deuterium-induced, differential isotope-shift values showed that peaks 7–10, 14–24, 28–29, 36, 49, and 50 in Fig. 3 have no measurable, or very small (<0.06 p.p.m.), chemical-shift changes. Thus, they must correspond to carbon atoms having no (directly attached) free hydroxyl groups[17]. Not counting the carbonyl and methyl regions, the rest of the peaks characteristically shifted by 0.1–0.2 p.p.m. from their original positions, thus marking them as free-hydroxyl-carrying carbon atoms[17]. Because digestion of the α-NeuAc-(2→3) isomer by this particular enzyme proceeds 4 times as fast as that for the α-NeuAc-(2→6) isomer[26], and the residual peaks (marked with an asterisk in Fig. 4) correspond to only one of the isomers, as already discussed, it was concluded that these peaks are caused

by the (2→6) isomer only. Thus, it was possible to assign the corresponding resonances of the two isomers in those instances where chemical-shift differences did not allow unambiguous assignments.

CONCLUSIONS

The data, and the analysis given in the previous sections, provide a complete, structural determination for three urinary, sialyl trisaccharides. Two of the trisaccharides had been reported[1,27,28] to be present in normal urine, whereas the third trisaccharide, α-NeuAc-(2→3)-β-D-Gal-(1→4)-D-GlcNAc, has not, as far as is known, been previously reported.

These trisaccharides, in particular the two lactosamine isomers, are structurally related to the trisaccharide branches of "complex" carbohydrate side-chains in a variety of glycoproteins[29]. Their ^{13}C-n.m.r. chemical-shifts can thus serve as a useful data base for the study of more-complex oligosaccharides and carbohydrate side-chains of glycoproteins and glycopeptides. A note of caution must be sounded concerning the use of lower-molecular-weight oligosaccharides as model compounds for the purpose of comparing chemical shifts with those obtained for more-complex structures, as will be discussed next.

Inspection of Fig. 3 and Table I shows that the chemical shifts for the respective GlcNAc anomers of the two isomers of sialyllactosamine are different for every carbon atom, and are as large as 2.4 p.p.m. (for the respective C-4 β anomers). Clearly, these chemical-shift deviations must be a result of the different position of substitution for the terminal α-NeuAc groups, as was shown conclusively by the results of neuraminidase digestion. These long-range, chemical-shift effects are not generally expected, as the α-NeuAc groups are removed by one β-D-Gal residue from the GlcNAc residue. Unusual, chemical-shift effects are also found for C-1 of the two respective β-D-Gal residues. In the case of the (2→6) isomer, it was found to be ~0.6 p.p.m. Previous studies showed that, indeed, substitution on O-6 of the β-D-Gal residue should affect the chemical-shift value of C-1, compared with the chemical-shift value observed[23] for the unsubstituted β-D-Gal. Slightly different chemical-shifts for each of the C-2, C-3, and, possibly, C-6 resonances of the two α-NeuAc groups would be expected, but not for C-5 (0.2 p.p.m.) and C-7 (0.3 p.p.m.) of the same two groups.

These effects may be a result of different, intramolecular interactions between the acidic NeuAc groups and the GlcNAc residues in the two respective isomers, interactions whose origin is not yet fully understood. The same "anomalous", chemical-shift effects have been observed in the ^{13}C-n.m.r. spectrum of intact, human α_1-acid glycoprotein[20], whose carbohydrate side-chain structure carries the same trisaccharide branches[30]. These effects are also observed[25] in the ^{13}C-n.m.r. spectrum of the pentasaccharide IV^{6}NeuAcLcOse$_4$, which is found in human colostrum[31]. Clearly, the use of ^{13}C-n.m.r. data obtained from such model disaccharides as β-D-Gal-(1→4)-D-GlcNAc in order to predict the chemical shifts

for the GlcNAc residue in such structures as α-NeuAc-(2→6)-β-D-Gal-(1→4)-β-D-GlcNAc-X may not be adequate for the purpose of identifying this residue in the structure (*e.g.*, the chemical shift for C-4 β is 79.77 *vs.* 81.98 p.p.m.). Also, the use of ^{13}C-chemical-shift data obtained for asialylglycopeptides and oligosaccharides should be carefully and critically compared with those for the corresponding, sialyl homologs.

This report demonstrates the analytical capabilities and nondestructive nature of ^{13}C-n.m.r. spectroscopy. It constitutes a useful technique complementary to ^{1}H-n.m.r. spectroscopy and the more-conventional, wet-chemistry methods. ^{13}C-n.m.r. spectroscopy is particularly suitable for studies of the carbohydrate side-chains in intact glycoproteins, as overlap between the resonances for the carbohydrate region and for the peptide backbone is minimal[13,15,20]. This eliminates the need to remove the NeuAc groups before obtaining the respective glycopeptides by proteolytic digestion for the purpose of further structural studies. Finally, ^{13}C-n.m.r. spectroscopy also constitutes a very useful technique for analysis of mixtures of oligosaccharides or glycopeptides that are not readily separated by conventional methods.

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