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^{1}H-2D-NUCLEAR MAGNETIC RESONANCE APPLIED TO THE PRIMARY STRUCTURE DETERMINATION OF A NOVEL OCTASACCHARIDE GLYCOLIPID ISOLATED FROM THE SPERMATOZOA OF BIVALVES*

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ABSTRACT

High resolution, two-dimensional ^{1}H-n.m.r. spectroscopy has been used to confirm a proposed primary structure of a glycolipid having an octasaccharide head-group. Pure absorption and relay experiments were found to be particularly useful in establishing connectivities in poorly resolved regions of the spectrum. The spectral assignments, which indicate novel linkages including an internal fucopyranosyl residue as well as terminal xylosyl and 4-*O*-methylglucopyranosyluronic acid groups, add to a growing data base for structural characterization through n.m.r. spectroscopy.

INTRODUCTION

High-resolution, ^{1}H-n.m.r. spectroscopy has proven very useful as a tool for primary structure characterization of the oligosaccharide portion of glycolipids and glycoproteins. Most applications have relied extensively on chemical-shift correlations with model compounds containing similar glycosidic linkages. An extensive database of chemical shifts for carbohydrates from glycoproteins has, for example, been tabulated by Vliegenthart *et al.*[1]. Structural assignments from this database rely extensively on the shifts of a few well-resolved resonances, usually the

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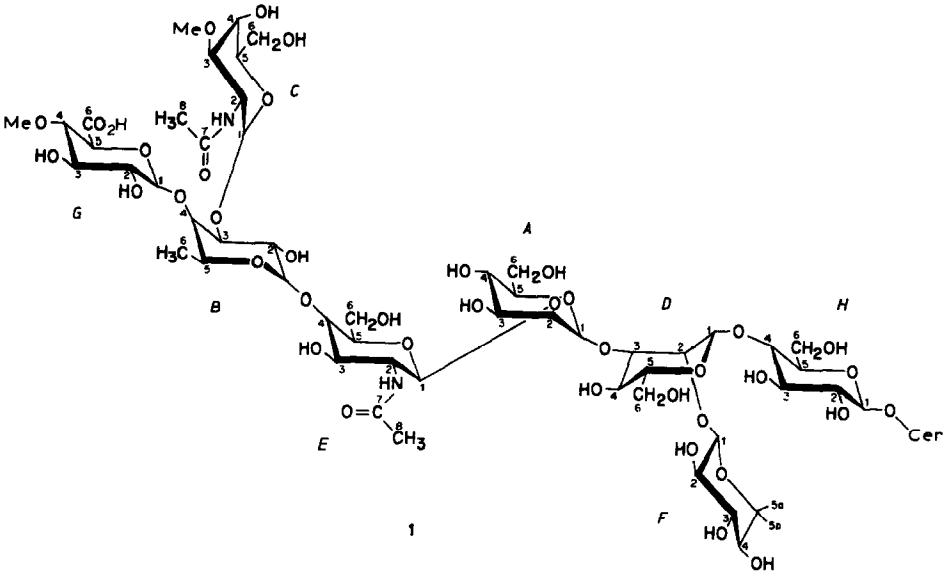
anomeric and H-2 resonances, on a given residue. Although this approach has been successful for a large number of cases, there are potential limitations in that it neglects possible departures from regular chemical-shift patterns due to factors such as remote group interactions[2], and it suffers from the absence of model compounds for rare or novel glycosidic linkages.

One possible way to supplement this approach is to characterize additional novel residues and extend the number of assigned proton resonances for a given residue. Two-dimensional n.m.r. spectroscopy, through various scalar-coupling-correlated experiments[3], has provided a means of extending assignments for a given residue. They have also provided an independent method of establishing linkage sites through cross-relaxation-correlated experiments which establish connectivities between protons that are spatially proximate. Applications of two-dimensional methods to glycolipids[2–5] and to the oligosaccharide portions of glycoproteins[6,7] are now frequent. Applications to molecules as large as decasaccharides have been attempted. However, as larger and more complex molecules are being investigated, it is clear that refinements of methodology will be required to characterize completely the primary structure.

We report herein the use of recent improvements in two-dimensional n.m.r. methodology which are very useful in spectral characterization of high-mol.wt. oligosaccharides. Pure-absorption, two-dimensional spectroscopy serves to improve the resolution over the conventional, magnitude presentation of data. Homonuclear, relayed coherence-transfer spectroscopy serves to lessen the ambiguities associated with spectral overlap through the elucidation of remote, as well as direct-scalar connectivities.



The application presented herein is to the primary structure determination of a glycolipid having an eight-sugar, negatively charged headgroup (**1**). This glycolipid was isolated from the spermatozoa of a fresh-water bivalve and is a member of a series having a four sugar Glc→Man→Man→GlcNAc core unit[8]. It is unusual in having an internal fucosyl residue and a terminal *O*-methylglucosyluronic acid. This glycolipid presents a challenging problem with respect to the characterization of primary structure, both because of its size and the lack of suitable model compound data for its novel constituents.

RESULTS AND DISCUSSION

The following section outlines the procedure for spectral assignment and structural analysis of the oligosaccharide portion of a glycolipid. In the section of a one-dimensional spectrum of Lipid-IV (L-IV; **1**) which contains most resonances from the oligosaccharide portion, the anomeric resonances were labelled *A–H* from low to high field* (Fig. 1). Higher- and lower-field regions contain information that allows characterization as a ceramide having a preponderantly unsaturated acyl chain composition. From the one dimensional spectrum (Fig. 1), it is possible to reach several preliminary conclusions concerning the sugar composition of **1**. From the anomeric region (δ 5.0–4.2) it was possible to estimate the number of sugar

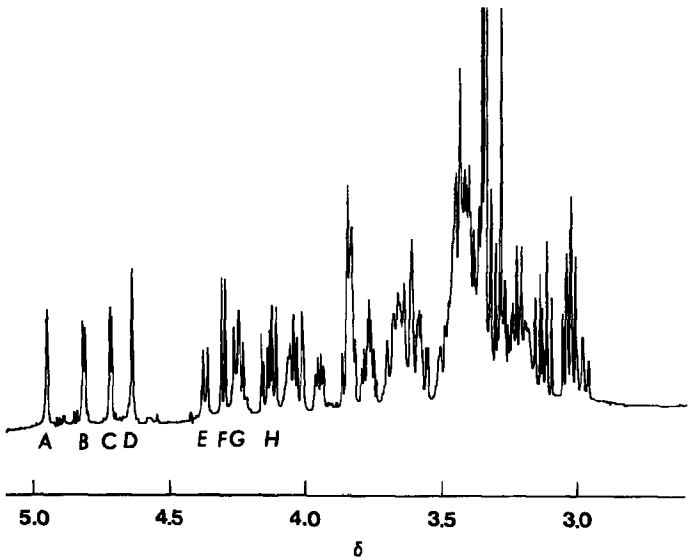


Fig. 1. Plot of region containing the resonances of the ring protons of **1** from a 490-MHz ^{1}H-n.m.r. spectrum. The H-1 protons of **1** are labeled *A–H*.

*The same letters are used for the corresponding sugar units.

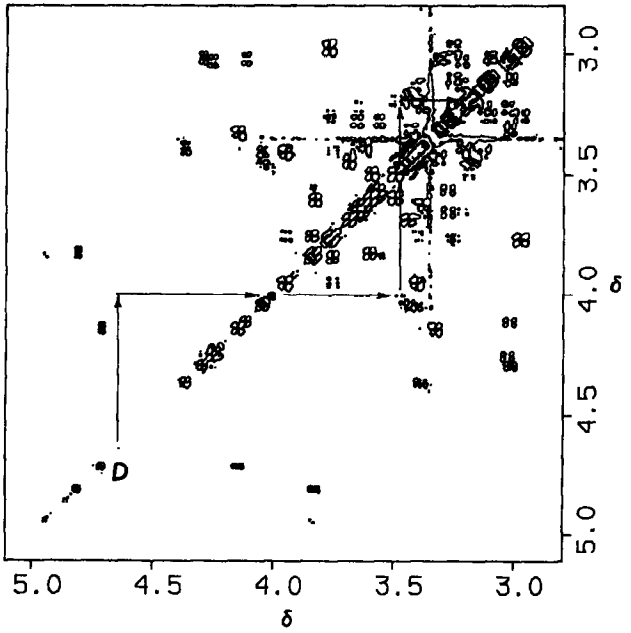


Fig. 2. Contour plot of the same region, as illustrated in Fig. 1, of multiple quantum-filtered COSY. The constructions indicating connectivities are shown for H-1, H-2, and H-3.

residues. Although it appears that there are doublets corresponding to nine anomeric protons, we shall show that one of these apparent doublets does not correspond to an anomeric proton and that there are actually eight sugars units. The presence of two mannose residues also appears on the basis of the small, poorly resolved H-1–H-2 splittings in the resonances of units *A* and *D* at δ 4.95 and 4.64. Two additional α-linked units (*B* and *C*) and four β-linked sugars are also present. In the region δ 4.0–3.0, two singlets, each of an intensity corresponding to three protons, were assigned to *O*-methyl groups (δ 3.28 and 3.35). In the region δ 2.0–1.5 (not shown), two resonances at δ 1.98 and 1.80, which are typical of *N*-$COCH_3$ protons, and a doublet at δ 1.16 which is characteristic of CH_3-6 protons of a fucose residue were observed.

On the contour plot of a pure-absorption COSY experiment (Fig. 2), both cross and auto peaks occur as alternating pairs of positive and negative peaks. These phase properties were very useful in identifying and resolving overlapping cross peaks. Positive and negative contour levels are not distinguished in Fig. 2 but were color-coded in the plots used for analysis. Anomeric resonances and their cross peaks were well resolved in the low-field region of the f_2 dimension and provided a convenient starting point for spectral analysis. Beginning at the diagonal, successive vertical and horizontal lines, which correspond to scalar connectivities, could be constructed to group resonances into subspectra belonging to a single residue. For example, beginning at δ 4.64 with a *D*-1 resonance, and

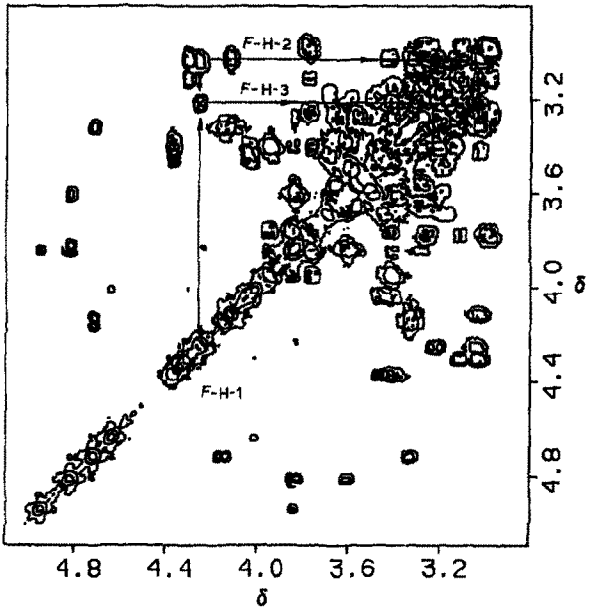


Fig. 3. Contour plot of the same region as illustrated in Fig. 1 from homonuclear, relayed-coherence transfer experiment. The constructions indicating connectivities are shown for *F*-H-1, *F*-H-2, and *F*-H-3. This illustrates the usefulness of this experiment in resolving ambiguities due to spectral overlap.

lowering the contour level somewhat, it was possible to find a weak cross peak showing a connectivity to the *D*-2 resonance at δ 4.01. That this connectivity is weak is a result of a small H-1–H-2 spin-coupling constant. From the *D*-2 resonance, a connectivity to the *D*-3 resonance at δ 3.48 was found, and from the *D*-3 resonance a connectivity to the *D*-4 resonance at δ 3.19. Thus, even in this rather difficult case, it was possible to assign resonances at δ 4.64, 4.01, 3.48, and 3.19 to H-1, -2, -3, and -4 of residue *D* (see Fig. 2). The shifts of resonances for H-1 and -2 are consistent with those tabulated by Vliegenthart *et al.*[1] for a β-D-mannose residue substituted at O-3. This result and the appearance of a (1→3) intraring cross-relaxation peak (see below) led us to conclude that the *D* spin-system corresponds to a β-D-mannose unit.

In some cases, assignment beyond the H-2 resonance is not possible owing to spectral overlap. For example, the *F* anomeric resonance at δ 4.30 could be connected to H-2 of the *F* unit at δ 3.01; however, the assignment was difficult to carry further owing to overlap from the H-2 resonances of the *G* and *H* units which also have resonances near δ 3.01.

A homonuclear, relayed-coherence, transfer-spectrum experiment is useful because it establishes remote as well as direct connectivities, which helps to resolve ambiguities due to spectral overlap (Fig. 3). For instance, in the case of the *F*–H-1 resonance at δ 4.30 described earlier, it was possible to bypass the *F*–H-2 resonance at δ 3.01 and directly assign H-3 of *F* to the resonance at δ 3.09. This resolves the

ambiguity due to the overlap of the H-2 resonances of *G* and *H* and allows connectivities from *F*-H-3 to *F*-H-5 to be established. The unique occurrence of scalar connectivities of H-4 to two H-5 resonances and comparison to model compound data[9] assign the spin-system *F* to a xylose unit.

The combination of pure-absorption COSY and relayed-coherence-transfer experiments allows the assignment of most of the ring protons of all carbohydrate residues. In most cases, sequential assignments are done from the anomeric end, as illustrated in the case of units *D* and *F*. For the galactose and fucose units, connectivities beyond the H-3 resonance are difficult to establish because of the small H-3–H-4 scalar coupling and resultant low-amplitude connectivity peak. In the case

TABLE I

CHEMICAL SHIFTS (δ) AND COMPARISON WITH LITERATURE VALUES FOR THE OLIGOSACCHARIDE PROTONS OF COMPOUND **1**

Unit	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*Structure*
A	4.95[a] 4.95[b]	3.83[a] 3.79	3.55[a] 3.60	3.26[a] 3.28	3.65[a] 3.61		(1→2)-α-D-Man*p*-(1→
B	4.81[a,c,d] 4.98[e]	3.82[a,c,d] 3.78	3.59[a,c,d] 3.81	3.82[c] 3.84	4.25[c] 4.35	1.16[c] 1.18	α-L-Fuc*p*
C	4.72[a] 4.79[e]	4.15[a] 4.17	3.32[a] 3.87	4.07[a] 4.03			α-D-Gal*p*NAc
D	4.64[a] 4.56[g]	4.01[a] 4.01	3.48[a]	3.19[a]	3.42[a,f]		(1→3)-β-D-Man*p*
E	4.36[a] 4.36[b]	3.38[a] 3.50	3.44[a] 3.47	3.67[a] 3.50	3.21[a,f] 3.34		(1→4)-β-D-Glc*p*NAc
F	4.30[a] 4.12[j]	3.01[a] 2.93	3.09[c] 3.09	3.27[a] 3.29	2.96[d,h] 3.76[a,i] 2.98 3.63		β-D-Xyl*p*
G	4.25[a] 4.29[k]	3.02[c] 3.02	3.20[c] ~3.1	3.37[a] ~3.1	3.61[a] ~3.5		β-D-Glc-*p*A
H	4.11[a] 4.14[l]	3.01[a] 3.08	3.33[b] 3.34	3.44[a] 3.29	3.18[a] 3.29		(1→4)-β-D-Glc*p*

[a]Connectivity observed to resonance in multiple quantum filtered COSY; some connectivities were obtained from data set obtained with 90°–*T*–180°–*T* + t_1–90°–90° pulse sequence (see text). [b]Chemical shift values from ref. 12 corrected by −0.24 p.p.m. for solvent and reference difference (Me_2SO–D_2O *vs.* D_2O–Na 4,4-Me_2-4-silapentane-1-sulfonate). [c]Connectivity observed to resonance in homonuclear, relayed-coherence-transfer experiment. [d]Connectivity observed to resonance in pure absorption NOESY experiment. [e]Chemical shift values from ref. 10, corrected by −0.24 p.p.m. for solvent and reference difference (Me_2SO–D_2O *vs.* D_2O–Na 4,4-Me_2-4-silapentane-1-sulfonate). [f]Some uncertainty over assignment due to spectral overlap. [g]Chemical shift values from ref. 1, corrected by −0.225 p.p.m. for solvent and reference difference (Me_2SO–D_2O *vs.* D_2O–Na 4,4-Me_2-4-silapentane-1-sulfonate). [h]Axial proton. [i]Equatorial proton. [j]Chemical shift values from ref. 9 corrected by −0.225 p.p.m. for solvent and reference difference. [k]Chemical shift values from ref. 14. [l]Chemical shift values from ref. 4.

of the fucose unit, the *B*-H-6 resonance at δ 1.16 provided a second useful starting point on the other side of the ring. Connectivities to the *B*-H-5 resonances at δ 4.25 and *B*-H-4 resonance at δ 3.83 were easily established. With the connection of the *B*-H-1, -H-2, and -H-3 resonances from the anomeric end and assignment to the fucose unit on the basis of shift correlation, the complete assignment of all fucose resonances was achieved. It is of interest that the *B*-H-5 resonance of the α-L-fucose unit falls in a region where normally anomeric proton resonances are observed. The assignment of this resonance to H-5 of the fucose unit leaves only eight anomeric protons in the anomeric-resonance region. Along with the integration data that eliminate the possibility of sugar units not having an anomeric proton, this result indicates that **1** contains an octasaccharide component.

The assignments of the resonances are summarized in Table I. As discussed, these assignments result from a number of factors including chemical-shift correlation, small- or large-scalar couplings as reflected in intensities of crosspeaks, and unique resonance positions or multiplicities as in the case of the protons of the xylose and fucose units, or H-5 of the glucuronic acid unit. Chemical shifts for analogous sites in previously assigned oligosaccharides are included in the Table. In most cases, the agreement was within 0.1 p.p.m., which is acceptable given the differences in solvent systems and the occurrence of some additional linkage sites in **1**.

Having assigned the various resonances and identified the sugar units, the

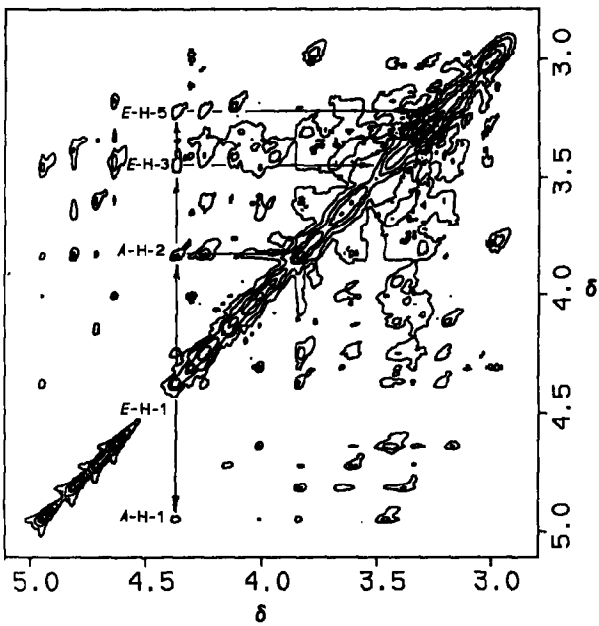


Fig. 4. Contour plot of the same region, as illustrated in Fig. 1, of pure absorption NOESY experiment. The constructions indicating dipolar connectivities are shown for the *E*-H-1 resonance.

remaining structural problem was the locations of the glycosyl linkages and of the *O*-methyl groups. Although some of this information could be deduced from the characteristic linkage shift seen in comparing model compound data to data for **1** (Table I), 2-D n.O.e. experiments which show connectivities based on cross relaxation were useful in solving this problem. When the data are accumulated for short mixing times, the peak intensities in the 2-D n.O.e. experiment are proportional to $1/r^6$ where r is the distance between proton pairs. As used in the present work, significant connectivities were established only for interproton distances <3 Å. Focusing on the anomeric resonance, one expects to see one or more intraring cross-peaks to resonances from protons on the same residue[2]. For β-linked sugar units, one sees axial 1–3 and 1–5 connectivities at 2.5 Å. In β-mannosides, one expects to see an additional 1–2 equatorial connectivity at 2.5 Å. For α-linked sugar residues, one sees only an equatorial 1–2 connectivity at 2.5 Å. In other words, except for β-mannose, one expects to see one or two intraring connectivities. The 2D n.O.e. experiment of **1** (Fig. 4, Table II), showed three or more connectivities for most rings. Consideration of the energetics of glycosidic rotational conformation by Lemieux, Bock, and associates[10–13] suggests that a small range of torsional angles are preferred and that in all of these cases the anomeric proton is expected

TABLE II

N.O.E. CONNECTIVITIES FOR COMPOUND **1**

H-1 of	*Intraring H*	*Interring H*	*Structure*
A	*A*-H-2	*D*-H-3 *E*-H-1[a]	α-D-Man*p*-(1→3)-D-Man
B	*B*-H-2	*E*-H-4[b]	α-L-Fuc*p*-(1→4)-GlcNAc
C	*C*-H-2	*B*-H-3	α-L-Glc*p*NAc-(1→3)-L-Fuc
D	*D*-H-2 *D*-H-3 *D*-H-5	*H*-H-4[b]	β-D-Man*p*-(1→4)-D-Glc
E	*E*-H-3 *E*-H-5	*A*-H-2[b] *A*-H-1[a]	β-D-Glc*p*NAc-(1→2)-D-Man
F	*F*-H-3 *F*-H-5	*D*-H-2	β-D-Xyl*p*-(1→2)-D-Man
G	*G*-H-3 *G*-H-5	*B*-H-4[b]	β-D-Glc*p*A-(1→4)-L-Fuc
H	*H*-H-3 *H*-H-5	Cer-H-1′	β-D-Glc*p*-(1→1′)-Cer

[a]A weaker, additional interring n.O.e. connectivity is consistent with the proposed structure. [b]The assignment is ambiguous. The one given is consistent with the proposed structure.

to be within 3 Å of a proton on the linkage site. Thus, an interring connectivity to the linkage site is expected to account for one of the unidentified cross-peaks at each anomeric position. Although there are possible exceptions to the rule[10], association of the largest, extra cross-peak with the linkage site provided a reasonably reliable method of sequencing **1**. For four of the eight anomeric protons, resolution was adequate to establish unambiguously the following linkages: α-D-Man*p*-(1→3)-β-D-Man*p*, α-D-Gal*p*NAc-(1→3)-α-L-Fuc*p*, β-D-Xyl-(1→2)-β-D-Man*p*, and β-D-Glc*p*-(1→1′)-Cer. In the case of H-1 of the β-D-Glc*p*NAc and β-D-GlcA units, three possible linkage sites were identified, *i.e.*, (1→2)-α-D-Man*p*, and (1→2)- or (1→4)-α-L-Fuc*p*. In the case of β-D-Glc*p*NAc, an additional weak cross-peak giving an unambiguous connectivity to the anomeric resonance of α-D-Man*p* suggested that the β-D-Glc*p*NAc-(1→2)-α-D-Man*p* linkage is correct. Of the two possible β-D-Glc*p*A and α-L-Fuc*p* connectivities, the structure β-D-Glc*p*A-(1→4)-α-L-Fuc*p* is consistent with the structure proposed by Hori *et al.*[8]. For the remaining two anomeric linkages, those of α-L-Fuc*p* and β-D-Man*p*, the strongest cross-peaks, not assignable to intraring n.O.e. values, do not correlate with the proposed linkages. It is possible that these cross-peaks correspond to unassigned H-5 or -6 protons. In the case of α-L-Fuc*p*, a weaker interring cross-peak correlated with H-4 of β-D-Glc*p*NAc, indicating a linkage consistent with the proposed structure. In the case of β-D-Man*p*, the cross-peak for the proposed linkage to O-4 of β-D-Glc*p* would lie under an observed intraring cross-peak. Thus, five of the linkages shown in **1** are independently established by the present work, and the remaining data are consistent with the other three linkages.

Since the methyl protons of the *O*-methyl groups are within 3 Å of the ring protons of the methylation site, some information on *O*-methylation sites, was also obtained from the 2-D cross-relaxation experiments. The CH_3 peaks at δ 3.28 and 3.35 were, however, very strong, sharp resonances and, as such, produced several false n.O.e. cross-peaks which resulted from the intersection of the low-amplitude tails on peaks in the f_1 dimension with tails of the methyl peak in the f_2 dimension, and vice-versa. For the methyl resonances at δ 3.28, the strongest cross-peak laid at δ 3.38, a position consistent with a proposed methyl group at O-4 of the D-glucuronic acid unit. The methyl resonance at δ 3.35 laid very close to a resonance from residual HDO in the sample, and a definitive assignment could not be made from the n.O.e. data. However, the large deviation of chemical shift from the model compound at O-3 of residue *C* (Table I) would be consistent with a proposed methyl group at O-3 position of the 2-acetamido-2-deoxy-D-galactose unit.

The data presented herein illustrate the possibility of determining large parts of the primary sequence of a rather complex oligosaccharide by use of two-dimensional n.m.r. methods. The combination of a pure-absorption, multiple-quantum filtered COSY experiment, which affords a significant improvement in resolution over the conventional magnitude presentation of two-dimensional data, and a homonuclear, relayed-coherence transfer experiment, which serves to eliminate the ambiguities due to spectral overlap, facilitated the nearly complete assignment of the ring protons of a given carbohydrate unit.

When combined with assignment of resonances to particular units, pure absorption NOESY spectra facilitated the direct identification of many linkage sites and allowed a partial determination of sequence. The structure derived herein for **1** is consistent with that determined by Hori *et al.*[8], who used classical methods for the sequencing of oligosaccharides, and was initially carried out without the knowledge of the complete structure. The determination has also been carried out in a nondestructive manner with a moderate investment of time. In addition, the present work has also significantly broadened the database of chemical-shift correlations with primary structure for use with one-dimensional n.m.r. assignment methods. In particular, unit *B*, assigned to α-L-fucose, occurs in a unique environment. Vliegenthart *et al.*[1] and Lemieux *et al.*[10] have studied several compounds containing a terminal L-fucosyl group. These studies allowed the comparison of the present shifts to the shifts for H-1 and -5, and sometimes the complete resonance assignments, in an L-fucose unit having various linkages. No comparison is completely satisfactory, but a set for an α-L-(1→4)-linked fucose residue is included in Table I. Here, the resonance for H-3 was shifted upfield by 0.22 p.p.m. as compared to the values reported by Lemieux *et al.*[10], and the resonances for H-1 and -5 also deviated slightly, by −0.17 and −0.10 p.p.m., respectively. The shift of the H-3 resonance was not unexpected for a residue substituted at O-3. In the present case, the L-fucose residue is substituted at O-3 by a 2-acetamido-2-deoxy-D-galactopyranosyl group.

Unit *G* corresponding to D-glucuronic acid gave quite unusual results. The shifts in Table I were compared to the shifts of the D-glucuronic acid unit in chondroitin sulfate[14] in which only 3 resonances were assigned. The *G*-H-4 shift is likely to be sensitive to an O-methyl group and it is only by the resonance at *G*-H-5 that the D-glucuronic acid unit could be distinguished from the D-glucose unit.

Unit *F* corresponding to D-xylose also gave rather unusual results. The identity of unit *F* as D-xylose was confirmed on the basis of the coupling pattern and the observation that the *F*-H-4 resonance showed direct scalar connectivities to two resonances at δ 2.96 and 3.76 This was significant as the database for D-xylose in glycolipids is very limited. The present data are obviously useful in the analysis of new glycolipids. They should be used, however, with caution for other systems as chemical shifts do vary with solvent and the present shifts were determined for solutions in dimethyl sulfoxide. This solvent is useful for glycolipids but the results may not exactly correlate with shifts for solutions in solvents common for oligosaccharides.

Besides extending the database for correlations between primary structure and chemical shifts, and offering a nondestructive method for the elucidation of oligosaccharide sequence, the present data offer the potential for the elucidation of secondary structures. In particular, the rate of buildup of n.O.e. cross-peaks for linkage sites, when compared to the rate of n.O.e. buildup for intraresidue pairs, at fixed distances, gives a quantitative measure of the interproton distance across the glycosidic bond. In cases where additional cross-peaks are seen, additional

distance constraints may be sufficient to characterize a secondary structure. Such peaks are seen in the present study, but given the unusual solvents used, it is not clear whether the derived conformation is particularly relevant.

EXPERIMENTAL

Lipid IV (L-IV, **1**) was obtained from the spermatozoa of bivalves, as described by Hori *et al.*[8], and dissolved in 49:1 (v/v) ($^2H_6$)Me_2SO–^2H_2O (0.4 mL). All solvents were of the highest isotopic purity and were obtained from Merck & Co. (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). All spectra were recorded with a home-built spectrometer based on an Oxford superconducting magnet operating at 11.5 T (490 MHz for ^1H). One-dimensional spectra were obtained with 256 transients and a recycle time of 1.2 sec using 90° pulses. A spectral width of 3205 Hz using 4K complex points in quadrature mode was employed.

Pure-absorption, multiple quantum-filtered COSY spectra were obtained by use of the 90°–t_1–90°–90°–t_2 pulse sequence and phase cycling of Rance *et al.*[15]. In order to achieve quadrature in the f_1 dimension, 512 real and 512 imaginary t_1 points were acquired and stored separately in a manner analogous to that of States *et al.*[16]. Each t_1 point was obtained by use of 1024 complex points, and a sweep width of 3205 Hz with 48 transients for a total acquisition time of 18 h. The data were processed with an F.t. n.m.r. program on a Vax 11/750 computer. Sine-bell weighting functions, shifted by 90°, were applied in both dimensions with zero filling in the t_1 dimension to yield a 1 K × 1 K data matrix.

Phasing parameters for the f_2 dimension were obtained from a single file (t_1 = 0) of an experiment performed with a 90°–τ–180°–τ + t_1–90°–90°–t_2 pulse sequence, which differs from the pulse sequence of Rance *et al.*[15] in that there is an additional delay τ (16 msec) followed by a 180° pulse, to allow for dephasing of multiplet components during early t_1 points. Such a sequence offers the advantages of improved sensitivity and easier phasing in the f_2 dimension. Two-dimensional data sets can be collected with this sequence, but phase errors are then introduced in multiplet components of the t_1 dimension, which complicate phase-sensitive displays.

Homonuclear relayed coherence transfer spectra were obtained by use of the 90°–t_1–90°–τ–180°–τ–90°–t_2 pulse sequence and the phase cycling of Wagner[17]. Each t_1 point was obtained by use of a 5814-Hz sweep width with the carrier frequency set at one end of the region of interest, 2 K complex points, a fixed delay (τ) of 0.018 sec and 96 transients with a 1.1-sec recycling rate; 512 t_1 points were acquired for a total acquisition time of 17 h. The data were processed with sine-bell window functions with zero filling in the t_1 dimension to yield a 2 K × 2 K data matrix.

Pure absorption NOESY spectra were obtained by use of the 90°–t_1–90°–τ_m–90°–t_2 pulse sequence and phase cycling of States *et al.*[16]; 192 t_1 points were obtained with 1024 complex points, 3205-Hz sweep width, a mixing time τ_m of 0.125 sec, and 64 transients with a 1.2-sec recycling rate for a total acquisition time of 10 h. The

data were processed with a cosine-bell window function in both dimensions with zero filling in the t_1 dimension to yield a 1 K × 1 K data matrix.

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