Carbohydrate Research, 184 (1988) 47–65
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

THE DETERMINATION OF COMPLEX CARBOHYDRATE STRUCTURE BY USING CARBONYL CARBON RESONANCES OF PERACETYLATED DERIVATIVES*

WARREN J. GOUX

Department of Chemistry, University of Texas, P. O. Box 830688, Richardson, Texas 75083-0688 (U.S.A.)

(Received January 8th, 1988; acccepted for publication in revised form, June 5th, 1988)

ABSTRACT

Carbonyl carbon resonances have been assigned to specific acetyl substituents in peracetylated derivatives of a variety of di- and tri-saccharides that occur as substructures of *N*- and *O*-linked glycoprotein glycans. Assignments were made by correlating shifts of these resonances to previously assigned pyranoid-ring proton and acetyl methyl proton shifts by means of 2D ^{13}C–^{1}H shifts correlation spectra. It was found that, when the shift assignment data for carbonyl carbon atoms, acetyl methyl protons, and pyranoid-ring protons are plotted in three dimensions, patterns appear that are unique to the different types of residues occurring in a parent structure. It is suggested that these shift data can complement existing ^{1}H- and ^{13}C-n.m.r. methods for determining primary structures of complex carbohydrates.

INTRODUCTION

Oligosaccharides constitute the most abundant and diverse group of compounds present in living systems. Their functions range from that of antigenic determinants, such as the ABO blood-group determinants of man, to the purported regulation of gene expression in plants[1,2]. Their physiological roles appear to be influenced by their tertiary and, ultimately, their primary structure[3–11]. Their structural complexity arises from the large number of structurally unique residues present and from the variety of ring configurations and of glycosidic linkages that can be made to and from neighboring residues[12].

In recent years, ^{1}H-n.m.r. spectroscopy has been used[1] to determine the primary structures of oligosaccharides isolated, usually in limited quantities, from glycolipids, glycoproteins, yeast cell-walls, and the urine of patients suffering from glycoproteinosis[12–14]. As a basis for structural identification, "reporter-group"

*Data herein were initially presented at the 194th National ACS Meeting in New Orleans, LA, August 30–September 4, 1987.

0008-6215/88/$ 03.50

resonances, most arising from anomeric protons between 4.0 and 5.5 p.p.m., are compared with those observed in spectra of model compounds. Structural details, including pyranose or furanose ring-structure and glycosidic linkages made to and from neighboring residues, can be gleaned from proton chemical shifts and coupling constants. Possible complications inherent in the method include overlap of these reporter-group resonances among themselves or with other resonances in the spectrum, particularly those arising from the solvent (HOD or H_2O).

^{13}C-N.m.r. spectroscopy has also been used to determine primary structures of oligosaccharides and oligosaccharides covalently linked to peptides, proteins, and lipids[15–20]. In a manner analogous to the ^{1}H-n.m.r.-spectral method, structural details are inferred from the shifts of pyranose or furanose ring-carbon atoms. However, carbon spectra are inherently easier to interpret, owing to the much greater chemical-shift range over which resonances occur and the lack of complexities arising from spin–spin coupling and overlap of resonances with those arising from the solvent. Unfortunately, these advantages are usually overshadowed by the low sensitivity of the ^{13}C nucleus, making acquisition of spectra of samples isolated in limited quantities (1–5 mg) difficult or impossible.

In recent reports, we and others have shown that the carbonyl carbon resonances of peracetylated saccharides[21,22], the benzyl methylene carbon resonances of perbenzylated saccharides[23], and the methyl carbon resonances of permethylated saccharides[24,25] have shifts that are sensitive to primary structure. These resonances, which generally occur in a 2.5–3.0-p.p.m. range, can be assigned to specific pyranose or furanose ring substituents by using one- and two-dimensional, homo- and hetero-nuclear deoupling techniques or lanthanide shift reagents[26]. We had previously noted the possibility of ^{13}C-enrichment of carbon atoms giving rise to these resonances, forming the basis of a relatively sensitive ^{13}C-n.m.r.-spectral method capable of complementing existing ^{1}H-n.m.r.-spectral methods for determining complex carbohydrate structures. Furthermore, in the case of peracetylation, introduction of the ^{13}C isotope can be carried out in quantitative yield in a single-step reaction.

The goals of the present study were threefold. (*1*) To develop procedures for carrying out oligosaccharide peracetylation on small quantities (5–10 mg) of starting material using ^{13}C-enriched reagents. Of particular interest were those di- and tri-saccharides having primary structures that occur as substructures of *N*-linked and *O*-linked glycoprotein glycans. (*2*) To test the feasibility of using conventional 2D n.m.r. techniques to assign carbonyl carbon resonances in spectra of ^{13}C-carbonyl-enriched peracetylated oligosaccharides prepared in limited quantities. (*3*) To develop methods for handling the data in a comprehensive manner, so that patterns of carbonyl resonances in spectra of unknown compounds can be recognized and used to infer structural features.

EXPERIMENTAL

Materials and methods. — D-Galactose, L-fucose, *N*-acetyl-D-glucosamine, methyl α-D-mannopyranoside, methyl β-D-galactopyranoside, *N*-acetyl-D-neuraminic acid, chitobiose [β-GlcNAc-(1→4)-GlcNAc], lactose [β-Gal-(1→4)-Glc], lactosamine [β-Gal-(1→4)-GlcNAc], and (*N*-acetylneuraminyl)lactose from bovine colostrum [available as 85% of α-NeuAc-(2→3)-β-Gal-(1→4)-Glc plus 15% of α-NeuAc-(2→6)-β-Gal-(1→4)-Glc], α-Man-(1→3)-α-Man-OMe, α-Man-(1→4)-α-Man-OMe, and α-Man-(1→6)-α-Man-OMe were obtained from Sigma Chemical Co. (St. Louis, MO). β-Gal-(1→3)-GlcNAc was a gift from J. Montreuil (Université des Sciences et Techniques de Lille, Villeneuve d'Ascq, France). Pivaloyl chloride was obtained from Aldrich Chemical Co. (Milwaukee, WI). Methyl α-L-fucopyranoside (**13**) was prepared by standard procedures[27]. The methyl β-D-glycoside of methyl neuraminate (**14**) was prepared by the method of Czarniecki and Thornton[28]. Acetic-1-^{13}C pivalic anhydride was prepared from sodium (1-^{13}C)-acetate and pivaloyl chloride[29].

Peracetylation. — Neutral mono- and di-saccharides were acetylated by stirring the pre-dried solid (5–100 mg) in a 2-mL reaction-vessel with a 10–20-fold excess of dry pyridine and acetic-1-^{13}C pivalic anhydride. The reaction was usually complete within 72 h at room temperature (as determined by ^{13}C-n.m.r. spectroscopy). In the case of (*N*-acetylneuraminyl)lactose, it was necessary to add a small volume of *N,N*-dimethylformamide (200 μL) in addition to the pyridine and acetic-1-^{13}C pivalic anhydride and warm the reaction mixture for 5 h at 50°. Following the reaction, the product mixture was warmed gently (50°) under diminished pressure, in order to remove the solvent, and the excess of reactants. Despite these efforts, resonances arising from acetic-1-^{13}C pivalic anhydride and pivalic acid were visible between 0 and 2 p.p.m. in the ^{1}H-n.m.r. spectra of the products. However, no further purification steps were undertaken, as the presence of these impurities did not interfere with the assignments of pyranoid-ring protons or carbonyl carbon resonances.

N.m.r.-spectral conditions. — Normal F.t. ^{1}H- and phase-sensitive ^{1}H COSY spectra[30] were recorded at room temperature for solutions in sample tubes (5 mm o.d.) with a Bruker AM-300 spectrometer (7 T). Typically, *J*-correlated (COSY) spectra were acquired by using a sweep width of ±800 Hz in both frequency dimensions. Each spectrum was collected by using 2 "dummy" and 8 normal acquisitions, with a delay time of 4 s between acquisitions; 1024 and 512 real data points were collected in the second frequency dimension arising from transient acquisition (F2) and first frequency dimension arising from variable evolution period (F1), respectively. Processing was carried out by zero-filling to 2048 data points in each dimension, and Fourier-transforming using a sine-bell, weighting function with no offset.

^{13}C-N.m.r. spectra were recorded for solutions in sample tubes (10-mm o.d.) at 50.1 MHz with a Bruker AM-200 spectrometer modified to minimize thermal

gradients in the direction of the magnetic field[31]. The proton decoupler was set to the frequency of the acetyl methyl protons, and ^{13}C-n.m.r. spectra were acquired with less than 0.25 W decoupling power, using the WALTZ-16 pulse sequence[32,33]. In this configuration, 0.03–0.05-Hz carbonyl carbon linewidths were routinely obtained. Carbon-detected ^{13}C–^{1}H shift-correlation spectra were acquired by using sweepwidths of ±75 Hz in F2 and ±500 Hz in F1. Broadband decoupling was applied during acquisition. The fixed delay-times before (Δ_1) and following (Δ_2) the final mixing pulse were 70 and 35 ms, in order to emphasize long-range couplings of carbonyl carbon atoms to pyranose-ring protons[34]. Each spectrum was an average of 16 acquisitions with a 2-s delay between acquisitions. 1024 data points in F2 and 256 data points in F1 were zero-filled to 2048 and 512 data points prior to Fourier transformation. Spectra were transformed by using Gaussian line-broadening of 0.1 Hz in both dimensions.

Chemical shifts ^{1}H and ^{13}C are reported with respect to internal Me_4Si (1% in $CDCl_3$). All n.m.r. experiments were carried out at Los Alamos National Laboratory, Los Alamos, NM.

RESULTS

The carbonyl carbon resonances of compounds **9** and **15–18** had already been assigned[21]. With the exception of compounds **4a** and **4b** (see later), the ^{13}C- and ^{1}H-n.m.r. spectra suggested that all of these compounds were completely acetylated and lacked any free hydroxyl groups.

The carbonyl carbon resonances of each of the compounds studied give a unique spectral pattern between 168.5 and 172 p.p.m. Each of the resonances can be assigned to a specific acetyl substituent by correlating their chemical shifts to shifts of previously assigned pyranosyl-ring protons[21]. The individual steps used in making these assignments are illustrated in Fig. 1 for octa-*O*-acetyl-α-lactose (**3**). Pyranosyl proton resonances were first assigned by using homonuclear correlated spectroscopy (COSY). In the COSY contour plot shown in Fig. 1a, resonances on either of the two axes also lie along the diagonal running from the lower left to the upper right. Off-diagonal contours, lying either above or below the diagonal, arise from sets of coupled spins. Having assigned the doublets at 6.26 and 4.55 p.p.m. to H-1 of the α-Glc and β-Gal residues on the basis of their unique chemical shifts[35], all sets of resonances can be assigned by tracing out the coupling network. Fig. 1b shows the ^{13}C–^{1}H shift-correlation spectrum of **3**, where fixed delay-times before and following the final mixing pulse have been adjusted to emphasize long-range ^{13}C–^{1}H coupling. Carbonyl carbon resonances can be assigned to specific substituents from cross-peaks arising from the coupling to nearest neighboring pyranosyl protons. For example, the carbonyl carbon resonance lying farthest upfield can be assigned* to C1-Ac, as it is seen to correlate with a proton doublet at 6.26

*Key: C1-Ac, acetoxyl carbonyl carbon atom substituted on C-1; H1-AcMe, acetoxyl methyl protons substituted on C-1.

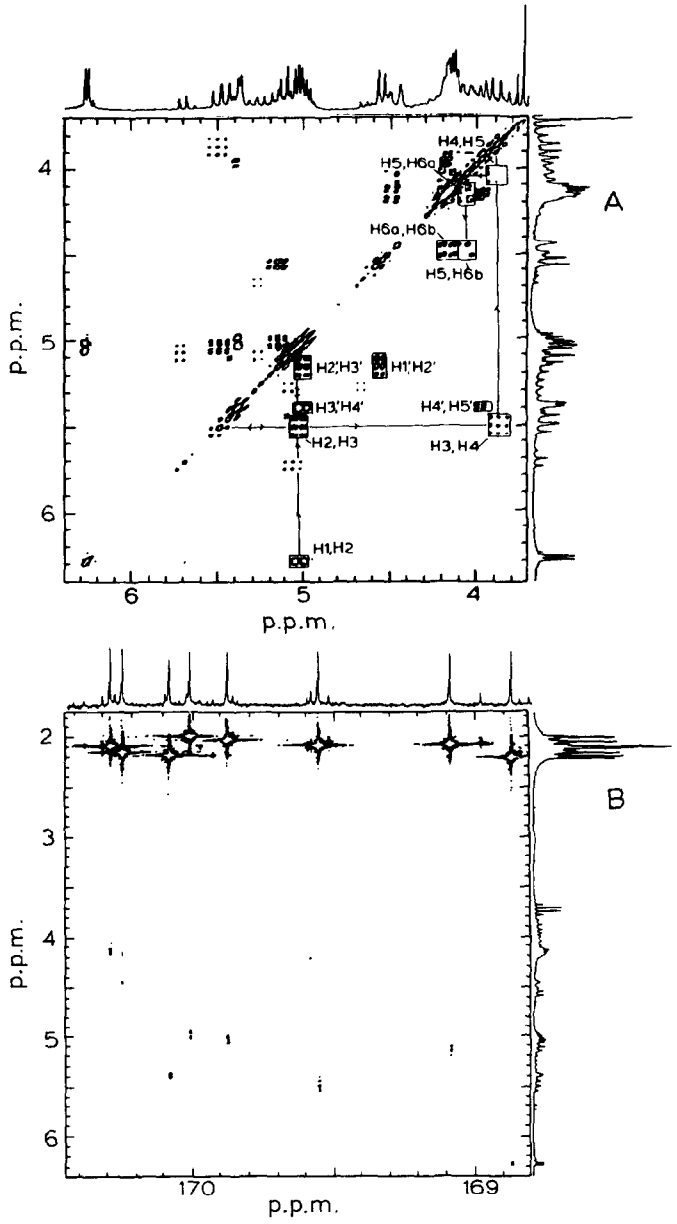
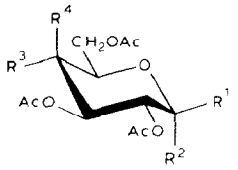


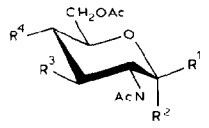
Fig. 1. (a) The COSY spectrum (contour plot) of octa-*O*-acetyl-α-lactose (**3**) (5 mg/mL). [The normal, F.t., ^{1}H-n.m.r. spectrum is shown along both of the chemical-shift axes. Arrows in the plot illustrate how the D-glucopyranosyl-ring proton resonances can be assigned by tracing out their coupling network. Unlabeled, off-diagonal contours arise from pyranosyl-ring protons of octa-*O*-acetyl-β-lactose. (b) The ^{13}C–^{1}H shift-correlation spectrum of **3** (3 mg/mL). Normal, F.t., proton-decoupled ^{13}C-n.m.r. and ^{1}H-n.m.r. spectra are shown along the horizontal and vertical axes.]

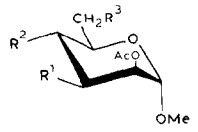


1a $R^1 = R^3 = H, R^2 = R^4 = OAc$
1b $R^1 = R^4 = OAc, R^2 = R^3 = H$
2 $R^1 = OMe, R^2 = R^3 = H, R^4 = OAc$
3 $R^1 = R^4 = H, R^2 = OAc, R^3 = 2,3,4,6\text{-}Ac_4\text{-}\beta\text{-}Gal$
4a $R^1 = R^4 = H, R^2 = OAc, R^3 = 4,7,8,9\text{-}Ac_4\text{-}\alpha\text{-}NeuAc\,2 \rightarrow 3$ $4,6\text{-}Ac_2\text{-}\beta\text{-}Gal$
4b $R^1 = OAc, R^2 = R^4 = H, R^3 = 4,7,8,9\text{-}Ac_4\text{-}\alpha\text{-}NeuAc\,2 \rightarrow 3$ $4,6\text{-}Ac_2\text{-}\beta\text{-}Gal$

p.p.m., previously assigned to the anomeric proton of the α-Glc residue. Carbonyl carbon resonances also are seen to correlate with proton resonances arising from acetyl methyl protons (1.95–2.25 p.p.m.). Chemical shifts of these protons have been shown to depend on pyranosyl ring structure[21,22].

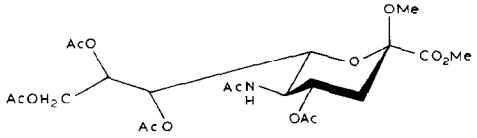
Shift and assignment data for the carbonyl carbon atoms, their nearest-neighboring pyranosyl-ring protons, and acetyl methyl protons are summarized in Table I. A few of the assignments deserve special comment. The H-2, H-3, and H-4 resonances of tetra-*O*-acetyl-α-Man-OMe (**9**) all lie in a narrow region of the ^{1}H-n.m.r. spectrum, between 5.25 and 5.31 p.p.m. This near-degeneracy made it impossible to assign carbonyl carbon resonances to C2-Ac, C3-Ac, and C4-Ac on a one-to-one basis from ^{1}H–^{13}C shift-correlation spectra (even from data acquired at 9.4 T). Instead, assignments were made from the splitting patterns observed in spectra of the corresponding (1-^{13}C)- and (2-^{13}C)-enriched compounds[21]. The data in Table I show that similar assignment problems arise in peracetylated mannose disaccharides. For example, H-2, H-3′, and H-4′ of **10**, and H-2, H-3, and H-3′ of **11** all have chemical shifts between 5.19 and 5.25 p.p.m. All pyranosyl-ring proton resonances of **12**, except H-6, have shifts between 5.22 and 5.26 p.p.m. It should, however, be noted that, for those compounds in which the carbonyl carbon resonances can be assigned from ^{13}C–^{1}H shift-correlation spectra, nearest-neighboring acetyl methyl protons give rise to resonances having shifts that are characteristic of their assigned substitution sites and which depend less on the specific structure of the parent compound. For example, the acetyl methyl protons of acetyl groups substituted at O-4 of galactose give rise to resonances with shifts between 2.14 and 2.16 p.p.m. in compounds **1a, 1b, 2, 3, 7,** and **8**. In the same set of compounds, the acetyl methyl protons H1-AcMe, H2-AcMe, and H3-AcMe give rise to resonances with shifts between 2.12 and 2.16, 2.02 and 2.06, and 1.96 and 2.04 p.p.m. Similarly, the acetyl methyl proton resonances of H1-AcMe, H3-AcMe and H4-AcMe of GlcNAc residues in compounds **5, 6a, 6b, 7**, and **8** have shifts lying





5 $R^1 = H, R^2 = R^3 = R^4 = OAc$
6a $R^1 = H, R^2 = R^3 = OAc, R^4 = 3,4,6\text{-}Ac_3\text{-}\beta\text{-}GlcNAc$
6b $R^1 = R^3 = OAc, R^2 = H, R^4 = 3,4,6\text{-}Ac_3\text{-}\beta\text{-}GlcNAc$
7 $R^1 = H, R^2 = R^3 = OAc, R^4 = 2,3,4,6\text{-}Ac_4\text{-}\beta\text{-}Gal$
8 $R^1 = H, R^2 = R^4 = OAc, R^3 = 2,3,4,6\text{-}Ac_4\text{-}\beta\text{-}Gal$

9 $R^1 = R^2 = R^3 = OAc$
10 $R^1 = 2,3,4,6\text{-}Ac_4\text{-}\alpha\text{-}Man, R^2 = R^3 = OAc$
11 $R^1 = R^3 = OAc, R^2 = 2,3,4,6\text{-}Ac_4\text{-}\alpha\text{-}Man$
12 $R^1 = R^2 = OAc, R^3 = 2,3,4,6\text{-}Ac_4\text{-}\alpha\text{-}Man$



14

between 2.06 and 2.19, 1.96 and 2.10, and 1.97 and 2.04 p.p.m. Accordingly, carbonyl carbon resonances in the mannose-containing compounds **10–12**, which could not be assigned on a one-to-one basis from shift-correlation data were, instead, assigned by comparing shifts of nearest-neighboring acetyl methyl proton resonances with shifts of corresponding resonances of compound **9**. For example, the carbonyl carbon atoms of **10**, which are nearest to ring protons with shifts of 5.21, 5.19, and 5.23 p.p.m. are also nearest to methyl protons with shifts of 2.19, 1.96, and 2.03 p.p.m. These can be compared to methyl protons of **9** having shifts of 2.15, 1.98, and 2.04 p.p.m., and that are accordingly assigned to acetyl methyl protons H2-AcMe, H3-AcMe, and H4-AcMe. Similar strategy was used in assigning ambiguous carbonyl resonances of compounds **11** and **12**.

Fig. 2 shows the carbonyl carbon resonances of acetylated neuraminyl-lactose

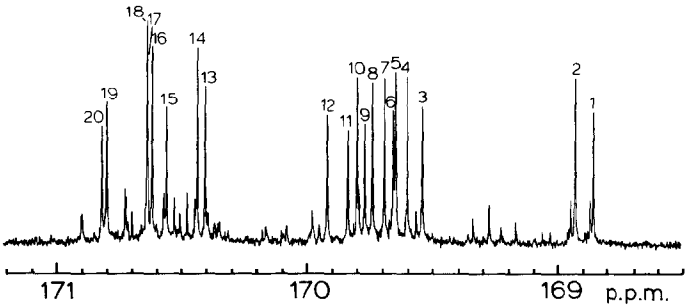


Fig. 2. Carbonyl carbon resonances in the ^{13}C-n.m.r. spectrum of a mixture of **4a** and **4b** (3 mg of derivatized oligosaccharide/mL). [The spectrum represents an average of 32 transients, each with an acquisition time of 30 s and 10-s wait-time between successive acquisitions.]

TABLE I

SUMMARY OF CHEMICAL SHIFTS OF CARBONYL CARBON ATOMS AND NEAREST PYRANOSYL-RING PROTONS AND ACETYL METHYL PROTONS ON PERACETYLATED CARBOHYDRATE DERIVATIVES[a]

Acetoxyl substituent on	*Compound*								
	1a *α-Gal*			**1b** *β-Gal*			**2** *β-Me-Gal*		
	$\delta^{13}C$	$\delta^{1}H$-*PR*	$\delta^{1}H$-*AcMe*	$\delta^{13}C$	$\delta^{1}H$-*PR*	$\delta^{1}H$-*AcMe*	$\delta^{13}C$	$\delta^{1}H$-*PR*	$\delta^{1}H$-*AcMe*
C-1	168.81	6.37	2.12	168.85	5.77	2.16			
C-2	169.76	5.29	2.02	169.26	5.32	2.05	169.41	5.21	2.06
C-3	170.00	5.31	2.00	169.82	5.18	1.99	170.05	5.05	1.98
C-4	170.04	5.51	2.16	170.01	5.44	2.16	170.18	5.40	2.15
C-6	170.23	4.09, 4.13	2.04	170.22	4.17	2.04	170.31	4.19, 4.17	2.04
	3 *α-Lactose*			**4a** *α-NeuAc Lactose*			**4b** *β-NeuAc Lactose*		
	$\delta^{13}C$	$\delta^{1}H$-*PR*	$\delta^{1}H$-*AcMe*	$\delta^{13}C$	$\delta^{1}H$-*PR*	$\delta^{1}H$-*AcMe*	$\delta^{13}C$	$\delta^{1}H$-*PR*	$\delta^{1}H$-*AcMe*
C-1	168.87	6.25	2.18	168.85	6.25	2.16	168.93	5.69	2.09
C-2	169.87	5.01	2.00	169.92	4.99	1.98	169.60	5.00	2.00
C-3	169.56	5.46	2.06	169.54	5.50	2.05	169.69	5.28	2.05
C-6	170.25	4.47, 4.15	2.13	170.64	4.51, 4.45	2.10	170.62	4.54, 4.58	2.09
C-2′	169.08	5.12	2.05	[b]	4.72	[b]	[b]	4.72	[b]
C-3′	170.00	5.07	1.97						
C-4′	170.08	5.37	2.16	169.77	5.43	2.19	169.80	5.43	2.20
C-6′	170.28	4.14	2.06	170.41		2.06	170.44		2.05
C-4″				170.82	5.38	1.98	170.80	5.38	1.98
C-7″				169.65	5.24	2.06	169.64	5.24	2.06
C-8″				169.83	5.11	2.09	169.74	5.11	2.07
C-9″				170.56	4.26, 4.03	2.05	170.64		2.04

	5 *α-GlcNAc*			**6a** *β-GlcNAc-(1→4)-α-GlcNAc*			**6b** *β-GlcNAc-(1→4)-β-GlcNAc*		
	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe
C-1	168.61	6.18	2.14	168.91	6.07	2.16	169.47	5.55	2.06
C-3	171.47	5.32	2.04	171.43	5.19	2.02	171.01	5.06	2.03
C-4	169.04	5.19	2.04						
C-6	170.06	4.26, 4.22	2.07	171.30	4.40, 4.15	2.11	170.54[c]		2.09
C-3′				170.83	5.12	1.98	170.93	5.18	1.99
C-4′				169.34	5.02	1.98	169.36	5.00	1.97
C-6′				170.57	4.35, 3.97	2.05	170.60[c]		2.05
	7 *α-Lactosamine*			**8** *β-Gal-(1→3)-α-GlcNAc*			**9** *α-Man-OMe*[d]		
	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe
C-1	168.66	6.10	2.18	168.56	6.05	2.19			
C-2							169.91	5.25	2.15
C-3	171.33	5.26	2.10				169.76	5.33	1.98
C-4				168.90[c]	5.02	2.04	169.62	5.31	2.04
C-6	170.25	4.38, 4.12	2.11	170.71	4.23, 4.09	2.07	170.51	4.25, 4.34	2.10
C-2′	169.22	5.12	2.05	169.51[c]	5.04	2.05			
C-3′	169.93	4.99	1.96	170.20	4.95	1.96			
C-4′	169.99	5.37	2.15	170.03	5.35	2.14			
C-6′	170.26	4.12, 3.91	2.06	170.40	4.25, 4.03	2.06			
	10 *α-Man-(1→3)-α-Man-OMe*			**11** *α-Man-(1→4)-α-Man-OMe*			**12** *α-Man-(1→6)-α-Man-OMe*		
	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe
C-2	170.43[e]	5.21[d]	2.19[e]	169.66[e]	5.23	2.08	170.11[e]	5.23	2.12
C-3				169.78[e]	5.25	2.02	169.83[ce]	5.22	1.96
C-4	169.88	5.27	2.09				169.79[e]	5.23	2.02
C-6	170.70	4.21, 4.08	2.08	170.43	4.25, 4.23	2.09			
C-2′	169.99	4.98	2.11	169.66	5.07	2.11	169.92[e]	5.26	2.12
C-3′	169.56[e]	5.19[d]	1.96	169.51[e]	5.21[d]	1.95	169.67[ce]	5.22	1.95
C-4′	169.86[e]	5.23[d]	2.03	169.52	5.29	2.01	169.71[e]	5.26	2.01
C-6′	170.65	4.26, 4.08	2.09	170.44	4.23, 4.10	2.07	170.56	4.23, 4.10	2.08

TABLE I *(continued)*

Acetoxyl substituent on	*Compound*								
	13 *α-L-Fuc-OMe*			**14** *β-NeuAc-CO_2Me-OMe*					
	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe	$\delta^{13}C$	$\delta^{1}H$-PR	$\delta^{1}H$-AcMe
C-2	170.42	5.15	2.10						
C-3	169.97	5.36	1.99						
C-4	170.59	5.30	2.19	170.92	5.20	2.03			
C-7				170.09	5.35	2.17			
C-8				170.64	5.29	2.09			
C-9				170.52	5.17	2.05			

[a]^{1}H-PR and ^{1}H-AcMe are shifts for pyranosyl ring and acetyl methyl proton resonances. Proton chemical shifts were taken from ^{13}C–^{1}H shift-correlation spectra. H-6 and H-6′ resonances shifts not appearing in the Table could not be assigned from the 2D contour map. [b]The H-2′ atom resonances of compounds **4a** and **4n** did not appear as cross-peaks in the ^{13}C–^{1}H shift-correlation spectra of these compounds. [c]Carbonyl carbon resonances in compounds **6b, 8, 11**, and **12** which could not be assigned on a one-to-one basis. [d]Assignments for carbonyl carbon resonances of **9** were taken from ref. 21. [e]Carbonyl carbon resonances in **10–12** which were assigned by comparing acetyl methyl proton shifts to those of compound **9**.

(**4**). Numbered resonances arise from the mixture of acetylated anomers, while the smaller unnumbered peaks arise from acetylated oligosaccharide impurities [the commercially purchased sample contained 85% of α-NeuAc-(2→3)-β-Gal-(1→4)-Glc and 15% of α-NeuAc-(2→6)-β-Gal-(1→4)-Glc)]. Peaks 1, 3, 6, 9, 11, 12, 13, 15, 17, and 20 appear slightly smaller than other peaks lying nearby, and can probably be assigned to the unfavorable anomer. It should be note that the sum of numbered resonances, namely, 20 (10 from each of the two anomers) is inconsistent with the expected 22 resonances which would arise from a mixture of peracetylated α-NeuAc-(2→3)-β-Gal-(1→4)-Glc anomers. In the ^{13}C–^{1}H shift-correlation spectrum of the mixture, there are no carbonyl resonances which can be correlated with proton resonances at 4.72 p.p.m., the shift assigned to Gal H-2, from COSY data (see Table I). It appears that the hydroxyl group at Gal C-2′ is blocked from acetylation. This may result from steric hindrance from the nearby glycosidic linkage at Gal C-3′, or, more likely, from the participation of Gal O-2′ in the formation of a β-lactone with the α-carboxyl group of the nonreducing, terminal NeuAc. This derivative has not previously been observed for other saccharides containing similar structures which have been acetylated under acidic conditions[36].

DISCUSSION

Fig. 3 shows the carbonyl carbon rsonances of compounds **2**, **3**, **7**, and **8**. Resonances assigned to the carbonyl carbon atoms of galactose are so designated in the Figure. Clearly, the shifts of these resonances change as the glycosidic substitution site of the neighboring residue is changed (compare spectrum C of **7** with spectrum D of **8**) or the type of neighboring residue to which the galactose is glycosidically linked (compare spectrum B of **3** to spectrum C of **7**). The way in which changes in structure affect the resonance shifts can be emphasized by subtracting, from the galactose carbonyl resonances, shifts of corresponding carbonyl carbon atoms of compound **2**, the peracetylated methyl galactoside. These differences are shown in Table II, along with similarly calculated shift-differences for other peracetylated disaccharides. While the shift differences, taken collectively, are certainly unique for each of the compounds listed, it is as yet unclear as to how they can be interpreted in terms of structure.

One of the goals of the present study was to investigate whether the pattern of carbonyl resonances observed in the spectrum of a peracetylated oligosaccharide of unknown structure could be used to yield information as to (*a*) what types of peracetylated residues were present, and (*b*) how they were glycosidically linked to other residues. Fig. 4 shows the ranges of assigned carbonyl carbon resonance shifts of all compounds studied to date (over 30). In a few cases, there are carbonyl resonances which are characteristic of specific residue types. For example, carbonyl carbon resonances present between 171.3 and 171.5 p.p.m. in the spectrum of an acetylated unknown can be assigned to the OAc-3-group of acetylated *N*-acetyl-α-D-glucosamine residues. A single resonance between 169.1 and 169.3 p.p.m. and

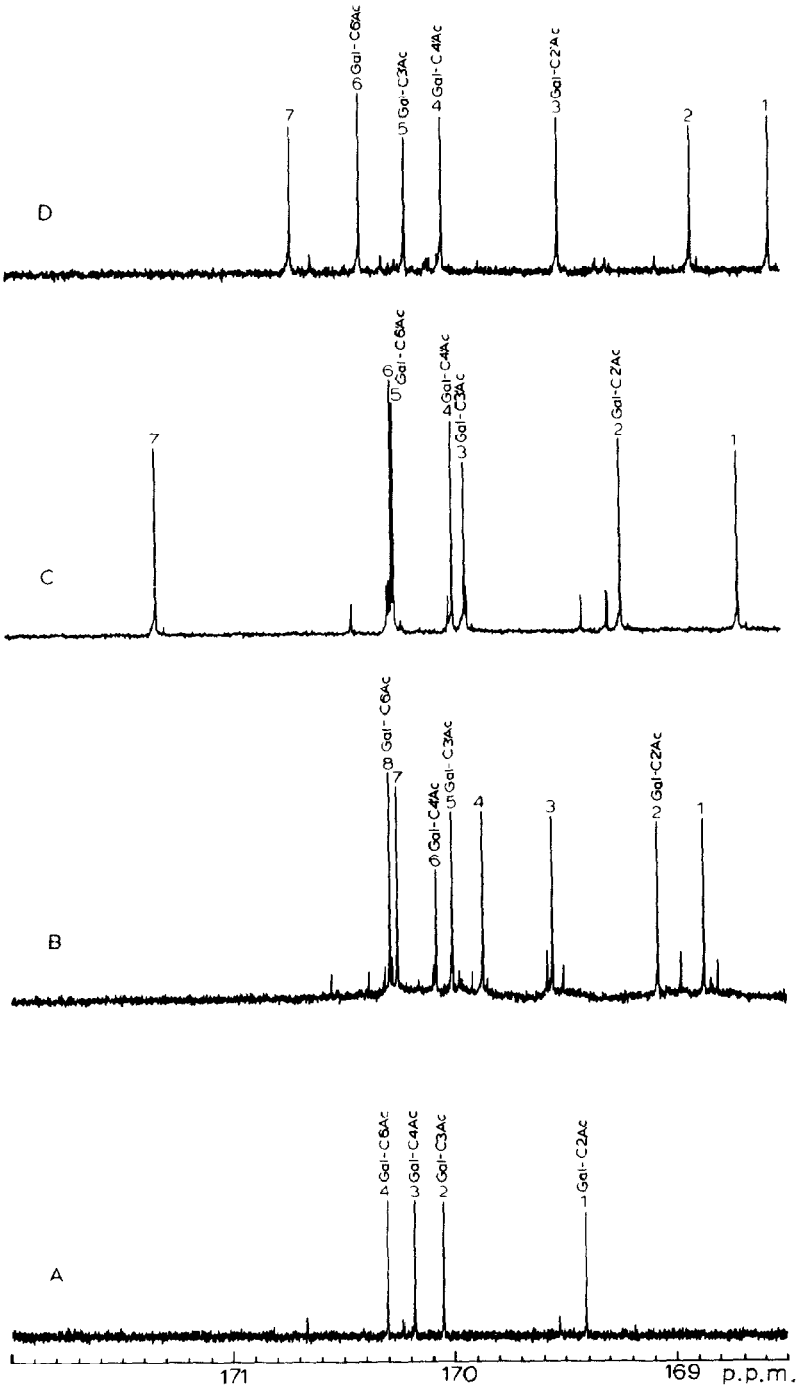


Fig. 3. Carbonyl carbon resonances in the ^{13}C-n.m.r. spectra of (A) **2**, (B) **3**, (C) **7**, and (D) **8**. [Spectra are an average of 32 transients, each with an acquisition time of 30 s and a 10-s wait time between successive acquisitions.]

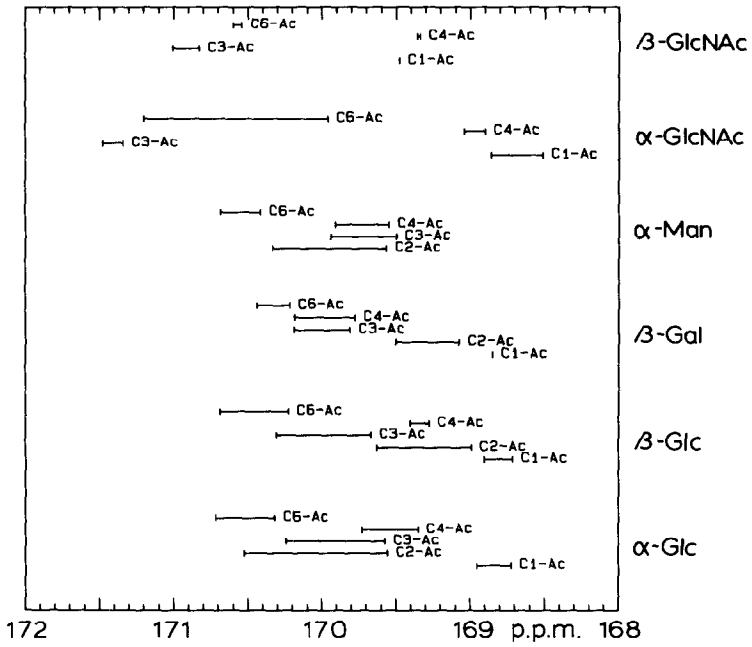


Fig. 4. Shift ranges of carbonyl resonances in peracetylated residues contained in a variety of parent structures. [Shift data for α- and β-D-glucose were taken from spectra of compounds **3**, **4**, and **15–18**, and **1** and **9** of ref. 21; data for β-D-galactose were taken from spectra of compounds **1b**, **2**, **3**, **7**, and **8**; data for α-D-mannose were taken from spectra of compounds **9–12**, and **4a** of ref. 21.]

two resonances between 169.8 and 170.2 p.p.m. appear to be a characteristic spectral pattern of acetylated β-galactose residues. Although the acquisition of n.m.r. data on a greater number of peracetylated compounds may help further to confirm the uniqueness of these spectral patterns, it should be apparent from Fig. 4 that ambiguities will persist. For example, three carbonyl carbon resonances between 169.5 and 169.9 p.p.m. might be indicative of the presence of either acetylated α-glucose or α-mannose residues. These types of degeneracies can, however, be removed if the shifts of resonances arising from pyranosyl-ring and acetyl methyl protons coupled to each carbonyl carbon atom are also considered. Rather than considering each of the resonances in Fig. 4 as a data point in one-dimensional "shift space", they now become data points in three-dimensional shift space. The comprehensive carbonyl carbon chemical-shift data shown in Fig. 4 can then be plotted as shown in Fig. 5. Each of the pseudo-three-dimensional cubes shown contains shift data for the acetyl groups of four different types of residue occurring in a variety of parent compounds. Different types of data points represent the assignments made from ^{13}C–^{1}H shift-correlation spectra. Open circles, filled circles, open triangles, filled triangles, and open diamonds represent shift data for acetyl groups substituted at O-1, O-2, O-3, O-4, and O-6.

TABLE II

CHEMICAL SHIFT DIFFERENCES BETWEEN CARBONYL CARBON RESONANCES AND CORRESPONDING CARBON RESONANCES OF PERACETYLATED GLYCOPYRANOSES AND METHYL GLYCOPYRANOSIDES[a]

Assignment	*Compound*			
	3 *β-Gal-(1→4)-α-Glc*	**7** *β-Gal-(1→4)-α-GlcNAc*	**8** *β-Gal-(1→3)-α-GlcNAc*	**10** *α-Man-(1→3)-α-Man-OMe*
C1Ac	0.14	0.05	−0.05	
C2Ac	0.26			0.52
C3Ac	−0.59	−0.14		
C4Ac			−0.14*	0.26
C6Ac	−0.27	0.19	0.65	0.19
C2′Ac	−0.33	−0.19	−0.10*	0.07
C3′Ac	−0.05	−0.12	0.15	−0.20
C4′Ac	−0.10	−0.19	−0.15	0.24
C6′Ac	−0.04	−0.05	0.09	0.14
	11 *α-Man-(1→4)-α-Man-OMe*	**12** *α-Man-(1→6)-α-Man-OMe*	**15** *β-Glc-(1→4)-β-Glc*	
C1Ac			−0.08	
C2Ac	−0.25*(−0.13)	0.20	0.25	
C3Ac	0.02*(−0.10)	0.07*	−0.37	
C4Ac		0.17		
C6Ac	−0.08		−0.28	
C2′Ac	0.52	0.01	−0.41	
C3′Ac	−0.21	−0.09*	−0.19	
C4′Ac	−0.10	0.09	−0.13	
C6′Ac	−0.07	0.05	−0.29	

	16 *α-Glc-(1→4)-β-Glc*	**17** *β-Glc-(1→6)-α-Glc*	**18a** *α-Glc-(1→6)-α-Glc*	**18b** *α-Glc-(1→6)-β-Glc*
C-1	−0.12	−0.07	0.06	−0.04
C-2	0.39	0.04	0.04*	0.07
C-3	0.04	0.06	−0.07	0.10
C-4		0.05	0.02	0.00
C-6	−0.10			
C-2′	0.39	0.11	0.13	0.18
C-3′	−0.24	−0.06	−0.08	−0.14
C-4′	−0.21	−0.02	0.07*	0.04
C-6′	−0.22	−0.06	−0.07	−0.04

[a]Chemical-shift differences were calculated by subtracting carbonyl carbon chemical shifts of the most structurally similar peracetylated monomer from corresponding shifts of the parent compounds. For example, shift differences for **7** were calculated by subtracting the carbonyl carbon shifts of compounds **2** and **5** from shifts of corresponding carbon atoms in compound **7**. Data for compounds **15–18** were taken from Table III of ref. 21.

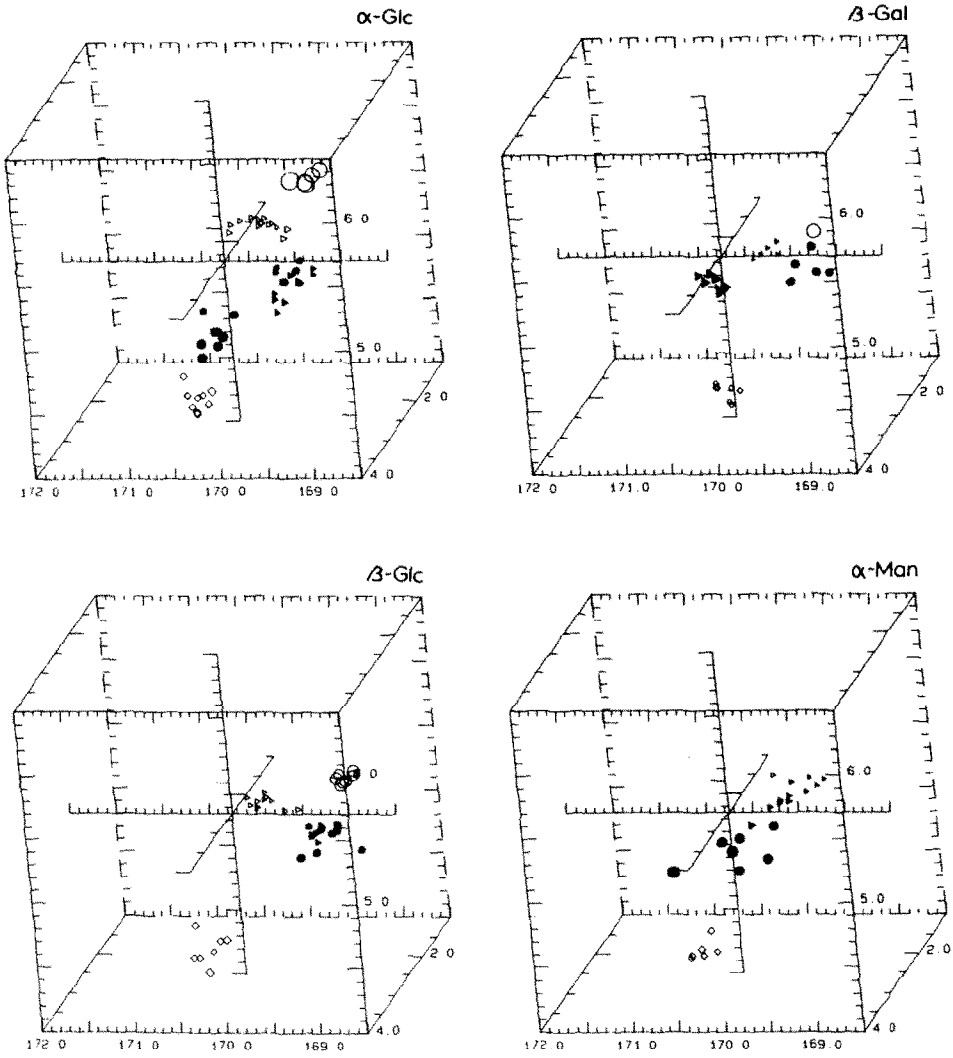


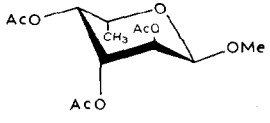
Fig. 5. Resonance shifts of carbonyl carbon atoms and nearest-neighboring acetyl methyl protons and pyranosyl-ring protons, taken from the data of Table I, and Tables II, III, and IV in ref. 21. [Different data-point types represent assigned substituents. Key: (○) 1-*O*-acetyl, (●) 2-*O*-acetyl, (▽) 3-*O*-acetyl, (▼) 4-*O*-acetyl, and (◊) 6-*O*-acetyl. Points are plotted in pseudo-three-dimensional shift-space with larger points lying nearer towards the front of the cube.]

Close inspection of the data in Fig. 5 reveals two general conclusions that can be made, as well as some interesting "anomalies". For each residue type there is a clustering of different types of data points. The physical interpretation of this is that a particular acetyl group of a particular residue type has a limited range of shifts associated with it, irrespective of the parent compound in which it occurs. Whether this is due to the limited number of compound studied or is a result of the

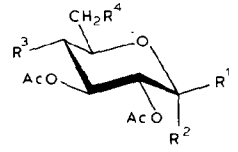
insensitivity of shifts associated with a particular acetyl group to structural variations is a question that cannot be answered completely at present. It may also be seen that, when all of the clusters are considered together, the pattern formed is unique to each type of residue. For example, the shift data for the acetyl group substituted at O-2 in β-glucose (filled circles) appears to overlap corresponding data of β-galactose. However, the region of shift space occupied by the acetyl group substituted at O-4 (filled triangles) is clearly different in the two types of residue.

In some cases, there appear to be data points in Fig. 5 which lie in a region of shift space apart from a cluster of data points of the same type. These "anomalies" usually have as a basis the unique structures of parent compounds for which the data were taken. For example, one of the data points representing the 2-*O*-acetyl group of α-mannose (filled circle) appears to be removed from the region of space occupied by similar data points. Reference to the original data shows that the parent compound from whose spectrum the "anomalous" data were taken is glycosidically linked to a neighboring residue through the nearby 3-hydroxyl group (compound **10**). Apparently, the proximity of this linkage perturbs the shifts of AcO-2 and H2-AcMe. Data points representing shifts of 2-*O*-acetyl groups of acetylated α-glucose residues fall into two regions of space. Reference back to the raw data shows that one of the subgroups was taken from shift data of 2-*O*-acetyl groups neighboring 1-*O*-acetyl groups, while the other subgroup arises from compounds glycosidically linked at C-1.

In the shift difference data of Table II, trends appear which suggest that the shifts of carbonyl carbon resonances in any residue (with respect to a reference peracetylated glycoside) can be correlated with the type of glycosidic linkage made to other residues. For example, all carbonyl resonances of peracetylated β-Gal and β-Glc are shifted upfield with respect to corresponding reasonances of peracetylated β-Gal-OMe and β-Glc-OMe when linked from their anomeric carbon atoms to O-4 of an adjoining residue, as in **3, 7** and **15**. Similarly, when a (1→4) linkage is made from peracetylated α-Glc or α-Man, as in **11** and **16**, the AcO-2′ resonance is shifted downfield and those of AcO-3′, AcO-4′ and AcO-6′ are shifted upfield with respect to peracetylated α-Glc-OMe and α-Man-OMe. With a few



13



15 $R^1 = R^4 = OAc, R^2 = H, R^3 = 2,3,4,6\text{-}Ac_4\text{-}\beta\text{-}Glc$

16 $R^1 = R^4 = OAc, R^2 = H, R^3 = 2,3,4,6\text{-}Ac_4\text{-}\alpha\text{-}Glc$

17 $R^1 = R^3 = OAc, R^2 = H, R^4 = 2,3,4,6\text{-}Ac_4\text{-}\beta\text{-}Glc$

18 a $R^1 = H, R^2 = R^3 = OAc, R^4 = 2,3,4,6\text{-}Ac_4\text{-}\alpha\text{-}Glc$

18 b $R^1 = R^3 = OAc, R^2 = H, R^4 = 2,3,4,6\text{-}Ac_4\text{-}\alpha\text{-}Glc$

exceptions, similar trends are apparent for acetylated α-Glc and α-Man which are joined (1→6) to a neighboring residue, as in **12, 18a**, and **18b**. It is also true that the shift data used in constructing Table II is one dimension of the data used in constructing Fig. 5. Although not obvious from the Figure, due to the limited amount of data, subclusters of data points should appear according to linkage types made to, or from, peracetylated residues in the parent compound. Hence, data similar to those shown in Fig. 5 may ultimately be used in a pattern-recognition manner for determining structures of unknowns from their ^{13}C–^{1}H shift-correlation spectra.

CONCLUSIONS

The peracetylation of complex carbohydrates appears to offer several advantages insofar as the determination of primary structures by n.m.r. spectroscopy is concerned. For all peracetylated compounds studied so far, pyranosyl-ring proton resonances are well resolved and can be easily assigned by using COSY spectra. By comparison, shifts of only a few reporter-group proton resonances of nonderivatized oligosaccharides occur in a relatively narrow region of the spectrum, free from other overlapping pyranosyl-ring proton or solvent resonances. In order to determine primary structure, the shifts and coupling constants of these few reporter-group resonances are relied on. In contrast the method that has been presented makes use of the shifts (and coupling constants) of all pyranosyl-ring protons, acetyl methyl protons, and carbonyl carbon resonances. When and if two or more structures or substructures have resonances that overlap in one of these chemical-shift dimensions, they usually do not overlap in another. This gives rise to a unique set of data for each type of residue occurring in a parent carbohydrate structure.

Finally, the feasibility of acquiring ^{13}C–^{1}H shift-correlation data on reasonably small quantities of material (5–10 mg) has been demonstrated. We anticipate that similar data could be obtained on even smaller quantities (1–5 mg) by utilizing ^{1}H-detected ^{13}C–^{1}H shift-correlation experiments[37] at higher magnetic-field strengths.

ACKNOWLEDGMENTS

The author thanks Dr. Clifford Unkefer for his help in preparing many of the compounds used in this study and Dr. J. Montreuil for his donation of β-Gal-(1→3)-GlcNAc. This work was supported by the Robert A. Welch Foundation (AT-885), by Associated Western Universities, and by the NIH (RR02231)-USDOE/OHER Stable Isotope Resource at Los Alamos, NM.

REFERENCES

1 W. M. WATKINS, in A. GOTTSCHALK (Ed.), *Glycoproteins*, Part B, 2nd edn., Elsevier, Amsterdam, 1972, pp. 830–891.
2 A. G. DARVILL AND P. ALBERSHEIM, *Annu. Rev. Plant Physiol.*, 35 (1984) 243–275.
3 C. A. BUSH, A.-Y. YAN, AND B. N. N. RAO, *J. Am. Chem. Soc.*, 108 (1986) 6168–6173.
4 E. BERMAN, *Biochemistry*, 23 (1984) 3754–3759.
5 J.-R. BRISSON AND J. P. CARVER, *Biochemistry*, 22 (1983) 1362–1368.
6 J.-R. BRISSON AND J. P. CARVER, *Biochemistry*, 22 (1983) 3671–3680.
7 J.-R. BRISSON AND J. P. CARVER, *Biochemistry*, 22 (1983) 3680–3686.
8 R. U. LEMIEUX, K. BOCK, L. T. J. DELBAERE, S. KOTO, AND V. S. RAO, *Can. J. Chem.*, 58 (1980) 631–653.
9 H. J. JENNINGS, C. LUGOWSKI, AND D. L. KASPER, *Biochemistry*, 20 (1981) 4511–4518.
10 S. W. HOMANS, R. A. DWEK, J. BOYD, M. MAHMOUDIAN, W. G. RICHARDS, AND T. W. RADEMACHER, *Biochemistry*, 25 (1986) 6342–6350.
11 J. P. CARVER, *Biochem. Soc. Trans.*, 12 (1984) 517–519.
12 J. MONTREUIL, *Adv. Carbohydr. Chem. Biochem.*, 37 (1980) 157–223.
13 J. F. G. VLIEGENTHART, H. VAN HALBEEK, AND L. DORLAND, *Pure Appl. Chem.*, 53 (1981) 45–77.
14 J. F. G. VLIEGENTHART, L. DORLAND, H. VAN HALBEEK, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 209–374.
15 A. ALLERHAND AND E. BERMAN, *J. Am. Chem. Soc.*, 106 (1984) 2400–2412.
16 A. ALLERHAND AND E. BERMAN, *J. Am. Chem. Soc.*, 106 (1984) 2412–2420.
17 E. BERMAN, A. ALLERHAND, AND A. L. DEVRIES, *J. Biol. Chem.*, 255 (1980) 4407–4410.
18 K. DILL, E. BERMAN, AND A. A. PAVIA, *Adv. Carbohydr. Chem. Biochem.*, 43 (1985) 1–49.
19 L. O. SILLERUD, R. K. YU, AND D. E. SCHAFER, *Biochemistry*, 21 (1982) 1260–1271.
20 T. A. GERKEN AND N. JENTOFT, *Biochemistry*, 26 (1987) 4689–4699.
21 W. J. GOUX AND C. J. UNKEFER, *Carbohydr. Res.*, 159 (1987) 191–210.
22 M. L. APPLETON, C. E. COTTRELL, AND E. J. BEHRMAN, *Carbohydr. Res.*, 158 (1986) 227–235.
23 S. N. DHAWAN, T. L. CHICK, AND W. J. GOUX, *Carbohydr. Res.*, 172 (1988) 297–307.
24 M. HAVERKAMP, H. DE BIE, AND J. F. G. VLIEGENTHART, *Carbohydr. Res.*, 37 (1974) 111–125.
25 J. HAVERKAMP, J. P. C. M. VAN DONGEN, AND J. F. G. VLIEGENTHART, *Tetrahedron*, 29 (1973) 3431–3439.
26 L. PONCINI, *Bull. Soc. Chim. Belg.*, 90 (1981) 57–62.
27 M. L. WOLFROM AND A. THOMPSON, *Methods Carbohydr. Chem.*, 2 (1963) 211–215.
28 M. F. CZARNIECKI AND E. R. THORNTON, *J. Am. Chem. Soc.*, 99 (1977) 8273–8278.
29 D. G. OTTO, *Synthesis with Stable Isotopes*, Wiley, New York, 1981, pp. 36–37.
30 D. MARION AND K. WUTHRICH, *Biochem. Biophys. Res. Commun.*, 113 (1983) 967–974.
31 A. ALLERHAND, R. E. ADDLEMAN, AND D. OSMAN, *J. Am. Chem. Soc.*, 107 (1985) 5809–5810.
32 A. J. SHAKA, J. KEELER, T. FRENKIEL, AND R. J. FREEMAN, *Magn. Reson.*, 52 (1983) 335–338.
33 A. J. SHAKA, J. KEELER, AND R. J. FREEMAN, *J. Magn. Reson.*, 53 (1983) 313–340.
34 A. BAX, *Two-Dimensional Nuclear Magnetic Resonance in Liquids*, Delft University Press, Delft, Holland, 1982, pp. 50–68.
35 D. Y. GAGNAIRE, F. R. TARAVEL, AND M. R. VIGNON, *Carbohydr. Res.*, 51 (1976) 157–168.
36 A. DELL AND P. R. TILLER, *Biochem. Biophys. Res. Commun.*, 135 (1986) 1126–1134.
37 A. BAX AND M. F. SUMMERS, *J. Am. Chem. Soc.*, 108 (1986) 2093–2094.