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Nuclear magnetic resonance spectroscopy of peracetylated oligosaccharides having ^{13}C-labeled carbonyl groups in lieu of permethylation analysis for establishing linkage substitutions of sugars☆

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Abstract

Peracetylation of free hydroxyl groups in model saccharides with [^{13}C-carbonyl]acetic anhydride resulted in additional splittings of sugar ring proton signals in NMR spectra, due to 3-bond *J* couplings between each acetyl carbonyl carbon and a sugar ring proton at that position. Quantification of 144 of these 3-bond coupling constants in different saccharide structures showed a range between 2.5 and 4.7 Hz, whereas all possible 4-bond couplings between sugar ring protons and acetyl carbonyl carbons were within linewidth (<0.5 Hz). Therefore, further splitting of sugar ring proton signals in the range of 2.5–4.7 Hz upon acetylation with a [^{13}C-carbonyl]acetyl group identifies that position as (formerly) having a free hydroxyl group. This performs the same basic function as permethylation analysis, but does not require hydrolysis of glycosidic linkages. Additionally, proton-detected 2D heteronuclear multiple bond correlation (HMBC) experiments or proton-detected heteronuclear correlation spectroscopy (hetCOSY) enabled ring proton–carbonyl-^{13}C 3-bond *J* connectivities to be correlated with high sensitivity. Modified NMR pulse sequences are reported that include frequency selective decoupling schemes to enable coupling constants to be determined from 2D data. The tailored pulse sequences resulted in higher spectral resolution and sensitivity for [^{13}C-carbonyl]–ring proton correlations.

Keywords: Carbohydrates; Oligosaccharides; Peracetylation; NMR; Linkage analysis; Sugar ring ^{1}H-acetyl carbonyl ^{13}C *J* couplings

1. Introduction

Permethylation analysis of carbohydrates has been an important tool for establishing linkage substitutions of monosaccharides in oligosaccharides [3–6]. The basic logic of the methods so developed is that, by converting every free hydroxyl group into a methyl ether, the original positions of all free hydroxyl groups can be established. By default, all other positions are either involved in a glycosidic linkage, a ring cyclization (pyranoside or furanoside), or are substituted with a functional group that is stable to the methylation conditions.

NMR has been used extensively for primary structural elucidation of oligosaccharides having natural abundance isotopes [7–10], where the goals are essentially twofold. First, assignment and quantification of through-bond

☆ Preliminary reports have been presented [1,2].

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homonuclear couplings ($^2J_{H,H}$ and $^3J_{H,H}$), via 2D experiments such as COSY and TOCSY yields, in principle, the information required to assign each monosaccharide spin system to a characteristic stereochemistry, including anomeric configuration and ring form (excluding the enantiomer). Second, observation of any true through-bond correlations of nuclei between monosaccharide residues yields the information needed to establish the sites of linkages between them.

In practice, the major impediments in meeting the foregoing two goals are first, that spectral dispersion in two dimensions is often inadequate to permit quantitation of $^2J_{H,H}$ and $^3J_{H,H}$ couplings from multiplet patterns because of severe overlap. Second, establishing linkage positions between sugars requires that heteronuclear multiple-bond correlations ($^3J_{C,H}$, HMBC) across glycosidic linkages be observed. These are limited in sensitivity due to the low ^{13}C natural abundance. The strongest nuclear Overhauser enhancements observed between monosaccharides may not be between two protons directly across a glycosidic bond [11], which also applies to long range interresidue $^1H-^1H$ couplings sometimes seen in gradient COSY experiments [12] and so neither are adequate for proof of linkage.

Peracetylated oligosaccharides have been studied with interest in the additional spectral dispersion observed for sugar ring protons as compared with the non-acetylated structures [13,14]. Goux and co-workers [15–17] prepared peracetylated carbohydrate derivatives having ^{13}C-labeled carbonyl groups. They were interested in assignments of chemical shifts of ^{13}C signals, and used ^{13}C NMR spectroscopy to record carbon frequencies and ^{13}C-detected heteronuclear NMR to detect $^{13}C-^1H$ correlations. As they detected ^{13}C signals, the quantities of sugar samples they required were in the range of 5–100 mg [16], and they focused primarily on chemical shift pattern recognition methods [17]. They did not report proton-detected heteronuclear experiments and did not report values for specific $^3J_{C,H}$ coupling constants, $^4J_{C,H}$ couplings, or ranges of these coupling constants, the knowledge of which is crucial to making positional assignments.

The aims of this study were: (1) To prepare a series of peracetylated carbohydrate structures with [^{13}C-carbonyl]-labeled acetyl groups, and their natural abundance counterparts. (2) To establish the range in the values of $^3J_{C,H}$ coupling constants between carbonyl carbons and ring protons at the same position on the sugar ring. (3) To establish the range in the values of $^4J_{C,H}$ coupling constants between carbonyl carbons and ring protons at adjacent positions on the sugar ring. (4) To develop ^1H-detected heteronuclear 2D NMR methods to assign and quantitate $^1H-^{13}C$ couplings between sugar ring protons and acetyl carbonyl carbons. Based on the data of this study, it is evident that the methods provide the same basic information as permethylation analysis, i.e., by converting every free hydroxyl group into a [^{13}C-carbonyl]acetyl ester, the original positions of all free hydroxyl groups can be established. By default, other positions are either involved in a glycosidic linkage, a ring cyclization, or are substituted with a functional group that is stable to peracetylation.

2. Results and discussion

Peracetylation was performed by standard procedures using acetic anhydride or [^{13}C-carbonyl]-labeled acetic anhydride and pyridine, washed once with $CDCl_3$, and transfered to an NMR tube in $CDCl_3$. To examine for exchangable amide protons, present in some compounds, spectra were directly acquired. However, the chemical shifts of these and other proton signals were not highly reproducible in $CDCl_3$ (without D_2O), which may be due to variable amounts of dissolved water that were difficult to control. As shown in Fig. 1(a) and (b), addition of 10 μL of D_2O to samples saturated the $CDCl_3$, which resulted in exchange of amide protons and gave very reproducible ^1H chemical shifts (± 0.002 ppm, relative to Me_4Si at 27 °C).

All compounds were prepared with standard acetic anhydride and, in separate experiments, [^{13}C-carbonyl]-labeled acetic anhydride, and their 1D spectra were compared.

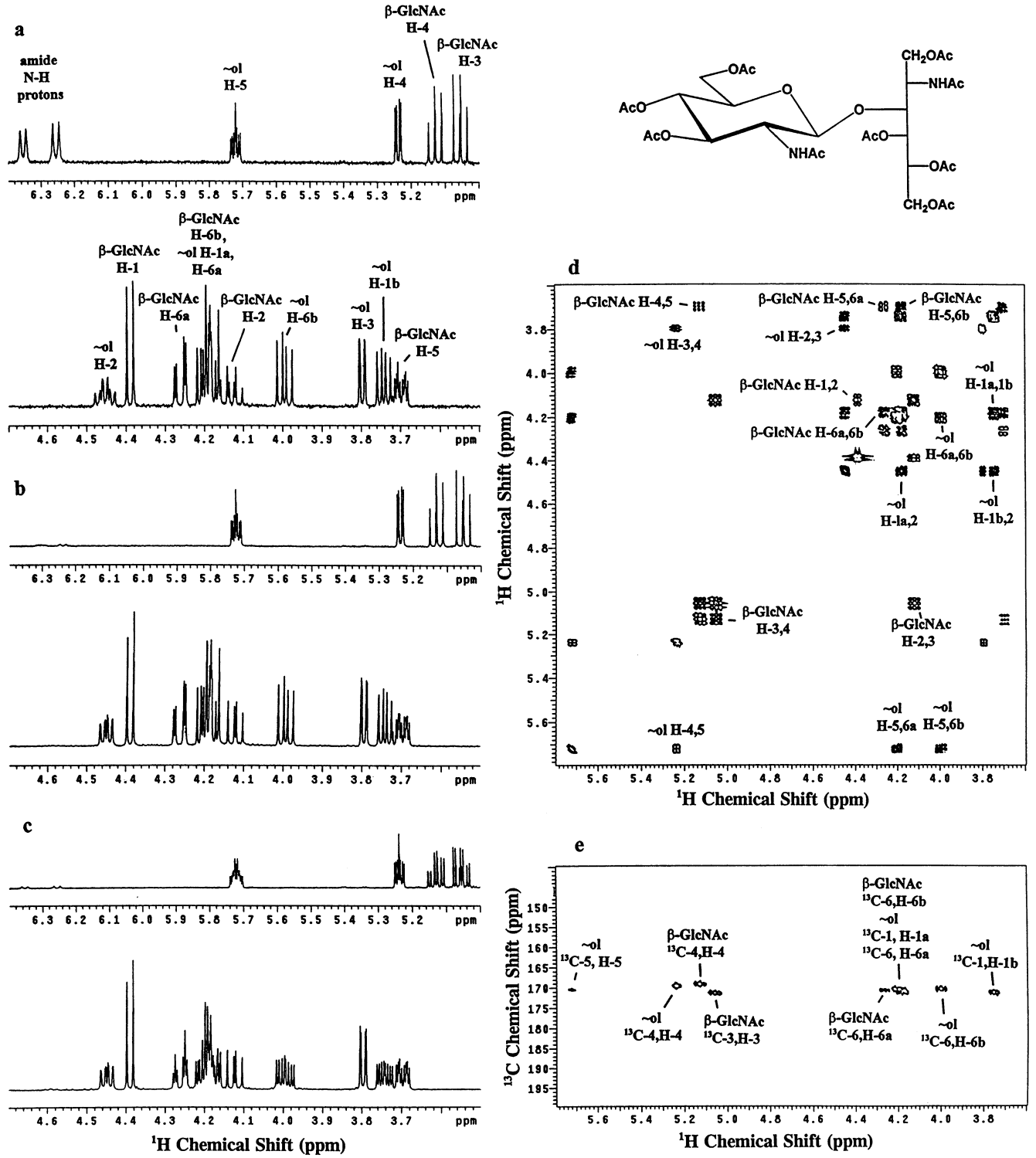

Fig. 1. NMR spectra of 200 μg of the peracetylated disaccharide-alditol, β-D-Glc*p*NAc-(1→3)-D-GalNAc-ol. Shown are the regions of ^{1}H frequencies containing the sugar backbone proton signals and for (e), the carbonyl carbon frequencies. (a) and (b) The 1D spectra of the compound containing natural abundance carbon in acetyl groups, immediately after addition of D_2O (a) and after 48 h (b). (c) The 1D spectrum of the compound with the *O*-acetyl groups containing [^{13}C-carbonyl] carbons, after exchange of amide protons. (d) The 2D gradient COSY spectrum of the compound having natural abundance carbon in acetyl groups, after exchange of amide protons. (e) The 2D gHMBC spectrum of the compound with the *O*-acetyl groups containing [^{13}C-carbonyl] carbons, after exchange of the amide protons. Pertinent 1D signal assignments and 2D crosspeak assignments are indicated. The ^{13}C carbons indicated refer to the acetyl carbonyl carbons, selectively enriched.

Gradient COSY (gCOSY) spectra of all standard peracetylated products were carried out to determine proton assignments, and in regions of spectral overlap in 1D spectra, to establish those proton coupling constants. A comparison of the 1D spectra of the non-labeled versus ^{13}C-labeled products is shown for two peracetylated compounds, β-D-Glc*p*NAc-(1→3)-D-GalNAc-ol, and β-D-Glc*p*-(1→4)-D-Glc-ol (Fig. 1(b) and (c), and Fig. 2(a) and (b)). Immediately apparent in these comparisons are the additional ^{13}C–^{1}H couplings, which for compounds of this size, enabled most of the ^{13}C–^{1}H couplings to be directly quantified. gCOSY spectra of the non-labeled compounds yielded the $^{2}J_{H,H}$ and $^{3}J_{H,H}$ couplings, along with the standard 1D spectra (Figs. 1 and 2, Tables 1–3).

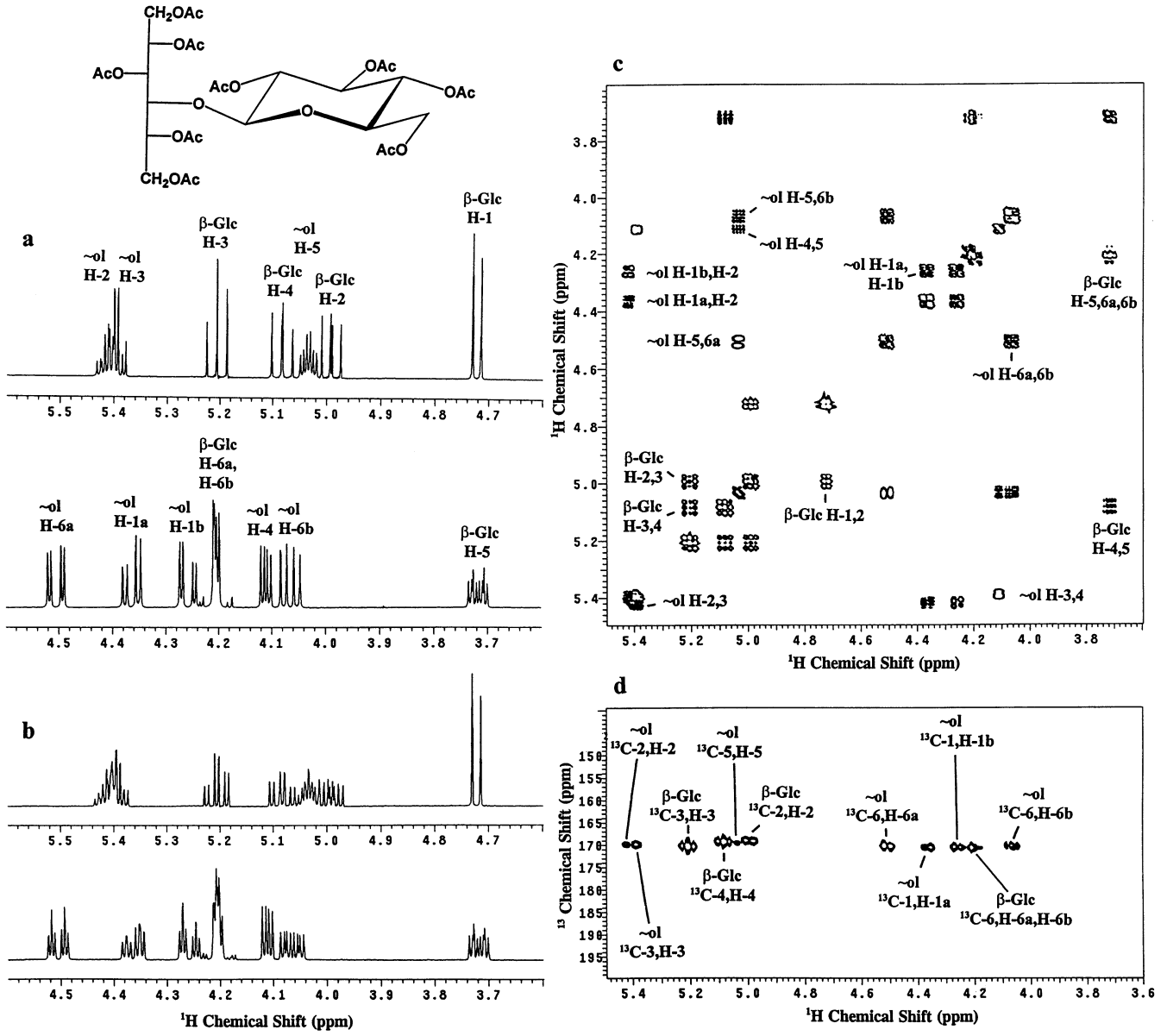

Fig. 2. NMR spectra of 500 μg of the peracetylated disaccharide-alditol, β-D-Glc*p*(1→4)-D-Glc-ol. Shown are the regions of ^{1}H frequencies containing the sugar backbone proton signals, and for (d), the carbonyl carbon frequencies. (a) The 1D spectrum of the compound containing natural abundance carbon in acetyl groups. (b) The 1D spectrum of the compound with the *O*-acetyl groups containing [^{13}C-carbonyl] carbons. (c) The 2D gCOSY spectrum of the compound having natural abundance carbon in acetyl groups. (d) The 2D gHMBC spectrum of the compound with the *O*-acetyl groups containing [^{13}C-carbonyl] carbons. Pertinent 1D signal assignments and 2D crosspeak assignments are indicated. The ^{13}C carbons indicated refer to the acetyl carbonyl carbons, selectively enriched.

Table 1
^{1}H chemical shifts, $^{2or3}J_{H,H}$ coupling constants between ring protons, and $^{3}J_{C,H}$ coupling constants between ^{13}C-carbonyl carbons and ring protons for five [^{13}C-carbonyl]-labeled peracetylated disaccharide-alditols having 2-acetamido-2-deoxy sugars[a,b]

Chemical shift, δ, ppm	($^{2or3}J_{H,H}$), Hz	[$^{3}J_{C,H}$], Hz	Structure													
			β-D-Glc*p*NAc-(1→3)-D-GalNAc-ol			β-D-Gal*p*-(1→3)-D-GalNAc-ol			α-D-Gal*p*NAc-(1→3)-D-GlcNAc-ol			β-D-Glc*p*NAc-(1→4)-D-GlcNAc-ol			β-D-Glc-*p*-NAc-(1→6)-D-Gal-ol		
Alditol																	
H-1a	($J_{1a,1b}$)	[$J_{1,1a}$]	4.181	(−10.6)	[3.1]	3.951	[c]	[3.0]	4.174	(−11.2)	[3.0]	4.340	(−11.8)	[3.1]	4.282	(−11.7)	[3.1]
H-1b	($J_{1a,2}$)	[$J_{1,1b}$]	3.741	(9.4)	[3.0]	3.951	(7.6)[c]	[3.0]	4.091	(6.7)	n.d.[d]	4.019	(5.3)	[3 1]	3.848	(4.7)	[3.8]
H-2	($J_{1b,2}$)	[$J_{2,2}$]	4.450	(5.9)	n.c.o.[e]	4.456	(7.6)[c]	n.c.o.	4.588	(6.5)	n.c.o.	4.699	(4.3)	n.c.o.	5.277	(7.3)	[4.1]
H-3	($J_{2,3}$)	[$J_{3,3}$]	3.794	(0.8)	n.c.o.	3.964	(0.8)	n.d.[d]	3.994	(2.8)	n.c.o.	5.154	(7.9)	[4.4]	5.308[f]	(<1.0)	[2.8][f]
H-4	($J_{3,4}$)	[$J_{4,4}$]	5.237	(6.7)	[4.7]	5.216	(7.2)	[4.6]	5.187	(2.8)	[4.2]	4.147	(3.5)	n.c.o.	5.308[f]	n.d.[g]	[2.8][f]
H-5	($J_{4,5}$)	[$J_{5,5}$]	5.723	(2.2)	[3.9]	5.666	(2.2)	[3.9]	5.346	(5.0)	[3.8]	5.316	(7.3)	[3.8]	5.220	(<1.0)	[4.1]
H-6a	($J_{5,6a}$)	[$J_{6,6a}$]	4.201	(4.9)	[3.0]	4.208	(5.2)	[3.0]	4.347	(4.8)	[2.9]	4.480	(2.9)	[3.0]	3.835	(5.1)	n.c.o.
H-6b	($J_{5.6b}$)	[$J_{6,6b}$]	3.994	(7.1)	[3.3]	3.996	(6.9)	[3.2]	4.074	(6.0)	[3.5]	4.167	(5.9)	[3.0]	3.488	(7.2)	n.c.o.
	($J_{6a,6b}$)			(−11.7)			(−11.7)			(−12.0)			(−12.3)			(−10.8)	
Pyranoside																	
H-1	($J_{1,2}$)		4.389	(8.3)	n.c.o.	4.544	(7.8)	n.c.o.	5.028	(3.7)	n.c.o.	5.089	(8.4)	n.c.o.	4.795	8.3	n.c.o.
H-2	($J_{2,3}$)	[$J_{2,2}$]	4.121	(10.8)	n.c.o.	5.180	(10.5)	[3.9]	4.714	(11.7)	n.c.o.	3.852	(10.5)	n.c.o.	3.585	(10.5)	n.c.o.
H-3	($J_{3,4}$)	[$J_{3,3}$]	5.052	(9.4)	[3.7]	5.045	(3.4)	[3.4]	5.080	(3.1)	[3.4]	5.369	(9.3)	[3.9]	5.411	(9.2)	[3.8]
H-4	($J_{4,5}$)	[$J_{4,4}$]	5.131	(9.9)	[4.0]	5.406	(1.1)	[4.0]	5.399	(1.1)	[4.1]	5.001	(10.0)	[4.0]	5.019	(10.0)	[4.1]
H-5	($J_{5,6a}$)		3.696	(2.5)	n.c.o.	3.979	(6.8)[h]	n.d.[d]	4.273	(6.6)	n.c.o.	3.721	(2.4)	n.c.o.	3.701	(4.8)	n.c.o.
H-6a	($J_{5,6b}$)	[$J_{6,6a}$]	4.263	(4.0)	[2.5]	4.169	(6.8)[h]	[3.2]	4.114	(6.6)	[3.0]	4.212	(5.8)	[2.7]	4.254	(2.5)	[3.3]
H-6b	($J_{6a,6b}$)	[$J_{6,6b}$]	4.178	(−12.4)	[3.4]	4.169	[h]	[3.2]	4.072	(−11.2)	[3.7]	4.156	(−12.3)	n.d.[d]	4.112	(−12.3)	[2.5]
N-acetyl methyls																	
			1.952			1.938			2.026			1.942			1.952		
			1.955						2.031			2.005					
O-acetyl methyls[b]																	
			2.023			1.999			2.012			2.028			2.015		
			2.031			2.040			2.056			2.028			2.021		
			2.058			2.055			2.061			2.073			2.021		
			2.094			2.077			2.072			2.076			2.071		
			2.094			2.096			2.114			2.087			2.084		
			2.094			2.105			2.131			2.090			2.088		
			2.117			2.137			2.175			2.112			2.093		
						2.183									2.114		

[a] For a solution in $CDCl_3$ saturated with D_2O at 27 °C, relative to internal Me_4Si at 0.000 ppm.
[b] Only the *O*-acetyl groups were [^{13}C-carbonyl]-labeled. They were distinguishable from *N*-acetyl methyl groups by their ca. 7 Hz coupling between the carbonyl carbon and the methyl protons.
[c] H-1a and H-1b were tightly coupled; $\frac{1}{2}(J_{1a,2}+J_{1b,2}) = 7.6$ Hz
[d] n.d., not determined. C–H coupling, if present, was not determinable from 1D spectra, due to spectral overlap.
[e] n.c.o., for this entire table, no additional coupling observed; in comparing the ^{13}C-labeled compound with the ^{12}C (natually abundant) isotope. Any $^{4}J_{C,H}$ couplings were within linewidth.
[f] Additional splitting of the tightly coupled signal was observed in the [^{13}C-carbonyl]-labeled compound.
[g] n.d., not determined; as H-3 and H-4 were tightly coupled.
[h] H-6a and H-6b were tightly coupled; $\frac{1}{2}(J_{5,6a}+J_{5,6b}) = 6.8$ Hz

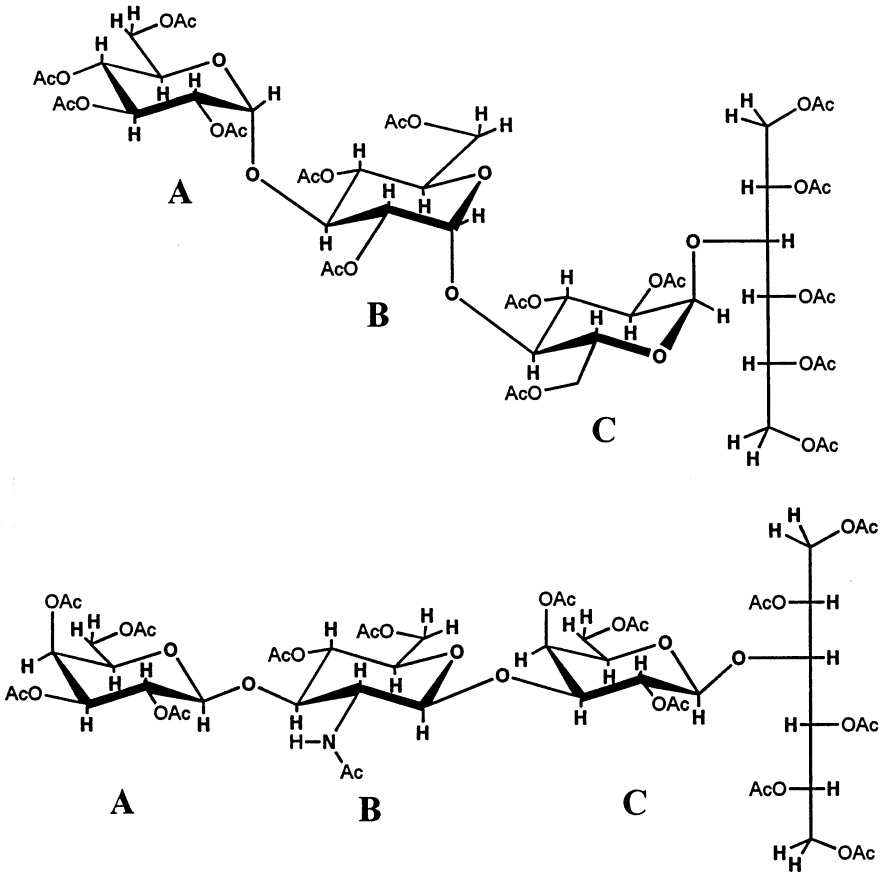

Fig. 3. Structures of peracetylated oligosaccharide alditols described herein. At the top is α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-D-Glc-ol, and at the bottom is β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc-ol. Individual pyranoside rings are labeled for referencing crosspeak multiplets in Table 3 and Figs. 4, 9 and 10.

Two oligosaccharide-alditols (Fig. 3) were also peracetylated with ^{13}C-carbonyl and natural abundance acetyl groups. A comparison of the gCOSY spectra of unlabeled and [^{13}C-carbonyl]-labeled, peracetylated α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-D-Glc-ol is shown in Figs. 4 and 5. A number of crosspeak multiplet patterns differed between the two spectra. Those protons having a $^{3}J_{C,H}$ coupling to a ^{13}C-carbonyl group in the labeled compound showed an additional splitting as compared with the natural abundance compound. In addition, the gCOSY pulse sequence was modified through the inclusion of the GARP decoupling scheme at the carbonyl ^{13}C frequency for the time period shown in Fig. 6(a). The resultant carbon-decoupled gCOSY (cdgCOSY) pulse sequence eliminated the ^{13}C–^{1}H couplings and resulted in the same spectrum as observed for the natural abundance compound (compare multiplets, Fig. 5(a)–(c) to (g)–(i), respectively). Furthermore, application of the GARP decoupling scheme at the carbonyl ^{13}C frequency for a standard ^{1}H 1D experiment during the 90° pulse and acquisition period eliminated the ^{13}C–^{1}H coupling, giving rise to a 1D spectrum identical to the unlabeled compound (data not shown). These experiments were applied to all the compounds described herein with the same results: the 3-bond ^{13}C–^{1}H coupling constants were observed in 1D and 2D gCOSY experiments and these couplings could be eliminated through inclusion of the GARP decoupling scheme in 1D or cdgCOSY pulse sequences.

For [^{13}C-carbonyl]-labeled compounds, gradient HMBC (gHMBC) experiments showed the sugar-ring proton–^{13}C-carbonyl couplings as crosspeaks (Figs. 1(e) and 2(d)). One major advantage to using the ^{13}C label is that gHMBC crosspeaks were detected about 90 times more sensitively than the same experiments using natural abundance carbon. The gHMBC experiment was performed with all

the structures reported herein with similar results. Under the conditions used, only 3-bond ring-proton–^{13}C-carbonyl couplings were observed. No 4-bond couplings were discernible above t_1 noise. One disadvantage of HMBC experiments in general results from ^{1}H–^{1}H J modulation of the net magnetization during the long dephasing/rephasing periods of the pulse sequence, resulting in unresolved ^{1}H–^{13}C multiplet patterns and poor F_1 (^{13}C) resolution. For this reason, direct transfer of ^{13}C magnetization with ^{1}H detection was far preferable for good F_1 resolution. A modified phase-sensitive hetCOSY pulse sequence, with or without a hyperbolic secant decoupling scheme applied at the methyl proton frequency during the t_1 period, is shown in Fig. 6(b). Due to the narrow spectral range of the carbonyl ^{13}C frequencies, high spectral resolution was achievable because the F_1 spectral width could be restricted to 400 Hz. A comparison of spectra obtained without and with the application of the decoupling scheme are demonstrated in Figs. 7 and 8, respectively, using peracetylated β-D-Glc*p*-(1→4)-D-Glc-ol having ^{13}C-carbonyl groups. The 1:3:3:1 splitting pattern of the ^{13}C frequencies due to their coupling to the methyl protons of acetyl groups, as shown in Fig. 7(a), is completely eliminated through methyl proton decoupling (Fig. 8(a)). The complex multiplets are converted, along the ^{13}C axis, to a single splitting, which resulted from a single coupling, the ^{13}C-carbonyl nucleus to a sole ring proton. The selective proton-decoupled hetCOSY (spdhetCOSY) experiment permitted an accurate measurement of these ^{13}C–^{1}H coupling constants. For positions containing *gem*-coupled protons (i.e., C-6), two splittings of the ^{13}C-carbonyl frequency remained, which resulted from the coupling of the ^{13}C-carbonyl nucleus to both protons at that position (compare Fig. 7(b) to Fig. 8(b)). This clearly enabled positions having *gem*-coupled protons to be identified, in addition to the fact that most *gem*-coupled pairs showed two proton chemical shifts which correlated to a single ^{13}C-carbonyl frequency. Two other advantages of using the spdhetCOSY pulse sequence were that resolution between different crosspeak multiplets along the ^{13}C axis and sensitivity were increased due to lack of further splitting of multiplets by methyl protons coupled to the ^{13}C-carbonyl nuclei.

Application of the spdhetCOSY pulse sequence to the two oligosaccharide-alditols of Fig. 3 is shown in Figs. 9 and 10. The excellent F_1 resolution permitted the ^{13}C–^{1}H 3-

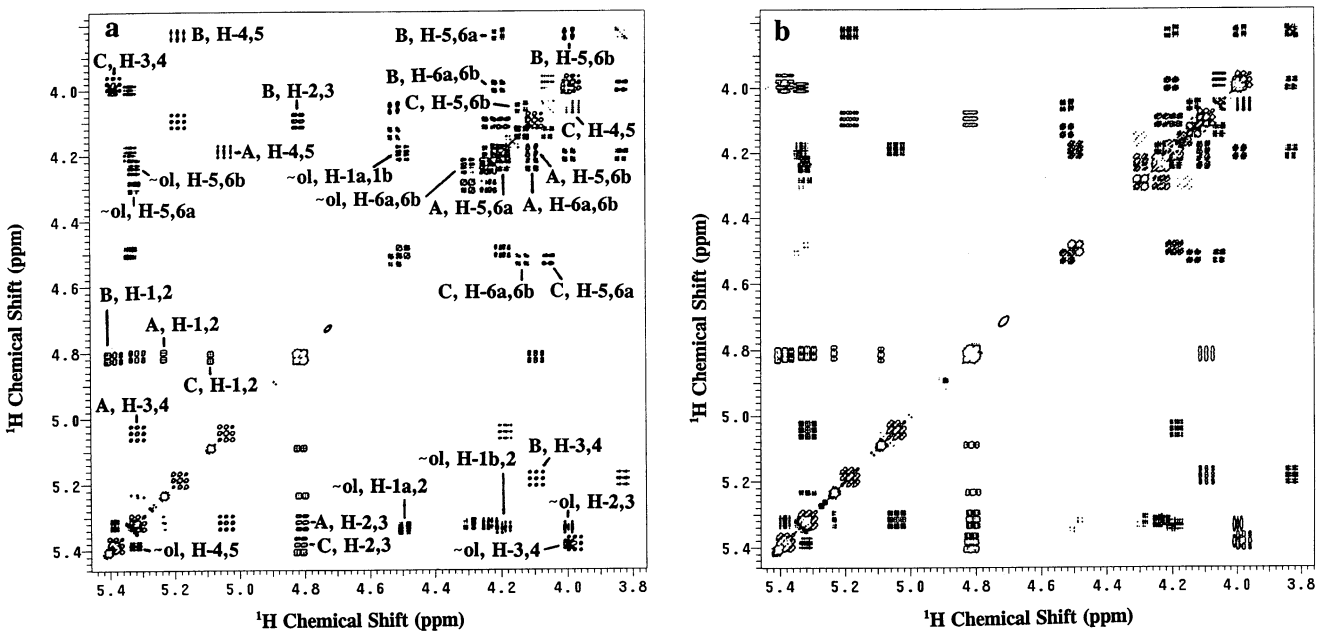

Fig. 4. Gradient COSY spectra of the peracetylated tetrasaccharide alditol, α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-D-Glc-ol. Assignments of crosspeak multiplets are indicated. Shown are the spectra of (a), the compound having natural abundance carbon in acetyl groups and (b), the compound having [^{13}C-carbonyl] acetyl groups.

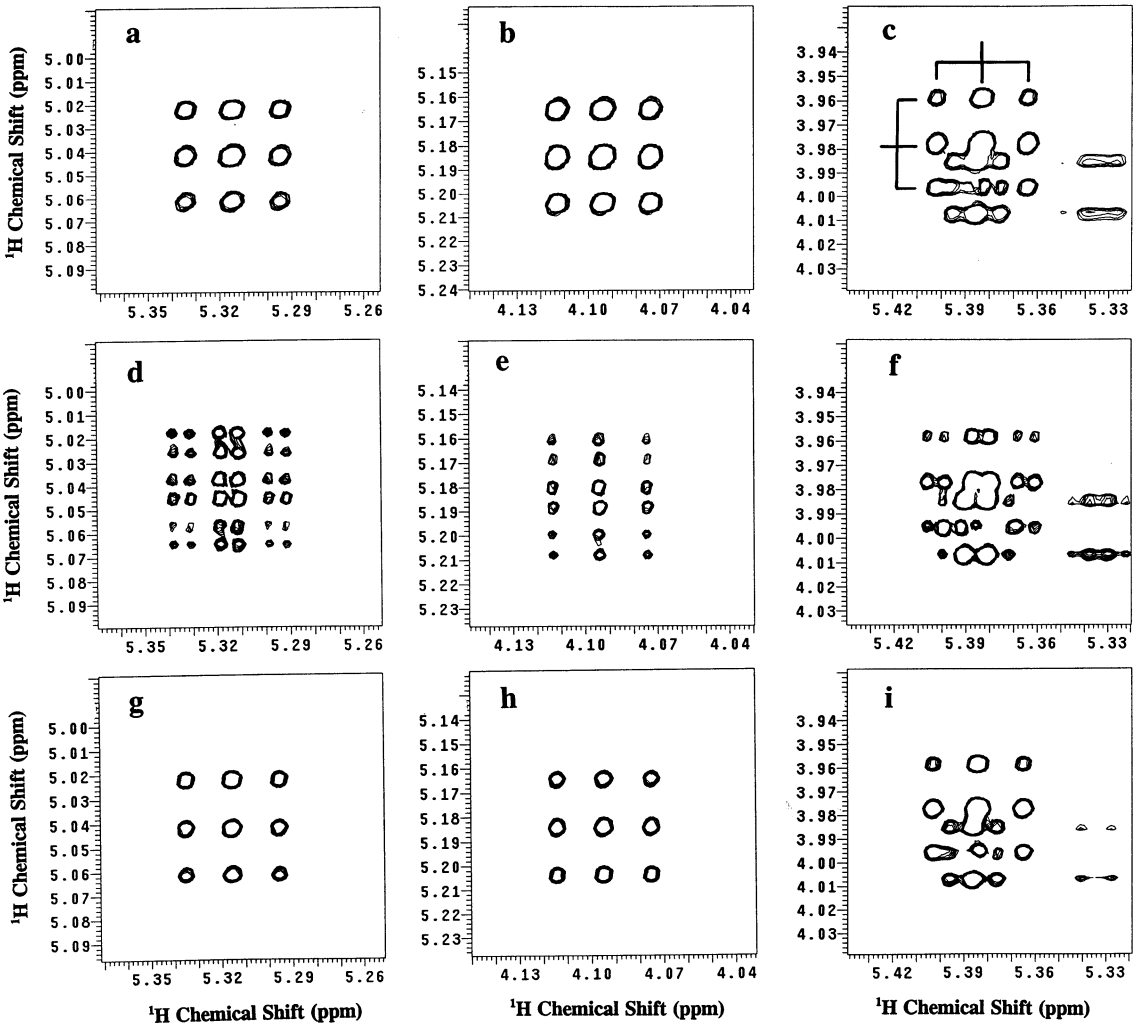

Fig. 5. Expansions of multiplet patterns of crosspeaks of gCOSY spectra of the peracetylated tetrasaccharide alditol α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-D-Glc-ol. Shown are the H-3/H-4 multiplet patterns for: (a)–(c), the standard compound having natural abundance carbon in acetyl groups (expanded from Fig. 4(a)); (d)–(f), the compound having [^{13}C-carbonyl] acetyl groups (expanded from Fig. 4(b)); and (g)–(i) the compound having [^{13}C-carbonyl] acetyl groups, using the cdgCOSY pulse sequence (Fig. 6(a)). All crosspeaks are shown with the H-3 frequency along the horizontal axis and the H-4 frequency along the vertical axis. The multiplet from glucopyranoside A (Fig. 3) is shown in the column (a), (d) and (g), that from glucopyranoside B in (b), (e) and (h), and that from glucopyranoside C in (c), (f) and (i). The H-3/H-4 multiplet pattern for glucopyranoside C partially overlapped with the alditol H-3/H-4 multiplet; crosspeaks arising from the pyranoside are indicated for both dimensions in (c).

bond coupling constants to be determined from the single splitting along the ^{13}C axis, except for the positions having *gem*-coupled pairs, where cancellation in the center of multiplets along the ^{13}C axis occurred as a result of the two similar couplings of both protons to the single carbon nucleus. The latter ^{13}C–^{1}H coupling constants were determined either from the 1D spectra, where possible, or by comparing the gCOSY experiments performed on the compounds with or without [^{13}C-carbonyl]-labeled acetyl groups, or by comparing the gCOSY and cdgCOSY experiments on the compound having the ^{13}C label. Coupling constants obtained were consistent between experiments, with an important criterion being high digital resolution in both dimensions for the 2D experiments.

Evident from Tables 1–3 are two key observations. First, determination of 144 3-bond couplings between ^{13}C-carbonyl carbons and their respective ring protons (square brackets) showed the range of these couplings to be from 2.5 to 4.7 Hz. Second, no additional 4-bond splittings were observed; any 4-bond ^{13}C-carbonyl–ring proton couplings were

Table 2
^{1}H chemical shifts, $^{2\text{ or }3}J_{H,H}$ coupling constants between ring protons, and $^{3}J_{C,H}$ coupling constants between ^{13}C-carbonyl carbons and ring protons for five [^{13}C-carbonyl]-labeled, peracetylated disaccharide-alditols[a]

Chemical shift, δ (ppm)	($^{2\text{ or }3}J_{H,H}$) (Hz)	[$^{3}J_{C,H}$] (Hz)	Structure													
			β-D-Glc*p*-(1→4)-D-Glc-ol			β-D-Gal*p*-(1→4)-D-Glc-ol			α-D-Gal*p*-(1→6)-D-Glc-ol			α-D-Glc*p*-(1→3)-D-Glc-ol			α-D-Gal*p*-(1→4)-D-Gal-ol		
Alditol																	
H-1a	($J_{1a,1b}$)	[$J_{1,1a}$]	4.364	(−12.4)	[3.4]	4.336	(−12.4)	[3.3]	4.386	(−12.1)	[3.0]	4.470	(–12.1)	[3.0]	4.273	(−11.8)	[3.0]
H-1b	($J_{1a,2}$)	[$J_{1,1b}$]	4.258	(4.4)	[2.9]	4.286	(4.7)	[3.0]	3.992	(3.6)	[3.5]	4.200	(3.4)	[3.6]	4.020	(5.4)	[3.2]
H-2	($J_{1b,2}$)	[$J_{2,2}$]	5.417	(3.2)	[3.5]	5.450	(3.5)	[3.6]	5.337	(6.8)	[3.9]	5.312	(7.3)	[3.7]	5.474	(6.1)	[3.9]
H-3	($J_{2,3}$)	[$J_{3,3}$]	5.388	(7.5)	[3.8]	5.406	(7.2)	[3.9]	5.45[b]	(5.2)	[>3.5][c]	4.023	(5.2)	n.c.o.	5.321	(3.7)	[4.4]
H-4	($J_{3,4}$)	[$J_{4,4}$]	4.111	(3.4)	n.c.o.[d]	4.120	(3.5)	n.c.o.	5.44[b]	n.d.[e]	[>3.5][c]	5.382	(5.3)	[4.2]	4.079	(4.6)	n.c.o.
H-5	($J_{4,5}$)	[$J_{5,5}$]	5.033	(6.1)	[4.4]	5.058	(6.1)	[3.7]	5.045	(5.5)	[3.7]	5.301	(4.7)	[3.6]	5.305	(4.6)	[3.2]
H-6a	($J_{5,6a}$)	[$J_{6,6a}$]	4.505	(3.1)	[3.0]	4.497	(3.1)	[2.9]	3.725	(5.2)	n.c.o.	4.303	(3.4)	[2.6]	4.302[f]	[f]	[3.1]
H-6b	($J_{5,6b}$)	[$J_{6,6b}$]	4.065	(5.7)	[3.6]	4.086	(5.8)	[3.5]	3.665	(5.4)	n.c.o.	4.230	(7.1)	[3.2]	4.302[f]	[f]	[3.1]
	($J_{6a,6b}$)			(−12.4)			(−12.4)			(−11.0)			(−12.1)			[f]	
Pyranoside																	
H-1	($J_{1,2}$)		4.722	(8.0)	n.c.o.	4.665	(7.9)	n.c.o.	5.124	(3.7)	n.c.o.	5.190	(3.9)	n.c.o.	5.318	(3.8)	n.c.o.
H-2	($J_{2,3}$)	[$J_{2,2}$]	4.992	(9.6)	[4.3]	5.177	(10.5)	[4.1]	5.145	(10.5)	[3.5]	4.967	(10.4)	[3.6]	5.164	(11.0)	[3.5]
H-3	($J_{3,4}$)	[$J_{3,3}$]	5.206	(9.4)	[3.8]	5.009	(3.5)	[3.5]	5.312	(3.3)	[3.2]	5.397	(9.6)	[3.7]	5.304	(3.2)	[3.3]
H-4	($J_{4,5}$)	[$J_{4,4}$]	5.082	(10.0)	[4.1]	5.384	(1.0)	[3.8]	5.479	(1.2)	[4.2]	5.083	(9.7)	[4.1]	5.491	(1.3)	[4.2]
H-5	($J_{5,6a}$)		3.718	(3.1)	n.c.o.	3.924	(6.4)	n.c.o.	4.203	(6.5)	n.c.o.	4.12[g]	(4.7)	n.d.[h]	4.454	(6.9)	n.c.o
H-6a	($J_{5,6b}$)	[$J_{6,6a}$]	4.22[i]	(4.4)	[2.9]	4.17[i]	(6.4)	[3.3]	4.11[i]	(6.5)	[3.0]	4.266	[h]	[3.1]	4.150	(6.7)	[3.1]
H-6b	($J_{6a,6b}$)	[$J_{6,6b}$]	4.19[i]	(−12.4)	[3.1]	4.15[i]	(−11.4)	[3.1]	4.09[i]	n.d.[e]	[3.1]	4.12[g]	(−13.1)	n.d.[h]	4.121	(−11.3)	[2.8]
Acetyl methyls																	
			2.007			1.986			1.973			1.999			1.990		
			2.032			2.064			2.033			2.036			2.056		
			2.062			2.066			2.050			2.056			2.059		
			2.066			2.073			2.050			2.065			2.061		
			2.069			2.079			2.069			2.068			2.075		
			2.074			2.083			2.121			2.085			2.088		
			2.077			2.089			2.124			2.091			2.119		
			2.096			2.094			2.132			2.114			2.130		
			2.105			2.187			2.138			2.132			2.141		

[a] For a solution in $CDCl_3$ saturated with D_2O at 27 °C, relative to internal Me_4Si at 0.000 ppm.
[b] Chemical shifts of H-3 and H-4 were only assigned to two decimal places due to their partial tight coupling.
[c] H-3 and H-4 showed additional splittings ($^{3}J_{C,H}$) of a minimum of 3.5 Hz. More exact determination was not possible due to their partial tight coupling.
[d] n.c.o., for this entire table, no additional coupling observed; in comparing the ^{13}C-labeled compound with the ^{12}C (naturally abundant) isotope. Any $^{4}J_{C,H}$ couplings were within linewidth.
[e] n.d., not determined, due to partial tight coupling of H-3/H-4, or H-6a/H-6b proton pairs.
[f] The alditol H-6a and H-6b were tightly coupled; $\frac{1}{2}(J_{5,6a}+J_{5,6b}) = 5.3$ Hz.
[g] Chemical shifts of H-5 and H-6b were only assigned to two decimal places due to their partial tight coupling.
[h] n.d., not determined, due to tight coupling of H-5 and H-6b.
[i] Chemical shifts of H-6a and H-6b were only assigned to two decimal places due to their partial tight coupling.

Table 3
^{1}H chemical shifts, $^{2\text{ or }3}J_{H,H}$ coupling constants between ring protons, and $^{3}J_{C,H}$ coupling constants between ^{13}C-carbonyl carbons and ring protons for two [^{13}C-carbonyl]-labeled, peracetylated oligosaccharide-alditols[a]

Chemical shift, δ (ppm)	($^{2\text{ or }3}J_{H,H}$) (Hz)	[$^{3}J_{C,H}$] (Hz)	Structure and monosaccharide										
Pyranoside or alditol			α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-D-Glc-ol										
			α-Glc (A)			α-Glc (B)			α-Glc (C)			Glc-ol		
H-1a	($J_{1a,1b}$)	[$J_{1,1a}$]										4.493	(−12.1)	[3.1]
H-1b	($J_{1a,2}$)	[$J_{1,1b}$]										4.192	(3.4)	[3.8]
	($J_{1b,2}$)												(7.4)	
H-1	($J_{1,2}$)		5.233	(3.7)	n.c.o.[b]	5.406	(3.9)	n.c.o.	5.089	(3.9)	n.c.o.			
H-2	($J_{2,3}$)	[$J_{2,2}$]	4.808	(10.5)	[3.4]	4.814	(10.2)	[3.6]	4.813	(10.3)	[3.5]	5.333	(5.3)	[3.4]
H-3	($J_{3,4}$)	[$J_{3,3}$]	5.315	(9.4)	[3.5]	4.094	(9.3)	n.c.o.	5.383	(8.7)	[3.6]	3.996	(5.6)	n.c.o.
H-4	($J_{4,5}$)	[$J_{4,4}$]	5.042	(10.1)	[4.0]	5.185	(10.3)	[4.1]	3.977	(10.0)	n.c.o.	5.385	(4.2)	[4.0]
H-5	($J_{5,6a}$)	[$J_{5,5}$][c]	4.186	(4.1)	n.c.o.	3.830	(3.3)	n.c.o.	4.055	(2.3)	n.c.o.	5.321	(3.4)	[3.2]
H-6a	($J_{5,6b}$)	[$J_{6,6a}$]	4.229	(3.4)	[3.2]	4.202	(3.5)	[3.3]	4.519	(3.8)	[2.8]	4.295	(7.3)	[2.5]
H-6b	($J_{6a,6b}$)	[$J_{6,6b}$]	4.102	(−12.2)	[2.6]	3.990	(−12.4)	[2.5]	4.132	(−12.2)	[2.5]	4.232	(−12.2)	[3.1]
			β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc-ol										
			β-Gal (A)			β-GlcNAc (B)			β-Gal (C)			Glc-ol		
H-1a	($J_{1a,1b}$)	[$J_{1,1a}$]										4.333	(−12.4)	[3.5]
H-1b	($J_{1a,2}$)	[$J_{1,1b}$]										4.267	(4.8)	[2.9]
	($J_{1b,2}$)												(3.5)	
H-1	($J_{1,2}$)		4.461	(7.9)	n.c.o.	5.139	(8.1)	n.c.o.	4.583	(7.9)	n.c.o.			
H-2	($J_{2,3}$)	[$J_{2,2}$]	5.040	(9.9)	[4.1]	2.850	(10.5)	n.c.o.[d]	5.060	(10.0)	[3.9]	5.437	(7.3)	[3.6]
H-3	($J_{3,4}$)	[$J_{3,3}$]	4.943	(3.4)	[3.3]	4.606	(9.2)	n.c.o.	3.811	(3.6)	n.c.o.	5.382	(3.6)	[3.9]
H-4	($J_{4,5}$)	[$J_{4,4}$]	5.334	(1.0)	[4.0]	4.932	(9.3)	[4.2]	5.359	(0.8)	[4.0]	4.123	(6.0)	n.c.o.
H-5	($J_{5,6a}$)	[$J_{5,5}$][c]	3.851	(6.7[e])	n.c.o.	3.625	(2.7)	n.c.o.	3.818	(5.0)	n.c.o.	5.066	(3.1)	[3.4]
H-6a	($J_{5,6b}$)	[$J_{6,6a}$]	4.090	(6.7[e])	[3.1]	4.373	(4.0)	[3.1]	4.171	(7.4)	[3.1]	4.472	(5.9)	[2.9]
H-6b	($J_{6a,6b}$)	[$J_{6,6b}$]	4.090	[e]	[3.1]	4.012	(−12.2)	[3.2]	4.026	(−11.7)	[3.2]	4.100	(−12.4)	[3.5]

[a] For a solution in $CDCl_3$ saturated with D_2O at 27 °C, relative to internal tetramethylsilane at 0.000 ppm. Pyranosides are lettered as in Fig. 3.

[b] n.c.o., for this entire table = no additional coupling observed, in comparing the ^{13}C-labeled compound with the ^{12}C (natually abundant) isotope. Any $^{4}J_{C,H}$ couplings were within linewidth.

[c] For the pyranosides, no such coupling was possible as position 5 cannot be acetylated.

[d] For the β-GlcNAc H-2, coupling to the amide proton was observed prior to deuterium exchange. The position did not contain a ^{13}C-carbonyl acetyl group.

[e] The β-Gal (A) H-6a and H-6b were tightly coupled; $\frac{1}{2}(J_{5,6a}+J_{5,6b}) = 6.7$ Hz.

within the spectral linewidth ($J \leq 0.5$ Hz). For example, for all pyranoside H-1 and H-5 signals, positions to which there were obviously no directly attached acetyl groups, no additional splittings were observed in comparing the ^{13}C-labeled and non-labeled compounds in 1D spectra or gCOSY spectra. Those positions having a glycosidic substituent, and those positions having an acetamido (unlabeled) group also showed no additional splitting. For all positions (55 in number) not capable of having a 3-bond ^{13}C–^{1}H coupling, no additional splittings were observed. Also, none of these positions were detected in gHMBC experiments or in spdhetCOSY experiments, as they were not ^{13}C-acetylated. This leads to the conclusion that the positions of free hydroxyl groups, after acetylation with ^{13}C-carbonyl acetic anhydride, can be directly determined by determining the 3-bond ^{13}C–^{1}H coupling constants between the ring proton and the carbonyl carbon. Furthermore, the gHMBC and spdhetCOSY experiments are just two of several ways to transfer and select for transfer processes, either coherent or incoherent, between heteronuclei [18]. Hence, the enrichment of the acetyl [^{13}C-carbonyl] nucleus over natural abundance endows it as a selective tag for identifying the sites of acetylation and for elucidation of J coupled spin systems using other potential proton-detected 2D or multidimensional experiments.

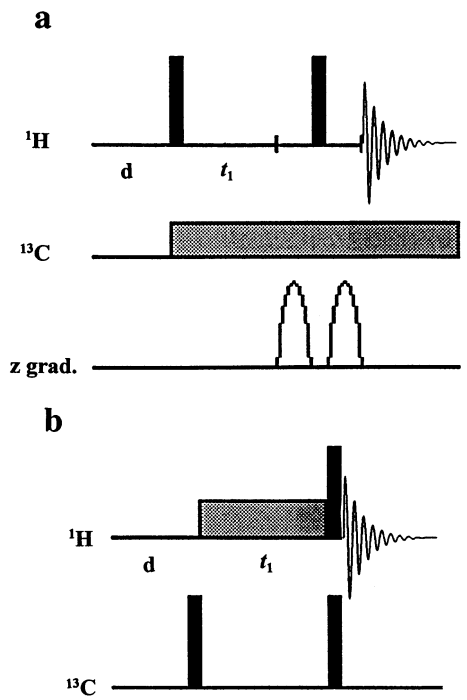

Fig. 6. Modified NMR pulse sequences. Solid black rectangles represent 90° pulses for ^{1}H or ^{13}C, and parabolic shapes show the application of z gradients. Gray rectangles refer to decoupling pulse schemes. (a) The carbon-decoupled gradient COSY (cdgCOSY) pulse sequence. This was a standard gCOSY pulse sequence, modified with the inclusion of the GARP decoupling scheme at the ^{13}C frequency during the time interval shown. The delay between transients (d) and the evolution time (t_1) are indicated. (b) The selective proton-decoupled heteronuclear COSY (spdhetCOSY) pulse sequence. This was a phase-sensitive hetCOSY pulse sequence modified by inclusion of the hyperbolic secant decoupling scheme applied at the methyl proton frequency during t_1.

Chemical shifts of the acetyl methyl protons for the disaccharide alditols are reported in Tables 1 and 2. For peracetylated α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-D-Glc-ol, *O*-acetyl methyl proton shifts (δ) were: 2.130, 2.124, 2.114, 2.107, 2.104, 2.089, 2.085, 2.081, 2.068, 2.067, 2.056, 2.051, 2.010, 2.006 and 1.986. For peracetylated β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc-ol, the *N*-acetyl methyl proton shift was 2.001 and the *O*-acetyl methyl proton shifts were 2.158, 2.147, 2.140, 2.106, 2.091, 2.077, 2.071 (two methyl groups), 2.063, 2.061 (three methyl groups), 2.045 and 1.966. These chemical shifts do not permit unambigous structural assignments to be made, as was previously concluded by Goux and Weber [17]. Their main value is that they permit a direct count of the number of acetylated positions and that they enable the number of *N*-acetyl groups and *O*-acetyl groups to be readily distinguished (Table 1, footnote b).

3. Conclusions

The ability to sensitively assign the sites of acetyl group substitutions based on the 3-bond coupling constant of a sugar ring proton and [^{13}C-carbonyl]-carbon of the acetyl group provides the same basic information given by permethylation analysis. However, there are some significant advantages to using the NMR methods reported herein. First, permethylation analysis requires cleavage of glycosidic linkages, whereas 'peracetylation analysis' by NMR permits substitution positions to be determined on intact structures.

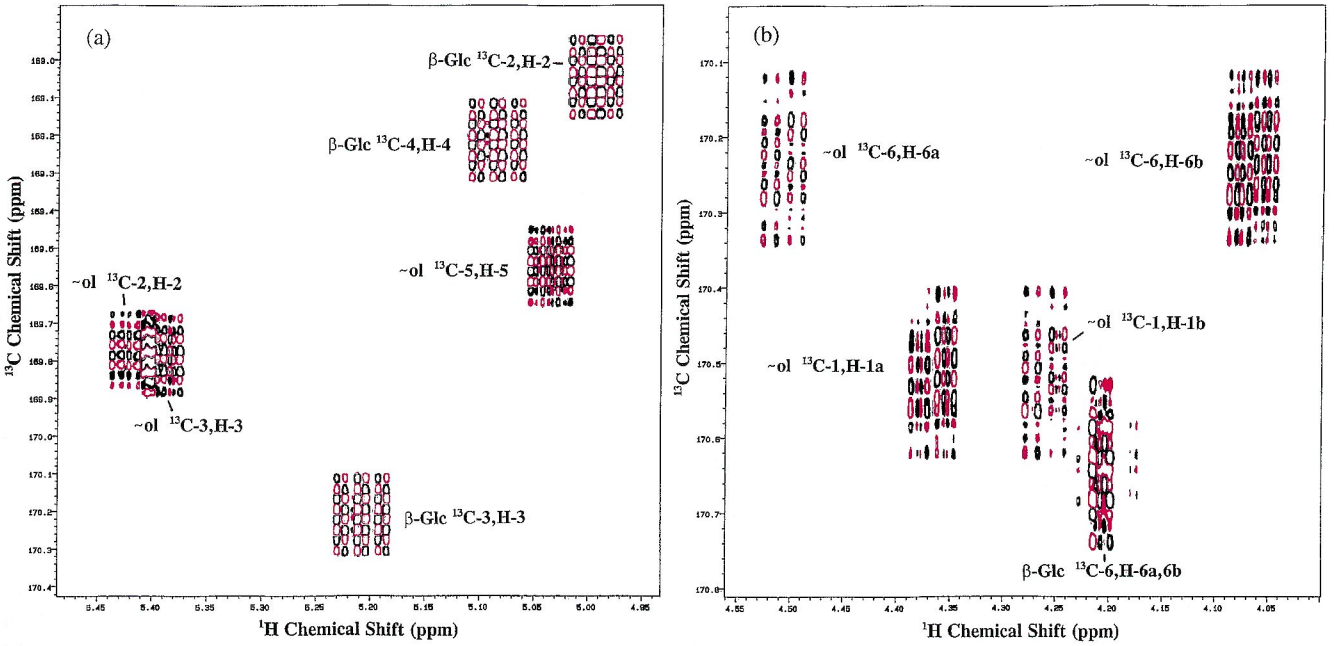

Fig. 7. A standard phase-sensitive proton-detected hetCOSY spectrum of peracetylated β-D-Glc*p*-(1→4)-D-Glc-ol having [^{13}C-carbony]acetyl groups, without application of the hyperbolic secant decoupling scheme (Fig. 6(b)). Multiplet patterns are shown with negative contours in red and positive contours in black. (a) Expansion of the region containing ^{13}C-carbonyl–^{1}H crosspeaks for H-2 to H-5 of the pyranoside or alditol. (b) Expansion of the region containing ^{13}C-carbonyl–^{1}H crosspeaks for the *gem*-coupled proton sets. Assignments of crosspeak multiplets are indicated.

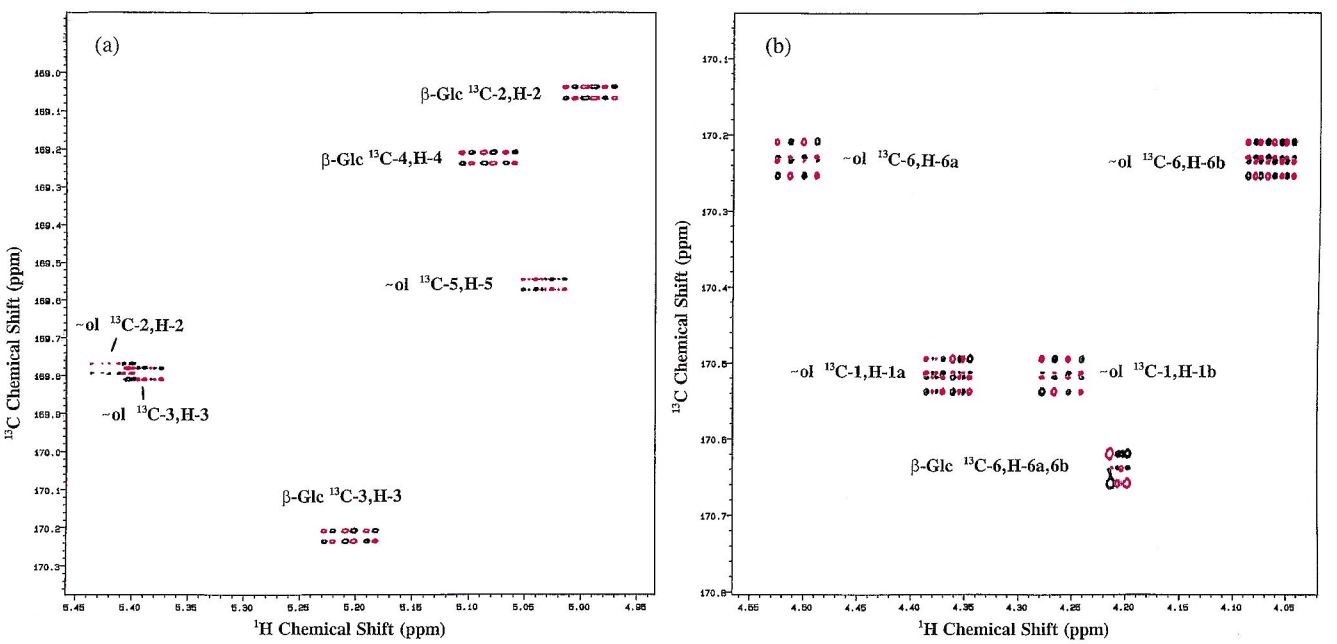

Fig. 8. A spdhetCOSY spectrum of [^{13}C-carbonyl] peracetylated β-D-Glc*p*-(1→4)-D-Glc-ol, with decoupling at the methyl proton frequency (pulse sequence in Fig. 6(b)). Multiplet patterns are shown with negative contours in red and positive contours in black. (a) Expansion of the region containing ^{13}C-carbonyl–^{1}H crosspeaks for H-2 to H-5 of the pyranoside or alditol. (b) Expansion of the region containing ^{13}C-carbonyl–^{1}H crosspeaks for the *gem*-coupled proton sets. Assignments of crosspeak multiplets are indicated.

Second, knowledge of the position of ring cyclization is straightforward, spectral dispersion permitting, because the position involved in cyclization (either O-4 or O-5 for most sugars) cannot be acetylated and hence does not demonstrate an additional ^{1}H–^{13}C coupling upon peracetylation. Third, the procedure should permit assignment of the sites of

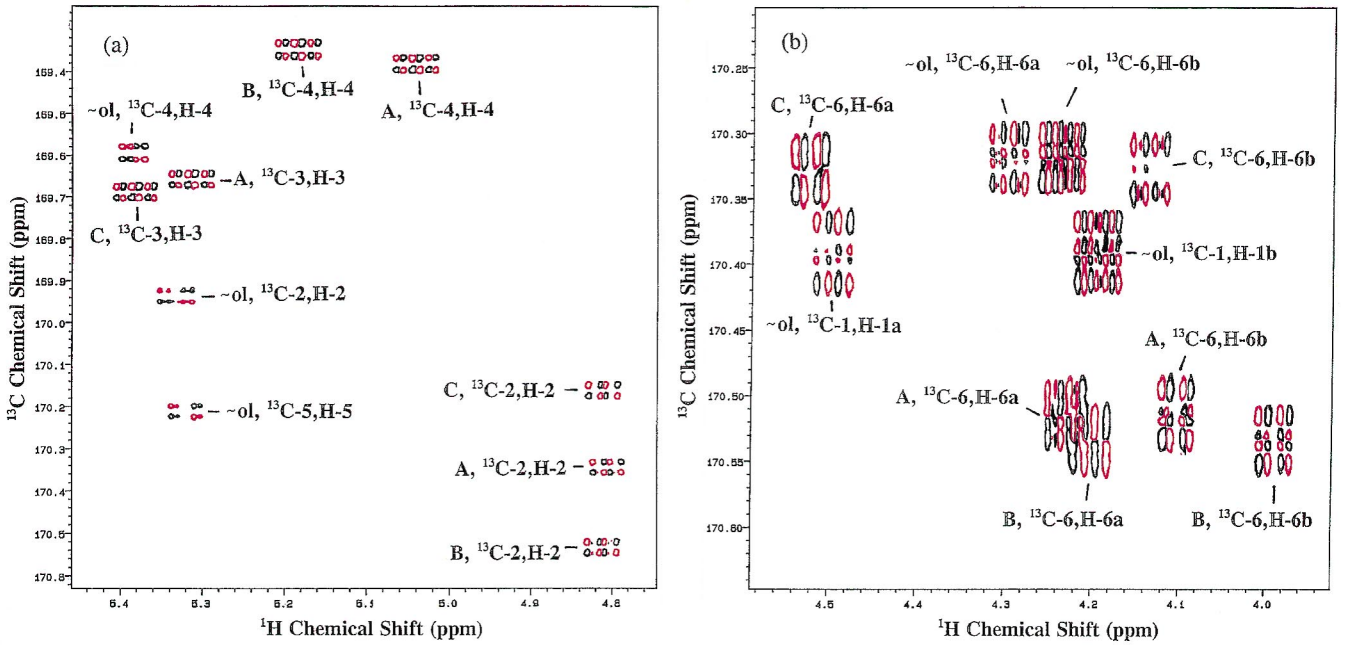

Fig. 9. A spdhetCOSY spectrum of [^{13}C-carbonyl]-peracetylated α-D-Glc*p*-(1→3)-α-D-Glc*p*-(1→4)-α-D-Glc*p*-(1→3)-D-Glc-ol, with decoupling at the methyl proton frequency. Multiplet patterns are shown with negative contours in red and positive contours in black. (a) Expansion of the region containing ^{13}C-carbonyl–^{1}H crosspeaks for H-2 to H-5 of the pyranoside or alditol. (b) Expansion of the region containing ^{13}C-carbonyl–^{1}H crosspeaks for the *gem*-coupled proton sets. Assignments of crosspeak multiplets are indicated.

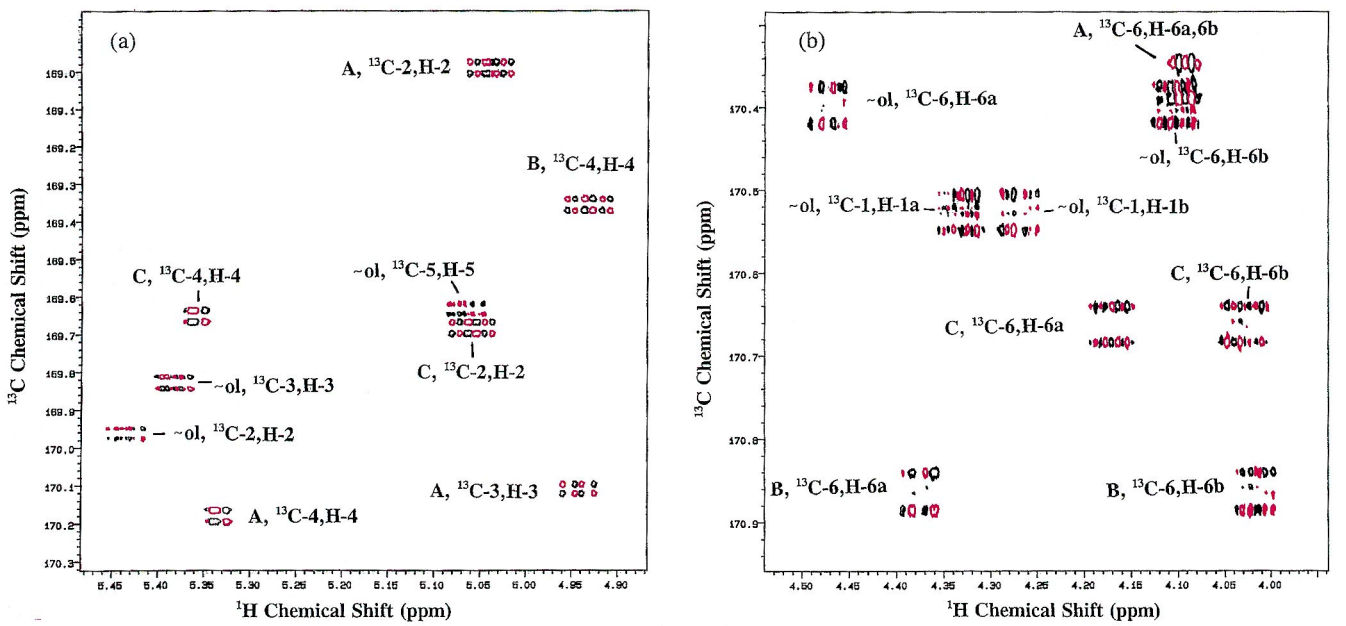

Fig. 10. A spdhetCOSY spectrum of [^{13}C-carbonyl]-peracetylated β-D-Gal*p*-(1→3)-β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-Glc-ol, with decoupling at the methyl proton frequency. Multiplet patterns are shown with negative contours in red and positive contours in black. (a) Expansion of the region containing ^{13}C-carbonyl–^{1}H crosspeaks for H-2 to H-5 of the pyranoside or alditol. (b) Expansion of the region containing ^{13}C-carbonyl–^{1}H crosspeaks for the *gem*-coupled proton sets. Assignments of crosspeak multiplets are indicated.

substitution of many non-carbohydrate groups, such as sulfates, for example. Particularly relevant is the potential to sensitively assign the positions of functional groups that do not survive permethylation conditions, such as acetyl groups. Fourth, for many structures, the original saccharide should be recoverable because mild conditions are available

for *O*-deacetylation [19,20]. Fifth, peracetylation is one of the most simple, mild and reproducible derivatization methods available in carbohydrate chemistry.

With regard to advantages over NMR of underivatized carbohydrates in D_2O, several are evident. Spectral dispersion of ring protons other than anomeric protons is about twofold greater, which is well known [13,14]. The ability to sensitively assign positions of linkages is possible as indicated herein. In addition, the ^{13}C nucleus may be used with a greater than 90-fold increase in sensitivity for providing spectral dispersion in a ^{13}C dimension in proton-detected 2D or 3D experiments, and should enable many more $^{1}H-^{1}H$ couplings to be measured in larger molecules. Based on the spectral resolution achievable with the two tetrasaccharide-alditols in the spdhetCOSY experiment (Figs. 9 and 10), it is estimated that oligosaccharide substitution positions should be possible to determine for structures having up to ca. 8–10 monosaccharides using only 2D experiments. Furthermore, the exchange of acetamido protons can be monitored, and H-2 can be easily assigned for 2-acetamido-2-deoxy sugars. This should be useful in cases where an aldose and a 2-acetamido-2-deoxyaldose having the same configuration are found in the same oligosaccharide and where ambiguity may exist as to which spin system corresponds to which sugar. Also, use of the [^{13}C-carbonyl]acetyl group may find valuable uses in synthetic carbohydrate chemistry, in determining the positions of anhydro rings, ester, ether or cyclic acetal substituents, etc. Simply by *O*-acetylating any free hydroxyl groups, their positions can be located on the sugar ring and by default, the other substituents must be located at other sites.

Credence also needs to be paid to the inherent caveats of this procedure. First, the method is strictly useful only as a primary structural tool and obviously bears no relevance to conformational studies of underivatized molecules. Second, it does not give information as to the order (sequence) of monosaccharides in a sugar chain, as interresidue through-bond couplings are not assigned. However, NOEs across glycosidic linkages have been observed in peracetylated glycolipids by NOESY and peracetylated oligosaccharide-alditols by ROESY experiments [13,14]. Combined with the through-bond [^{13}C-carbonyl]-carbon–ring proton couplings to unambiguously establish the positions of (formerly free) hydroxyl groups, the structure of many carbohydrates in the range of 2–10 monosaccharides should be capable of being solved without a requirement for permethylation. Third, for somewhat larger structures (namely, 10–20 sugars), 2D experiments will yield the inevitable overlap of crosspeaks and, as with underivatized structures, strong couplings. It is clear that spectral dispersion will be needed in additional dimensions. Where structures are very large, such as polysaccharides having multiple repeating units, permethylation analysis may remain the best approach. Fourth, the method does not identify individual monosaccharide enantiomers, which still requires cleavage of glycosidic bonds.

It is also apparent that virtually any functional group having a ^{13}C atom covalently bound to the oxygen atom at various sugar positions, such as ^{13}C-methyl ethers, [^{13}C-carbonyl]benzoyl esters, etc., could be used, in principle, for this type of analysis. Even other nuclei, such as ^{29}Si in trimethylsilyl ethers, could be envisioned to be of utility, provided derivatizations are complete, and that the ranges of 3- and 4-bond coupling constants of these nuclei to sugar ring protons do not overlap.

4. Experimental

Compounds.—Disaccharide-alditols were obtained either by isolation from bovine submaxillary mucin by alkaline borohydride treatment [21], or were prepared by reduction of disaccharides or tetrasaccharides (purchased from Sigma) with $NaBH_4$ ([22], as modified in [23]). Structures having a reducing hexose were also reduced with $NaBD_4$ in a separate experiment, so that the 'endedness' of the assignments of the alditol could be made unambiguously after peracetylation by direct comparison of $NaBH_4$ and $NaBD_4$ reduced samples.

Analyses.—Peracetylation was carried out essentially by the standard method [24]. Disaccharide-alditols (100–500 μg) in 1.5 mL vials having teflon-lined septa were dissolved in 70 μL pyridine (Pierce, silylation grade) followed by addition of 30 μL of acetic anhydride (Aldrich) or [^{13}C-carbonyl] acetic anhydride (Cambridge Isotopes, acetic anhydride 1,1′-$^{13}C_2$, 99%). Samples were mixed and left for 24 h between 25 and 50 °C under Ar, then solvents were removed under vacuum in a Speed-Vac rotor (Savant). Following one wash with $CDCl_3$, the sample was transfered in $CDCl_3$ to an NMR tube and a trace of Me_4Si was added. Deuterium oxide (10 μL, 99.996%, Cambridge Isotopes) was added, the sample mixed and the phases separated with a small hand centrifuge. Spectra were recorded at 27 °C on a Varian Inova 500 spectrometer equipped with a pulsed field gradient module and a triple resonance probe. Chemical shifts were recorded relative to Me_4Si (for ^{1}H, and ^{13}C, δ 0.000, indirectly determined for ^{13}C using [^{13}C-carbonyl]acetone (Cambridge Isotopes), δ 207.0 at 27 °C in $CDCl_3$ saturated with D_2O, from a 1D carbon-detected spectrum run separately). For 1D spectra, the spectral width was 5000 Hz, collected over 16,000 data points, with a preparatory delay of 2 s. gCOSY spectra [25] were recorded with 4–16 transients per t_1 increment and 1.75 s relaxation delay, with a phase = 1 (echo N-type coherence selection). Gradients were rectangular and applied in the z-direction of strength 6 G/cm and 2 ms duration, with gradient rise and fall times of 100 μs. Spectral widths were 2500 Hz in both dimensions, acquiring 3520 points × 2048 t_1 increments. The cdgCOSY experiment employed the GARP decoupling scheme [26] applied during the period shown in Fig. 6(a). The decoupler offset was set to the ^{13}C-carbonyl frequency using minimal power to achieve complete decoupling. Three hundred steady-state transients were run to ensure temperature equilibrium prior to acquisition, during which time z shims were reoptimized. gHMBC experiments ([27], standard Varian pulse sequence employing pulsed field gradients) were acquired as 2048 points × 512 t_1 increments. Spectral widths were 2500 and 12,000 Hz in F_2 and F_1, respectively. Four to eight transients were accumulated per t_1 increment with a 1.427 s preparatory delay, a fixed delay (taumb) of 63–93 ms, and the parameter J ($^1J_{X,H}$) set between 120 and 140 Hz. Data were processed with a combination of phase-shifted Gaussian and Gaussian multiplication before the first Fourier transformation and sinebell and sinebell-shifted multiplication before the second. Proton-detected phase-sensitive hetCOSY experiments were performed with a simple pulse sequence employing time-proportional phase incrementation as shown in Fig. 6(b). The spdhetCOSY experiment employed an adiabatic hyperbolic secant decoupling scheme [28,29] applied selectively at the methyl proton frequencies during the evolution time as shown in Fig. 6(b). Eight to sixteen transients were collected per t_1. The spectral width was 1500–2000 Hz in the F_2 dimension. Two separate experiments were performed with each compound, one over an F_1 spectral width of 8000 Hz, to include the internal [^{13}C-carbonyl]acetone frequency at 207.0 ppm for frequency referencing, and one over a 400 Hz spectral width, collecting 4096 × (400 × 2) hypercomplex files for both separate experiments. Data was processed using sinebell, Gaussian and phase-shifted Gaussian multiplication before the first Fourier transformation and sinebell and sinebell-shifted multiplication before the second.

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