Carbohydrate Research, 172 (1988) 297–307
Elsevier Science Publishers B.V., Amsterdam - Printed in The Netherlands

Note

The assignment of the signals of benzyl methylene carbon atoms in ^{13}C-n.m.r. spectra of per-*O*-benzylated methyl glycopyranosides

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(Received April 24th, 1987; accepted for publication in revised form, July 2nd, 1987)

In recent years, high-field ^{1}H- and ^{13}C-n.m.r. spectroscopies have been used to determine the primary structures of complex carbohydrates[1–4]. Chemical shifts of ^{1}H and ^{13}C resonances in spectra of these compounds yield information as to the types of residues present, their ring form, and the types of glycosidic linkages to and from neighboring residues. Whereas ^{1}H-n.m.r. spectroscopy is inherently the more sensitive, ^{13}C-n.m.r. spectroscopy offers a much greater chemical-shift range, making it possible to resolve carbon resonances free from the complexities of spin–spin coupling and from interference from the solvent resonances. A form of n.m.r. spectroscopy that shares some of the sensitivity advantages of ^{1}H-n.m.r. and the resolution advantage of ^{13}C-n.m.r spectroscopy would clearly be of benefit in determining complex carbohydrate structures.

Recently, we showed that carbonyl carbon resonances of peracetylated mono-, di-, and tri-saccharides have chemical shifts that are sensitive to primary and, to a lesser degree, secondary structure[5]. The acetylation reaction is easily carried out, and offers the opportunity for ^{13}C-enrichment. Each type of residue present gives rise to a set of carbonyl resonances, with those resonances arising from carbonyl groups nearest to substitution sites being the most sensitive to the configuration of the glycosidic linkages present. However, because all members of such a set of carbonyl resonances are sensitive to structure, it is necessary to create a large library of shifts of known peracetylated oligosaccharides before the method can be used to determine unknown structures.

In the present study, we found that resonances arising from the methylene carbon substituents of perbenzylated methyl glycopyranosides are equally as sensitive to structure as are carbonyl resonances of peracetylated derivatives. The benzyl methylene resonances, like the carbonyl resonances, can be assigned to specific substituents. However, the perbenzylated derivatives have two advantages. (*1*) The types of

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0008-6215/88/$ 03.50

residues present in an oligosaccharide and their sites of hydroxyl substitution (either *via* hemiacetal-ring formation or interresidue glycosidic linkage participation) can be determined by carrying out benzylation with ^{13}C-enriched and nonenriched reagents before and following hydrolysis and reduction. The same approach has been used to help identify complex carbohydrate structures by mass-spectral identification of partially methylated, acetylated alditol derivatives[6]. The use of benzyl rather than acetyl derivatives avoids potential acetyl migration to a neighboring substitution site following hydrolysis[7]. (*2*) Perbenzylated oligosaccharides separated by h.p.l.c. methods can conveniently be detected by their u.v. absorbance.

EXPERIMENTAL

Materials. — 2-Acetamido-2-deoxy-D-glucose, methyl α- and β-D-glucopyranoside, methyl α- and β-D-galactopyranoside, methyl α-D-mannopyranoside, methyl β-D-xylopyranoside, and methyl α-D-arabinopyranoside were obtained from Sigma Chemical Co. (St. Louis, MO) and were used without purification. Methyl 2-acetamido-2-deoxy-α- and β-D-glucopyranoside, methl β-D-mannopyranoside, methyl α-D-lyxopyranoside, and methyl β-L-rhamnopyranoside were prepared by standard procedures[8,9].

Perbenzylation of methyl glycopyranosides was carried out by using a modification of the procedure described by Hakomori[10] for the preparation of permethylated carbohydrate derivatives. In a typical preparation, 1 g (~ 5 mmol) of the methyl glycopyranoside was dissolved in 20 mL of dimethyl sulfoxide (dried with calcium hydride) under a nitrogen atmosphere. To this was added 4.1 equiv. of sodium methylsulfinylmethanide (sodium dimsyl), prepared according to Corey and Chaykovsky[11]. After 10 min, a slight excess of freshly distilled benzyl chloride was added, and the mixture was stirred for an additional 30 min. The reaction was quenched by addition of ice–water, and the product was extracted into chloroform. The extract was dried (anhydrous sodium sulfate), and evaporated under diminished pressure, giving a light-yellow oil which was chromatographed in a column (1.5 × 50 cm) of silica gel packed with 1:1 petroleum ether (b.p. 35–60°)–chloroform. Elution with this solvent gave unreacted benzyl chloride. Subseqent elution of the column with pure chloroform (250 mL) gave the pure methyl tetra-*O*-benzyl-glycopyranoside as a light-yellow oil* in 75–85% yield.

N.m.r. spectral conditions. — Perbenzylated sugars were dissolved in $CDCl_3$ (1.0–1.5 g in 3 mL) containing 1% Me_4Si. Normal F.t., DEPT[12], and INADEQUATE[13] ^{13}C-n.m.r. spectra were acquired in the presence of broad-band decoupling at 4.7 T, using a JEOL FX-200 spectrometer. INADEQUATE experiments were carried out at 55°, using a $\pi/2$-τ-π-τ-$\pi/2$-Δ-acq pulse sequence, where τ and Δ were 5.2 ms and 2 μs, respectively. A relaxation delay time of 2 s was used. ^{13}C-^{1}H-shift

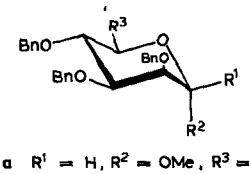
* Methyl 2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranoside was obtained as a solid which was recrystallized from methanol (m.p. 82–83°).

correlation spectra[13] were acquired with a Bruker AM-200 spectrometer, using a sweepwidth of 1000 Hz in the F_2 (^{13}C shift) dimension and ±250 Hz in the F_1 (^1H shift) dimension (region of aromatic protons not included). The fixed delay times, before and following the final mixing pulse (Δ), were set to 3.3 ms or to 55 ms, to emphasize short- and long-range couplings, respectively. Each spectrum was an average of 8 acquisitions when Δ was 3.3 ms, or 64 acquisitions when Δ was 55 ms. A relaxation delay of 2 s was used. ^{13}C–^1H-Correlation experiments were carried out at ambient probe temperature (~30°) in the presence of broad-band decoupling.

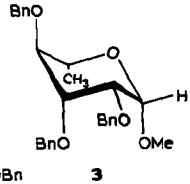
Pyranoid ring-crabon assignments and ^{13}C–^{13}C coupling constants were determined by fitting experimental INADEQUATE spectra to simulated spectra generated by LAOCOON[14] running on an IBM PC-compatible microcomputer.

RESULTS AND DISCUSSION

Chemical shifts (^{13}C- and ^1H-) of the following structures were determined: methyl 2,3,4,6-tetra-*O*-benzyl-α- and β-D-mannopyranoside (**1a,1b**), methyl 2,3,4-tri-*O*-benzyl-α-D-lyxopyranoside (**2**), methyl 2,3,4-tri-*O*-benzyl-β-L-rhamnopyranoside (**3**), methyl 2,3,4-tri-*O*-benzyl-β-D-xylopyranoside (**4**), methyl 2,3,4,6-tetra-*O*-benzyl-α- and β-D-glucopyranoside (**5a,5b**), methyl 2,3,4,6-tetra-*O*-benzyl-α- and β-D-galactopyranoside (**6a,6b**), methyl 2,3,4-tri-*O*-benzyl-α-D-arabinopyranoside (**7**), and methyl 2-acetamido-2-deoxy-α- and β-D-glucopyranoside (**8a,8b**). Benzyl methylene carbon atoms and protons will be designated by the pyranosyl carbon atom at which they are substituted, being differentiated from the latter group by having the suffix "MBzl" (e.g. C-2MBzl).



1a R^1 = H, R^2 = OMe, R^3 = CH OBn
1b R^1 = OMe, R^2 = H, R^3 = CH_2OBn
2 R^1 = H, R^2 = OMe, R^3 = H
Bn = $PhCH_2$



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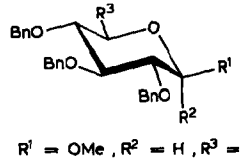
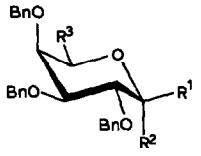
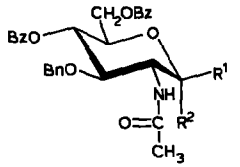


4 R^1 = OMe, R^2 = H, R^3 = H
5a R^1 = H, R^2 = OMe, R^3 = CH_2OBn
5b R^1 = OMe, R^2 = H, R^3 = CH_2OBn



6a R^1 = H, R^2 = OMe, R^3 = CH_2OBn
6b R^1 = OMe, R^2 = H, R^3 = CH_2OBn
7 R^1 = H, R^2 = OMe, R^3 = H



8a R^1 = H, R^2 = OMe
8b R^1 = OMe, R^2 = H

The strategy used in the past to assign carbonyl carbon resonances of peracetylated carbohydrate derivatives was first to assign completely resonances in their ^{1}H-n.m.r. spectra to pyranosyl-ring protons, and the next to correlate these resonances to nearby carbonyl carbon atoms by using ^{13}C–^{1}H shift-correlation spectra[5]. However, in some cases, the proton spectra could not be completely assigned due to strong coupling among two or more protons. For this reason, a different strategy has been used to assign benzyl methylene carbon atoms in the compounds of interest. First, the pyranosyl-ring carbon atoms will be assigned on the basis of their unique shifts and ^{13}C–^{13}C coupling constants. The pyranosyl-ring carbon atoms and the benzyl methylene carbon atoms will then be correlated to the pyranosyl-ring protons by using ^{13}C–^{1}H shift-correlation experiments optimized for short- and long-range couplings. This allows the methylene carbon atoms to be correlated indirectly to the nearest pyranosyl-ring carbon atoms, because they are both coupled to the same ring proton.

Pyranosyl ring carbon assignments and ^{13}C–^{13}C coupling constants. — The proton-decoupled, ^{13}C-n.m.r. spectrum of **2** shows resonances arising from the pyranosyl ring and the benzyl methylene carbon atoms between ~70 and 100 p.p.m. By virtue of their unique chemical shifts, resonances at 99.9 and 61.4 p.p.m. can be assigned to C-1 and C-5, respectively[15]. Fig. 1a shows the three remaining pyranosyl-ring carbon resonances, arising from C-2, C-3, and C-4. The INADEQUATE spectrum[13] in Fig. 1b shows the ^{13}C–^{13}C satellites appearing in an antiphase arrangement on either side of the central, ^{13}C resonances. (In some cases, a small central resonance also appears as a result of pulse imperfections and incomplete phase-cycling.) The satellites of the anomeric carbon resonance (not shown) and one

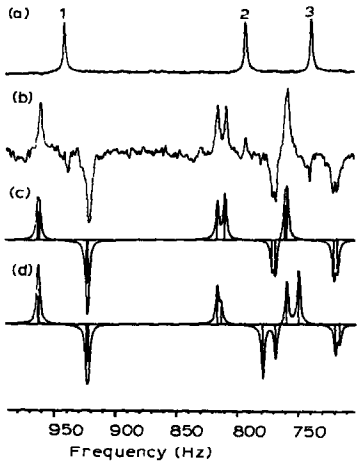


Fig. 1. (a) Proton-decoupled ^{13}C-n.m.r. spectrum of **2** in $CDCl_3$. [Only the pyranosyl-ring carbon resonances arising from C-2, C-3, and C-4 are shown.] (b) INADEQUATE spectrum of **2**. (c) Computer-simulated, "best fit", INADEQUATE spectrum of **2**, assuming that resonance 1 is assigned to C-3. (d) Computer simulation, assuming that resonance 1 is assigned to C-4.

set of satellites of resonance 2 are separated by 47.5 Hz, whereas the satellites of the other pyranosyl carbon resonances in the spectrum are separated by 38–42 Hz. Hence, resonance 2 can be assigned to C-2 by virtue of its unique coupling to C-1. Figs. 1c and

TABLE I

CHEMICAL SHIFTS OF RING CARBON ATOMS OF PERBENZYLATED METHYL PYRANOSIDES[a]

Compound		*Assigned shift*						
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*Me*
α-Man	**1a**	98.93	74.46	74.87	71.66	80.24	69.26	54.78
β-Man	**1b**	102.72	75.16[b]	75.92[b]	74.87	82.17	69.61	57.17
α-Lyx	**2**	99.93	75.39	78.90	74.81	61.44		55.07
β-Rha	**3**	99.05	74.70	80.19	67.86	80.47	17.99	54.60
β-Xyl	**4**	105.30	81.99	83.69	77.90	63.89		57.05
α-Glc	**5a**	98.23	79.84	82.17	77.67	70.08	68.44	55.19
β-Glc	**5b**	104.66	82.29	84.57	77.79	75.00	68.86	57.11
α-Gal	**6a**	98.76	76.39	79.08	75.11	69.14	69.03	55.31
β-Gal	**6b**	104.95	79.60	82.11	73.53	73.35	68.80	57.00
α-Ara	**7**	99.40	76.39	77.32	74.11	60.27		55.48
α-GlcNAc	**8a**	98.73	52.67	80.32	78.49	70.86	68.70	54.93
β-GlcNAc	**8b**[c]	101.25	56.17	81.16	78.56	74.84	70.00	56.43

[a]All chemical shifts were measured digitally from 1% Me_4Si in deuteriochloroform. Assignments are based on best fit of INADEQUATE spectra acquired at 4.7 T. [b]Assignments were made on the basis of similarity of shifts with compound **1a**. [c]Assignments for **8b** were made on the basis of similarity of shifts with those for compound **8a**.

TABLE II

^{13}C–^{13}C COUPLING CONSTANTS FOR PERBENZYLATED METHYL PYRANOSIDES[a]

Compound		*Coupling constant (Hz)*				
		$J_{C\text{-}1,C\text{-}2}$	$J_{C\text{-}2,C\text{-}3}$	$J_{C\text{-}4,C\text{-}5}$	$J_{C\text{-}5,C\text{-}6}$	
α-Man	**1a**	47.6		40.7	40.1	43.1
β-Man	**1b**	45.1			41.0	44.1
α-Lyx	**2**	47.8	38.7	41.3	40.0	
β-Rha	**3**	47.6	38.2	39.6	41.0	
β-Xyl	**4**	47.1	39.9	41.1	40.0	
α-Glc	**5a**	47.1	39.6	40.6	41.3	44.1
β-Glc	**5b**	47.0	40.5	40.2	39.5	44.5
α-Gal	**6a**	46.3	40.2	39.1	39.0	
β-Gal	**6b**	47.0	41.8	39.0	39.0	47.0
α-Ara	**7**	46.1		39.0	37.3	
α-GlcNAc	**8a**	44.3	38.1	40.1	40.7	46.7

[a]Determined by fitting experimental INADEQUATE spectra using, LAOCOON[15]. Values not listed were not clearly observable in the spectra, due to poor signal-to-noise ratios or partially overlapping resonances.

1d show simulated INADEQUATE spectra, assuming that resonance 1 can be assigned to C-3 (see Fig. 1c), or assuming that it can be assigned instead to C-4 (see Fig. 1d). Both spectra represent "best fits" to experimental data, assuming the shifts determined from Fig. 1a. Particularly in the region of resonances 2 and 3, the simulated spectra differ markedly, with Fig. 1c more closely fitting the experimental spectrum. On this basis, resonances 1 and 3 can be assigned to C-3 and C-4, respectively. Resonance assignments made in this manner for all other compounds studied are summarized in Table I, and ^{13}C–^{13}C coupling constants obtained from best-fit simulated spectra are given in Table II. Assigned shifts for **5a** and **6b** are in agreement with previous assignments for these compounds[16]. The ^{13}C–^{13}C coupling constants reported for perbenzylated derivatives are, in general, about 1 Hz larger than corresponding coupling constants for the underivatived methyl glycopranosides[17].

Assignment of benzyl methylene carbon atoms. — Figs. 2a–d show the proton-decoupled, ^{13}C-n.m.r. DEPT spectra[12] of **1a, 1b, 5a,** and **5b** in the region of benzyl methylene carbon resonances. In general, the resonances in each of the spectra form a pattern unique to each of the derivatives over an ~5-p.p.m. shift range. The chemical shift nonequivalence between benzyl methylene carbon resonances arising from substituents at different pyranose ring substitution sites is similar to that seen for carbonyl carbon resonances in peracetylated methyl glycoside derivatives[5]. Some of the methylene carbon resonances of these structurally related compounds appear to have similar shifts. For example, all spectra in Fig. 2 have a resonance lying between 73.3 and 73.5 p.p.m. and one between 74.8 and 75.0 p.p.m. Compounds **1a** and **1b** have a resonance between 71.4 and 72.1 p.p.m. whereas **5a** and **5b** have a resonance at ~75.5 p.p.m.

Fig. 3a shows the ^{13}C–^{1}H shift-correlation map of **5a**, where the crosspeaks

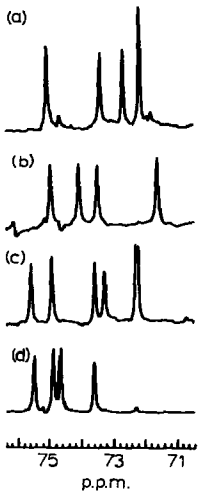


Fig. 2. ^{13}C-N.m.r. DEPT spectra, showing only benzyl methylene carbon resonances of (a) **1a**, (b) **1b**, (c) **5a**, and (d) **5b**. [The resonance at 72.1 p.p.m. in spectrum c arises from an unidentified impurity.]

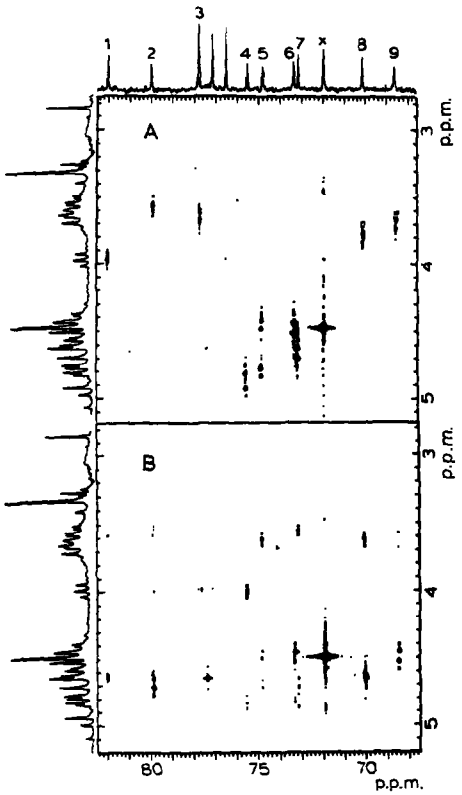


Fig. 3. ^{13}C–^{1}H Shift-correlation contour spectra of **5a** in which data were collected with (a) a delay time, Δ, of 3.2 ms. Contours arise from those carbon atoms directly attached to protons. (b) A delay time, Δ, of 55 ms. Contours arise from carbon atoms coupled to protons two or three bonds away. [^{13}C- and ^{1}H-Spectra are shown along the horizontal and vertical axes. Peak x in the ^{13}C spectrum arises from an unidentified impurity. The group of three resonances lying between 76 p.p.m. and 78 p.p.m. arises from the solvent ($CDCl_3$). Resonance 3 overlaps the solvent resonance lying farthest downfield.]

appearing in the map arise from one-bond, ^{13}C–^{1}H couplings. Resonances 1–3, 8, and 9 in Fig. 3a arise from pyranose-ring carbon atoms and are all coupled to protons between 3.5 and 4.0 p.p.m. The benzyl methylene carbon atoms (resonances 4–7) all appear coupled to protons appearing as AB quartets between 4.4 and 5.0 p.p.m. Fig. 3b is a similar contour map where crosspeaks arising from long-range, ^{13}C–^{1}H couplings (3–5 Hz) have been emphasized. The carbon atom giving rise to resonance 2 in Fig. 3b is clearly coupled to methylene protons having the same chemical shifts and splitting pattern as the protons to which the methylene carbon atom giving rise to resonance 7 in Fig. 3a is coupled. Similarly, the carbon atom giving rise to resonance 9 in Fig. 3b is coupled long-range to the same methylene protons as the methylene carbon atom giving rise to resonance 6 in Fig. 1a. Although it is unclear from Fig. 3b as to which methylene proton resonances carbon resonances 1 and 3 are correlated

TABLE III

CHEMICAL SHIFTS FOR BENZYL METHYLENE CARBON ATOMS OF PERBENZYLATED METHYL PYRANOSIDES[a]

Compound		*Chemical shift*			
		C-2MBzl	*C-3MBzl*	*C-4MBzl*	*C-6MBzl*
α-Man	**1a**	72.59	72.13[b]	75.10[b]	73.35
β-Man	**1b**	73.87	71.42[b]	74.87[b]	73.47
α-Lyx	**2**	73.11	72.71	72.71	
β-Rha	**3**	72.76	75.34	72.13	
β-Xyl	**4**	74.87	75.63	73.41	
α-Glc	**5a**	73.53	75.80	75.10	73.53
β-Glc	**5b**	74.75	75.69	74.75	73.46
α-Gal	**6a**	73.53	73.24	74.70	73.53
β-Gal	**6b**	75.16	72.94	74.40	73.35
α-Ara	**7**	73.70	72.83	71.83	
α-GlcNAc	**8a**		74.73	74.91	73.45
β-GlcNAc	**8b**		74.58[c]	74.58[c]	73.45[c]

[a]Assignments were made indirectly by correlating the benzyl methylene carbon atom to assigned ring-carbon atoms by observing the protons to which both types of carbon atoms are coupled. Proton–carbon correlations were made by using ^{13}C-^{1}H shift-correlation spectra, as described in the text. [b]Assignments were made on the basis of similarity of shifts with those of compounds **5a–6b**. [c]Assignments were made on the basis of similarity of shifts with those of compound **8a**.

with, the reverse correlations can be made. Hence, the methylene carbon atoms giving rise to resonances 4 and 5 are coupled long-range to the same pyranose-ring protons as the carbon atoms giving rise to resonances 1 and 3. Because all of the pyranose-ring carbon atoms have been assigned (see Table I), resonances 4–7 can be assigned to benzyl methylene carbon atoms C-3MBzl, C-4MBzl, C-6MBzl, and C-2MBzl. Benzyl methylene carbon resonance assignments for the remaining compounds studied are summarized in Table III.

Trends in the shift data of Table III are emphasized in Table IV, where differences in shifts of corresponding methylene carbon atoms are shown. Shift differences reported are intended to demonstrate the effects of changing a single pyranose ring substituent from an axial to an equatorial orientation. A few general trends appear from the data summarized in Tables III and IV. (*1*) Perbenzylated derivatives of methyl gluco- and manno-pyranosides all have C-4MBzl resonances lying between 74.75 and 75.10 p.p.m. All perbenzylated methyl hexopyranosides studied have C6-MBzl resonances between 73.35 and 73.55 p.p.m. (*2*) A benzyl methylene carbon atom substituted axially gives rise to a resonance that lies upfield of a corresponding equatorially substituted methylene carbon resonance. The magnitude of the shift appears to depend on the position of the substition. The effect for groups substituted at C-2 lies between ~0.6 and 0.9 p.p.m. (for C-2MBzl of **1b–5b** and **1a–5a**), whereas the effect is much less pronounced when the benzyl substituent moves from an axial to an equatorial position at C-4 (0.35–0.4 p.p.m. for C-4MBzl of **6b–5b** and **6a–5a**). (*3*) There is a downfield shift of a benzyl methylene carbon resonance when the nearest-

TABLE IV

CHEMICAL-SHIFT DIFFERENCES OF BENZYL METHYLENE CARBON RESONANCES ARISING FROM STRUCTURAL DIFFERENCES BETWEEN COMPOUND[a]

Assignment	*Calculated, ^{13}C chemical-shift differences*						
	$\delta_c(1a)-\delta_c(5a)$	$\delta_c(1b)-\delta_c(5b)$	$\delta_c(6a)-\delta_c(5a)$	$\delta_c(6b)-\delta_c(5b)$	$\delta_c(1a)-\delta_c(1b)$	$\delta_c(5a)-\delta_c(5b)$	$\delta_c(6a)-\delta_c(6b)$
C-2MBzl	−0.94	−0.64	0.00	0.41	−1.28	−1.22	−1.63
C-3MBzl	−3.67	−4.27	−2.56	−2.75	0.71	0.11	0.30
C-4MBzl	0.00	0.12	−0.40	−0.35	0.23	0.35	0.30
C-6MBzl	−0.20	0.01	0.00	−0.11	−0.12	0.07	0.18

[a]Chemical-shift differences were calculated from chemical shifts of resonances listed in Table III.

neighbor substituent is moved from an axial to a equatorial orientation. The magnitude of the shift depends on the pyranose-ring position of the nearest-neighbor substituent. However, the nearest-neigbor effect is always greater than the effect on a benzyl methylene carbon shift seen when the benzyl group containing the same carbon atom changes configuration. For instance, the C-2MBzl resonance is shifted ~1.2 to 1.6 p.p.m. downfield when the glycoside methyl group is moved from axial to equatorial, whereas similar changes in configuration of the neighboring C-2 and C-4 substituents shift the C-3MBzl resonance by ~3.7–4.3 and 2.5–2.8 p.p.m. respectively (**1a–5a**, **1b–5b**, **6a–5a**, and **6b–5b**).

CONCLUSIONS.

In summary, the assignment of benzyl methylene carbon resonances in the ^{13}C-n.m.r. spectra of perbenzylated methyl aldopyranosides has shown that only the C-6MBzl resonances has a shift independent of pyranosyl-ring configuration. Resonances arising from benzyl methylene carbon atoms undergo substantial downfield shifts when the substituent at an adjacent center is changed from axial to equatorial, while smaller shift perturbations are observed for other benzyl methylene resonances. This suggests that these resonances may be used to determine structures of oligosaccharides, once a library of shifts has been created. The use of ^{13}C-enriched reagent in the preparation of such oligosaccharide derivatives should make the method applicable to biological material isolated in limited quantities. Furthermore, the method may have advantages over ^{1}H-n.m.r. methods now used, in that the ^{13}C-n.m.r. spectrum is uncomplicated by extensive overlap of signals, each having a complex spin–spin splitting pattern.

ACKNOWLEDGMENTS

W. J. G. acknowledges support from Robert A. Welch grant AT-885. S. N. D. thanks Kurukshetra University, Kurukshetra, India, for granting him study leave.

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