10, **12**, and **13** were kindly supplied by Professor Clinton E. Ballou, Department of Biochemistry, University of California, Berkeley. Samples of **7**, **11**, and **13** were kindly provided by Dr. Philip A. J. Gorin of the Prairie Regional Laboratory, National Research Council, Saskatoon, Saskatchewan, Canada. Compound **3** was prepared as described below. The ^{13}C chemical shifts of **4** and **5** wre taken directly from the report of Allerhand et al.[16]

Preparation of Ethyl α-D-Mannopyranoside. Gaseous HCl (about 5 g) was bubbled into a solution of 5 g of D-mannose in 100 mL of anhydrous ethanol. Then the solution was refluxed for about 1 h, during which its color darkened. After cooling to room temperature, silver carbonate was added until it no longer caused effervescence. The resulting mixture was filtered and lyophilized. The residue was dissolved in 100 mL of H_2O, treated with 5 g of charcoal, filtrered, and freeze dried.

Purification of Manα1→6Man. An impure sample of **11** was subjected to gel filtration chromatography on Biogel P2, 200–400 mesh (1.7 × 50 cm column), using H_2O as eluant (6 mL/h). The elution profile was monitored at 206 nm and at 540 nm (after orcinol/acid test). The fraction of lowest molecular weight which had a positive orcinol/acid test was collected and lyophilized.

Carbon-13 NMR Spectroscopy. The ^{13}C NMR spectra were recorded at 67.9 MHz, under conditions of proton decoupling, on a Fourier transform NMR spectrometer equipped with a Bruker 63.4-kG high-resolution superconducting magnet, Brucker 10- and 15-mm probes, "home-built" radiofrequency electronics (with quadrature detection), and a Nicolet 1085 computer. The probe temperature was about 32 °C. Unless otherwise indicated (see below), time-domain spectra were accumulated in 8192 addresses for each of the two detection channels, with a total spectral width of 6250 Hz (which corresponds to a digital resoltuion of 0.0112 ppm), and a recycle time of 1.32 s. The spectral width was 5000 Hz for Figure 4C, and it was 12 500 Hz for Figures 2A,D and 4A. However, a spectral width of 6250 Hz was used for all the spectra of samples in H_2O and D_2O which were used to get the Δ values of Table III. Furthermore, except in the case of **9**, all spectra used for getting Δ values were processed with 0.0056 ppm digital resolution, by adding a tail of 8192 zero addresses to each block of 8192 time-domain data points. In the cases of **11** and **12**, which yielded several very closely spaced peaks, the one-to-one connections between resonances of samples in H_2O and in D_2O were established by also using mixtures of H_2O and D_2O as the solvent. Most spectra were recorded on solutions which contained a trace of dioxane as an internal reference (taken at 67.86 ppm downfield from the ^{13}C resonance of Me_4Si). However, in some cases, especially when the oligosaccharide had a resonance close to that of dioxane, spectra were recorded for samples with and without dioxane. Chemical shifts of spectra without dioxane (such as Figures 2C,D, 3, and 4A,C) were obtained by using a secondary reference after the chemical shifts of the sample with dioxane had been determined. All chemical shifts were measured digitally.

Acknowledgment. This research was supported by the National Science Foundation (Grant PCM 79-16899). We thank Professor Clinton E. Ballou and Dr. Philip A. J. Gorin for supplying the oligosaccharides used in this study. We thank Dr. Kilian Dill for carrying out the preparation of **3**.

Systematic Approach to the Analysis of Carbon-13 NMR Spectra of Complex Carbohydrates. 2. Application to the High Mannose Glycopeptides of Hen Ovalbumin

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Contribution from the Department of Chemistry, Indiana University, Bloomington, Indiana 47405. *Received October 12, 1982*

Abstract: Seven glycopeptides of the type $(Man\alpha)_nMan\beta1\rightarrow4GlcNAc\beta1\rightarrow4GlcNAc\beta1\rightarrow Asn$, where $n = 0$, 2, 3, 4, 5, and 6, are studied by ^{13}C NMR spectroscopy at 67.9 MHz. Even the spectrum of the compound with $n = 6$ yields well-resolved single-carbon resonances for most carbons. Nearly all the resonances of all seven compounds are assigned to specific carbons with the use of the following methods: (i) the empirical parameters based on small oligosaccharides presented in the preceding paper of this issue; (ii) comparisons of spectra within the series of glycopeptides from $n = 0$ to 6; (iii) comparisons of chemical shifts of compounds in H_2O with those of the same compounds in D_2O; (iv) the effect of pH on chemical shifts; (v) spin–lattice relaxation times; and (vi) values of directly bonded ^{13}C–^1H scalar coupling constants. It is shown that the ^{13}C NMR spectrum of a glycopeptide with n α-D-mannopyranose residues can be predicted with great accuracy from a knowledge of the spectrum of any related glycopeptide with $n - 1$ residues, with the use of the parameters presented in the preceding paper of this issue.

In the preceding paper of this issue[1] we presented chemical shifts and assignments for the ^{13}C resonances of simple mannose oligosaccharides. Here we present the interpretation of the ^{13}C NMR spectra of the high mannose glycopeptides from hen ovalbumin (and some derivatives) which are shown in Figure 1. Structures 4–7 of Figure 1 occur commonly in side chains of glycoproteins.[2]

Results and Discussion

Figure 2 shows the spectra of **1**, 4, **5**, **6**, and **7** (Structures of Figure 1). We did not isolate compounds **2** and **3**, but we found them as transient products during the α-mannosidase digestion of **5** and **6**.[3] We recorded ^{13}C NMR spectra during the α-mannosidase digestion and then we digitally subtracted the spectrum of free D-mannose in order to remove the resonances of the monosaccharide. In this fashion we were able to obtain the spectra of **2** and **3**. Details have been published.[3]

We used a combination of the following information for assigning the ^{13}C resonances: (i) The chemical shifts of the oligosaccharides of the preceding paper.[1] (ii) Internal comparisons of the spectra of **1**–**7**. (iii) Deuterium isotope effects on the chemical shifts of **1**, 4, **5**, and **7**. (iv) The pH dependence of the chemical shifts of **1** and 4. (v) One-bond carbon–hydrogen scalar coupling constants of 4. (vi) Spin–lattice relaxation times of the ^{13}C resonances of **5**.

We shall refer to the carbohydrate residues by their designations in Figure 1. A number following a residue designation indicates

(1) Allerhand, A.; Berman, E. *J. Am. Chem. Soc.* **1984**, *106*, preceding paper in this issue.
(2) Montreuil, J. *Adv. Carbohydr. Chem. Biochem.* **1980**, *37*, 157–223.
(3) Berman, E.; Allerhand, A. *J. Biol. Chem.* **1981**, *256*, 6657–6662.

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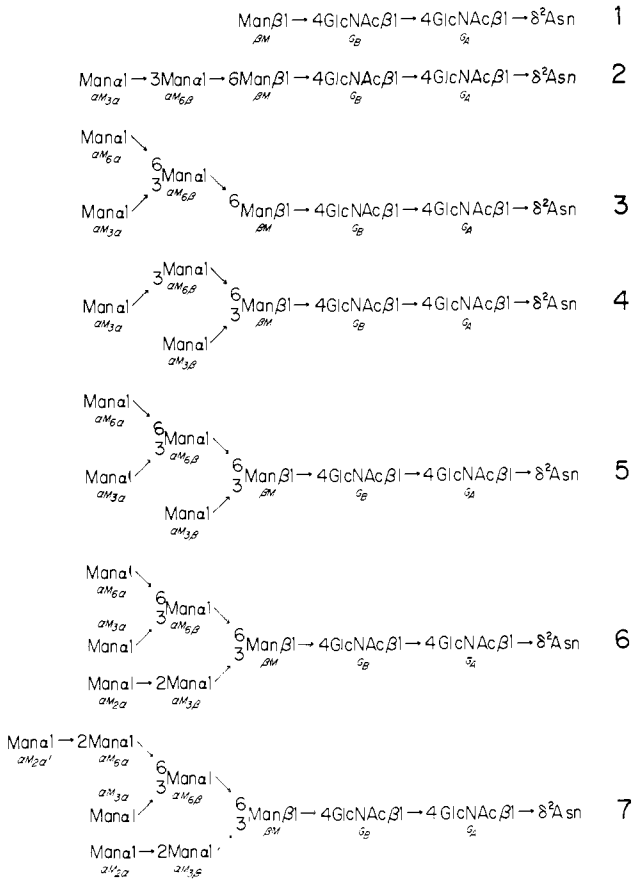

Figure 1. Structures and compound designations of the glycopeptides whose ^{13}C NMR spectra are reported and analyzed in this report.

a specific carbon of that residue.

Consider first the spectrum of Manβ1→4GlcNAcβ1→4GlcNAcβ1→Asn (**1**), shown in Figure 2A. A fairly good model for the assignment of the βM resonances of **1** is the mannose residue of Manβ1→4Glc, whose ^{13}C chemical shifts (in D_2O) and assignments have been reported by Usui et al.[4] In order to "convert" these values into ^{13}C chemical shifts in H_2O, we added 0.2 ppm to the reported value of C-3 and 0.1 ppm to the ones of C-2, C-4, and C-6 (on the basis of Table III of ref 1). Fairly good predictions for the ^{13}C chemical shifts of G_B of **1** can be made by starting with the chemical shifts of the nonreducing residue of GlcNAcβ1→4GlcNAc(β) and correcting for substitution at C-4 of that residue. We have used the chemical shifts of GlcNAcβ1→4GlcNAc(β) in D_2O reported by Nunez and Barker,[5] except that we added 0.2 ppm to the value of C-3 and 0.1 ppm to the values of C-2, C-4, and C-6 of the nonreducing residue, in order to make the chemical shifts compatible with our values of H_2O solutions. We obtained the correction factors for substitution at C-4 (by β-D-mannopyranose) by subtracting the ^{13}C chemical shifts of GlcNAc(β) in D_2O reported by Nunez and Barker[5] from those of the GlcNAc residue of Manα1→3Manβ1→4GlcNAc(β) in D_2O reported by Nunez et al,[6] except that we sutracted 0.1 ppm from the correction factor for C-4 because when going from D_2O to H_2O the chemical shift of C-4 of free GlcNAc (but not that of C-4 of the GlcNAc residue of Manα1→3Manβ1→4GlcNAc) increases by about 0.1 ppm. Fairly good predictions for the ^{13}C chemical shifts of G_A of **1** can be made by starting with the ^{13}C chemical shifts of the GlcNAc residue of GlcNAcβ1→Asn in H_2O reported by Dill and Allerhand[7] and correcting for the substitution at C-4 by another

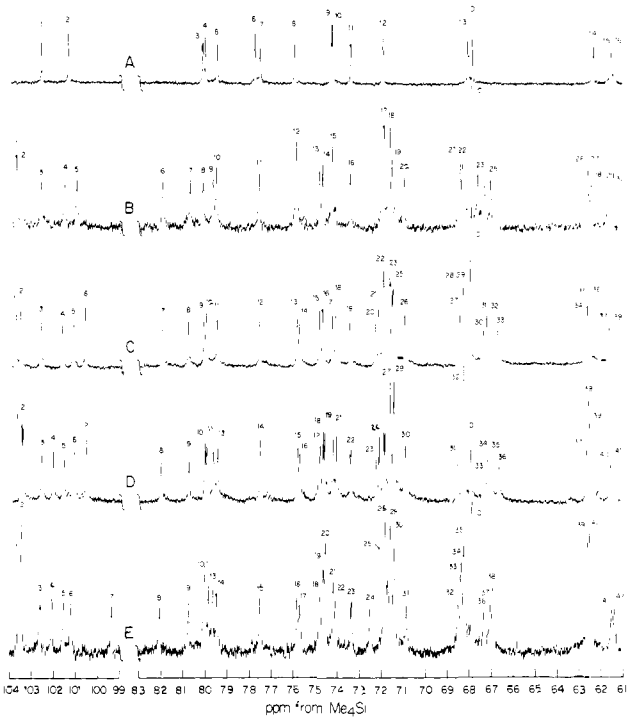

Figure 2. Region of most aliphatic carbons in the proton-decoupled ^{13}C NMR spectra (at 67.9 MHz) of **1** (A), **4** (B), **5** (C), **6** (D), and **7** (E). The resonances of the asparagine and those of C-2 and the acetyl groups of the GlcNAc residues are not in the spectral range shown. The chemical shifts of all resonances are contained in the bodies and footnotes of the tables. Peaks are numbered consecutively from *left* to *right*. All spectra were obtained on samples in H_2O at 32 °C, with a 10-mm probe and a recycle time of 1.32 s. Other conditions were as follows: (mM concentration, pH, number of scans, digital broadening in Hz) **1** (25, 7.0, 18 700, 0.2), **4** (7.3, 6.9, 43 000, 1.0), **5** (19, 7.8, 17 500, 0.2), **6** (50, 7.3, 14 000, 0), **7** (12, 7.1, 18 500, 0.5). The peak labeled D is the ^{13}C resonance of internal dioxane.

GlcNAc(β) residue. We obtained the correction factors by subtracting the reported ^{13}C chemical shifts of GlcNAc(β) in D_2O[5] from those of the corresponding reported values of the reducing residue of GlcNAcβ1→4GlcNAc(β) in D_2O.[5] We subtracted 0.1 ppm from the correction factor for C-4 in order to make all the correction factors compatible with ^{13}C chemical shifts in H_2O.

Table I shows the predicted chemical shifts for **1** based on the basis of reported ^{13}C chemical shifts of the above model compounds.[4-7] A comparison of these values with the experimental chemical shifts of **1** (also shown in Table I) yields unambiguous one-to-one assignments for all carbons except the following four groups: (G_A4, G_B4), (βM5, G_A5), (βM3, G_A3, G_B3), and (G_A6, G_B6). We have placed asterisks on the pairs (G_A4, G_B4) and (G_A6, G_B6) to indicate a lack of one-to-one assignments in this report. We have used *italics* for the chemical shifts of the other two sets to indicate that the given *one-to-one* assignments do not come from the calculated chemical shifts but from other evidence (see below). A similar use of asterisks and italic numbers will be used in all other tables.

On the basis of Table I, peak 6 (77.73 ppm) and peak 7 (77.51 ppm) of **1** must be the resonances of *βM5* and *G_A5*. However, the chemical shift of peak 6 remains invariant in the pH range 1.5–9.5, while peak 6 moves upfield about 0.1 ppm when going from pH 1.5 to 9.5, which suggests that peak 7 arises from G_A, the closest carbohydrate residue to the titrating asparagine. Also, the chemical shifts of peaks 6 and 7 exhibit deuterium isotope effects (Δ values) of 0.03 and 0.00 ppm, respectively (Table I),

(4) Usui, T.; Mizuno, T.; Kato, K.; Tomoda, M.; Miyajima, G. *Agric. Biol. Chem.* **1979**, *43*, 863–865.
(5) Nunez, H. A.; Barker, R. *Biochemistry* **1980**, *19*, 489–495.
(6) Nunez, H. A.; Matsuura, F.; Sweeley, C. C. *Arch. Biochem. Biophys.* **1981**, *212*, 638–643.
(7) Dill, K.; Allerhand, A. *FEBS Lett.* **1979**, *107*, 26–29. This report does not present one-to-one assignments for the closely spaced resonances of C-1 and C-5 of GlcNAcβ1→Asn. However, there is recent strong evidence that the downfield peak arises from C-1. See: Blumberg, K.; Bush, C. A. *Anal. Biochem.* **1982**, *119*, 397–406.

Table I. Observed and Predicted ^{13}C Chemical Shifts of 1 and 2

carbon[a]	Δ[b]	chemical shift[c] 1, exptl[d]	1, calcd[e]	2, exptl[f]	2, calcd[g]
$\alpha M_3\alpha 1$				103.42 (1)	103.46
G_B1	−0.01	102.55 (1)	102.9	102.56 (2)	102.55
$\beta M1$	−0.01	101.36 (2)	101.7	*101.64* (3)	101.52
$\alpha M_6\beta 1$				*101.06* (4)	100.80
G_A4*	0	80.11 (3)	80.5	80.54 (5)	80.11
G_B4*	−0.01	79.99 (4)	80.4	80.07 (6)	79.99
$\alpha M_6\beta 3$				*79.64* (7)	79.45
G_A1	+0.09	79.48 (5)	79.3	*79.44* (8)	79.48
$\beta M5$	+0.03	*77.73* (6)	78.1	75.80 (11)[h]	75.85
G_A5	0	*77.51* (7)	77.5	77.46 (9)	77.51
G_B5	+0.01	75.92 (8)	76.1	76.00 (10)[h]	75.92
$\alpha M_3\alpha 5$				74.54 (12)	74.67
$\beta M3$	+0.15	*74.25* (9)	74.8	74.11 (13)	74.37
G_A3	+0.11	*74.21* (10)	74.0	74.11 (13)	74.21
$\alpha M_6\beta 5$				74.11 (13)	74.05
G_B3	+0.10	*73.37* (11)	73.6	73.30 (14)	73.37
$\beta M2$	+0.12	71.92 (12)	72.4	71.81 (15)	71.86
$\alpha M_3\alpha 3$				71.81 (15)	71.82
$\alpha M_3\alpha 2$				71.50 (16)	71.46
$\alpha M_6\beta 2$				70.83 (17)	70.88
$\alpha M_3\alpha 4$				68.18 (18)	68.31
$\beta M4$	+0.12	68.06 (13)	68.5	67.84 (19)	67.93
$\alpha M_6\beta 4$				67.43 (20)	67.50
$\beta M6$	+0.12	62.35 (14)	62.5[i]	67.43 (20)	67.15
$\alpha M_3\alpha 6$				62.27 (21)	62.33
$\alpha M_6\beta 6$				62.27 (21)	~62.4
G_A6*	+0.11	61.54 (15)	61.2	61.48 (22)	61.54
G_B6*	+0.10	61.34 (16)	61.7	61.30 (23)	61.34
G_B2	+0.10	56.49 (17)	56.7	56.40 (24)	56.49
G_A2	+0.14	55.05 (18)	54.9	55.01 (25)	55.05

[a] Carbohydrate residues are designated as in Figure 1. The number following the residue designation is the carbon number. *Asterisks* on two vertically adjacent carbons indicate a lack of one-to-one assignments for these two carbons (experimental and calculated values). [b] Deuterium isotope effects on the ^{13}C chemical shifts of 1, in ppm. Each Δ value was obtained by subtracting a chemical shift of 1 in D_2O from the corresponding value of 1 in H_2O. [c] In ppm downfield from Me_4Si. [d] Obtained from the spectrum of Figure 2A (1 in H_2O, pH 7.0, 32 °C). Numbers in parentheses are the peak designations of Figure 2A. Resonances which are not given in the table had the following chemical shifts in a spectrum recorded at pH 8.1 and 33 °C, using a larger spectral window (and lower digital resolution) than for Figure 2A: 52.58 (Asn C^α), 36.73 (Asn C^β), 23.52 (CH_3), 23.47 (CH_3); the carbonyl resonances were at 176.19, 175.88, 174.55, and 174.01 ppm. [e] Calculated values were obtained as described in the text. Reported ^{13}C chemical shifts of model compounds in D_2O were corrected for direct comparison of our values in H_2O by adding 0.1 ppm to the chemical shift of every carbon with a free hydroxyl, and an additional 0.1 ppm was added to the chemical shift of C-3 if C-2 and C-4 both had free hydroxyl groups. [f] Obtained from the spectrum whose anomeric carbon resonances are shown in Figure 3G of ref 3 and whose region of nonanomeric carbons is shown in Figure 4B of ref 3 (20 mM 2 in H_2O, pH 4.4, 40 °C, 50 mM sodium acetate, 1 mM zinc sulfate). Numbers in parentheses are the peak designations of Figure 4B of ref 3. [g] Calculated values were obtained by taking the experimental values of 1 and correcting for the addition of the Manα1→3Man(α) disaccharide unit to C-6 of βM, as described in the text. [h] The assignments of peaks 10 and 11 to βM5 and G_B5 of 2 have not been made on a one-to-one basis. [i] Because of a lack of one-to-one assignments for C-6 of the mannose and glucose residues of Manβ1→4Glc,[4] another possible value is 62.1 ppm.

which *suggests* that peak 6 should be assigned to βM5, because this carbon has two "γ effects" (hydroxyl group on carbon adjacent to the one under consideration) while G_A5 has only one "γ effect".[8] The use of deuterium isotope effects on ^{13}C chemical shifts (caused by replacement of labile hydrogen by deuterium) for assignment of resonances has been discussed in the preceding paper.[1] Please note that the Δ values were obtained from spectra recorded with a digital resolution of 5.6 parts per *billion* (see Experimental Section), which yields more than adequate precision for the use of Δ values in making assignments.[1] The precision of the Δ values is illustrated in Figure 3, which shows the changes in the chemical shifts of most of the resonances of two relatively simple compounds, Manα1→6Man(α) and Manα1→6Man(β), when going from pure H_2O to a mixture of H_2O and D_2O (0.4 mol fraction D_2O) and to pure D_2O. The applicability of Δ values for assigning resonances is illustrated in Figure 4, which shows the structure of Manα1→6Man(α) and the Δ value of each carbon in parts per billion (ppb). There are three ring carbons with one "β effect" (directly bonded OH group) and two "γ effects", and these three carbons exhibit Δ values of 149, 155, and 160 ppb. There are four ring carbons with one "β effect" and one "γ effect", and they show Δ values of 110, 124, 121, and 121 ppb. Carbon 6 of the nonreducing mannopyranose residue, which has one "β effect" and no "γ effect", exhibits Δ = 115 ppb, but a C-6 is expected to show a slightly larger "β effect" than the ring carbons.[8] One carbon has no "β effect" but *two* "γ effects" (C-5 of the nonreducing ring) and it yields a Δ value of 34 ppb. When there is no "β effect" and zero or one "γ effect" the Δ values diminish to about 20 ppb or less (Figure 4).

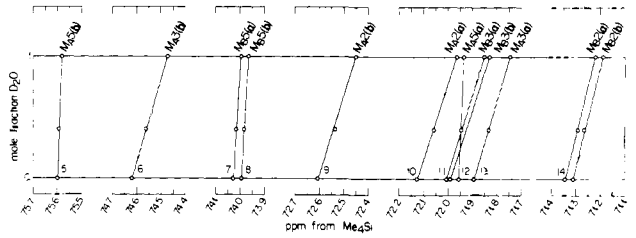

Figure 3. Effect of the mole fraction of D_2O on the ^{13}C chemical shifts of aqueous Manα1→6Man. Experimental conditions are those of the preceding paper.[1] M_A and M_B refer to the reducing and nonreducing mannopyranose residues, respectively, while (a) and (b) refer to Manα1→6Man(α) and Manα1→6Man(β), respectively. The number just above each chemical shift in pure H_2O is the peak number of Figure 4B of the preceding paper.[1]

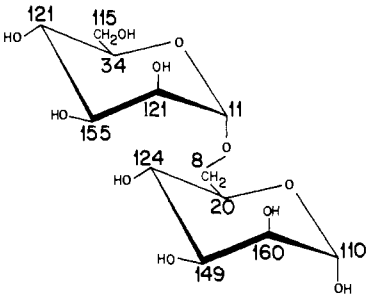

Figure 4. Effects of going from H_2O to D_2O on the ^{13}C chemical shifts of Manα1→6Man(α). The indicated values (Δ effects), in parts per billion, are chemical shifts in H_2O minus the corresponding ones in D_2O, taken from Figure 3. Experimental details are given in the preceding paper.[1]

The calculated chemical shifts for βM3, G_A3, and G_B3 of **1** (Table I) indicate that peaks 9, 10, and 11 should be assigned to these carbons, but not on a one-to-one basis. However, specific assignments follow from the effects of pH and D_2O. The Δ values of peaks 9, 10, and 11 are 0.15, 0.11, and 0.10 ppm, respectively. All three carbons under consideration have one β effect, but G_A3 and G_B3 have one γ effect while βM3 has two. Therefore, peak 9 (Δ = 0.15 ppm) must be assigned to βM3. Also, the resonance of peak 10 moves about 0.2 ppm downfield when going from pH 1.5 to 7.9, while the chemical shifts of peaks 9 and 11 are invariant in the pH range 1.5–9.5. Therefore, we assign peak 10 to G_A3. By elimination, peak 11 is assigned to G_B3. This completes the assignments for **1** presented in Table I.

Some of the one-to-one assignments for **1** which follow directly from the calculated values in Table I can be verified from independent evidence. For example, the specific assignment for G_A1

(8) Pfeffer, P. E.; Valentine, K. M.; Parrish, F. W. *J. Am. Chem. Soc.* **1979**, *101*, 1265–1274.

(peak 5), relative to those of the nearby resonances of G_A4 and G_B4, is confirmed by a comparison of chemical shifts in H_2O and D_2O. Peak 5 has a Δ value of 0.09 ppm while peaks 3 and 4 have Δ values of 0.00 and −0.01, respectively. On this basis, peak 5 must arise from G_A1 and not G_A4 or G_B4, because G_A1 is the only one of these carbons which has a "β effect" from a directly bonded OH or NH group (in this case it is NH). The calculated chemical shifts for **1** (Table I) indicate that peak 17 (56.49 ppm) and peak 18 (55.05 ppm) arise from G_B2 and G_A2, respectively. These one-to-one assignments can be confirmed by means of the effect of D_2O and, independently, from the pH dependence of the chemical shifts. The Δ values of peaks 17 and 18 are 0.10 and 0.14 ppm, respectively, which indicates that peak 18 arises from G_A2, which has two γ effects (one from the NH on C-1), and not from G_B1, which has just the one γ effect from the OH on C-3. When going from pH 1.5 to 9.5, peaks 17 and 18 move upfield by about 0.05 and 0.20 ppm, respectively, confirming that peak 18 arises from G_A.

The chemical shifts of the asparagine and of the acetyl groups of G_A and G_B will not be discussed throughout this paper; they do not differ significantly from the corresponding values reported for GlcNAcβ1→Asn[7] and GlcNAc oligosaccharides.[5,6]

Consider now the experimental ^{13}C chemical shifts of **2** given in Table I, which were obtained from ref 3. The structure of **2** is obtained from **1** by the addition of a Manα1→3Man(α) disaccharide residue to C-6 of βM of **1** (Figure 1). We considered two options for calculating the chemical shifts of **2**: (i) an ab initio method would involve using the *calculated* chemical shifts of **1**, obtained as described above, as the starting point before applying the corrections caused by the addition of the Manα1→3Man(α) moiety. (ii) An alternative "incremental" method would use the *experimental* values of **1** as the starting point. We chose the latter method, because if the motivation is to assign the resonances of **2**, then clearly the second method should yield better predictions. In practice, the two methods do not differ in a major way, because the observed and calculated chemical shifts of **1** do not differ greatly. Furthermore, the ab initio method yields good predictions for the observed ^{13}C NMR spectra of **2**–**7** (see, for example, Figures 6 and 7 of the preceding paper[1]). A referee commented that "the incremental method has the inherent weakness that any error made in the assignments of the starting compound of the series would be perpetuated throughout". This is undoubtedly true.

The corrections for the presence of the Manα1→3Man(α) group were carried out as follows: (i) the calculated chemical shifts for $\alpha M_{3\alpha}$ were taken from Table VI of ref 1 (1→3α listing). (ii) For the calculated values of $\alpha M_{6\beta}$, the starting point was the set of chemical shifts listed for 1→6β_R in Table VI of ref 1, and these values were corrected for substitution at C-3 with the use of the 3α listings of Table V of ref 1. (iii) The chemical shifts of βM of **1** were corrected for substitution at C-6 with the use of the 6β_R values of Table V of ref 1. The resulting calculated chemical shifts of **2** agree remarkably well with the set of experimental values (see Table I), and permit one-to-one assignments by inspection for most resonances. The lack of one-to-one assignments for the pairs (G_A4, G_B4) and (G_A6, G_B6) carries over from **1** to **2** and to all other compounds in this report. Our work has not yielded one-to-one assignments for the pair (βM5, G_B5) of **2**. Although the calculated chemical shifts of **2** do not yield one-to-one assignments for the pairs (βM1, $\alpha M_{6\beta}1$) and ($\alpha M_{6\beta}3$, G_A1), the specific assignment for G_A1 follows from the chemical shifts of G_A1 of **1** (Table I) and 4 (see below), and the one-to-one assignments for βM1 and $\alpha M_{6\beta}1$ of **2** follow from the corresponding assignments of 4, which were made from measurements of the one-bond ^{13}C–^1H scalar coupling constants of βM1 and $\alpha M_{6\beta}1$ (see below).

Consider now the observed chemical shifts of **3**, taken from ref 3, which are compared in Table II with predicted values taken to be the experimental chemical shifts of **2** (Table I) modified as follows: The chemical shifts for the additional residue $\alpha M_{6\alpha}$ were taken from Table VI of the preceding paper[1] for a nonreducing terminal (1→6(α))-linked α-D-mannopyranose residue.

Table II. Observed and Predicted ^{13}C Chemical Shifts of **3**

carbon[a]	chemical shift[b] exptl[c]	chemical shift[b] calcd[d]	carbon[a]	chemical shift[b] exptl[c]	chemical shift[b] calcd[d]
$\alpha M_{3\alpha}1$	103.48 (1)	103.42	$\alpha M_{6\alpha}3$	*72.06* (17)	72.04
G_B1	102.61 (2)	102.56	βM2	71.86 (18)	71.81
βM1	101.78 (3)	101.64	$\alpha M_{3\alpha}3$	71.86 (18)	71.81
$\alpha M_{6\beta}1$	101.26 (4)	101.04	$\alpha M_{3\alpha}2$	71.55 (19)	71.50
$\alpha M_{6\alpha}1$	100.63 (5)	100.63	$\alpha M_{6\alpha}2$	71.39 (20)	71.42
G_A4*	80.74 (6)	80.54	$\alpha M_{6\beta}2$	70.87 (21)	70.80
G_B4*	*80.13* (7)	80.07	$\alpha M_{6\alpha}4$	68.22 (22)	68.21
$\alpha M_{6\beta}3$	*80.02* (8)	79.86	$\alpha M_{3\alpha}4$	68.22 (22)	68.18
G_A1	79.48 (9)	79.44	βM4	67.88 (23)	67.84
G_A5	77.50 (10)	77.46	βM6	*67.64* (24)	67.43
G_B5	75.84 (11)	76.00[e]	$\alpha M_{6\beta}4$	*67.11* (25)	67.33
βM5	75.84 (11)	75.80[e]	$\alpha M_{6\beta}6$	66.64 (26)	66.86
$\alpha M_{3\alpha}5$	74.60 (12)	74.54	$\alpha M_{3\alpha}6$	62.40 (27)	62.27
G_A3	74.15 (13)	74.11	$\alpha M_{6\alpha}6$	62.40 (27)	62.37
βM3*	74.15 (13)	74.11	G_A6*	61.52 (28)	61.48
$\alpha M_{6\alpha}5$*	74.04 (14)	74.07	G_B6*	61.34 (29)	61.30
G_B3	73.37 (15)	73.30	G_B2	56.47 (30)	56.40
$\alpha M_{6\beta}5$	*72.27* (16)	72.11	G_A2	55.05 (31)	55.01

[a] Carbohydrate residues are designated as in Figure 1. The number following the residue designation is the carbon number. *Asterisks* on two vertically adjacent carbons indicate that their assignments have not been made on a one-to-one basis. [b] In ppm downfield from Me_4Si. [c] Obtained from the spectrum whose anomeric region is shown in Figure 3C of ref 3 (~20 mM **3** in H_2O, pH 4.4, 40 °C), after digital subtraction of the resonances of free D-mannose.[3] Numbers in parentheses are peak designations. [d] Calculated values were determined by taking the values of **2** as the starting point and correcting for the addition of $\alpha M_{3\alpha}$ at C-3 of $\alpha M_{3\beta}$, as described in the text. [e] The calculated values for G_B5 and βM5 are not given on a one-to-one basis.

The chemical shifts of $\alpha M_{6\beta}$ were corrected for substitution at C-6 with the use of Table V of the preceding paper.[1] As in the case of **2**, a comparison of the experimental and predicted values for **3** (Table II) yields one-to-one assignments for nearly all resonances. However, our one-to-one assignments within the pairs (G_B4, $\alpha M_{6\beta}3$), ($\alpha M_{6\beta}5$, $\alpha M_{6\alpha}3$), and (βM6, $\alpha M_{6\beta}4$) do not follow from Table II but are based on specific assignments derived for **4** and **5** (see below).

It should be noted at this point that in this report we are using a different philosophy for predicting the ^{13}C chemical shifts of complex oligosaccharides than in the preceding paper,[1] where in our calculations for **3** and **4** (designated M_4B and M_4A, respectively, in ref 1) we used the experimental spectrum of **1** as the starting point, together with Tables V and VI of ref 1, without the use of experimental spectra of molecules larger than **1**. In fact, such an approach is feasible for **5**, **6**, **7**, and even larger "high mannose" glycopeptides. However, in the preceding paper[1] the motivation was to test the predictive powers of Tables V and VI of that paper. In this report we are concerned with making specific assignments of the ^{13}C resonances of **3**, **4**, **5**, **6**, and **7**. We find that, even though the ab initio procedure of the preceding paper has fairly good predictive power (see Figures 6 and 7 of ref 1), we get even better overall agreement between the calculated and experimental chemical shifts if we use as the starting point for the calculations the *experimental* ^{13}C chemical shifts of the largest possible precursor, preferably a precursor with only one less carbohydrate residue than the molecule under condideration. This method is feasible once the assignments of the ^{13}C resonances of the precursor have been carried out. Thus, when we completed our assignments for the ^{13}C resonances of **2**, we used the experimental data of **2** as the starting point for the analysis of the spectrum of **3**. In the same manner, we shall now use **2**, **4**, **5**, and **6** as the starting points for the analysis of **4**, **5**, **6**, and **7**, respectively.

Consider the spectrum of **4** shown in Figure 2B. Experimental and predicted chemical shifts are shown in Table III. The predicted values were taken to be the *experimental* ones of **2** modified to take into account the additional $\alpha M_{3\beta}$ residue by adding the chemical shifts of the 1→3β entries of Table VI of

Table III. Observed and Predicted ^{13}C Chemical Shifts of 4

carbon[a]	Δ[b]	chemical shift[c] exptl[d]	chemical shift[c] calcd[e]	carbon[a]	Δ[b]	chemical shift[c] exptl[d]	chemical shift[c] calcd[e]
$\alpha M_{3\beta}1$	+0.01	*103.74* (1)	103.7	$\alpha M_{3\alpha}3$	+0.16	71.85 (17)	71.81
$\alpha M_{3\alpha}1$	+0.01	*103.50* (2)	103.42	$\alpha M_{3\beta}3$	+0.16	71.85 (17)	72.0
G_B1	−0.01	102.64 (3)	102.56	$\beta M2$	+0.07	*71.56* (18)	71.4
$\beta M1$	−0.02	101.56 (4)	101.6	$\alpha M_{3\alpha}2$*	+0.12	*71.56* (18)	71.50
$\alpha M_{6\beta}1$	−0.01	101.01 (5)	101.06	$\alpha M_{3\beta}2$*	+0.12	*71.45* (19)	71.8
$\beta M3$	+0.06	81.93 (6)	81.9	$\alpha M_{6\beta}2$	+0.09	70.90 (20)	70.83
G_A4*	−0.03	80.67 (7)	80.54	$\alpha M_{3\beta}4$	+0.11	68.33 (21)	68.4
G_B4*	+0.01	80.09 (8)	80.07	$\alpha M_{3\alpha}4$	+0.13	68.23 (22)	68.18
$\alpha M_{6\beta}3$	+0.07	79.63 (9)	79.64	$\alpha M_{6\beta}4$	+0.07	*67.51* (23)	67.43
G_A1	+0.08	79.48 (10)	79.44	$\beta M6$	−0.05	*67.23* (24)	67.5
G_A5	0	77.51 (11)	77.46	$\beta M4$	+0.07	*66.92* (25)	67.2
G_B5	+0.01	75.83 (12)	75.8[f]	$\alpha M_{3\alpha}6$	+0.11	62.56 (26)*	62.27
$\beta M5$	+0.01	75.83 (12)	75.9[f]	$\alpha M_{3\beta}6$	+0.11	62.46 (27)*	62.6
$\alpha M_{3\beta}5$	+0.04	74.76 (13)	74.8	$\alpha M_{6\beta}6$	+0.11	62.31 (28)*	62.27
$\alpha M_{3\alpha}5$	+0.03	74.63 (14)	74.54	G_A6*	+0.10	61.47 (29)	61.48
G_A3	+0.08	*74.18* (15)	74.11	G_B6*	+0.09	61.35 (30)	61.30
$\alpha M_{6\beta}5$	+0.04	*74.14*[h]	74.11	G_B2	+0.11	56.42 (31)	56.40
G_B3	+0.11	73.37 (16)	73.30	G_A2	+0.13	55.04 (32)	55.01

[a] Carbohydrate residues are designated as in Figure 1. The number following the residue designation is the carbon number. *Asterisks* on two vertically adjacent carbons indicate a lack of one-to-one assignments for these two carbons. [b] Value of the deuterium isotope effect for this resonance, in ppm. Each Δ value was obtained from the listed chemical shift by subtracting the corresponding value of the sample in D_2O. [c] In ppm downfield form Me_4Si. *Asterisks* on a group of two or more vertically adjacent chemical shifts indicate that all the corresponding assignments refer to the group as a whole and are not one-to-one assignments. [d] Obtained from the spectrum of Figure 2B (4 in H_2O, pH 6.9, 32 °C). Numbers in parentheses are the peak designations of Figure 2B. Resonances which are not given in the table had the following chemical shifts in a spectrum recorded at pH 6.3 and 32 °C, using a larger spectral window (and lower digital resolution) than for Figure 2B: 52.44 (Asn C^α), 36.46 (Asn C^β), 23.56 (CH_3), 23.45 (CH_3); the carbonyl resonances were at 176.21, 175.79, 174.19, and 173.94 ppm. [e] Calculated values were obtained by taking the experimental chemical shifts of 2 and correcting for the addition of $\alpha M_{3\beta}$ at C-3 of βM, as described in the text. The discrepancies between the experimental and calculated values for $\alpha M_{3\beta}$ and βM are expected to be considerably greater than for the other carbohydrate residues, because the calculated values for $\alpha M_{3\beta}$ (taken from Table VI of the preceding paper[1]) and the corrections for substitution at C-3 of βM (taken from Table V of ref 1) are based on chemical shift data from the literature and are therefore not as directly comparable with our values as the rest of the calculated values, which are based on chemical shifts measured in our laboratory. For an illustration of the problem of comparing ^{13}C chemical shifts from different laboratories, see Table IX-XI of ref 1. [f] Because G_B5 and $\beta M5$ of 2 have not been assigned on a one-to-basis (Table I), another set of predicted values for G_B5 and $\beta M5$ of 4 is 76.0 and 75.7 ppm, respectively. [g] The resonances of C-6 of $\alpha M_{3\alpha}$, $\alpha M_{3\beta}$, and $\alpha M_{6\beta}$ have not been assigned on a one-to-basis. [h] Shoulder on the upfield side of peak 15.

the preceding paper[1] and by correcting the chemical shifts of βM for C-3 substitution with the use of the 3β correction factors of Table V of ref 1. It should be noted here that the agreement between experimental and calculated values is expected to be less good for $\alpha M_{3\beta}$ and βM than for the other residues of 4, because the values of the $1\rightarrow3\beta$ listing of Table VI of ref 1 and the 3β corrections of Table V of ref 1 are based on chemical shift data from the literature and are therefore not as directly comparable with our chemical shifts as the rest of the entries of Table V and VI of ref 1, which are based on measurements in our laboratory. The reader is referred to Tables IX–XI of ref 1 for illustrations of the problems which arise when comparing ^{13}C chemical shifts from different laboratories. In any case, a comparison of the experimental and predicted values of Table III clearly yields all the listed one-to-one assignments except that of $\beta M2$ and those for the sets ($\alpha M_{3\beta}1$, $\alpha M_{3\alpha}1$), (G_A3, $\alpha M_{6\beta}5$), and ($\alpha M_{6\beta}4$, $\beta M6$, $\beta M4$). The specific assignments of peak 1 (103.74 ppm) and peak 2 (103.50 ppm) to $\alpha M_{3\beta}1$ and $\alpha M_{3\alpha}1$, respectively, are based on the following evidence: (i) Only the peak at 103.5 ppm is present in the spectrum of **3** (Table II). (ii) The α-D-mannosylation of $\alpha M_{3\alpha}2$ (going from **5** to **6**) causes an upfield shift of the peak at 103.7 ppm but does not affect the one at 103.5 ppm. The specific assignment of peak 15 to G_A3 is based on the effects of pH and D_2O: Peak 15 (74.18 ppm) undergoes a 0.2 ppm downfield shift when going from pH 1.8 to 10.0, while the shoulder at 74.14 ppm has a pH-independent chemical shift. Also, peak 15 has Δ = 0.08 ppm while the shoulder has Δ = 0.04 ppm (Table III), which indicates that peak 15 arises from the hydroxyl-bearing G_A3. The specific assignment for $\beta M2$ (relative to $\alpha M_{3\alpha}2$ and $\alpha M_{3\beta}2$) and one-to-one assignments within the set ($\alpha M_{6\beta}4$, $\beta M6$, $\beta M4$) follow from the effect of D_2O (see below).

As mentioned in our discussion of **2**, the specific assignment for $\beta M1$ is based on values of one-bond $^{13}C-^1H$ scalar coupling constants ($^1J_{CH}$) which we determined only for 4. We observed values of 171 ± 2, 172 ± 2, 165 ± 2, 160 ± 2, and 171 ± 2 Hz for peaks 1, 2, 3, 4, and 5, respectively. The high values for peaks 1, 2, and 5 are only consistent with α-pyranose anomers, while those of peaks 3 and 4 indicate that these are the resonances of the two residues with β-pyranose configurations which yield anomeric carbon resonances in the range of peaks 1–5 (G_B and βM).[9] Since we have firmly assigned peak 3 (at 102.64 ppm) to G_B1 on the basis of evidence presented for 1, peak 4 (at 101.56 ppm) is assigned to $\beta M1$. This removes the uncertainty of the relative assignments of $\beta M1$ and $\alpha M_{6\beta}1$ presented above when discussing **2**.

The effects of D_2O (Table III) yield independent evidence for some of the assignments of Table III. In the range of peaks 6–10, the relatively small Δ values of peaks 7 and 8 (~0.01 ppm) are consistent with assignments to G_A4 and G_B4 (not on a one-to-one basis), because each lacks a β effect and has only one γ effect. In contrast, $\beta M3$ and $\alpha M_{6\beta}3$ have no β effects but each has two γ effects, so that the resulting Δ values of about 0.06 and 0.07 ppm of peaks 6 and 9, respectively, are consistent with the assignments of Table III. Also, G_A1 has one β and one γ effect (both from NH groups), which is consistent with Δ = 0.08 ppm for peak 10. In the range of peaks 11–16, we have two relatively large Δ values for peak 15 (0.08 ppm) and peak 16 (0.11 ppm) and small Δ values (≲0.04 ppm) for all other resonances. This is consistent with our assignments of peaks 15 and 16 to G_A3 and G_B3, the only carbons in this range which have a β effect.

In the range of peaks 17–20, peak 17 (a two-carbon resonance) is the only one with the very large Δ value of 0.16 ppm and must therefore be assigned to $\alpha M_{3\alpha}3$ and $\alpha M_{3\beta}3$, the only two carbons in this range with a β and *two* γ effects. Of the remaining four contributors in this range, $\alpha M_{3\alpha}2$ and $\alpha M_{3\beta}2$ have one β and one γ effect, while $\beta M2$ and $\alpha M_{6\beta}2$ have one β and no γ effect.

(9) Bock, K.; Lundt, I.; Pedersen, C. *Tetrahedron Lett.* **1973**, 1037–1040. Bock, K.; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 293–297.

Table IV. Observed and Predicted ^{13}C Chemical Shifts of **5**

carbon[a]	Δ[b]	chemical shift[c] exptl[d]	chemical shift[c] calcd[e]	carbon[a]	Δ[b]	chemical shift[c] exptl[d]	chemical shift[c] calcd[e]
$\alpha M_3\beta 1$	+0.01	103.77 (1)	103.74	$\alpha M_3\alpha 3$	+0.16	71.84 (22)	71.85
$\alpha M_3\alpha 1$	+0.01	103.55 (2)	103.50	$\alpha M_3\beta 3$	+0.16	71.84 (22)	71.85
$G_B 1$	−0.02	102.63 (3)	102.64	$\beta M2$	+0.10	*71.59*[f]	71.56
$\beta M1$	−0.03	101.64 (4)	101.56	$\alpha M_3\alpha 2$*	+0.12	*71.56* (23)	71.56
$\alpha M_6\beta 1$	−0.01	101.13 (5)	100.99	$\alpha M_3\beta 2$*	+0.12	71.44 (24)	71.45
$\alpha M_6\alpha 1$	0	100.60 (6)	100.63	$\alpha M_6\alpha 2$	+0.12	71.39 (25)	71.42
$\beta M3$	+0.06	81.97 (7)	81.93	$\alpha M_6\beta 2$	+0.09	70.86 (26)	70.87
$G_A 4$*	−0.03	80.78 (8)	80.67	$\alpha M_3\beta 4$	+0.12	68.32 (27)	68.33
$G_B 4$*	−0.01	80.06 (9)	80.09	$\alpha M_3\alpha 4$	+0.13	68.21 (28)*	68.23
$\alpha M_6\beta 3$	+0.06	79.96 (10)	79.85	$\alpha M_6\alpha 4$	+0.12	68.18 (29)*	68.21
$G_A 1$	+0.08	79.48 (11)	79.48	$\beta M6$	−0.05	*67.26* (30)	67.23
$G_A 5$	0	77.51 (12)	77.51	$\alpha M_6\beta 4$	+0.08	*67.10* (31)	67.41
$G_B 5$	+0.01	75.81 (13)*	75.83	$\beta M4$	+0.06	*66.89* (32)	66.92
$\beta M5$	0	75.70 (14)*	75.83	$\alpha M_6\beta 6$	−0.02	*66.58* (33)	66.90[g]
$\alpha M_3\beta 5$	+0.03	74.76 (15)	74.76	$\alpha M_3\beta 6$	+0.11	62.56 (34)*	*h*
$\alpha M_3\alpha 5$	+0.03	74.63 (16)	74.63	$\alpha M_3\alpha 6$	+0.11	62.45 (35)*	*h*
$G_A 3$	+0.10	74.18 (17)	74.18	$\alpha M_6\alpha 6$	+0.11	62.38 (36)*	62.37
$\alpha M_6\alpha 5$	+0.03	74.03 (18)	74.07	$G_A 6$*	+0.10	61.47 (37)	61.47
$G_B 3$	+0.10	73.38 (19)	73.37	$G_B 6$*	+0.10	61.33 (38)	61.35
$\alpha M_6\beta 5$	+0.01	72.22 (20)	72.14	$G_B 2$	+0.10	56.42 (39)	56.42
$\alpha M_6\alpha 3$	+0.15	72.05 (21)	72.04	$G_A 2$	+0.13	55.03 (40)	55.04

[a] Carbohydrate residues are designated as in Figure 1. The number following the residue designation is the carbon number. *Asterisks* on two vertically adjacent carbons indicate a lack of one-to-one assignments for these two carbons (experimental and calculated values). [b] Deuterium isotope effects on the ^{13}C chemical shifts of **5**, in ppm. Each Δ value was obtained by subtracting a chemical shift of **5** in D_2O from the corresponding value of **5** in H_2O. [c] In ppm downfield from Me_4Si. *Asterisks* on a group of two or more vertically adjacent chemical shifts indicate that all the corresponding assignments refer to the group as a whole and are not one-to-one assignments. [d] Obtained from the spectrom of Figure 2C (**5** in H_2O, pH 7.8, 32 °C). Numbers in parentheses are the peak designations of Figure 2C. Resonances which are not given in the table had the following chemical shifts in a spectrum recorded at pH 6.5 and 28 °C, using a larger spectral window (and lower digital resolution) than for Figure 2C: 52.44 (Asn C^α), 36.49 (Asn C^β), 23.59 (CH_3), 23.47 (CH_3); the carbonyl resonances were at 176.19, 175.76, 174.26, and 173.92 ppm. [e] Calculated values were obtained by taking the experimental ones of 4 and correcting for the addition of $\alpha M_{6\alpha}$ at C-6 of $\alpha M_{6\beta}$, as described in the text. [f] Shoulder on the upfield portion of peak 22 of Figure 2C. [g] Because of the uncertainty in the assignment for $\alpha M_6\beta 6$ of 4, two other possible values are 67.05 and 67.15 ppm. [h] Three possible values are 62.56, 62.46, and 62.31 ppm, because of a lack of one-to-one assignments in the spectrum of 4.

Clearly, peak 19 and one-half of peak 18 (with Δ = 0.12 ppm) should arise from $\alpha M_{3\alpha}2$ and $\alpha M_{3\beta}2$, while peak 20 (Δ = 0.09 ppm) and the other half of peak 18 ($\Delta \approx$ 0.07 ppm) should be assigned to $\beta M2$ and $\alpha M_{6\beta}2$ (not on a one-to-one basis). However, since the specific assignment of peak 20 to $\alpha M_{6\beta}2$ follows directly from the calculated chemical shifts of Table III, we conclude that $\beta M2$ gives rise to one-half of peak 18.

In the range of peaks 23–25 of 4, peak 24 (Δ slightly *negative*) must arise from $\beta M6$, which lacks β and γ effects altogether, while peaks 23 and 25 (both with Δ = 0.07 ppm) should be assigned to $\alpha M_{6\beta}4$ and $\beta M4$, which have one β and no γ effect. Once peak 24 has been assigned to $\beta M6$, the one-to-one assignments for $\alpha M_{6\beta}4$ and $\beta M4$ to peaks 23 and 25, respectively, do follow from the calculated chemical shifts of Table III. They also follow from evidence presented below for **5**.

Consider now the spectrum of **5** shown in Figure 2C. The observed chemical shifts are compared in Table IV with predicted values obtained by taking the *experimental* values of 4, with corrections for the addition of $\alpha M_{6\alpha}$ at C-6 of $\alpha M_{6\beta}$, as described above for the prediction of the chemical shifts of **3** from the experimental values of **2**. A comparison of the experimental and calculated values in Table IV clearly yields all the given one-to-one assignments except that of $\beta M2$ and those for the set ($\beta M6$, $\alpha M_{6\beta}4$, $\beta M4$, and $\alpha M_{6\beta}6$), which are presented below.

On the basis of the calculated chemical shifts for **5**, either peak 23 (71.56 ppm) or the shoulder at 71.59 ppm (Table IV) must be assigned to $\beta M2$. However, because of its relatively small Δ value (Table IV), the shoulder at 71.59 ppm is assigned to $\beta M2$, on the basis of arguments analogous to those used above for assigning $\beta M2$ of 4.

The slightly *negative* Δ values of peaks 30 and 33 (Table IV) indicate that they arise from C-6 (and not C-4) of βM and $\alpha M_{6\beta}$, because C-6 has no β and γ effects at all, while C-4 has a β effect. We can also distinguish between C-4 and C-6 on the basis of line widths, because the methylene carbon of a *glycosidically linked* C-6 should have about twice the line width of a methine carbon of the pyranose ring,[10a] in the absence of complications caused by internal rotations,[10a] an assumption justified for *internal* residues on the basis of the T_1 values of the tetrasaccharide stachyose.[10b] Indeed, peaks 30 and 33 of Figure 2C are considerably broader than peaks 31 and 32. Furthermore, the spin–lattice relaxation times (T_1) of $\beta M6$ and $\alpha M_{6\beta}6$ should be about half as long as those of $\beta M4$ and $\alpha M_{6\beta}4$.[10a] Indeed, the inversion recovery spectra of Figure 5 indicate that peaks 30 and 33 have about half the T_1 values of peaks 31 and 32, because peaks 30 and 33 null when $\tau \simeq$ 0.05 s while peaks 31 and 32 null at $\tau \simeq$ 0.10 s. Having established that peaks 30 and 33 arise from C-6 while peaks 31 and 32 arise from C-4 (of βM and $\alpha M_{6\beta}$), the one-to-one assignments for these carbons follow from the calculated chemical shifts of Table IV.

A large number of the specific assignments which follow directly from the calculated chemical shifts of Table IV can be confirmed from the effects of D_2O (see Δ values in Table IV). Furthermore, T_1 values also confirm various assignments. For example, consider the inversion recovery spectra of the anomeric carbon resonances (peaks 1–6 in Figure 5). Clearly, peaks 1, 2, and 6 as a group have longer T_1 values than peaks 3–5 as a group. Therefore, we can assign the first group of resonances to the three terminal residues $\alpha M_{3\beta}$, $\alpha M_{3\alpha}$, and $\alpha M_{6\alpha}$ (not on a one-to-one basis), on the basis of arguments about the effects of internal rotations on T_1 values, as follows. It has been shown[10a] that if the rate of overall molecular tumbling is considerably greater than the experimental resonance frequency, a condition which is valid here,[10a] then internal motion (faster than overall tumbling) causes a monotonic increase in T_1 relative to the value expected in the absence of internal motion. Therefore, terminal carbohydrate residues will have longer T_1 values than those of internal residues in the same molecule, if internal rotation is fast enough.[10a] The experimental verification of these conclusions has been amply documented, and the results obtained for stachyose[10b] are particularly pertinent here.

(10) (a) Allerhand, A.; Doddrell, D.; Komoroski, R. *J. Chem. Phys.* **1971**, *55*, 189–198. (b) Allerhand, A.; Doddrell, D. *J. Am. Chem. Soc.* **1971**, *93*, 2777–2779.

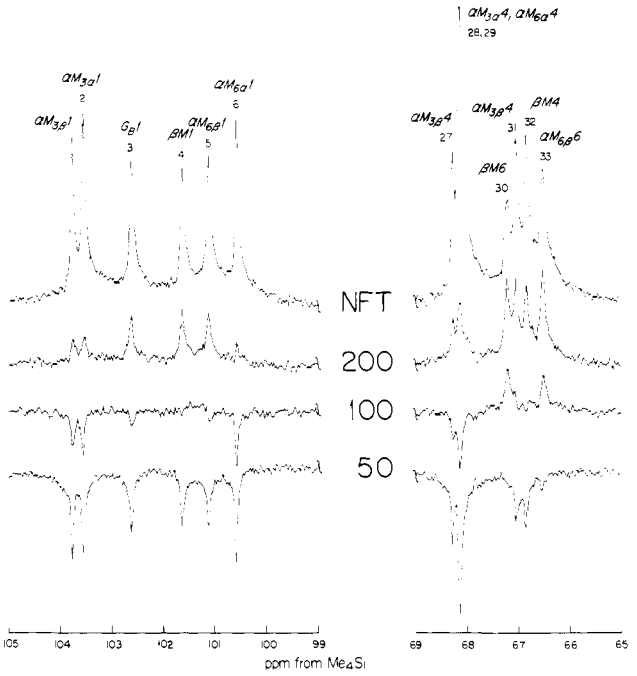

Figure 5. Portions of the inversion–recovery proton-decoupled ^{13}C NMR spectra (at 67.9 MHz) of **5** (20 mM in H_2O, pH 6.5). The spectrum labeled NFT is a normal Fourier transform NMR spectrum, with the peaks numbered as in Figure 2C. The other three are inversion–recovery spectra labeled with the interval (τ, in milliseconds) between each 180° radio-frequency pulse and the following 90° pulse. The NFT spectrum was recorded at 28 °C with a recycle time of 2.0 s and 8192 scans. The inversion–recovery spectra were recorded at 32 °C with a recycle time of τ + 1.5 s and 10 000 scans per spectrum. The vertical amplitudes of all spectra have been normalized to make a direct peak height comparison possible. All spectra were obtained in a 15-mm probe.

Consider now the spectrum of **6** (Figure 2D). Experimental and calculated chemical shifts are given in Table V. The calculated chemical shifts were obtained by using the experimental values of **5** as a starting point and then correcting for the addition of $\alpha M_{2\alpha}$ at C-2 of $\alpha M_{3\beta}$, with the use of Tables V and VI of ref 1. There is remarkable agreement between the experimental and predicted chemical shifts, and actually *all* the specific assignments given in Table V follow readily from a comparison of the two sets of values.

Finally, Figure 2E shows the spectrum of **7**. The experimental and predicted chemical shifts are given in Table VI, together with measured Δ values. The calculated chemical shifts were obtained from the experimental values of **6** by correcting for the addition of $\alpha M_{2\alpha'}$ at C-2 of $\alpha M_{6\alpha}$. Again, there is very good agreement between the experimental and calculated chemical shifts, except for C-5 and C-6 of $\alpha M_{6\beta}$ (see below). All the assignments presented in Table VI, except the relative assignments of peaks 10 and 11, follow readily from a comparison of the experimental and theoretical chemical shifts. The specific assignment of peak 11 (and not peak 10) to $\alpha M_{6\beta}3$ comes from the observation that peak 10 has Δ = 0.09 ppm (while peak 11 has a negligible value of Δ), which is consistent with the presence of hydroxyl groups on the two carbons adjacent to C-3 of $\alpha M_{6\beta}$. The Δ values of Table VI confirm many other specific assignments.

It is interesting that the experimental chemical shifts of $\alpha M_{6\beta}5$ and $\alpha M_{6\beta}6$ of **7** are larger than the corresponding calculated values by 0.30 and 0.35 ppm, respectively. The Δ value of the resonance we assigned to $\alpha M_{6\beta}5$ (0.02 ppm), together with the large Δ values of all other resonances in its vicinity (Table VI), clearly confirms our assignment for $\alpha M_{6\beta}5$. The specific assignment for $\alpha M_{6\beta}6$ is also the only one consistent with the calculated chemical shifts and Δ values of Table VI. We believe that the discrepancies of about 0.3 ppm between the observed and calculated chemical shifts of $\alpha M_{6\beta}5$ and $\alpha M_{6\beta}6$ are caused by long-range interactions (between nonadjacent carbohydrate residues) which come into play when going from **6** to **7**. An analogous effect probably explains the shift of peak 3 (80.11 ppm) or peak 4 (79.99 ppm) of **1**, assigned to G_A4 or G_B4, to 80.54 ppm in the spectrum of **2**, 80.74 ppm in the spectrum of **3**, 80.67 ppm in the spectrum of **4**, and 80.78 ppm in the spectrum of **5**, with no further significant changes when going to **6** and **7**.

Table V. Observed and Predicted ^{13}C Chemical Shifts of **6**

carbon[a]	chemical shift[b] exptl[c]	chemical shift[b] calcd[d]	carbon[a]	chemical shift[b] exptl[c]	chemical shift[b] calcd[d]
$\alpha M_{3\alpha}1$	103.55 (1)*	103.55	$\alpha M_{3\alpha}3$	71.84 (25)[e]	71.84
$\alpha M_{2\alpha}1$	103.51 (2)*	103.47	$\alpha M_{2\alpha}3$	71.78 (26)[e]	71.83
G_B1	102.63 (3)	102.63	βM2	71.55 (27)*	71.59
$\alpha M_{3\beta}1$	102.10 (4)	102.14	$\alpha M_{3\alpha}2$	71.55 (27)*	71.56[f]
βM1	101.62 (5)	101.64	$\alpha M_{3\beta}3$	71.51 (28)*	71.47
$\alpha M_{6\beta}1$	101.15 (6)	101.13	$\alpha M_{6\alpha}2$	71.37 (29)	71.39
$\alpha M_{6\alpha}1$	100.60 (7)	100.60	$\alpha M_{2\alpha}2$	71.37 (29)	71.40
βM3	82.05 (8)	81.97	$\alpha M_{6\beta}2$	70.86 (30)	70.86
G_A4*	80.77 (9)	80.78	$\alpha M_{3\beta}4$	68.49 (31)	68.54
G_B4*	80.04 (10)	80.06	$\alpha M_{3\alpha}4$	68.18 (32)	68.21*
$\alpha M_{6\beta}3$	79.96 (11)	79.96	$\alpha M_{6\alpha}4$	68.18 (32)	68.18*
$\alpha M_{3\beta}2$	79.65 (12)	79.78[g]	$\alpha M_{2\alpha}4$	68.18 (32)	68.27
G_A1	79.47 (13)	79.48	βM6	67.26 (33)	67.26
G_A5	77.51 (14)	77.51	$\alpha M_{6\beta}4$	67.09 (34)	67.10
G_B5*	75.80 (15)	75.81	βM4	66.90 (35)	66.89
βM5*	75.70 (16)	75.70	$\alpha M_{6\beta}6$	66.56 (36)	66.58
$\alpha M_{3\beta}5$	74.77 (17)	74.77	$\alpha M_{3\beta}6$	62.53 (37)*	*h*
$\alpha M_{3\alpha}5$	74.63 (18)	74.63	$\alpha M_{3\alpha}6$	62.43 (38)*	*i*
$\alpha M_{2\alpha}5$	74.55 (19)	74.55	$\alpha M_{6\alpha}6$	62.43 (38)*	*i*
G_A3	74.17 (20)	74.18	$\alpha M_{2\alpha}6$	62.37 (39)*	62.41
$\alpha M_{6\alpha}5$	74.02 (21)	74.03	G_A6*	61.46 (40)	61.47
G_B3	73.37 (22)	73.38	G_B6*	61.32 (41)	61.33
$\alpha M_{6\beta}5$	72.20 (23)	72.22	G_B2	56.42 (42)	56.42
$\alpha M_{6\alpha}3$	72.05 (24)	72.05	G_A2	55.01 (43)	55.03

[a] Carbohydrate residues are designated as in Figure 1. The number following each residue designation is the carbon number. *Asterisks* on two or more vertically adjacent carbons indicate a lack of one-to-one assignments for this group of carbons (experimental and calculated values). [b] In ppm downfield from Me_4Si. *Asterisks* on a group of two or more vertically adjacent chemical shifts indicate that all the corresponding assignments refer to the group as a whole and are not one-to-one assignments. [c] Obtained from the spectrum of Figure 2D (6 in H_2O, pH 7.3, 32 °C). Numbers in parentheses are the peak designations of Figure 2D. Resonances which are not given in the table had the following chemical shifts in a spectrum recorded at pH 5.0 and 32 °C, using a larger spectral window (and lower digital resolution) than for Figure 2D: 52.44 (Asn C^α), 36.44 (Asn C^β), 23.61 (CH_3), 23.47 (CH_3); the carbonyl resonances were at 176.19, 175.76, 174.19, and 173.90 ppm. [d] Calculated values were obtained by taking the experimental chemical shifts of **5** and correcting for the addition of $\alpha M_{2\alpha}$ at C-2 of $\alpha M_{3\beta}$ as described in the text. [e] The experimental values for $\alpha M_{3\alpha}3$ and $\alpha M_{2\alpha}3$ have not been assigned on a one-to-one basis. [f] Because of a lack of one-to-one assignments for $\alpha M_{3\alpha}2$ and $\alpha M_{3\beta}2$ of **5**, another possible value for $\alpha M_{3\alpha}2$ of 6 is 71.44 ppm. [g] Because of a lack of one-to-one assignments for $\alpha M_{3\alpha}2$ and $\alpha M_{3\beta}2$ of **5**, another possible value for $\alpha M_{3\beta}2$ of 6 is 79.90 ppm. [h] Because of a lack of one-to-one assignments for C-6 of $\alpha M_{3\beta}$, $\alpha M_{3\alpha}$, and $\alpha M_{6\alpha}$ of **5**, together with an uncertainty in the correction factor for C-6 upon substitution at C-2 of an α-D-mannopyranosyl residue (Table V of ref 1), several values in the range of 62.3–62.7 ppm are possible. [i] Because of a lack of one-to-one assignments in the spectrum of **5**, possible values are 62.56, 62.45, and 62.38 ppm.

Conclusions

It is quite remarkable that even a large glycopeptide such as **7**, which contains nine carbohydrate residues out of which *six* are α-D-mannopyranosyl residues (Figure 1), should yield a ^{13}C NMR spectrum which contains mainly resolved or partly resolved single-carbon resonances. Equally satisfying is the fact, demonstrated in this paper, that it is realistic to expect that one can assign such a jungle of ^{13}C resonances on a one-to-one basis (for the most part) to specific carbons.

Experimental Section

Pronase Digestion of Ovalbumin. For the purpose of obtaining compounds **5–8**, we used a procedure which is a slight modification of a

Table VI. Observed and Predicted ^{13}C Chemical Shifts of 7

carbon[a]	Δ[b]	chemical shift[c] exptl[d]	chemical shift[c] calcd[e]	carbon[a]	Δ[b]	chemical shift[c] exptl[d]	chemical shift[c] calcd[e]
$\alpha M_{3\alpha}1$	*f*	103.56 (1)*	103.55*	$\alpha M_{3\alpha}3$	*g*	71.85 (25)*	71.84*
$\alpha M_{2\alpha}1$	0	103.50 (2)*	103.51*	$\alpha M_{2\alpha}3$	+0.16	71.81 (26)*	71.78*
$\alpha M_{2\alpha'}1$	0	103.50 (2)*	103.47	$\alpha M_{2\alpha'}3$	+0.16	71.81 (26)*	71.83
G_B1	−0.01	102.65 (3)	102.63	$\alpha M_{6\alpha}3$	+0.12	71.70 (27)	71.68
$\alpha M_{3\beta}1$	−0.02	102.10 (4)	102.10	$\beta M2^*$	+0.11	71.56 (28)	71.55
$\beta M1$	−0.01	101.58 (5)	101.62	$\alpha M_{3\alpha}2^*$	+0.11	71.56 (28)	71.55
$\alpha M_{6\beta}1$	−0.02	101.24 (6)	101.15	$\alpha M_{3\beta}3^*$	+0.12	71.51 (29)[h]	71.51
$\alpha M_{6\alpha}1$	−0.02	99.37 (7)	98.97	$\alpha M_{2\alpha}2$	+0.11	71.39 (30)	71.37
$\beta M3$	+0.07	82.09 (8)	82.05	$\alpha M_{2\alpha'}2$	+0.11	71.39 (30)	71.40
G_A4^*	−0.01	80.76 (9)	80.77	$\alpha M_{6\beta}2$	+0.10	70.86 (31)	70.86
G_B4^*	−0.01	*80.04* (10)	80.04	$\alpha M_{3\beta}4$	+0.14	68.50 (32)	68.49
$\alpha M_{6\beta}3$	+0.09	*80.02* (11)	79.96	$\alpha M_{6\alpha}4$	*i*	68.37 (33)*	68.40
$\alpha M_{6\alpha}2$	−0.02	79.87 (12)	79.71	$\alpha M_{3\alpha}4$	*i*	68.32 (34)*	68.18
$\alpha M_{3\beta}2$	−0.01	79.67 (13)	79.65	$\alpha M_{2\alpha}4$	−0.13	68.20 (35)*	68.18
G_A1	+0.09	79.48 (14)	79.47	$\alpha M_{2\alpha'}4$	+0.13	68.20 (35)*	68.27
G_A5	+0.01	77.52 (15)	77.52	$\beta M6$	−0.03	67.31 (36)	67.26
G_B5^*	+0.04	75.82 (16)	75.80	$\alpha M_{6\beta}4$	+0.10	67.02 (37)	67.09
$\beta M5^*$	+0.01	75.71 (17)	75.70	$\beta M4$	+0.06	66.91 (38)	66.90
$\alpha M_{3\beta}5$	+0.02	74.77 (18)	74.77	$\alpha M_{6\beta}6$	+0.03	66.91 (38)	66.56
$\alpha M_{3\alpha}5$	+0.03	74.63 (19)	74.63	$\alpha M_{3\beta}6$	+0.12	62.55 (39)*	*j*
$\alpha M_{2\alpha}5$	+0.04	74.55 (20)	74.55	$\alpha M_{3\alpha}6$	+0.12	62.55 (39)*	*j*
$\alpha M_{2\alpha'}5$	+0.04	74.55 (20)	74.55	$\alpha M_{2\alpha}6$	+0.12	62.45 (40)*	*j*
G_A3	+0.12	74.18 (21)	74.17	$\alpha M_{6\alpha}6$	+0.12	62.45 (40)*	*k*
$\alpha M_{6\alpha}5$	+0.04	74.08 (22)	74.03	$\alpha M_{2\alpha'}6$	+0.12	62.45 (40)*	62.41
G_B3	+0.11	73.36 (23)	73.37	G_A6^*	+0.12	61.47 (41)	61.46
$\alpha M_{6\beta}5$	+0.02	72.50 (24)	72.20	G_B6^*	+0.11	61.34 (42)	61.32

[a] Carbohydrate residues are designated as in Figure 1. The number following the residue designation is the carbon number. *Asterisks* on two or more vertically adjacent carbons indicate a lack of one-to-one assignments for this group of carbons (experimental and calculated values). [b] Deuterium isotope effects on the ^{13}C chemical shifts of 7, in ppm. Each Δ value was obtained by subtracting a chemical shift of 7 in D_2O from the corresponding value of 7 in H_2O. [c] In ppm downfield from Me_4Si. *Asterisks* on a group of two or more vertically adjacent chemical shifts indicate that all the corresponding assignments refer to the group as a whole and are not one-to-one assignments. [d] Obtained from the spectrum of Figure 2E (7 in H_2O, pH 7.1, 31 °C). Numbers in parentheses are the peak designations of Figure 2E. Resonances which are not given in the table had the following chemical shifts in a spectrum recorded at pH 6.8 and 32 °C, using a larger spectral window than for Figure 2E: 56.43 (G_B2), 55.04 (G_A2), 52.50 (Asn C^α), 36.52 (Asn C^β), 23.59 (CH_3), 23.46 (CH_3); the carbonyl resonances were at 176.17, 175.74, 174.20, and 173.93 ppm. [e] Calculated values were obtained by taking the experimental chemical shifts of 6 and correcting for the addition of $\alpha M_{2\alpha'}$ at C-2 of $\alpha M_{6\alpha}$, as described in the text. [f] The poor resolution of this peak in the spectrum of 7 in D_2O yields a Δ value in the range −0.06 to −0.03 ppm. [g] The poor resolution of this peak in the spectrum of 7 in D_2O yields $\Delta \gtrsim 0.15$ ppm. [h] Peak 29 is a shoulder on the upfield portion of peak 28. [i] The poor resolution of this peak in the spectrum of 7 in D_2O yields $\Delta \gtrsim 0.10$ ppm. [j] Because of a lack of one-to-one assignments in the spectrum of 6, the calculated values can be 62.37, 62.43, or 62.53 ppm. [k] Because of a lack of one-to-one assignments in the spectrum of 6, together with an uncertainty in the correction factor for C-6 upon substitution at C-2 of an α-D-mannopyranosyl residue (Table V of ref 1), several values in the range 62.3–62.7 ppm are possible.

published one.[11,12] As a first step, 30 g of ovalbumin (Type V from Sigma Chemical Co.) was dialyzed extensively against deionized water, 4 times for a total of 48 h, and then adjusted to 600 mL in H_2O (15 mM $CaCl_2$, pH 8.5). While this solution was stirred and maintained at 37 °C, three successive additions of protease (Type VI from Sigma) were made (200 mg each time) at 6 h intervals. The pH of the reaction mixture was monitored and maintained at 8.5. The reaction was carried out for a total of 24 h.

The resulting mixture was concentrated under vacuum to about one-half its volume and centrifuged at 11000*g*. The supernatant was further concentrated under vacuum to about 100 mL and centrifuged again. The resulting residues were combined and shaken with 50 mL of water and centrifuged again. All supernatants were combined. The insoluble residue was found to contain less than 0.5% of the original carbohydrate content.

The combined supernatant was applied in 50-mL fractions to a Sephadex G25-150 column (61 × 3 cm) which was equilibrated with 0.1 M acetic acid and run at a rate of 120 mL/h at 4 °C. Typically, 8-mL fractions were collected, monitored at 254 nm, and analyzed for hexoses (orcinol–sulfuric acid method at 540 nm). The fractions containing hexoses were collected and freeze-dried. This procedure was repeated three times for a total of 100 g of ovalbumin.

The combined hexose-containing material was dissolved in 15 mM $CaCl_2$ solution (300 mL) and redigested again for a total of 48 h, with four additions of 100 mg protease. The resulting reaction mixture was concentrated under vacuum to 150 mL and centrifuged. The insoluble residue was suspended in 50 mL of the buffer solution and then spun again. The supernatants were combined and fractionated on the same column as before. The digestion procedure was repeated with four 50 mg additions of protease over 48 h and then with four 25 mg additions of protease over 48 h. Finally the collected material (7 g) was cleaned again on the same column as before to give 4.7 g of a glycopeptide mixture which contained about 90% of the original hexose content.

Fractionation of Glycopeptides. The glycopeptide mixture obtained above was fractionated on an ion exchange column (Dowex 50Wx2-400, 2 × 145 cm, 50 mM sodium acetate, pH 2.6, rate 28 mL/h at 4 °C) which had been equilibrated with the buffer solution for at least a week. Fractions containing up to 300 mg of hexoses were separated. When the last fraction was eluted, the buffer was changed to 50 mM sodium acetate, pH 6.0, in order to remove those fractions which contain polypeptide chains. The elution pattern was monitored with the orcinol–sulfuric acid test (at 420 nm), and it was found to be essentially the same as reported.[11,13,14]

Some of the fractions were rechromatographed on the same column as described above but at slow rates (20–23 mL/h). The fractions were desalted and purified by gel filtration (BioGel P4, 200–400 mesh, 25 mL/h, elution with water) with the same column dimensions as above. Further purifications of each fraction were achieved by gel filtration at even slower rates (about 10–15 mL/h). The purity of each fraction was checked by means of its ^{13}C NMR spectrum. Compounds **5**–**7** were obtained by this procedure. The proof of structure for compounds **5** and **6** has been reported by Conchie and Strachan.[13] Tai and co-workers have shown the structure of **7**.[14,15] In any case, the ^{13}C NMR spectra of **1**–**7**, as reported here, provide sufficient proof of structure *plus* the assignments of the ^{13}C resonances.[1] Compounds **2** and **3** were transient species

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during the α-mannosidase digestion of **5** and **6**.[3] Compounds **1** and **4** were prepared by glycosidase digestions of related glycopeptides, as described below.

Glycosidase Digestions. Compound **1** was obtained as the final product of the exhaustive digestions of **5** and **6** with jack bean α-mannosidase, as described.[3] It was purified by gel filtration chromatography with Bio-Gel P4. The starting point for the preparation of **4** was a hen ovalbumin glycopeptide related to **5** but with two extra GlcNAc(β) residues, one linked to C-4 of βM and the other to C-2 of $\alpha M_{3\beta}$. This compound was first digested with jack bean α-mannosidase, which removed only $\alpha M_{6\alpha}$. Then a digestion with β-*N*-acetylglucosaminidase yielded **4**. Details will be given elsewhere.[16]

Carbon-13 NMR Spectroscopy. The ^{13}C NMR spectra were recorded at 67.9 MHz, essentially as described.[1] For the spectra of Figure 2, we used 16384 time-domain addresses (two segments of 8192 addresses for quadrature detection) and a total spectral width of 6250 Hz, which resulted in a digital resolution of 0.0112 ppm. A spectral width of 12500 Hz (0.0225 ppm digital resolution) was used for Figure 3 and some other spectra, in order to cover the whole range of ^{13}C chemical shifts. The measured chemical shifts and Δ values of **1**, **4**, **5**, **6**, and **7** (Tables I and III–VI) were taken from spectra recorded with 0.0112 ppm digital resolution, while the experimental chemical shifts of **2** and **3** (Tables I and II) came from spectra with 0.0225 ppm digital resolution. However, the spectra which were used to obtain the deuterium isotope effects (Δ values) for **1** and **5** were processed with 0.0056 ppm digital resolution, by converting each time-domain spectrum of 16384 data points (0.0112 ppm digital resolution) into 32768 data points by means of zero filling (a tail of 8192 zero addresses was added to each of the two quadrature-detected segments of 8192 time-domain points). The beneficial effect of this zero filling on resolution and, more importantly from our standpoint, on the precision of chemical shift measurements has been thoroughly demonstrated.[17-21] A trace of internal dioxane was used as a reference (taken at 67.86 ppm downfield from the ^{13}C resonance of Me_4Si) for the chemical shift determinations. The values of directly bonded $^{13}C-^{1}H$ scalar coupling constants were measured from spectra recorded under conditions of gated decoupling set up to give the full nuclear Overhauser enhancement without affecting $^{13}C-^{1}H$ scalar coupling.[22] Inversion–recovery spectra were obtained with the use of the 180°–τ–90° pulse sequence.[22]

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