Carbohydrate Research, 142 (1985) 11–20
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL, DYNAMIC, AND METAL-ION BINDING STUDIES OF THE CORE GLYCOPEPTIDES β-D-Gal-(1→3)-α-D-GalNAc→Ser, Thr BY ^{13}C-N.M.R. SPECTROSCOPY

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(Received November 2nd, 1984; accepted for publication in revised form, February 28th, 1985)

ABSTRACT

^{13}C-N.m.r. spectral data as well as spin–lattice relaxation times (T_1 values) are presented for the core glycopeptides β-D-Gal-(1→3)-α-D-GalNAc→Ser, Thr. The binding of Gd^{3+} to these model compounds containing N-terminal blocking groups and esterified carboxyl groups indicates that the disaccharide contains a rather weak, but unique, binding-site in the vicinity of C-2 of α-D-GalNAc (possibly involving N-2′, the acetamido carbonyl group, O-3′ and/or possibly the glycosidic oxygen atom (O-3)).

INTRODUCTION

In the past few years, considerable use has been made of ^{13}C-nuclear magnetic resonance spectroscopy (^{13}C-n.m.r.) in order to gain structural and dynamic information about the carbohydrate residues of glycoproteins[1–5] and about metal-ion–carbohydrate interactions of relevant carbohydrates, glycopeptides, and glycoproteins[6–11]. These studies were made possible because many of the ^{13}C-spectral resonance assignments in these studies were based on published ^{13}C-n.m.r. work of relevant glycopeptides[12–17].

In order to further promote utilization of ^{13}C-n.m.r. spectroscopy for the study of glycoprotein structure and metal-ion–glycoprotein (carbohydrate) interactions, we present in this paper structural and dynamic information about the core glycopeptides β-D-Gal-(1→3)-α-D-GalNAc→Ser [methyl ester] (**1**), and β-D-Gal-(1→3)-α-D-GalNAc→Thr [methyl ester] (**3**) and the metal-ion binding studies of related compounds (**2**) and (**4**). These core glycopeptides are known to occur in many glycoproteins[18,19], especially in glycophorin A, the major erythrocyte transmembrane glycoprotein, which is of interest to our respective research groups[20–25].

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0008-6215/85/$ 03.30

1 R = H
2 R = benzyloxycarbonyl (Z)

3 R = H
4 R = benzyloxycarbonyl (Z)

5 R = Z

6 R = Z

7 R = Z

8 R = Z

9 R = Z

10 R = Z

EXPERIMENTAL

Material. — Gadolinium oxide (99.9%) was purchased from Alfa Products, Danvers, MA., and converted into gadolinium chloride.

General methods. — ^{13}C-N.m.r. spectra were obtained on a Jeol-FX90Q instrument operating at 22.5 MHz (2.1 T) in the f.t. mode, as described previously[6]. A stock solution of Gd^{3+} was also prepared as previously described[9]. Chemical shifts were obtained from ^{13}C-chemical-shift data available for related model compounds[7–10,14–17], and are given relative to Me_4Si.

Samples of the model compounds were routinely treated with Chelex-100 (H^+ form) and then freeze-dried. Preparation of the model compound samples for n.m.r. use involved dissolving them in de-ionized, distilled H_2O and adjusting the pH to 6.0–7.0. For the Gd^{3+}-binding studies, additions of Gd^{3+} stock solution to the samples were made by using an Eppendorf pipet, with the total additions ranging from 6 to 12 μL. Spin–lattice relaxation times (T_1 values) of the various carbon atoms were determined by using the PRFT method[26] with two sets of 6 τ values. The estimated precision is ±0.1 s.

Optical rotations were recorded with a Perkin–Elmer model 241 polarimeter. Capillary melting points were determined with a Büchi apparatus and are reported uncorrected. Thin–layer chromatography (t.l.c.) was performed on silica gel plates (Merck F 254), and the components were monitored by either u.v. light or by spraying with a 5% sulfuric acid–ethanol solution followed by heating at 100°. Column chromatography was performed on Merck 60, 70–230 mesh, silica gel or 230–400 mesh in the case of flash chromatography.

3-O-(2-Acetamido-2-deoxy-α-D-galactopyranosyl)-N-(benzyloxycarbonyl)-L-serine methyl ester (**5**) *and -L-threonine methyl ester* (**6**). — 3-*O*-(3,4,6-Tri-*O*-acetyl-2-azido-2-deoxy-α-D-galactopyranosyl)-*N*-(benzyloxycarbonyl)-L-serine (or -L-threonine) methyl ester were prepared using the method previously described by Ferrari and Pavia[27]. The reduction and acetylation of the azido group was performed according to the method of Paulsen[28]. *O*-Deacetylation of the D-galactosyl residue was achieved by treatment with triethylamine–methanol mixture to provide compounds **5** and **6**. The physical constants for compounds **5** and **6** were similar to those described in the literature[27].

3-O-[2-Acetamido-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-4,6-O-benzylidene-2-deoxy-α-D-galactopyranosyl]-N-benzyloxycarbonyl-L-serine methyl ester (**7**) *and -L-threonine methyl ester* (**8**). — Compound **5** (0.67 g, 1.47 mmol) was dissolved in a mixture of acetonitrile (27 mL) and *N,N*-dimethylformamide (3 mL). α,α-Dimethoxytoluene (0.8 mL) and *p*-toluenesulfonic acid (0.015 g) were added and the mixture was stirred overnight. Dichloromethane (50 mL) was added and the mixture was then washed with saturated sodium hydrogencarbonate, dried over sodium sulfate, and evaporated. Recrystallization of the residue from ether provided 3-*O*-(2-acetamido-4,6-*O*-benzylidene-2-deoxy-α-D-galactopyranosyl)-*N*-benzyloxycarbonyl-L-serine methyl ester in 70% yield (0.56 g); m.p. 196–198°, $[\alpha]_D^{20}$ +100.9° (*c* 1, $CHCl_3$).

The foregoing compound (0.35 g, 0.64 mmol) was dissolved in acetonitrile (10 mL). Mercuric cyanide (1 g) and dry calcium sulfate (1 g) were added. The mixture was stirred under nitrogen for 30 min, and tetra-*O*-acetyl-α-D-galactopyranosyl bromide (0.57 g, 1.4 mmol) was then added. After 8 h, more glycosyl bromide (0.5 g), mercuric cyanide (1 g), and calcium sulfate (0.5 g) were added. The mixture was stirred for an additional 16 h, diluted with CH_2Cl_2 (50 mL), and filtered through Celite 545. The filtrate was washed successively with solutions of potassium iodide (10%), saturated sodium thiosulfate, saturated sodium hydrogencarbonate, and water; it was then dried (sodium sulfate) and evaporated. The residue was purified by flash chromatography (65:35 toluene–acetone) to afford 0.32 g (60% yield) of compound **7** as a white foam; $[\alpha]_D^{20}$ +78° (*c* 0.8, $CHCl_3$).

Compound **8** was prepared, by the same method used to prepare compound **7**, in 62% yield; m.p. 103–105° (dec.), $[\alpha]_D^{20}$ +86.6° (*c* 0.8, $CHCl_3$).

3-O-[2-Acetamido-3-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-2-deoxy-α-D-galactopyranosyl]-N-benzyloxycarbonyl-L-serine methyl ester (**9**) *and -L-threonine methyl ester* (**10**). — Compound **7** (0.38 g, 0.43 mmol) was dissolved in 80% acetic acid (10 mL) and heated at 75° for one h. The mixture was evaporated and toluene was evaporated from the residue, which was purified by flash chromatography (2:3 CH_2Cl_2–acetone) to afford 0.31 g (91%) of the desired product; m.p. 170–175°, $[\alpha]_D^{20}$ +63.6° (*c* 1, $CHCl_3$).

Compound **10** was prepared in the identical manner used to prepare **9**, yield 92%; m.p. 104–105°, $[\alpha]_D^{20}$ +58° (*c* 0.5, $CHCl_3$).

3-O-[2-Acetamido-3-(β-D-galactopyranosyl)-2-deoxy-α-D-galactopyranosyl]-N-benzyloxycarbonyl-L-serine methyl ester (**2**) *and -L-threonine methyl ester* (**4**). — Acetylated compound **9** (0.275 g, 0.354 mmol) was dissolved in methanol (15 mL). Triethylamine (1.5 mL) was added, the mixture was stirred for 8 hours, evaporated, and toluene was evaporated from the residue, which was then dissolved in methanol and shaken with IRC-50 (H^+) resin for 2 h. After filtration and evaporation, the oily residue obtained crystallized from ether to give **2** as a white powder; yield 93% (0.2 g); m.p. 210° (dec.); $[\alpha]_D^{20}$ +84.3° (*c* 1, H_2O).

Compound **4** was similarly prepared starting with **10**. The yield was 92%; m.p. 170–175° (dec.), $[\alpha]_D^{20}$ +94° (*c* 0.5, H_2O).

Compounds **1** and **3** were prepared by the hydrogenation of **2** and **4** in ethanol–water (10% Pd/C under H_2 at a pressure of 0.5 MPa)[8].

RESULTS AND DISCUSSION

Tables I and II give the ^{13}C chemical-shift data and resonance assignments for the carbon atoms of compounds **1** and **3**. Figs. 1 and 2 show the effects of added Gd^{3+} on the ^{13}C resonances of compounds **2** and **4**. The ^{13}C resonance assignments in the tables and figures are based on the literature ^{13}C data available for glycoproteins[2] and glycopeptides[14–17] containing α-Gal*p*NAc and β-Gal*p*. The ^{13}C chemical-shift data and resonance assignments determined for compound **3** are

TABLE I

^{13}C-N.M.R. SPECTRAL DATA FOR **1**[a]

Carbon atom	*Chemical shift*[b]	T_1 *(s)*[c]
Ac & Ser CO	176.0 173.9	
β-Gal C-1	106.0	.36
α-GalNAc C-1	99.5	.18
α-GalNAc C-3	78.3	.30
β-Gal C-5	76.4	.33
β-Gal C-3	74.1	.39
α-GalNAc C-5 & β-Gal C-2	72.2	.29[d]
β-Gal C-4 & α-GalNAc C-4	70.2	.28[d]
Ser C-3	68.1	.32
α-GalNAc C-6 & β-Gal C-6	62.6	.25[d]
Ser C-2	56.1	.18
Ser (OMe)	54.6	.67
α-GalNAc C-2	50.0	.24
CH_3 (Ac)	23.6	.60

[a]Compound **1** was 135mM in H_2O, pH 7.04. The probe temperature was 30°. [b]The chemical shifts of the various resonances are referenced relative to C-6 of β-D-Gal*p* and α-D-Gal*p*NAc, taken to be 62.6 p.p.m. (see ref. 16). Estimated precision for the chemical shifts is ± 0.05 p.p.m. [c]Observed T_1 values. Determined only for protonated carbon atoms. [d]Average value.

almost identical to those reported for the disaccharide β-Gal-(1→3)-α-GalNAc→ found in fish antifreeze glycoproteins[2].

The anomeric configuration of carbohydrates **1** and **3** were unequivocally determined by the C-1′ coupling constants ($^1J_{CH}$)[14] and by the respective spectral patterns. The $^1J_{CH}$ values for anomeric carbon atoms of α-D-GalNAc and β-D-Gal of compounds **1** and **3** were found to be 168 and 160 Hz, respectively. Other features that are observed when comparing the ^{13}C data to that published for nonglycosylated Thr[17], Ser[19], and methyl α-galactopyranoside[30], are that Thr C-3, Ser C-3 and α-D-GalNAc C-3 shift approximately 7, 7, and 4 p.p.m. downfield, respectively, upon glycosylation.

Other important points concerning these compounds are that the resonance of α-D-GalNAc C-2 shifts ~1.2 p.p.m. upfield as a result of glycosylation at α-D-GalNAc C-3, whereas the resonance of α-D-GalNAc C-4 appears to be unaffected. Thus it would appear that this carbon atom is a good probe for detecting a substitution at α-D-GalNAc C-3. It should also be noted that the chemical shift of Thr C-4 does not appear to be affected by the 1→3 linkage of β-Gal to α-GalNAc. The $^1J_{CH}$ of methyl carbon atoms of the acetamido group of compounds **1** and **3** were found to be 128 and 129 Hz, respectively; these values are in excellent agreement with the $^1J_{CH}$ values observed for the acetamido methyl carbon atoms of α-GalNAc→Ser and α-GalNAc→Thr[14], but is much larger than the $^1J_{CH}$ value of 117 Hz observed

TABLE II

^{13}C-N.M R SPECTRAL DATA FOR **3**[a]

Carbon atom	*Chemical shift*[b]	T_1 *(s)*[c]
Ac & Thr CO	175.6 173.9	
β-Gal C-1	106.0	.38
α-GalNAc C-1	100.1	.28
α-GalNAc C-3	78.2	.30
β-Gal C-5 & Thr C-3	76.4	.38[d]
β-Gal C-3	74.1	.43
α-GalNAc C-5 & β-Gal C-2	72.2	.33[d]
β-Gal C-4 & α-GalNAc C-4	70.1	.34[d]
β-Gal C-6 & α-GalNAc C-6	62.6	.33[d]
Thr C-2	59.7	.20
Thr (OMe)	54.2	.78
α-GalNAc C-2	50.2	.23
CH_3 (Ac)	23.8	.79
Thr C-4	19.5	.28

[a]Compound **3** was 136mM in H_2O, pH 7.39. The probe temperature was 30°. [b]The chemical shifts of the various resonances are referenced relative to C-6 of β-D-Gal*p* and α-D-Gal*p*NAc, taken to be 62.6 p.p.m. (see ref. 16). Estimated precision for the chemical shifts is ± 0.05 p.p.m. [c]Observed T_1 values. Determined only for protonated carbon atoms. [d]Average value.

for the acetamido methyl carbon atom of the α-GalNAc residue of the core oligosaccharide found in fish antifreeze glycoproteins[2]. The lower coupling-constant observed for the fish antifreeze glycoprotein may be related to its function, as suggested by the authors[2].

The T_1 data for these model compounds give an indication as to the motional freedom of the specific carbon atoms[26]. For instance, it would be expected that most of the pyranoside carbon atoms (C-1′–C-6′) would have similar values T_1 values)[31]. These seem to be the case for our data, except for the carbon atoms involved in the glycosyl linkage (β-D-Gal C-1, α-D-GalNAc C-1 and C-3, Thr C-3, and Ser C-3). These are expected to show limited motion, by a decrease in the T_1 value. This is generally the case for our data. The limited motional freedom may also be detected by the decrease in the intensity of a given resonance (see Figs. 1 and 2), because of an increase in linewidths.

Figs. 1 and 2 show the effects of added Gd^{3+} on the ^{13}C-n.m.r. spectra of compounds **2** and **4**, respectively. We used Gd^{3+} because it is a relaxation probe that has been used to mimic Ca^{2+} in biological studies. The degree of line broadening observed for the carbohydrate carbon resonances may be used to gain quantitative information about the Gd^{3+}–carbohydrate carbon atom distances, because it has been shown that a dipolar mechanism dominates the ^{13}C–T_2

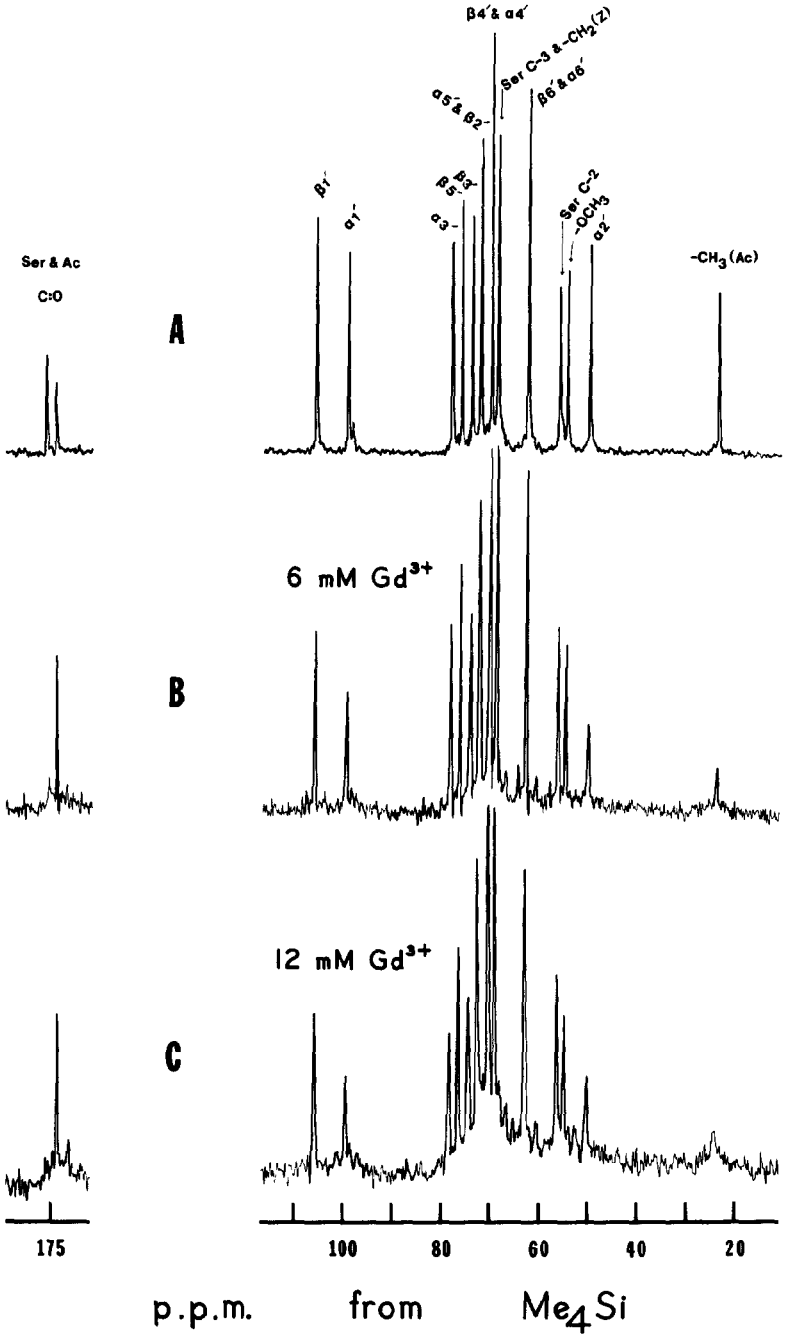

Fig. 1. The effect of Gd^{3+} on the ^{13}C resonances of the proton-decoupled, natural abundance, ^{13}C-n.m.r. spectrum of **2**. [Spectra were recorded with recycle times varying from 0.8–2.0 s. The concentration of compound **2** was 140mM in H_2O, pH ~7. The vertical gain of the spectra of solutions containing large portions of paramagnetic relaxation-reagent was increased slightly, so that broadening effects could be clearly observed. (A) Sample contained no Gd^{3+}, and required 23,703 accumulations. A line-broadening factor of 2.2 Hz was used during the data processing. (B) Sample contained 6.0mM Gd^{3+}, and required 31,498 accumulations. A line-broadening factor of 2.8 Hz was used during the data processing. (C) Sample contained 12.0mM Gd^{3+}, and required 30,210 accumulations. A line-broadening factor of 4.3 Hz was applied during the data processing.]

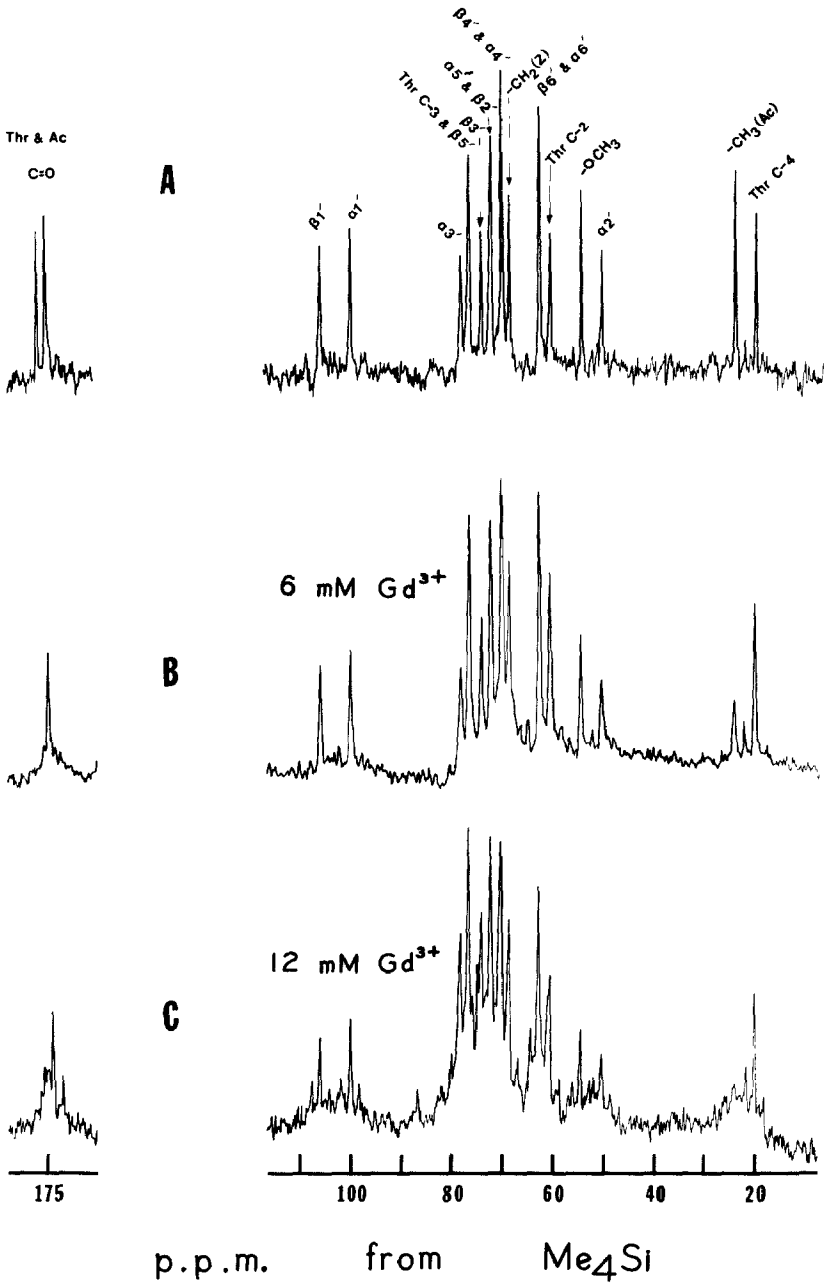

Fig. 2. The effect of Gd^{3+} on the ^{13}C resonances of the proton-decoupled, natural abundance, ^{13}C-n.m.r. spectrum of **4**. [Spectra were recorded with recycle times varying from 0.8–2.0 s. The concentration of compound **4** was 146mM in H_2O, pH ~7. The vertical gain of the spectra of solutions containing large portions of paramagnetic relaxation-reagent was increased slightly, so that broadening effects could be clearly observed. (A) Sample contained no Gd^{3+}, and required 20,198 accumulations. A line-broadening factor of 3.5 Hz was used during the data processing. (B) Sample contained 6.0mM Gd^{3+}, and required 13,311 accumulations. A line-broadening factor of 4.0 Hz was used during the data processing. (C) Sample contained 12.0mM Gd^{3+}, and required 22,640 accumulations. A line-broadening factor of 3.5 Hz was applied during the data processing.]

(electron–nuclear) relaxation in cyclitol–Gd^{3+} complexes[32]. Because of the overlapping resonances and the fact that the metal-ion binding to the carbohydrate appears to be "weak", no metal-ion–carbohydrate distance information was obtained. Our data does, however, give a qualitative view of the type(s) of carbohydrate–Gd^{3+} interactions that occur with these glycopeptides.

Previous work concerning Gd^{3+}–glycopeptide interactions were done with simple model compounds in which the amino acid(s) were glycosylated with only a single saccharide. The current work allows us to determine whether a disaccharide provides a better binding site, or chelation structure, for certain metal ions. Our studies used compounds containing blocked amino acids (OMe, *N*-benzyloxycarbonyl) because we have previously shown that free carboxyl and amino groups bind such metal ions as Gd^{3+} strongly[8]. Therefore in this work, we have removed any strong metal-ion binding sites associated with these groups.

Fig. 1 indicates that the addition of Gd^{3+} to a solution of compound **2** specifically broadens the resonances of the acetyl carbon atoms (methyl and carbonyl), α-D-GalNAc C-2, and to some extent β-D-Gal C-3, and C-1 of α-D-GalNAc. The results seem to indicate a rather specific binding-site near the vicinity of α-D-GalNAc C-2, possibly involving N-2 and O-3 of α-D-GalNAc, and the glycosidic oxygen atom (O-3). Fig. 2 indicates that the addition of Gd^{3+} to a solution of compound **4** specifically broadens the ^{13}C resonances of the acetyl carbon atoms (methyl and carbonyl) and C-2 of α-D-GalNAc, indicating a specific binding-site near α-D-GalNAc C-2, possibly involving N-2′.

These results are somewhat different than those observed for the binding of Gd^{3+} to *O*-α- and β-D-galactosylated peptides[8–10] and α-D-GalNAc→Ser-(OMe)(NAc)[8]. For the studies dealing with binding of Gd^{3+} to *O*-α- and β-D-galactosylated peptides, unique Gd^{3+} binding sites were observed near C-1′ and C-6′[8–10]. In the single study dealing with the binding of Gd^{3+} to α-D-GalNAc→Ser(OMe)(NAc), binding sites for Gd^{3+} were observed that involved N-2′ as well as the acetyl protecting group on the serine[8]. Work is currently in progress dealing with the metal-ion binding studies of more complex oligosaccharides of glycopeptides.

REFERENCES

1 K. DILL AND A. ALLERHAND, *J. Biol. Chem.*, 254 (1979) 4524–4531.
2 E. BERMAN, A. ALLERHAND, AND A. L. DEVRIES, *J. Biol. Chem.*, 255 (1980) 4407–4410.
3 E. BERMAN, D. E. WALTERS, AND A. ALLERHAND, *J. Biol. Chem.*, 256 (1981) 3853–3857.
4 E. BERMAN AND T. L. JAMES, *Carbohydr. Res.*, 113 (1983) 141–150.
5 T. A. GERKEN AND D. G. DEARBORN, *Biochemistry*, 23 (1984) 1485–1497.
6 M. E. DAMAN AND K. DILL, *Carbohydr. Res.*, 102 (1982) 47–57.
7 M. E. DAMAN AND K. DILL, *Carbohydr. Res.*, 132 (1984) 335–338.
8 K. DILL, M. E. DAMAN, R. L. BATSTONE-CUNNINGHAM, J. M. LACOMBE, AND A. A. PAVIA, *Carbohydr. Res.*, 123 (1983) 123–135.
9 K. DILL, M. E. DAMAN, R. L. BATSTONE-CUNNINGHAN, M. DENARIÉ, AND A. A. PAVIA, *Carbohydr. Res.*, 123 (1983) 137–144.
10 K. DILL, M. E. DAMAN, R. L. BATSTONE-CUNNINGHAM, M. DENARIÉ, AND A. A. PAVIA, *Carbohydr. Res.*, 124 (1983) 11–22.

11 M. E. DAMAN AND K. DILL, *Carbohydr. Res.*, 111 (1983) 205–214.
12 K. DILL AND A. ALLERHAND, *FEBS Lett.*, 107 (1979) 26–29.
13 A. ALLERHAND, K. DILL, E. BERMAN, J. M. LACOMBE, AND A. A. PAVIA, *Carbohydr. Res.*, 97 (1981) 331–336.
14 K. DILL, J. M. LACOMBE, AND A. A. PAVIA, *Carbohydr. Res.*, 98 (1981) 132–138.
15 K. DILL, R. E. HARDY, J. M. LACOMBE, AND A. A. PAVIA, *Carbohydr. Res.*, 101 (1982) 330–334.
16 K. DILL, R. E. HARDY, M. E. DAMAN, J. M. LACOMBE, AND A. A. PAVIA, *Carbohydr. Res.*, 108 (1982) 31–40.
17 K. DILL, R. E. HARDY, J. M. LACOMBE, AND A. A. PAVIA, *Carbohydr. Res.*, 114 (1983) 147–152.
18 N. SHARON, *Complex Carbohydrates, Their Chemistry, Biosynthesis, and Functions*, Addison–Wesley, Reading, Massachusettes, 1978.
19 A. GOTTSCHALK (Ed.), *Glycoproteins, Their Composition, Structure and Function*, 2nd edn., Elsevier, Amsterdam, 1972.
20 R. E. HARDY, R. L. BATSTONE-CUNNINGHAM, AND K. DILL, *Arch. Biochem. Biophys.*, 222 (1983) 222–230.
21 R. L. BATSTONE-CUNNINGHAM, R. E. HARDY, M. E. DAMAN, AND K. DILL, *Biochim. Biophys. Acta*, 746 (1983) 1–7.
22 B. FERRARI AND A. A. PAVIA, *Int. J. Peptide Protein Res.*, 22 (1983) 549–559.
23 A. A. PAVIA AND B. FERRARI, *Int. J. Peptide Protein Res.*, 22 (1983) 539–548.
24 R. E. HARDY, R. L. BATSTONE-CUNNINGHAM, M. E. DAMAN, A. M. HOLBROOKS, AND K. DILL, *Int. J. Biol. Macromol.*, 6 (1984) 103–107.
25 K. DILL, R. E. HARDY, R. L. BATSTONE-CUNNINGHAM, M. E. DAMAN, B. FERRARI, AND A. A. PAVIA, *Carbohydr. Res.*, 128 (1984) 183–191.
26 E. OLDFIELD, R. S. NORTON, AND A. ALLERHAND, *J. Biol. Chem.*, 250 (1975) 6368–6380.
27 B. FERRARI AND A. A. PAVIA, *Carbohydr. Res.*, 79 (1980) C1–C7.
28 H. PAULSEN AND W. STENZEL, *Chem. Ber.*, 111 (1978) 2348–2357.
29 L. POGLIANI AND D. ZIESSOW, *Org. Magn. Reson.*, 17 (1981) 214–216.
30 K. BOCK, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.
31 M. F. CZARNIECKI AND E. R. THORNTON, *J. Am. Chem. Soc.*, 99 (1977) 8279–8282.
32 M. E. DAMAN AND K. DILL, *J. Magn. Reson.*, 60 (1984) 118–121.