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^{13}C-N.M.R.-SPECTRAL STUDY OF THE MODE OF BINDING OF Gd^{3+} TO VARIOUS GLYCOPEPTIDES

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ABSTRACT

Natural-abundance, ^{13}C-n.m.r. spectroscopy was used to study the mode of binding of Gd^{3+} to mono-*O*-glycosylated L-serine and tripeptides variously composed of Gly and L-Thr. When the amino and carboxyl groups of the amino acid are not blocked, strong interaction of Gd^{3+} with them is observed; this is also readily apparent with some related, nonglycosylated peptides. When the amino and carboxyl groups of the amino acid are blocked, noticeable interaction of Gd^{3+} with the glycosidic oxygen atom (O-3) and O-2′ for the glycopeptide containing α-D-Gal*p*, and with O-3 and N-2′ for the glycopeptide containing α-D-Gal*p*NAc, is observed. Weak interactions are also possible with O-4′ and O-6′ of the glycosyl groups. Although the amino acids were protected, these metal ion–carbohydrate interactions may still be mediated, to some extent, by the acetyl protecting the amino group and by the ester group on the amino acid.

INTRODUCTION

The interaction of metal ions with carbohydrates has been a topic of interest for a number of years. In the case of metal ion–monosaccharide interactions[1–5], this complexation is known to influence the anomeric population of some monosaccharides[1]. This phenomenon has also been used successfully to separate various methyl glycosides by ion-exchange chromatography[4].

More recently, metal ion–carbohydrate interactions have been investigated in the more-complex oligosaccharide structures of brain gangliosides and of glycophorin of the red-cell membrane[6–8]. In these specific cases, *N*-acetyl-α-neuraminic acid (α-NeuAc) was the prime constituent in the metal ion–oligosaccharide interactions. Apparently, other carbohydrate residues (D-Gal*p*, D-Gal*p*NAc) are also in-

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volved, to some degree, in the metal-ion-binding phenomena. To aid in the structural analysis of these complexations, Daman and Dill[9] recently showed the modes of interaction of Mn^{2+} and Gd^{3+} with various derivatives of α-NeuAc.

In order to gain knowledge about the interactions of glycoproteins with metal ions, we decided that it would be useful to study the interactions of metal ions with various small glycopeptides as model compounds. Therefore, we here present a study of the mode of interaction of Gd^{3+} with mono-*O*-glycosylated (α-D-Gal*p*- and α-D-Gal*p*NAc) L-serine and some tripeptides composed of Gly and L-Thr.

Our results indicate that Gd^{3+} binds to the amino and carboxyl groups of the amino acids. However, when these groups are blocked, Gd^{3+} seems to bind in the vicinity of C-1′ and C-2′ of the glycosyl groups. Moreover, a weak binding-site near C-4′ and C-6′ of the glycosyl groups has also been found. Gd^{3+} binding sites near C-1′ and C-2′ may be mediated by the acetyl blocking the amino group, and by the ester group on the amino acid.

EXPERIMENTAL

Material. — Gadolinium oxide (99.9%) was purchased from Alfa Products, Danvers, MA.

Synthesis of model compounds. — The synthesis of some of the glycopeptides discussed herein has been published[10,11], or has been achieved by using the stepwise, coupling strategy of Pavia and co-workers[12,13]. Unpublished syntheses of some of the peptides and glycopeptides were conducted as follows.

Methyl 3-O-α-D-galactopyranosyl-L-serinate. — A solution of methyl *N*-(benzyloxycarbonyl)-3-*O*-(2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl)-L-serinate[10] (0.2 g, 0.3 mmol) in a mixture of ethanol (20 mL), acetic acid (2 mL), and water (5 mL) was boiled overnight in the presence of 10% Pd/C (0.6 g) under H_2 at a pressure of 0.5 MPa. The mixture was filtered, the catalyst washed with water, and the filtrate and washings were combined, and evaporated *in vacuo* at 50°. Addition of water to the residue, filtration, and evaporation were repeated three times. The last washing was made with de-ionized water (10 mL), and followed by evaporation *in vacuo*. The residue was dissolved in acetone, from which crystallization occurred; the yield of product was 0.08 g (95%); m.p. 185° (dec.), $[\alpha]_D^{22}$ +47° (c 1.0, H_2O).

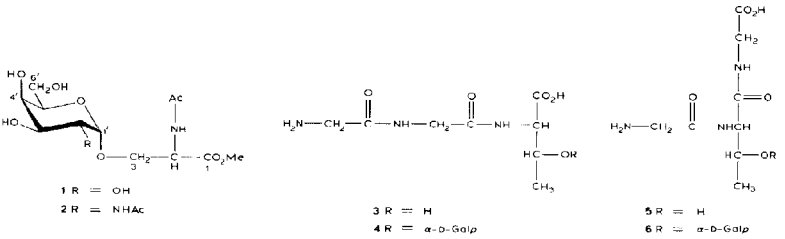
Methyl N-acetyl-3-O-α-D-galactopyranosyl-L-serinate (**1**). — The foregoing compound (0.08 g) was dissolved in a mixture of methanol (8 mL) and acetic anhydride (0.5 mL). After 30 min, the mixture was evaporated, and the volatile compounds were co-evaporated with toluene. The residue was dissolved in acetone, from which crystallization occurred; the yield of product was 0.085 g (97%); m.p. 178° (dec.), $[\alpha]_D^{22}$ +61.5° (c 1.0, H_2O).

Benzyl N-(benzyloxycarbonyl)glycyl-L-threonylglycinate. — The active ester *N*-hydroxysuccinimido *N*-(*tert*-butoxycarbonyl)-L-threoninate (Boc-Thr-OSu; 0.6

g, 2 mmol), prepared according to Storey *et al.*[14], was dissolved in CH_2Cl_2 (20 mL) containing glycine benzyl ester *p*-toluenesulfonate (0.7 g, 2.1 mmol) and triethylamine (0.25 g, 2.5 mmol), with stirring. After 4 h at room temperature, the mixture was evaporated *in vacuo*, to give Boc-Thr-Gly-OBzl as an oil that was used in the next step without purification. To remove the Boc protecting-group, this oil was dissolved in a mixture of CH_2Cl_2 (10 mL) and trifluoroacetic acid (10 mL). After 30 min at room temperature, the mixture was evaporated, and the residue was allowed to react with *N*-(benzyloxycarbonyl)glycine *o*-nitrophenyl ester (Z-Gly-*o*Np). The oily residue obtained on evaporation of the mixture was dissolved in 1,4-dioxane (20 mL); the solution was cooled to 0°, and NEt_3 (0.5 g), Z-Gly-*o*Np[15] (0.8 g), and benzotriazol-1-ol (HOBt) (0.05 g) were added. After 18 h at room temperature, the mixture was evaporated, and the residue dissolved in CH_2Cl_2; the solution was successively washed with 5% aqueous citric acid (2 × 20 mL), saturated sodium hydrogencarbonate (2 × 20 mL), and water, dried (sodium sulfate) and evaporated, to give a white powder (0.25 g, 45%) which crystallized from ethyl acetate–ethanol; m.p. 135–136°, $[\alpha]_D^{20}$ −35° (*c* 1.0, $CHCl_3$).

Glycyl-L-*threonylglycine* (**5**). — The foregoing compound (0.25 g), dissolved in methanol (20 mL), water (2 mL), and acetic acid (1 mL), was hydrogenated as previously described. After the usual processing, the title compound was obtained crystalline in a yield of 95%; m.p. 225–227°, $[\alpha]_D^{20}$ −31° (*c* 0.5, H_2O).

Benzyl N-*(benzyloxycarbonyl)glycylglycyl*-L-*threoninate*. — To a solution of benzyl L-threoninate hemioxalate (Bachem; 0.37 g, 1.5 mmol) in oxolane (THF; 10 mL) containing NEt_3 (0.2 mL, 1.5 mmol), and cooled to 0°, were added Z-Gly-Gly-*o*Np (Bachem; 0.6 g) and HOBt (0.05 g). After 24 h at room temperature, the mixture was evaporated, the residue dissolved in ethyl acetate, the solution successively washed with 5% citric acid (2 ×20 mL), saturated sodium carbonate (2 × 10 mL), and water (20 mL), dried, and evaporated, to give a white residue that crystallized from ethyl acetate. The yield was 0.45 g (51%); m.p. 127–128°, $[\alpha]_D^{20}$ +2.5° (*c* 0.7, MeOH).



Glycylglycyl-L-*threonine* (**3**). — Compound **3** was obtained by treatment of the foregoing compound with H_2 in the presence of Pd/C. The yield of product was 95%; m.p. 230° (dec.), $[\alpha]_D^{20}$ −6.5° (*c* 0.5, H_2O)

Methods. — Carbon-13 n.m.r. spectra were recorded with a JEOL-FX90Q instrument operated at 22.5 MHz (2.1 T) in the F.t. mode, as described previously[9]. Gd^{3+} stock solution was also prepared as previously described[9]; some of the chemical shifts were obtained by using internal 1,4-dioxane (added only when chemical shifts were determined), whose chemical shift was taken to be 67.86 p.p.m. downfield from Me_4Si. In some cases, chemical shifts were obtained from ^{13}C-chemical-shift data available for related model compounds[16].

Preparation of samples of the model compounds involved dissolving them in de-ionized, distilled H_2O and adjusting the pH to 6.0–7.0. Additions of Gd^{3+} stock solution to the samples were made by using an Eppendorf digital pipet, total additions ranging from 0.24 to 61 μL.

RESULTS AND DISCUSSION

Presented herein is the mode of interaction of Gd^{3+} with various glycopeptides, namely, mono-*O*-glycosylated L-serine and mono-*O*-glycosylated tripeptides composed of Gly and L-Thr. These compounds were *O*-glycosylated with α-D-Gal*p* and α-D-Gal*p*NAc.

In the case of L-serine, the carboxyl and amino groups were blocked, in order to minimize the known[17–19] interaction of Gd^{3+} with these groups. Thus, it should be possible to ascertain the mode of interaction of Gd^{3+} with the glycosyl groups

TABLE I

^{13}C-N.M.R. CHEMICAL-SHIFT DATA[a] FOR MODEL COMPOUNDS **1–6**

Carbon atom	**1**[b]	**2**[c]	**3**[d]	**4**[d]	**5**[f]	**6**[d]
1′	100.6	99.2		100.3		100.8
2′	69.7	51.4		69.9		69.8
3′	70.7	70.0		70.8		70.8
4′		68.9[e]				
5′	72.6	72.7		72.4		72.6
6′	62.5	62.7		62.6		62.7
Gly C-2 (N-terminal)			42.3	42.1	42.7	42.5
Gly C-2 (internal)			43.9	43.8		
Gly C-2 (C-terminal)					44.6	44.9
Thr C-3			69.3	76.9	68.2	75.9
Thr C-2			61.7	60.3	60.3	59.1
Thr C-4			20.5	19.6	19.8	19.0
Ser C-3	68.7	68.9[e]				
Ser C-2	54.6[f]	54.5[f]				
CH_3(Ac-2′)		23.5				
CH_3(Ac)	23.2	23.2				
CH_3(OMe)	54.6[f]	54.5[f]				

[a]Chemical shifts for these compounds are given at neutral pH. Estimated precision for the chemical shift is ±0.05 p.p.m. [b]216 mM in H_2O, pH 6.8; 15 250 accumulations. [c]125 mM in H_2O, pH 6.7, 13 600 accumulations. [d]Obtained from ref. 20. [e]A broad component due to the near overlap of two resonances. [f]Overlap of resonances.

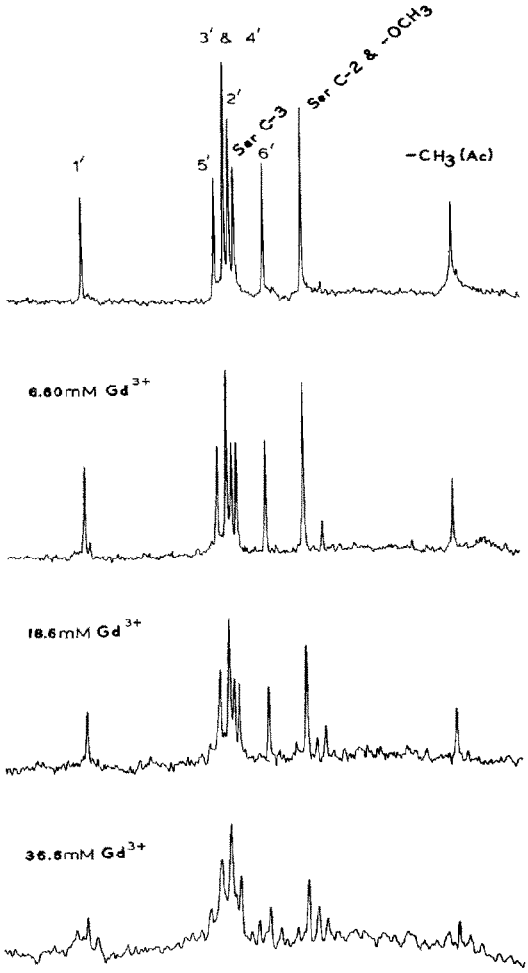


Fig. 1. The effect of added Gd^{3+} on the ^{13}C resonances of the aliphatic region of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of model compound **1**. [The concentration of compound **1** was 250mM (in H_2O), pH 6.8. A recycle time of 1.0–1.5 s was used to collect data. The vertical gain of the spectra containing the paramagnetic relaxation-reagent has been increased slightly, so that broadening effects may be clearly observed. (A) Sample contained no Gd^{3+}, and required 15 250 accumulations. A line-broadening factor of 3.0 Hz was applied during the data processing. (B) Sample contained 6.6mM Gd^{3+}, and required 21 723 accumulations. A line-broadening factor of 3.5 Hz was applied during the data processing. (C) Sample contained 18.6mM Gd^{3+}, and required 17 262 accumulations. A line-broadening factor of 5.0 Hz was applied during the data processing. (D) Sample contained 36.6mM Gd^{3+}, and required 35 690 accumulations. A line-broadening factor of 8.0 Hz was applied during the

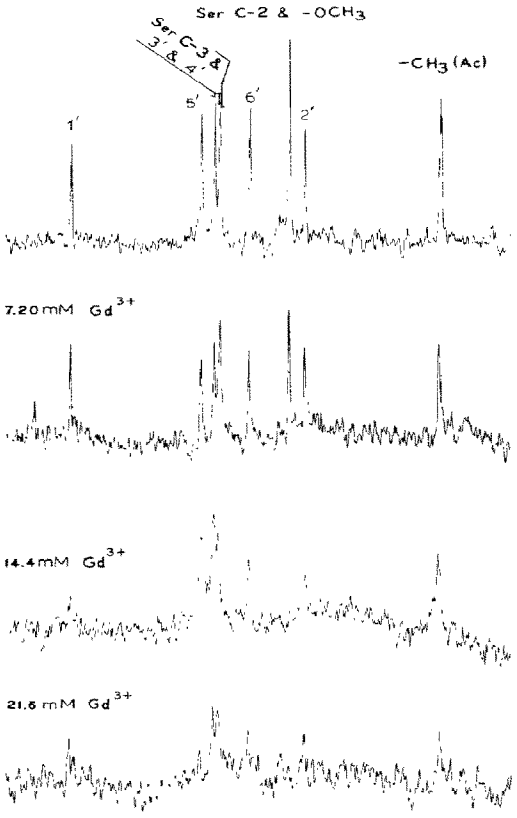


Fig. 2. The effect of added Gd^{3+} on the ^{13}C resonances of the aliphatic region of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of model compound **2**. [The concentration of compound **2** was 126mM (in H_2O), pH 6.6. A recycle time of 1.0–1.5 s was used to collect data. The vertical gain of the spectra containing the paramagnetic relaxation-reagent has been increased slightly, so that broadening effects may be clearly observed. (A) Sample contained no Gd^{3+}, and required 13 600 accumulations. A line-broadening factor of 3.0 Hz was applied during the data processing. (B) Sample contained 7.2mM Gd^{3+}, and required 43 407 accumulations. A line-broadening factor of 4.8 Hz was applied during the data processing. (C) Sample contained 14.4mM Gd^{3+}, and required 35 131 accumulations. A line-broadening factor of 6.0 Hz was applied during the data processing. (D) Sample contained 21 6mM Gd^{3+}, and required 31 492 accumulations. A line-broadening factor of 8.0 Hz was applied during the data processing.]

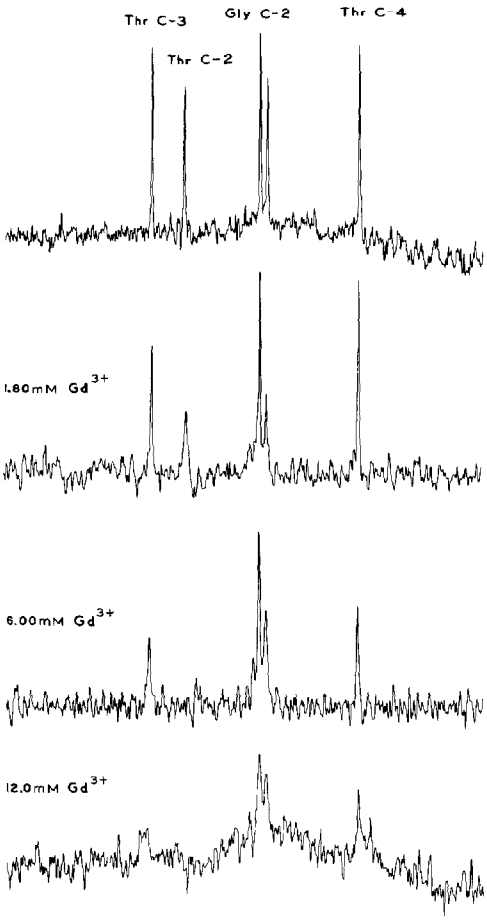


Fig. 3. The effect of added Gd^{3+} on the ^{13}C resonances of the aliphatic region of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of model compound **3**. [The concentration of compound **3** was 50mM (in H_2O), pH 7.2. A recycle time of 1.0–1.5 s was used to collect data. The vertical gain of the spectra containing the paramagnetic relaxation-reagent has been increased slightly, so that broadening effects may be clearly observed. (A) Sample contained no Gd^{3+}, and required 20 164 accumulations. A line-broadening factor of 4.0 Hz was applied during the data processing. (B) Sample contained 1.80mM Gd^{3+}, and required 10 533 accumulations. A line-broadening factor of 6.0 Hz was applied during the data processing. (C) Sample contained 6.00mM Gd^{3+}, and required 27 876 accumulations. A line-broadening factor of 7.0 Hz was applied during the data processing. (D) Sample contained 12.0mM Gd^{3+}, and required 41 561 accumulations. A line-broadening factor of 8.0 Hz was applied during the data processing.]

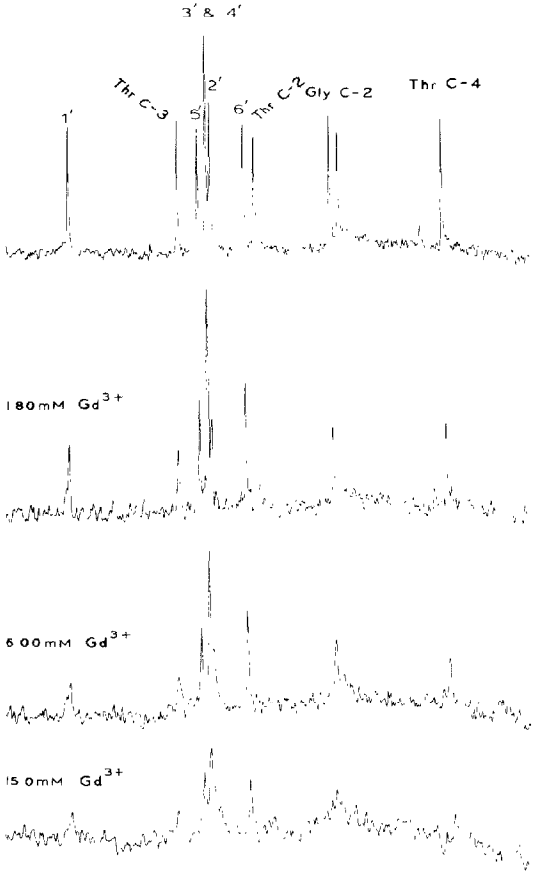


Fig. 4. The effect of added Gd^{3+} on the ^{13}C resonances of the aliphatic region of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of model compound **4** [The concentration of compound **4** was 62mM (in H_2O), pH 6.5. A recycle time of 1.0–1.5 s was used to collect data. The vertical gain of the spectra containing the paramagnetic relaxation-reagent has been increased slightly, so that broadening effects may be clearly observed. (A) Sample contained no Gd^{3+}, and required 38 651 accumulations. A line-broadening factor of 4.0 Hz was applied during the data processing. (B) Sample contained 1.80mM Gd^{3+}, and required 18 564 accumulations. A line-broadening factor of 5.0 Hz was applied during the data processing. (C) Sample contained 6.0mM Gd^{3+}, and required 32 327 accumulations. A line-broadening factor of 5.0 Hz was applied during the data processing. (D) Sample contained 15.0mM Gd^{3+}, and required 45 830 accumulations. A line-broadening factor of 8.0 Hz was applied during the data processing.]

without the interference of any strong, primary binding-sites on the amino acid. The chemical-shift data for the ^{13}C-n.m.r. spectra and the assignments of the resonances to specific carbon atoms are presented in Table I.

In order to obtain the interaction (or binding) of Gd^{3+} to these glycopeptides, the linewidths of the ^{13}C resonances of the glycopeptides as a function of added Gd^{3+} were monitored. This was feasible because Gd^{3+} is a relaxation probe (line-broadening agent) that will specifically broaden resonances at, or near, the metal-ion-binding site. Gd^{3+} has previously been used by us in order to gain knowledge about the metal-ion-binding sites of the monosaccharide α-NeuAc[9] and of glycophorin[7]. Moreover, it mimics La^{3+}, a metal ion used by Angyal[1] to study La^{3+}–carbohydrate complex-formation by means of ^1H-n.m.r. spectroscopy.

Fig. 1 shows the effects of added Gd^{3+} on the aliphatic region of the proton-decoupled, natural-abundance ^{13}C-n.m.r. spectrum of compound **1** [*O*-α-D-Gal*p*-(1→3)-L-Ser(OMe)(NHAc)]. The additions of Gd^{3+} appear to broaden the signals of C-2′ and, to some extent, those of the methyl group of the acetamido function, of C-1′, and of either Ser C-2 or the methyl group of the ester function. A weak interaction at O-6′ is possibly indicated by the signal of C-6′. All other resonances are eventually broadened, due to outer-sphere interactions with the metal ion. This result can be rationalized on the basis of one (or more) weak, metal-ion-binding sites in the vicinity of Ser C-2 and C-1 (involving the carbonyl), and the oxygen atoms on C-2′ and C-1′ or near Ser C-2 and Ser C-1 (involving the carbonyl) along with a second binding site involving the oxygen atom on C-6′.

In Fig. 2 are shown the effects of added Gd^{3+} on the aliphatic region of the proton-decoupled, natural-abundance ^{13}C-n.m.r. spectrum of compound **2** [*O*-α-D-Gal*p*NAc-(1→3)-L-Ser(OMe)(NHAc)]. Several resonances are already broadened by the addition of Gd^{3+}. These are those of Ser C-2, the methyl group of the ester function, one of the methyl groups of the acetamido functions, C-5′, possibly C-4′, and C-1′. A relatively strong binding-site in the vicinity of Ser C-2, and C-1 (involving the carbonyls of the ester and acetamido groups) can be invoked, and a second weak binding site near C-4′. These results clearly indicate that the metal-ion-binding of Gd^{3+} to compounds **1** and **2** differs somewhat.

In Fig. 3 are observed the effects of added Gd^{3+} on the proton-decoupled, natural abundance ^{13}C-n.m.r. spectrum of the tripeptide Gly-Gly-Thr (**3**). In this compound (unlike compound **5**), the Thr residue is at the C-terminal position. Gd^{3+} binding to this compound indicates a strong interaction with the C-terminal carboxyl group and the N-terminal amino group, resulting in the immediate broadening of the signals of Thr C-2 and Gly C-2, as expected.

The binding of Gd^{3+} to **4**, the *O*-D-galactosylated tripeptide **3**, is shown in Fig. 4. It would be expected that, for this model compound, strong Gd^{3+} interaction would occur with the free carboxyl and amino groups of the amino acid, as well as possible weak interactions with the α-D-galactosyl group. Aside from the signals of Thr C-2, Thr C-3, Thr C-4, and Gly C-2, which expectedly are broadened upon the addition of Gd^{3+}, the C-1′ and C-2′ resonances of the α-D-Gal*p* group are also

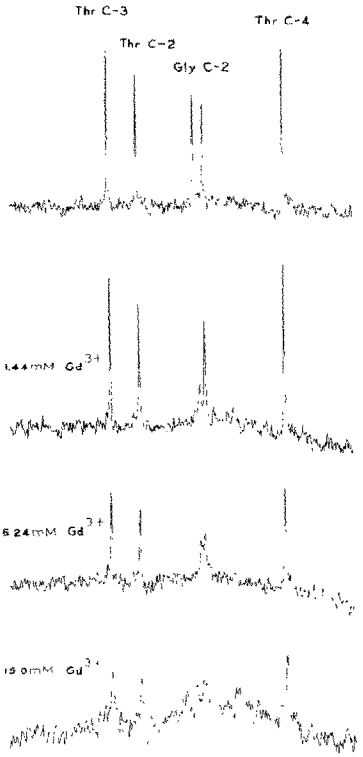


Fig. 5. The effect of added Gd^{3+} on the ^{13}C resonances of the aliphatic region of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of model compound **5**. [The concentration of compound **5** was 95mM (in H_2O), pH 5.8. A recycle time of 1.0–1.5 s was used to collect data. The vertical gain of the spectra containing the paramagnetic relaxation-reagent has been increased slightly, so that broadening effects may be clearly observed. (A) Sample contained no Gd^{3+}, and required 22 966 accumulations. A line-broadening factor of 3.0 Hz was applied during the data processing. (B) Sample contained 1.44mM Gd^{3+}, and required 22 698 accumulations. A line-broadening factor of 4.7 Hz was applied during the data processing. (C) Sample contained 6.24mM Gd^{3+}, and required 27 792 accumulations. A line-broadening factor of 6.0 Hz was applied during the data processing. (D) Sample contained 15.0mM Gd^{3+}, and required 42 913 accumulations. A line-broadening factor of 8.0 Hz was applied during the data processing.]

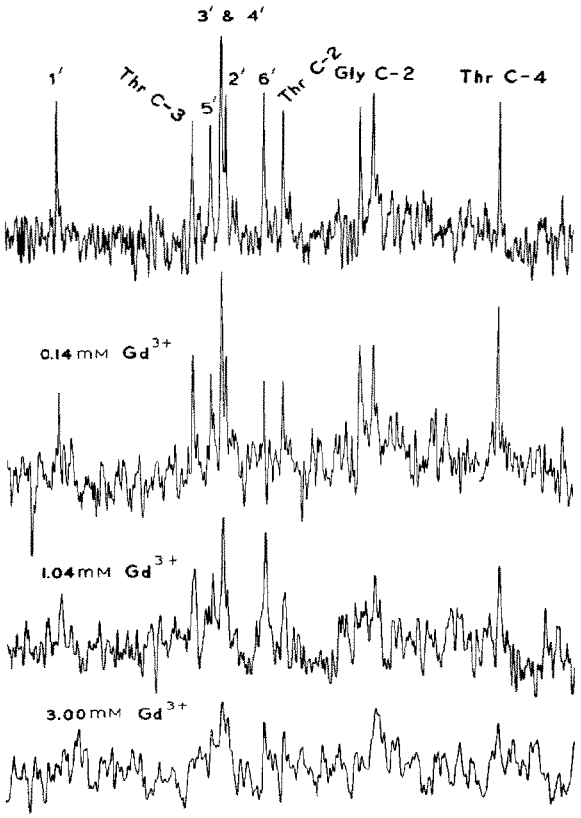


Fig. 6. The effect of added Gd^{3+} on the ^{13}C resonances of the aliphatic region of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of model compound **6**. [The concentration of compound **6** was ~30mM (in H_2O), pH 6.8. A recycle time of 1.0–1.5 s was used to collect data. The vertical gain of the spectra containing the paramagnetic relaxation-reagent has been increased slightly, so that broadening effects may be clearly observed. (A) Sample contained no Gd^{3+}, and required 52 000 accumulations. A line-broadening factor of 4.0 Hz was applied during the data processing. (B) Sample contained 0.14mM Gd^{3+}, and required 45 641 accumulations. A line-broadening factor of 5.0 Hz was applied during the data processing. (C) Sample contained 1.04mM Gd^{3+}, and required 73 500 accumulations. A line-broadening factor of 7.0 Hz was applied during the data processing. (D) Sample contained 3.0mM Gd^{3+}, and required 60 500 accumulations. A line-broadening factor of 8.0 Hz was applied during the data processing.]

broadened. This suggests that there is either a separate binding-site on the α-D-galactopyranosyl group, near C-1′ and C-2′, that is not influenced by the Gd^{3+} binding near the carboxyl and amino functions of the amino acids, or that the Gd^{3+} binding near these functions may mediate the interaction of Gd^{3+} with C-1′ and C-2′. The other carbon-atom resonances of the carbohydrate are only mildly affected.

Fig. 5 shows the effects of added Gd^{3+} on the proton-decoupled, natural abundance ^{13}C-n.m.r. spectrum of the tripeptide Gly-Thr-Gly (**5**). Because this species does not have the carboxyl or amino groups protected, the additions of Gd^{3+} immediately broaden the Gly C-2 resonances. The C-terminal Gly C-2 appears to broaden first, and the N-terminal Gly C-2 broadens second.

When O-3 of the Thr residue of **5** is glycosylated with α-D-Gal*p*, to give **6**, different modes of Gd^{3+} binding can be observed (see Fig. 6). Our analyses of the binding phenomena were limited by the small amount of sample available, resulting in a limited signal-to-noise ratio. However, it can definitively be seen that, in addition to the known interaction of Gd^{3+} with the C-terminal carboxyl group and the N-terminal amino group, the resonances of C-2′, C-1′, and, possibly, C-5′ are broadened, thus indicating a weak interaction of Gd^{3+} with these locations of the carbohydrate.

There are two conclusions that can be drawn from this work. (*i*) The binding of Gd^{3+} to the glycosyl group of a mono-*O*-glycosylated species is weak. In some cases, this binding may be aided by the N-terminal amino group or the C-terminal carboxyl group. (*ii*) The binding that does occur seems to involve O-3 and O-2′ of α-D-Gal*p*, and O-3 and N-2′ of α-D-Gal*p*NAc, and, possibly, O-6′ and O-4′ of both glycosyl groups.

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