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^{13}C-N.M.R.-SPECTRAL STUDY OF THE MODE OF INTERACTION OF Gd^{3+} AND Mn^{2+} WITH TWO VICINALLY DI-*O*-D-GALACTOSYLATED TRIPEPTIDES

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ABSTRACT

Natural-abundance, ^{13}C-n.m.r. spectroscopy was used to study the binding of Gd^{3+} and Mn^{2+} to two vicinally di-*O*-α- and -β-D-galactosylated tripeptides composed of Gly and L-Thr. $Gd^{3+}$and Mn^{2+} appear to interact with the α-D-Gal groups of the di-*O*-α-D-galactosylated tripeptide at two sites: near O-6′, and in the vicinity of O-2′ and Thr O-3. The metal-ion-binding to the β-D-Gal groups of the di-*O*-β-D-galactosylated tripeptide indicates that a strong binding-site exists near O-6′ and, possibly, several weak ones near O-3′ and Thr O-3. In the case of the di-*O*-α-D-galactosylated tripeptides, vicinal glycosylation appears to have little effect on the metal-ion-binding of the α-D-Gal groups.

INTRODUCTION

Because of the immense biological interest of metal-ion–carbohydrate interactions involving monosaccharides[1–4], oligosaccharides[5,6], glycopeptides[7–9], and glycoproteins[6], we have previously used ^{13}C-n.m.r. spectroscopy in order to begin studies elucidating the modes of interaction of such metal ions as Gd^{3+} and Mn^{2+} with *N*-acetyl-α-D-neuraminic acid (α-D-NeuAc)[3], oligosaccharides[6], glycopeptides[8,9], and a glycoprotein[6]. In the metal-ion–carbohydrate studies dealing with α-D-NeuAc, for an oligosaccharide containing α-D-NeuAc and a glycoprotein containing α-D-NeuAc, strong metal-ion–α-D-NeuAc interactions were observed. A weaker interaction was noted for simple glycopeptides and a tri-*O*-D-galactosylated hexapeptide.

In order to further our investigation of the mode of binding of metal ions to glycopeptides, we now present the results of studies on the modes of interaction of

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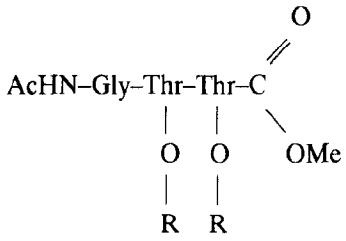
Gd^{3+} and Mn^{2+} with vicinally di-*O*-α- and -β-D-galactosylated tripeptides (**1** and **2**). Our results clearly establish the following. (*i*) Metal ions interact with blocked N-terminal and C-terminal amino acids. (*ii*) The two model compounds apparently contain similar, and different, metal-ion binding-sites. (*iii*) The modes of interaction of Gd^{3+} and Mn^{2+} are similar in the case of the glycopeptide containing only α-D-Gal groups, but may differ for the glycopeptide containing β-D-Gal groups.



1 R = α-D-Gal*p*
2 R = β-D-Gal*p*

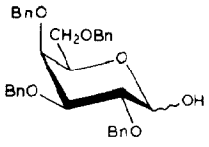
EXPERIMENTAL

Materials. — Gadolinium oxide (99.9%) was purchased from Alfa Products, Danvers, MA, and reagent-grade manganous chloride tetrahydrate, from Baker and Adamson.

Synthesis of model compounds. — The synthesis of the two vicinally di-*O*-α- and -β-D-galactosylated tripeptides (**1** and **2**) was achieved according to the general procedures described previously[10,11]. Elemental analyses and n.m.r.-spectral data were in agreement with the expected structures for all intermediates and products.

Glycosylation of protected and activated amino acids with 2,3,4,6-tetra-*O*-benzyl-D-galactopyranose (**3**) was performed in 1:1 dichloromethane–acetonitrile with a three-fold excess of both the amino acid and trifluoromethanesulfonic anhydride, at room temperature.

Methyl *N*-(fluorenylmethoxycarbonyl)-L-threoninate (**4**) was prepared from L-threonine methyl ester hydrochloride[12], according to the method described by Chang *et al.*[13], in 73% yield from dichloromethane–diethyl ether; m.p. 113–115°, $[\alpha]_D^{20}$ −13.1° (*c* 1, $CHCl_3$).



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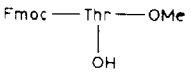
Bn = $PhCH_2$



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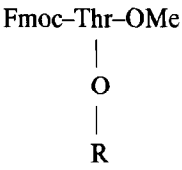
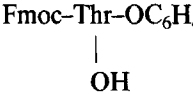
Condensation of compound **3** with compound **4** afforded a 7:3 (α:β) anomeric mixture of **5** in 59% yield. Pure galactosylamino acids **5α**, m.p. 94–95° (from diethyl ether–hexane), $[\alpha]_D^{20}$ +38.9° (*c* 1, $CHCl_3$), and **5β**, a white foam, $[\alpha]_D^{20}$ +5.0° (*c* 1, $CHCl_3$), were obtained after chromatography on a column of silica gel with 4:1 hexane–ethyl acetate.



5α R = 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl
5β R = 2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl

Glycosylation of the active ester *p*-nitrophenyl *N*-(fluorenylmethoxycarbonyl)-L-threoninate[14] (**6**) with compound **3**, according to the procedure mentioned earlier, gave an anomeric mixture (α:β = 9:11) in 85% yield; this was chromatographed on silica gel with 4:1 hexane–ethyl acetate to afford pure glycosylamino acids **7α**, a white foam, $[\alpha]_D^{20}$ +10.4° (*c* 1, $CHCl_3$); and **7β**, a white foam, $[\alpha]_D^{20}$ −20.5° (*c* 1, $CHCl_3$).



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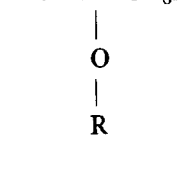


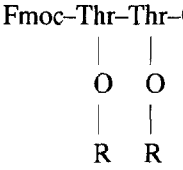
7α R = 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl
7β R = 2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl

Removal of the Fmoc protecting-groups was accomplished under mildly basic conditions by treatment with 3:10 piperidine–dichloromethane for 15 to 30 min.

Compound **5α** was deprotected, and the product acylated with the *p*-nitrophenyl ester **7α**. Acylation was achieved in 10:1 CH_2Cl_2–*N*,*N*-dimethylformamide in the presence of benzotriazol-1-ol (HOBT). Addition of diisopropylethylamine kept the pH of the mixture near 8.0. After 6 h, the solvent

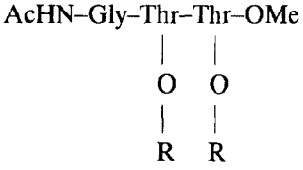
was evaporated *in vacuo*, and the residue purified by gel-filtration chromatography on Sephadex LH20 with 1:1 dichloromethane–methanol followed by chromatography on silica gel with 7:3 hexane–ethyl acetate, to afford the pure, di-*O*-D-galactosylated dipeptide **8α** as a white foam in 64% yield, $[\alpha]_D^{20}$ +54.5° (*c* 1, $CHCl_3$).

Deprotected compound **5β** was treated with active ester **7β**, using the previous method, to give the pure di-*O*-D-galactosylated dipeptide **8β** in 50% yield, $[\alpha]_D^{20}$ +16.7° (*c* 1, $CHCl_3$).



8α R = 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl
8β R = 2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl

After the removal of the Fmoc groups, the products from compounds **8α** and **8β** were acylated with *N*-acetyl-(*p*-nitrophenyl)glycinate, which led to pure di-*O*-D-galactosylated tripeptides **9α** in 82% yield after reaction for 3.3 h, $[\alpha]_D^{20}$ +70.8° (*c* 1, $CHCl_3$), and **9β** in 76% yield after reaction for ~12 h, $[\alpha]_D^{20}$ +26.9° (*c* 1, $CHCl_3$).



9α R = 2,3,4,6-tetra-*O*-benzyl-α-D-galactopyranosyl
9β R = 2,3,4,6-tetra-*O*-benzyl-β-D-galactopyranosyl

Hydrogenolysis of compounds **9** was achieved overnight in 12:2:1 (v:v:v) ethanol–water–acetic acid, in the presence of 10% palladium-on-charcoal as the catalyst, in a hydrogen atmosphere at 0.4 MPa. Di-*O*-D-galactosylated tripeptides **1** and **2** were obtained, after the usual processing, as white solid materials, in almost quantitative yield; **1**, hygroscopic, $[\alpha]_D^{20}$ +128.3° (*c* 1, H_2O); **2**, hygroscopic, $[\alpha]_D^{20}$ −8.2° (*c* 1, 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[3]. Gd^{3+} and Mn^{2+} stock solutions were also prepared as previously described[3]. ^{13}C-Chemical shifts were obtained for compounds **1** and **2** by using pub-

lished ^{13}C-chemical-shift data[15] for Gal C-3′ and Gal C-4′, and for[16] Gal C-6′of various glycopeptides containing these glycosyl linkages.

Sample preparation of the model compounds involved dissolving them in deionized, distilled H_2O, and adjusting the pH to 7.0–7.5. Additions of Gd^{3+} stock solutions to the sample were made by using an Eppendorf digital pipet (total additions ranged from 6.0 μL to 60 μL).

RESULTS AND DISCUSSION

Presented herein is the mode of interaction of Gd^{3+} and $Mn^{2+}$with vicinally di-*O*-α- and di-*O*-β-D-galactosylated tripeptides composed of Gly and L-Thr (**1** and **2**). In these compounds, the N-terminal amino group and the C-terminal carboxyl group are blocked, so that limited, metal-ion interactions would be expected with these groups[8,9]. Moreover, the points of glycosylation by α- and β-D-galactopyranosyl groups on the tripeptide are vicinal, which should allow determination of whether neighboring points of glycosylation may alter the metal-ion–D-Gal interactions. Also, because the glycopeptides contain either α-D-Gal*p* or β-D-Gal*p*, only, our studies will determine whether an anomeric change in the pyranose form of the carbohydrate affects metal-ion binding.

In order to obtain information concerning the binding sites of $Gd^{3+}$and Mn^{2+} on the glycopeptide, we monitored the line-widths of the ^{13}C resonances of the

TABLE I

^{13}C-N.M.R.-SPECTRAL DATA FOR THE VICINALLY DI-*O*-α-D-GALACTOSYLATED TRIPEPTIDE **1**

Carbon atom	*Peak number*[a]	*Chemical shift*[b]
Thr, Gly, and Ac CO	1	176.3
	2	173.2
1′ (C-terminal)	3	101.7
1′ (internal)	4	100.8
Thr C-3 (C-terminal)	5	77.0
Thr C-3 (internal)	6	76.5
5′	7	72.8
5′	8	72.6
3′ & 4′	9	70.8
2′	10	69.9
6′	11	62.8
Thr C-2 (C-terminal & internal)	12	58.7
CH_3 (OMe)	13	54.5
Gly C-2	14	44.2
CH_3 (Ac)	15	23.1
Thr C-4 (C-terminal)	16	19.5
Thr C-4 (internal)	17	18.9

[a]See Figs. 1 and 2 for peak-numbering system. [b]The chemical shifts of the various resonances are referenced relative to C-3′ and C-4′ of α-D-Gal*p*, taken to be 70.8 p.p.m. (see ref. 15). Estimated precision for the chemical shifts is ±0.05 p.p.m.

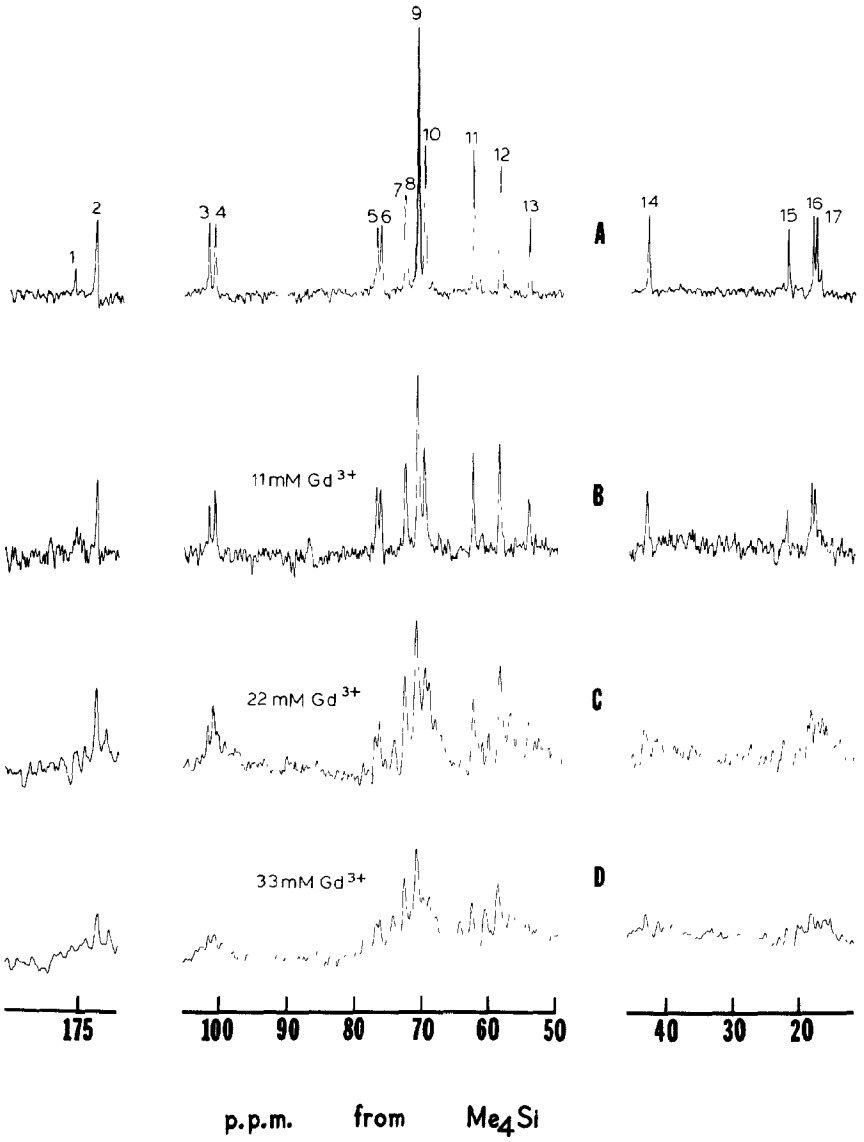


Fig. 1. The effect of added Gd^{3+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound **1**. [The concentration of **1** was 60mM (in H_2O), pH 7.5. Time-domain data were accumulated in 8192 addresses, with a recycle time of 1 to 1.5 s. The vertical 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 10,292 accumulations. A line-broadening factor of 2.9 Hz was applied during the data processing. (B) Sample contained 11mM Gd^{3+}, and required 15,910 accumulations. A line-broadening factor of 4.0 Hz was applied during the data processing. (C) Sample contained 22mM Gd^{3+}, and required 39,781 accumulations. A line-broadening factor of 6.0 Hz was applied during the data processing. (D) Sample contained 33mM Gd^{3+}, and required 41,170 accumulations. A line-broadening factor of 9.0 Hz was applied during the data processing.]

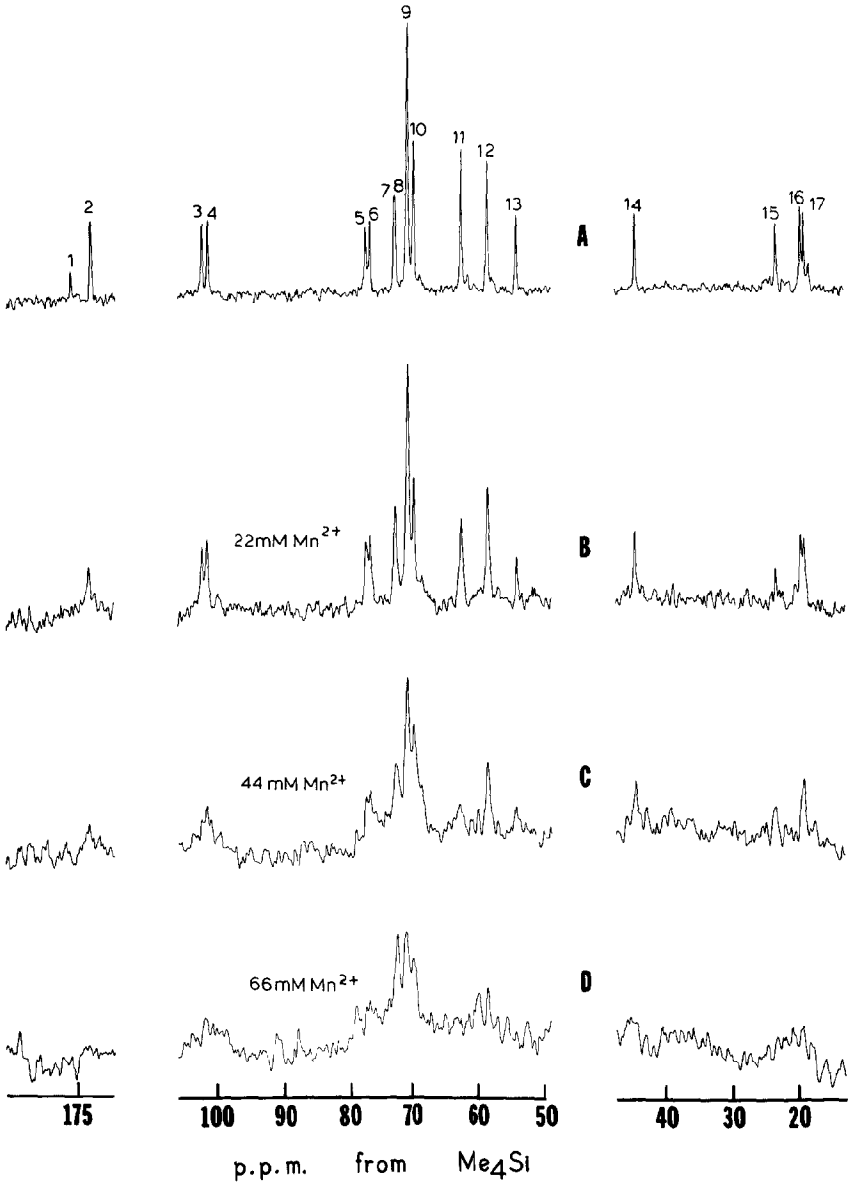


Fig. 2. The effect of added Mn^{2+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound 1. [Sample conditions and spectral accumulation parameters were the same as those described for Fig. 1. (A) Same as 1A. (B) Sample contained 22mM Mn^{2+}, and required 19,600 accumulations. A line-broadening factor of 5.0 Hz was applied during the data processing. (C) Sample contained 44mM Mn^{2+}, and required 46,835 accumulations. A line-broadening factor of 7.3 Hz was applied during the data processing. (D) Sample contained 66mM Mn^{2+}, and required 37,233 accumulations. A line-broadening factor of 8.3 Hz was applied during the data processing.]

glycopeptides as a function of the quantity of added Gd^{3+} and Mn^{2+}. This technique is feasible, because the metal ions used are relaxation probes (line-broadening agents) which will specifically broaden resonances at and near the metal-ion binding-site.

Figs. 1 and 2 show the effects of added Gd^{3+} and Mn^{2+}, respectively, on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of **1**. Table I gives the chemical shifts and assignments of the resonances labeled in Figs. 1A and 2A. All of the carbohydrate carbon atoms and the amino acid carbon atoms were readily assigned by using the ^{13}C chemical-shift data published for *O*-α-D-galactosylated[16] Thr and various *O*-α-D-galactosylated glycopeptides containing[8,9,15,16] Thr.

Gradual addition of Gd^{3+} to an aqueous solution of compound **1** (see Fig. 1) results in the broadening of carbon resonances corresponding to Gly C-2, the methyl carbon atom of the methyl ester, the acetamido methyl carbon atom, to some extent Thr C-2, and most of the carbonyl atoms. This result can be rationalized by the binding of Gd^{3+} to (or near) the blocked, N-terminal amino group and the methylated, C-terminal carboxyl group. These types of metal-ion-binding phenomena had previously been observed for other glycopeptides[8,9].

Carbohydrate carbon resonances which are clearly observed to broaden are C-1′, C-2′, C-6′, and, possibly, C-3′ or C-4′, of the α-D-Gal residues. Other carbon resonances that broaden are Thr C-3 and Thr C-4. These results can be rationalized in terms of the two metal-ion binding-sites associated with the carbohydrate: one involving O-2′ and the glycosidic oxygen atom (Thr O-3), and the other involving O-6′ and O-4′.

The gradual addition of Mn^{2+} to an aqueous solution of compound **1** yields metal-ion-binding results similar to those found with Gd^{3+}, although, in this case, the resonances of the carbonyl carbon atoms appear to broaden much quicker than the rest of the carbon atoms (compare Fig. 1 with Fig. 2). These results must, then, indicate that the mode of interaction of Gd^{3+} and Mn^{2+} with this vicinal di-*O*-α-D-galactosylated glycopeptide is similar, if not identical.

Figs. 3 and 4 show the effects of added Gd^{3+} and Mn^{2+}, respectively, on the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound **2**. Table II gives the chemical shifts and assignments of the resonances labeled in Figs. 3A and 4A. All of the carbohydrate carbon atoms and the amino acid carbon atoms were readily assigned by using the ^{13}C chemical-shift data published for *O*-β-D-galactosylated Thr and for various *O*-β-D-galactosylated glycopeptides[16] containing Thr.

Fig. 3 shows the effects of the gradual addition of Gd^{3+} on the ^{13}C resonances of an aqueous solution of compound **2**. The resonances that appear to be affected are Gly C-2, the methyl carbon atom of the methyl ester, the acetamido methyl carbon atom, Thr C-2, and the carbonyl carbon atoms. Again, this can be rationalized by the binding of Gd^{3+} to (or near) the blocked N-terminal amino group and the methylated C-terminal carboxyl group.

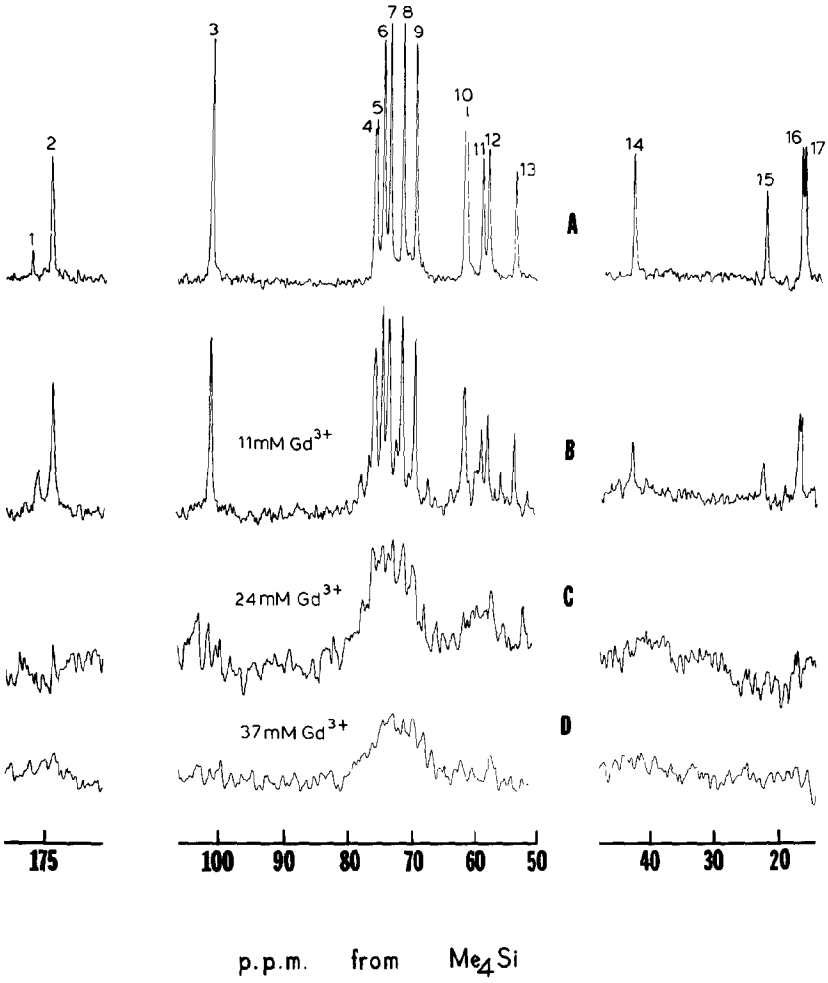


Fig. 3. The effect of added Gd^{3+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound **2**. [The concentration of compound **2** was 52mM (in H_2O), pH 7.2. Time-domain data were accumulated in 8192 addresses, with a recycle time of 1 to 1.5 s. The 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 16,621 accumulations. A line-broadening factor of 3.0 Hz was applied during the data processing. (B) Sample contained 11mM Gd^{3+}, and required 25,480 accumulations. A line-broadening factor of 4.0 Hz was applied during the data processing. (C) Sample contained 24mM Gd^{3+}, and required 51,478 accumulations. A line-broadening factor of 6.7 Hz was applied during the data processing. (D) Sample contained 36mM Gd^{3+}, and required 46,360 accumulations. A line-broadening factor of 8.0 Hz was applied during the data processing.]

The further addition of Gd^{3+} does not unambiguously show preferential binding of the metal ion to sites on the carbohydrate; many of the resonances appear to broaden at similar rates. However, C-1′ and C-6′ of β-D-Gal are broadened substantially, indicating a possible interaction of Gd^{3+} with O-3 and also with O-6′.

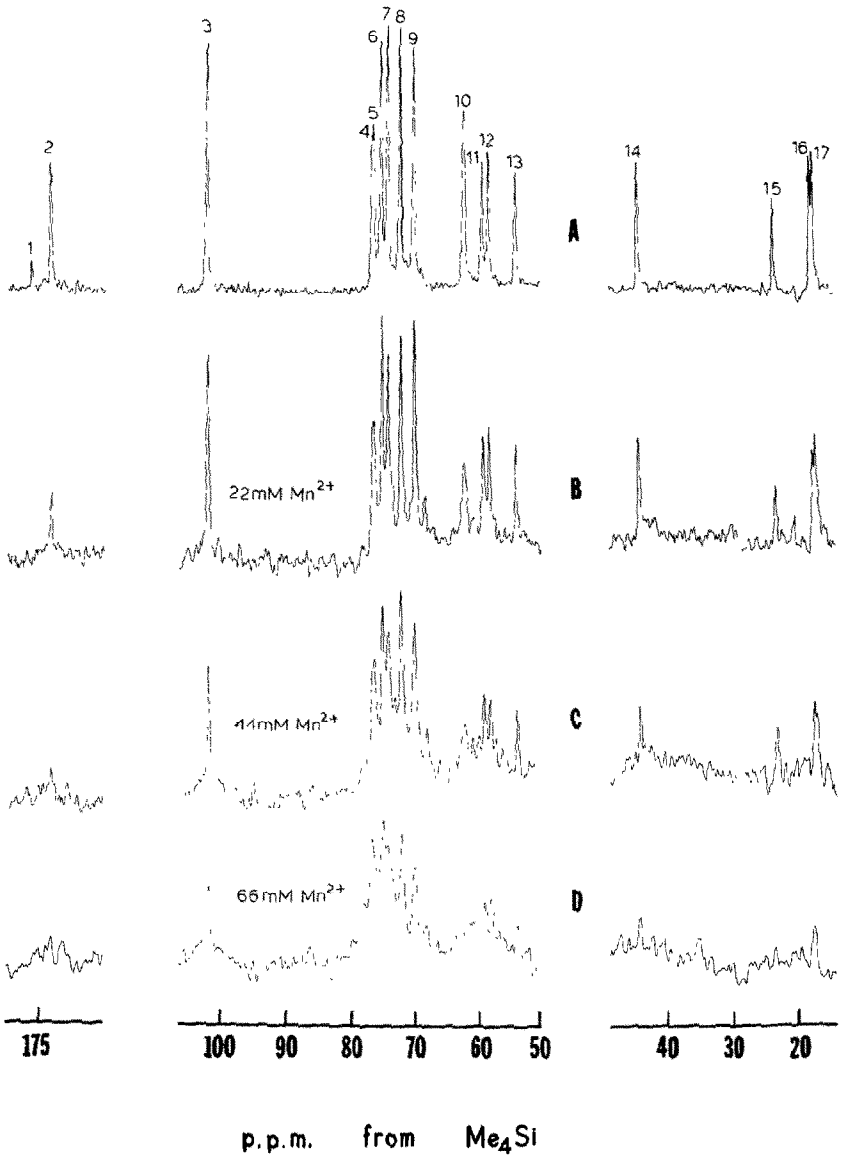


Fig. 4. The effect of added Mn^{2+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of compound **2**. [Sample conditions and spectral accumulation parameters were the same as those described in Fig. 3. (A) Same as 3A. (B) Sample contained 22mM Mn^{2+}, and required 47,909 accumulations. A line-broadening factor of 4.5 Hz was applied during the data processing. (C) Sample contained 44mM Mn^{2+}, and required 58,787 accumulations. A line-broadening factor of 6.0 Hz was applied during the data processing. (D) Sample contained 66mM Mn^{2+}, and required 41,708 accumulations. A line-broadening factor of 8.0 Hz was applied during the data processing.]

TABLE II

^{13}C-N.M.R.-SPECTRAL DATA FOR THE VICINALLY DI-*O*-β-D-GALACTOSYLATED TRIPEPTIDE **2**

Carbon atom	*Peak number*[a]	*Chemical shift*[b]
Thr, Gly, and Ac CO	1	176.4
	2	173.5
1′	3	101.9
Thr C-3 (C-terminal & internal)	4	76.7
	5	76.5
5′	6	75.4
3′	7	74.3
2′	8	72.4
4′	9	70.3
6′	10	62.6
Thr C-2 (C-terminal & internal)	11	59.7
	12	58.8
CH_3 (OMe)	13	54.7
Gly C-2	14	44.1
CH_3 (Ac)	15	23.3
Thr C-4 (C-terminal)	16	17.6
Thr C-4 (internal)	17	17.1

[a]See Figs. 3 and 4 for peak-numbering system. [b]The chemical shifts of the various resonances are referenced relative to C-6′ of β-D-Gal*p*, taken to be 62.6 p.p.m. (see ref. 16). Estimated precision for the chemical shifts is ±0.05 p.p.m.

Fig. 4 shows a more incisive picture of the mode of binding of Mn^{2+} to β-D-Gal*p* of glycopeptide **2**. Again, the gradual addition of Mn^{2+} to an aqueous solution of **2** results in the broadening of resonances associated with the C- and N-terminal carboxyl and amino groups, respectively, indicating binding at or near these groups.

Other carbon atoms whose resonances appear to broaden substantially are C-6′ and C-3′ of β-D-Gal, to some extent C-1′ of β-D-Gal, and, to some extent, Thr C-3 and Thr C-4. These results can be explained if there is a relatively strong, metal-ion binding-site at O-6′, and two weak sites involving O-3′ and O-3. It would appear, then, that, at least in the case of Mn^{2+}, a change in the anomeric configuration of the glycosyl group, resulting in a change in the geometry about the glycosidic linkage, affects the metal-ion binding of the D-galactosyl group. The case of Gd^{3+} is not so definitive.

Because the mode of interaction of Gd^{3+} with α-D-Gal of compound **1** resembles that of Gd^{3+} binding to simple α-D-galactosylated glycopeptides[8,9], it can be assumed that vicinal glycosylation with α-D-Gal has no effect on metal-ion-binding. This statement cannot be made for glycopeptide **2**, because no studies have as yet been done concerning metal-ion binding to simple glycopeptides containing a β-D-galactosyl linkage.

In summary, we have definitely shown that Gd^{3+} and Mn^{2+} bind to the blocked N-terminal amino group and the blocked C-terminal carboxyl group of

vicinally di-*O*-D-galactosylated tripeptides. Gd^{3+} and Mn^{2+} appear to bind by similar modes to the α-D-Gal groups of compound **1**, but may differ in their binding to the β-D-Gal groups of compound **2**. The metal-ion interaction with α-D-Gal and β-D-Gal differs considerably.

ACKNOWLEDGMENT

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