Carbohydrate Research, 123 (1983) 137–144
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

Note

^{13}C-N.m.r.-spectral study of the mode of binding of Gd^{3+} and Mn^{2+} to a tri-*O*-D-galactosylated hexapeptide

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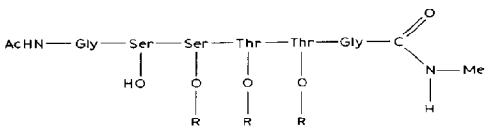
(Received May 11th, 1983; accepted for publication, June 2nd, 1983)

We have previously investigated the mode of binding of Gd^{3+} and Mn^{2+} to a monosaccharide[1], to oligosaccharides[2], and to glycopeptides[3] of biological interest. It was found[1] that Gd^{3+} and Mn^{2+} readily bind to the monosaccharide *N*-acetyl-α-neuraminic acid (α-NeuAc) in different modes, and that these metal ions also bind to α-NeuAc of oligosaccharides containing α-NeuAc groups[2]. In the study of mono-*O*-glycosylated amino acids and peptides, it was found that Gd^{3+} interacts weakly with the carbohydrate moiety (involving oxygen atoms attached to C-1′, C-2′, and, possibly, C-4′ and C-6′), and, that this binding may, to some degree, be mediated by free or blocked amino and carboxyl groups of the amino acids.

We now present studies detailing the mode of binding of Gd^{3+} and Mn^{2+} to a hexapeptide, composed of Gly, L-Thr, and L-Ser, that is vicinally tri-*O*-D-galactosylated (see formula **1**). This glycopeptide provides a unique structure for studying metal ion–carbohydrate interactions of small glycopeptides, because (*i*) the



1

R = α-D-Galp

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0008-6215/83/$ 03.00

points of glycosylation are at least one amino acid removed from the N- or C-terminal, and (*ii*) the three points of glycosylation are vicinal.

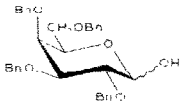
Our results indicated that there are weak metal ion–carbohydrate interactions of the glycohexapeptide with Gd^{3+} and Mn^{2+}, but that these interactions differ somewhat. The vicinal attachment of α-D-Gal*p* groups to the peptide does not appear to alter the metal-ion–glycohexapeptide interaction that we have previously observed for simple glycopeptides.

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 chemical synthesis of the tri-*O*-D-galactosylated hexapeptide **1** was performed according to a general procedure described by Ferrari and Pavia[4]. This synthesis involved a stepwise coupling strategy using appropriate, protected and activated amino acids and *O*-glycosylated amino acids. Fluorenylmethoxycarbonyl (Fmoc) was used as a temporary protecting group for the amino group of α-D-galactosyl-L-threonine and -L-serine derivatives.

The reaction sequence for synthesis of the tri-*O*-galactosylated hexapeptide involved preparation of 2,3,4,6-tetra-*O*-benzyl-D-galactopyranose[5] (**2**), *N*-(fluorenylmethoxycarbonyl)-*N*-(hydroxysuccinimyl)-L-threoninate[6] (**3**), *p*-nitrophenyl *N*-(fluorenylmethoxycarbonyl)-L-threoninate[7] (**4**), and *p*-nitrophenyl *N*-(fluorenylmethoxycarbonyl)-L-serinate[7] (**5**) as starting materials.



2

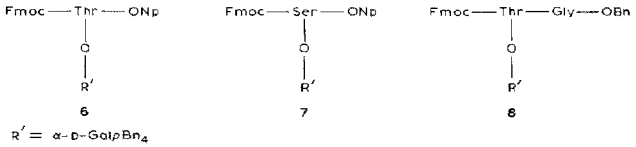
Bn = $PhCH_2$

Fmoc — Thr — OSu **3**

Fmoc — Thr — ONp **4**

Fmoc — Ser — ONp **5**

Condensation of compound **2** with the active esters **4** and **5**, respectively, was accomplished according to the general method of glycosylation reported by Pavia *et al.*[8] to give compound **6** ($\alpha:\beta = 9:11$) in 85% yield, $[\alpha]_D^{20}$ +10.4° (*c* 1, $CHCl_3$), and compound **7** ($\alpha:\beta = 3:2$) in 95% yield, $[\alpha]_D^{20}$ +11.3° (*c* 1, $CHCl_3$). The glycosylations were performed in 1:1 dichloromethane–acetonitrile with a three-fold excess of the amino acid, in the presence of trifluoromethanesulfonic acid, at room temperature. Pure **6α** and **7α** were obtained by chromatography of the respective anomeric mixtures on a column of silica gel. Compound **8** was obtained from compounds **2**, **3**, and benzyl glycinate according to the procedure described by Pavia *et al.*[8].

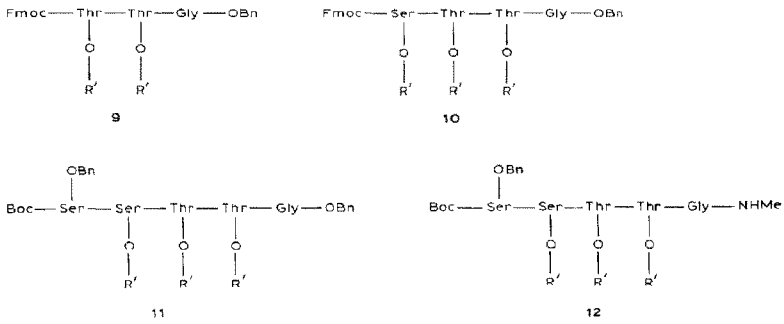


6 7 8

Removal of the fluorenylmethoxycarbonyl protecting group was achieved under mildly basic conditions by treatment of the protected glycopeptide with piperidine in dichloromethane. Acylations were performed in dichloromethane, in the presence of benzotriazol-1-ol as the catalyst.

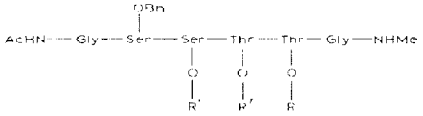
Compound **8** was treated with pure **6α**, to give, after column chromatography on Sephadex LH-20 and silica gel, the diglycosylated tripeptide **9** in 70% yield; $[\alpha]_D^{20}$ +59.1° (*c* 1, $CHCl_3$). The Fmoc group was removed from compound **9**, and the product was acylated with *p*-nitrophenyl ester **7α**. The usual processing afforded pure compound **10** in 78% yield; $[\alpha]_D^{20}$ +63.4° (*c* 1, $CHCl_3$). Deprotection of **10**, followed by acylation of the product with *O*-benzyl-*N*-(*tert*-butoxycarbonyl)-L-serine-*N*-hydroxysuccinimide (Boc-Ser$<^{OBzl}_{OSu}$; Bachem) led to compound **11**, which was purified by Sephadex gel-filtration and chromatography on silica gel; 86% yield; $[\alpha]_D^{20}$ +68.7° (*c* 1, $CHCl_3$). Compound **11** was treated with methylamine in 1:1 methanol–ethanol for 24 h at room temperature. After evaporation of the solvent *in vacuo*, and several co-evaporations with methanol, the tri-*O*-glycosylated pentapeptide **12** was obtained as a clear oil in 98% yield; $[\alpha]_D^{20}$ +71.2° (*c* 1, $CHCl_3$).

A solution of compound **12** in 1:2 trifluoroacetic acid–dichloromethane was stirred for 20 min at room temperature, and evaporated *in vacuo*, and the crude residue was used in the next step without purification. A solution thereof in dichloromethane was added dropwise to a mixture of *p*-nitrophenyl *N*-acetylglycinate (AcHN-Gly-ONp) and benzotriazol-1-ol in dichloromethane. After 30 min at room temperature, diisopropylethylamine was added, and then the reaction was al-



9 10 11 12

lowed to proceed for 2 h. The mixture was evaporated *in vacuo*, and the residue purified by gel filtration (Sephadex LH-20; 1:1 dichloromethane–methanol) followed by chromatography on silica gel (230–400 mesh) with ethyl acetate, to give pure tri-*O*-glycosylated hexapeptide **13** as a colorless syrup in 70% yield; $[\alpha]_D^{20}$ +69.1° (*c* 1, $CHCl_3$).



13

Compound **13** was dissolved in 12:2:1 (v/v/v) ethanol–water–acetic acid, and hydrogenolyzed overnight under a hydrogen atmosphere at 0.4 MPa in the presence of 10% palladium-on-charcoal as the catalyst. After filtration through a bed of Celite, and washing of the solid, evaporation of the combined filtrate and washings resulted in compound **1** as a white material in 100% yield; m.p. >72° (dec.), $[\alpha]_D^{20}$ +104.7° (*c* 1, $CHCl_3$). Elemental analyses and ^{13}C-n.m.r.-spectral data (see later) were in agreement with the assigned structure.

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[1]. Gd^{3+} and Mn^{2+} stock solutions were also prepared as described[1]. Chemical shifts were obtained by using, as references, ^{13}C chemical-shift data for C-3′ and C-4′ of related *O*-D-galactosylated model compounds[9–11]. 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+} and Mn^{2+} stock solutions to the sample were made in μL quantities by using an Eppendorf digital pipet, total additions ranging from 6.0 to 60 μL.

RESULTS AND DISCUSSION

The glycopeptide **1** with which we undertook the metal ion–carbohydrate binding studies is a rather simple glycopeptide, in that three of the amino acid residues are *O*-glycosylated with a monosaccharide, rather than a more-complex oligosaccharide. This simplifies analysis of the ^{13}C-n.m.r.-spectral data, and permits assigning of most of the resonances to specific carbon atoms on a one-to-one basis (see Figs. 1 and 2 and Table I).

This model compound provides a unique structure for studying the metal-ion interactions of small glycopeptides. (*i*) The points of *O*-glycosylation are at least one amino acid removed from the C- or N-terminal carboxyl or amino group. This is particularly important, because we have found that, for *O*-glycosylated amino acids or dipeptides, the C- or N-terminal carboxyl or amino group (free or blocked)

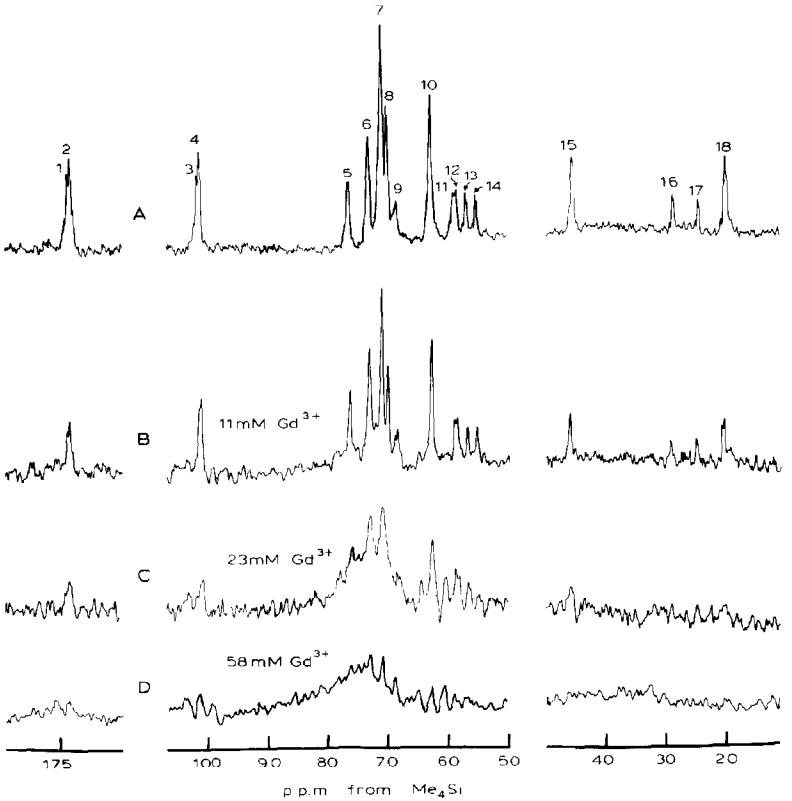


Fig. 1. The effect of added Gd^{3+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of the tri-*O*-D-galactosylated hexapeptide **1**. [The concentration of **1** was 35mM (in H_2O), pH 6.8. A recycle time of 1.0–1.5 s was used to collect the data. The vertical 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 28 278 accumulations. A line-broadening factor of 3.0 Hz was added during the data processing. (B) Sample contained 11mM Gd^{3+}, and required 26 421 accumulations. A line-broadening factor of 4.0 Hz was applied during the data processing. (C) Sample contained 23mM Gd^{3+}, and required 44 154 accumulations. A line-broadening factor of 6.0 Hz was applied during the data processing. (D) Sample contained 58mM Gd^{3+}, and required 46 689 accumulations. A line-broadening factor of 8.0 Hz was applied during the data processing.]

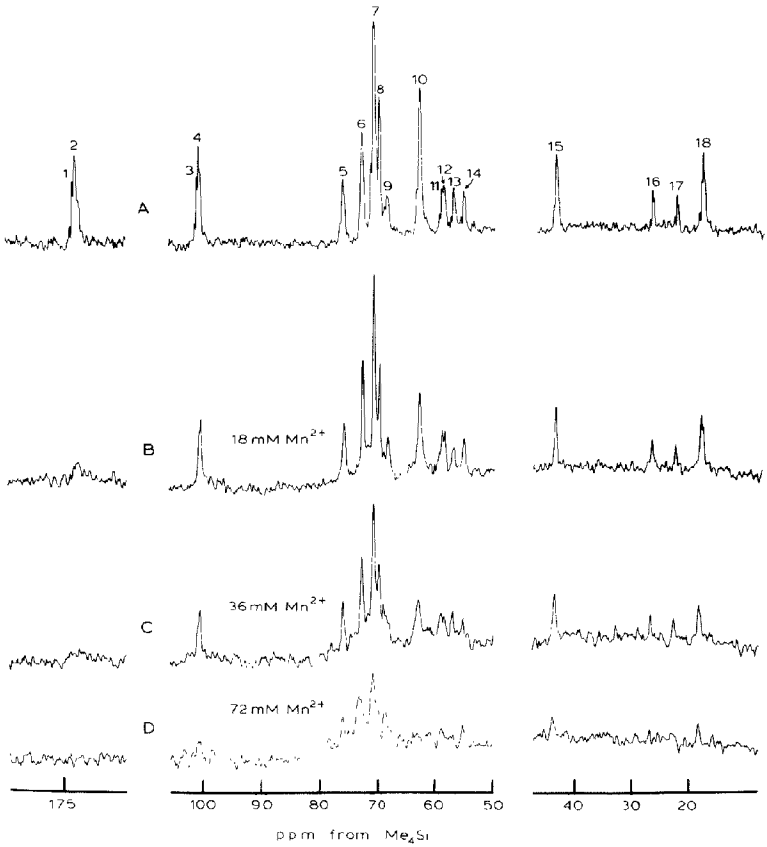


Fig. 2. The effect of added Mn^{2+} on the ^{13}C resonances of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of the tri-*O*-D-galactosylated hexapeptide **1**. [The concentration of **1** was 35mM (in H_2O), pH 6.8. A recycle time of 1.0–1.5 s was used to collect the data. The vertical 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 Mn^{2+}, and required 28 278 accumulations. A line-broadening factor of 3.0 Hz was added during the data processing. (B) Sample contained 18mM Mn^{2+}, and required 17 886 accumulations. A line-broadening factor of 4.0 Hz was applied during the data processing. (C) Sample contained 36mM Mn^{2+}, and required 38 039 accumulations. A line-broadening factor of 6.0 Hz was applied during the data processing. (D) Sample contained 72mM Mn^{2+}, and required 49 472 accumulations. A line-broadening factor of 9.0 Hz was applied during the data processing.]

TABLE I

^{13}C-N.M.R. SPECTRAL DATA FOR THE TRI-*O*-D-GALACTOSYLATED HEXAPEPTIDE **1**

Carbon atom	*Peak number*[a]	*Chemical shift*[b]
Thr, Ser, Gly, and CO of Ac	1	173.1
	2	172.8
1′ (α-D-Gal→Ser)	3	100.8
1′ (α-D-Gal→Thr)	4	100.5
Thr C-3	5	75.9
5′	6	72.6
3′ and 4′	7	70.7
2′	8	69.7
Ser C-3 (glycosylated)	9	68.1
6′ and Ser C-3 (nonglycosylated)	10	62.5
Ser C-2 (glycosylated), Ser C-2 (nonglycosylated), and Thr C-2	11	58.8
	12	58.4
	13	56.8
	14	55.1
Gly C-2	15	43.9
CH_3 (amide)	16	27.4
CH_3 (Ac)	17	23.1
Thr C-4	18	18.6

[a]See Figs. 1 and 2 for peak-numbering system. [b]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. 11).

may mediate metal-ion binding to the carbohydrate. (*ii*) The points of attachment of the three α-D-galactopyranosyl groups are consecutive. This may aid in the chelation of the metal ion. Previous observations for simpler systems indicated that, in small glycopeptides (containing only one point of *O*-glycosylation), the metal ion–carbohydrate interactions are weak.

Figs. 1 and 2 show the effects of added Gd^{3+} and Mn^{2+} on the ^{13}C resonances (aliphatic region) of the proton-decoupled, natural-abundance, ^{13}C-n.m.r. spectrum of the tri-*O*-D-galactosylated hexapeptide. Table I gives the chemical shifts and assignments for the resonances labeled in Figs. 1A and 2A.

The carbohydrate carbon atoms are readily assigned, because of the resonance overlap of all of the α-D-Gal*p* carbon atoms except C-1′. Most of the amino acid carbon atoms are clearly discernible, and could be readily assigned on a one-to-one basis, except for the region of peaks 11–14. This spectral region should contain the four resonances of the Ser C-2 (glycosylated and nonglycosylated), and Thr C-2.

Fig. 1 clearly shows that, upon gradual addition of Gd^{3+}, the atoms that appear to be noticeably affected are C-2′, C-3′ or C-4′, C-1′, Gly C-2, glycosylated Thr C-4, and, to some extent, the carbonyl atoms and C-4′. Because of the low signal–noise ratio, we could not determine whether the signal for Ser C-3 was, or was not, broadened due to Gd^{3+} interaction. These results seem to indicate that Gd^{3+} interacts with the blocked N-terminal and C-terminal Gly C-2. Moreover, there ap-

pears to be Gd^{3+} interaction with the oxygen atoms on C-1′, C-2′, and Thr C-3, and, possibly, a weak interaction with those on C-6′ and C-4′ (or C-3′).

Fig. 2 clearly shows that, upon gradual addition of Mn^{2+}, the carbohydrate carbon atoms and some related, amino acid carbon atoms that appear to be noticeably affected are those of the carbonyl groups, C-2′, C-6′, and, to a lesser extent, C-1′, Thr C-3, and, possibly, C-3′ or C-4′. These results show that the modes of interaction of Mn^{2+} and Gd^{3+} with the glycohexapeptide differ somewhat.

The results herein presented indicate that Gd^{3+} and Mn^{2+} bind in different modes to the glycohexapeptide. The binding of the metal ions to the carbohydrate residues appears to be weak. The fact that the glycohexapeptide contains vicinal points of glycosylation does not seem to alter the binding mode of Gd^{3+} to α-D-Gal, when compared to our previous work dealing with binding of Gd^{3+} to simple, D-galactosylated amino acids and peptides[3]. It is probable that the metal ions would interact more strongly with oligosaccharides (especially branched) attached to amino acids or peptides[7].

ACKNOWLEDGMENT

K. D. acknowledges the financial support of the Research Corporation for this project.

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