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AN APPROACH TO THE STRUCTURAL ANALYSIS OF OLIGOSACCHARIDES BY N M R SPECTROSCOPY

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ABSTRACT

The proton chemical shifts of the glycosidic protons have been measured for solutions of oligosaccharides in D_2O, to some degree, the shifts are diagnostic of both the nature of the sugar and of the type of linkage To determine the sequence of the sugar residues, the aldose or ketose is first treated with cyanide to produce a terminal carboxylic acid Then Gd^{3+} is added, which is bound on the carboxyl group, causing line broadening of the proton resonances and a decrease in the spin–lattice relaxation-time (T_1) of the glycosidic protons These effects fall off progressively along the oligosaccharide chain, hence allowing the sequence to be determined The method, which is illustrated for maltotriose, has been used at the milligram level, and may be applicable up to pentasaccharides Difficulties occur in the treatment of non-reducing sugars (1↔1 and 1↔2 glycosides), and with certain sugar residues that bind Gd^{3+} on three adjacent hydroxyl-groups of particular stereochemistry The possible usefulness of ^{13}C-n m r spectroscopy is also considered

INTRODUCTION

The determination of the types of linkages between sugar residues and their sequence in oligosaccharides presents considerable difficulties Various workers have used a combination of methods, including partial and complete hydrolysis with acid, hydrolysis with specific enzymes, periodate oxidation, and methylation, sometimes in combination with g l c and m s [1–5], which now appears to be very useful[4] We have recently studied the possible application of an n m r technique[6] that had been applied to the sequence determination of peptides[7–10]

The principle of the method involves firstly the identification of the various residues in the chain by assignment of the n m r resonances to specific residues The line width or the spin–lattice relaxation-time (T_1) of these resonances is then measured before and after the addition of a paramagnetic broadening probe (normally Gd^{3+}), which binds to the C-terminal carboxyl group of a peptide chain or a carboxyl group introduced at the end of an oligosaccharide chain. The extent of the increase in line width or decrease of T_1 brought about by the binding of Gd^{3+} is proportional to the inverse sixth-power of the distance between the bound, para-

magnetic metal ion and the nucleus that gives rise to the resonance[11] Thus, the effect drops off rapidly down the chain, and provided that the n m r resonances are correctly assigned, it is possible to determine the sequence of amino acids in a peptide chain[7-11] or of sugar residues in an oligosaccharide chain

In this paper, we have concentrated on the ^{1}H-n m r resonances of the glycosidic protons in oligosaccharides, the chemical shifts of which are, to some degree, characteristic of the sugar residue and also of the type of linkage Further information on the latter point may be obtained by ^{13}C-n m r spectroscopy It has been necessary to develop methods for the introduction of a terminal carboxyl group to act as a binding site

EXPERIMENTAL

The sugars were commercial samples and were used without further purification Deuterium oxide (>99 8%) was obtained from the Australian Atomic Energy Commission Solutions of known concentration of Gd^{3+} were prepared from 99 9% $GdCl_3$ $6H_2O$ (Ventron, Alfa Products)

^{1}H-N m r spectra at 100 MHz were recorded for ~10% solutions with a continuous-wave Jeol JNM-MH-100 spectrometer at an ambient probe-temperature of 26°, the chemical shift was measured against tetramethylsilane in $CDCl_3$ as the external standard in a sealed capillary tube A micro-tube (capacity, 0 05 ml) was used for milligram samples of sugars[7] ^{1}H-N m r spectra at 270 MHz and measurements of T_1 were obtained with a Bruker HX-270 spectrometer located at the National NMR Centre in Canberra ^{13}C-N m r measurements were also obtained with the latter instrument For measurement of T_1, the normal 180-τ-90° pulse sequence was used at ~8 different delay-times, τ The height (S_τ) of a particular resonance, at delay-time τ, was measured, and a graph drawn of $\ln(S_\infty - S_\tau)$ *vs* τ, where S_∞ is the height for an infinite delay-time (obtained from the normal spectrum using a 90° pulse) T_1 is obtained as the reciprocal of the gradient of the straight-line graph

Synthesis of carboxyl sugars — Four aldoses were converted into the corresponding carboxylic acids by oxidation with bromine[12,13] The sodium salt was recrystallised from an ethanol–water mixture and obtained in 70–90% yield Purity was checked by elemental analysis Since this method is confined to aldoses, a more-general method, useful for both aldoses and ketoses, was developed. In the cyanohydrin method[14-16], the sugar was dissolved in water, and a 10% molar excess of sodium cyanide was added The solution was kept for 30 h at room temperature in a stoppered flask, and the resulting cyanohydrin was then hydrolysed to the acid amide Heating at 100° for 6 h hydrolysed the amide to the corresponding carboxylic acid, which was precipitated as the sodium salt by the addition of ethanol The salt was collected and dissolved in water, and the solution lyophilised The method was used successfully on both a macro and a micro (1-mg) scale.

The identity of the product was checked in each case by ^{1}H-n m r. spectroscopy at 100 MHz, and the spectrum compared with that of the parent sugar The reso-

nances from the protons attached to the carbon atoms α and β to the carboxyl group moved ~0 4 and ~0 2 p p.m downfield, respectively, on decreasing the pH of the solution from ~6 to 1 5. This effect was useful in the assignment of these resonances and also allowed the n.m r titration of the carboxyl group An S-shaped titration curve of chemical shift of the resonance against pH-meter reading (measured in D_2O) was constructed, and the apparent dissociation constant K' obtained as previously described[17]. Such an experiment[6] on D-gluconic acid gave a pK' of 3 5

Non-reducing oligosaccharides — It is necessary to use special methods for non-reducing oligosaccharides, in order to generate a reducing group Thus, for raffinose, invertase, which specifically hydrolyses terminal β-D-fructofuranose residues[18] (with the production of D-fructose and an aldose or ketose that will react with cyanide), can be used

Raffinose [α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru, 400 mg] was dissolved in 10 ml of D_2O buffered at pH-meter reading of 5 1, and invertase (Koch–Light, 3 mg) was added After incubation at 30° for 24 h, ^{1}H-n m r spectroscopy showed that the formation of fructose and melibiose was complete Sodium cyanide (77 mg) was then added, and the mixture was kept at room temperature for 30 h, boiled under reflux for 6 h, and lyophilised That the product contained "fructoheptonic acid" and a small amount of invertase (which did not affect the sequencing procedure, see below) and also "6-*O*-α-D-galactopyranosyl-glucoheptonic acid" was confirmed by comparison of the ^{1}H-n m r spectrum at 100 MHz with spectra of samples of the acids previously prepared by treatment of D-fructose and melibiose by the cyanohydrin method

Stachyose [α-D-Gal-(1→6)-α-D-Gal-(1→6)-α-D-Glc-(1→2)-β-D-Fru] was treated by the same procedure, to give a mixture of a small amount of invertase, "fructoheptonic acid", and "6-*O*-α-D-galactopyranosyl-6-*O*-α-D-galactopyranosyl-glucoheptonic acid". This was examined by ^{1}H-n m r spectroscopy, as a function of pH as indicated previously, to check on the formation of carboxylic acids

RESULTS AND DISCUSSION

^{1}H-N m r chemical-shift data In Table I are given the chemical-shift data for the proton resonances that can be readily identified in the ^{1}H-n m r spectra of sugars The α- and β-anomeric proton resonances are identified as a pair of doublets with coupling constants of ~3 and 7 Hz, respectively, which are normally located downfield and upfield, respectively, from the HDO resonance The total area of the α- and β-anomeric peaks amounts to one proton The chemical shifts of the anomeric protons are the same (within experimental error) when they occur in a monosaccharide or an oligosaccharide, provided that the glycosidic linkage is not placed adjacent to the anomeric proton, *i e*, at the 2 position Since the signals for the H-2 protons occur in a complex region of overlapping peaks, their chemical shifts were obtained by spin decoupling with the corresponding anomeric or glycosidic protons The resonances from the glycosidic protons, like those from the anomeric protons, are well

TABLE I

CHEMICAL SHIFTS OF PROTON RESONANCES OF SUGARS IN D_2O AT 26°[a]

Sugar	*Anomeric protons*		*Glycosidic protons*		*H-2 protons*	
	α	β	α	β	α	β
D-Glucose	5 30	4 72	NA	NA	3 62	3 32
D-Galactose	5 30	4 62	NA	NA	3 87[b]	3 54
D-Mannose	5 20	4 92	NA	NA	3 94	3 94
D-Xylose	5 28	4 62	NA	NA	3 62	3 21
D-Fucose	5 23	4 52	NA	NA	3 62	3 52
D-Ribopyranose	5 52	5 34	NA	NA	—	—
D-Ribofuranose	4 94	5 04	NA	NA	—	—
2-Amino-2-deoxy-D-glucose	5 48	4 96	NA	NA	3 30	—
2-Amino-2-deoxy-D-galactose	5 50	4 94	NA	NA	—	3 28
Trehalose α-Glc-(1→1)-α-Glc	NA	NA	5 07	NA	—	—
Kojibiose α-Glc-(1→2)-Glc	5 20	—	5 54	NA	—	—
Sophorose β-Glc-(1→2)-Glc	5 52	—	—	4 68	—	—
Sucrose α-Glc-(1→2)-β-Fru	NA	NA	5 45	NA	3 61	—
Turanose α-Glc-(1→3)-Fru	NA	NA	5 40	NA	—	—
Maltose α-Glc-(1→4)-Glc	5 25	4 68	5 45	NA	3 61	—
Maltotriose α-Glc-(1→4)-α-Glc-(1→4)-Glc	5 26	4 70	5 45[c]	NA	—	—
Cellobiose β-Glc-(1→4)-Glc	5 29	4 72	NA	4 59	—	3 36
Isomaltose α-Glc-(1→6)-Glc	5 34	4 76	5 06	NA	—	—
Gentiobiose β-Glc (1→6)-Glc	5 29	4 72	NA	4 58	—	—
Lactulose β-Gal-(1→4)-β-Fru	NA	NA	NA	4 56	—	—
Lactose β-Gal-(1→4)-Glc	5 26	4 68	NA	4 47	—	3 52
Melibiose α-Gal-(1→6)-Glc	5 32	4 76	5 08	NA	—	—
Raffinose α-Gal-(1→6)-α-Glc-(1→2)-β-Fru	NA	NA	5 05[d] 5 46	NA	3 9 3 7	—
Stachyose α-Gal-(1→6)-α-Gal-(1→6)-α-Glc-(1→2)-β-Fru	NA	NA	5 04[e] 5 48	NA	—	—

[a]Chemical shifts measured at the centre of the doublet in p p m downfield from external tetramethylsilane NA = not applicable [b]Obtained from Ref 23 [c]This is the chemical shift of the two similar glycosidic protons which are not separated in ^{1}H-n m r at 270 MHz However, they are separable by using ^{13}C-n m r at 67 89 MHz [d]5 05, 3 9 = α-Gal-(1→6), 5 46, 3 7 = α-Glc-(1→2)

separated and readily observed For two glycosidic protons of a similar type, as in maltotriose, separation of the resonances was not possible even at 270 MHz

The results in Table I indicate that it may perhaps be possible to determine the nature of the sugar residue and the type of linkage from the chemical shift of the glycosidic proton resonance This approach is explored further in Table II, which summarises the information on the oligosaccharides shown in Table I, on the basis of a particular type of sugar residue and the nearest-neighbour sugar residue to the glycosidic proton Multiple entries in Table II indicate that data are available from more than one oligosaccharide, and it is noted that these values generally agree within experimental error (±0 02 p p m). The data in Table II are rather incomplete, because of the difficulty of obtaining oligosaccharides of defined structure

TABLE II

CHEMICAL SHIFTS (P P M) OF THE GLYCOSIDIC PROTONS OF SUGAR RESIDUES IN AN OLIGOSACCHARIDE[a]

Sugar residue	*Nearest, neighbouring sugar residue*	*Type of linkage*				
		(1→1)	*(1→2)*	*(1→3)*	*(1→4)*	*(1→6)*
α-Glucose	Glucose	5 07	5 54	U	5 45 (5 21)[b] 5 45 (5 26)[c]	5 06
α-Glucose	Fructose	U	5 45 5 46 5 48	5 40	U	U
β-Glucose	Glucose	U	4 68	U	4 59	4 58
α-Galactose	Glucose	U	U	U	U	5 04 (5 01)[c] 5 05 5 08 (5 10)[c]
α-Galactose	Galactose	U	U	U	U	5 04
β-Galactose	Glucose	U	U	U	4 47 (4 60)[b]	U
β-Galactose	Fructose	U	U	U	4 66 (4 59)[c]	U

[a]U = unavailable The numbers in brackets refer to the chemical shifts of the glycosidic protons when the neighbouring sugar residue has been modified chemically to contain a carboxyl group [b]Bromination method [c]Cyanohydrin method

It is not possible to distinguish between, for example, the α-Glc-(1→6)-Glc, α-Gal-(1→6)-Glc, and α-Gal-(1→6)-Gal structures solely on the basis of the chemical shift of their glycosidic protons However, the H-2 protons of α-Glc and α-Gal have different chemical shifts when present in monosaccharides or in oligosaccharides (see Table I), hence it is possible to distinguish between α-Glc and α-Gal when present in an oligosaccharide chain, by means of a double-resonance experiment A more difficult situation is that of β-Glc(1→4)-Glc and β-Glc(1→6)-Glc, which also cannot be decided on the basis of the chemical shifts of the glycosidic protons (Table II) In this case, we utilise the observation that when the adjacent sugar residue is modified to produce a carboxyl group, the resonance for the glycosidic proton shifts much more with a (1→4)- than with a (1→6)-linkage (see Table II) Thus, the two structures can be distinguished, provided that the β-Glc(1→4) or β-Glc(1→6) residue is adjacent to the terminal reducing-sugar residue

Sequence determination in oligosaccharides The first method is based on the sequential broadening of resonances, and the second on the decrease in T_1 of resonances that occurs on the addition of Gd^{3+} to a solution of the carboxylic acid derivative of the sugar. Typical results obtained from the use of the first method are shown in Fig 1 for the carboxylic acid produced *via* treatment of maltotriose with cyanide The spectrum of the trisaccharide carboxylic acid was obtained at pH 3 5 At this pH, resonance 3 is clearly separated from the envelope of sugar resonances at higher field Resonance 1 occurs as a doublet split by the adjacent CH proton, but resonances 2 and 3 appear more complex, presumably because the chain-elongation process used to form the carboxylic acid generates diastereoisomers

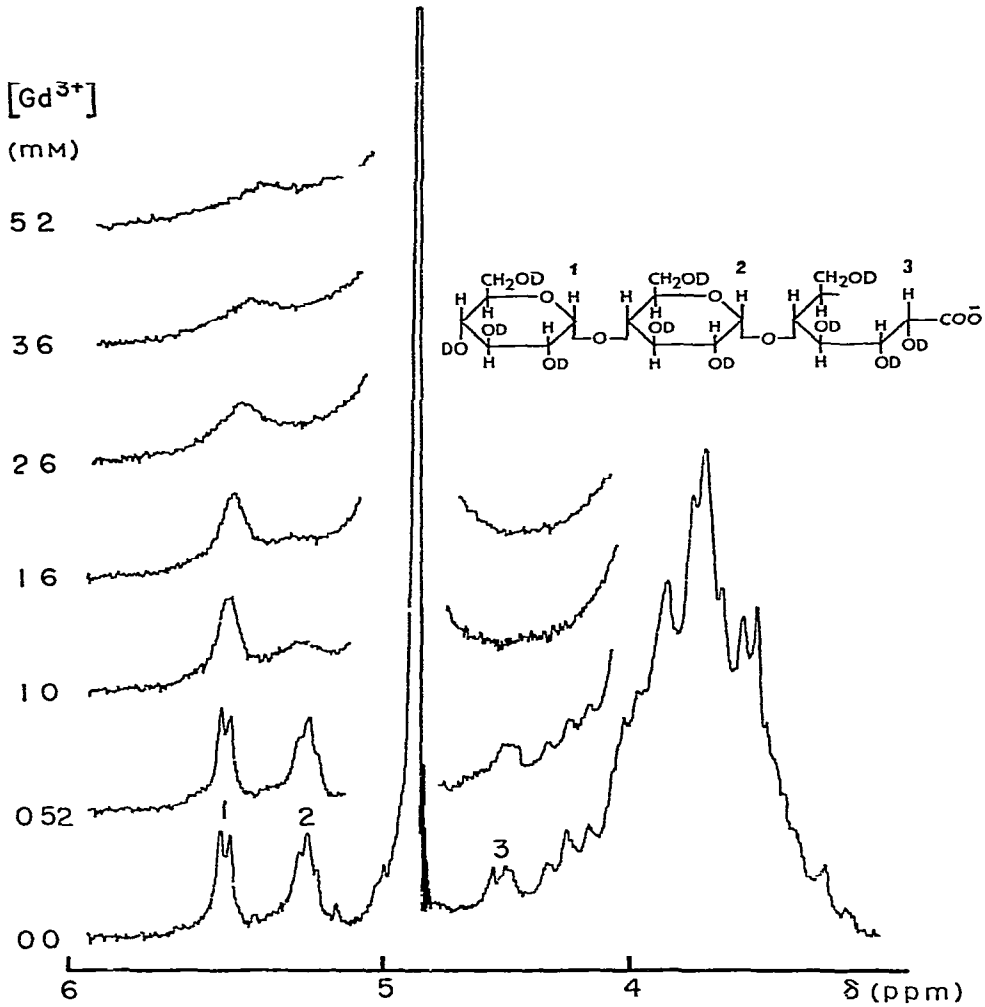


Fig 1 ^{1}H N m r spectrum at 100 MHz of "α-Glc-(1→4)-α-Glc-(1→4)-Glucoheptonic acid" in D_2O at 26° and pH-meter reading 3 5 after the addition of Gd^{3+} at the concentrations shown The resonances numbered 1, 2, and 3 arise from the labelled protons on the chemical structure

Addition of successive amounts of Gd^{3+} to the solution in the n m r tube, using a micrometer syringe containing a relatively concentrated solution of $GdCl_3$, causes the broadening of resonances 3, 2, and 1 in that order (see Fig 1) This shows the potential of the method for determining the sequence of an oligosaccharide, provided that the appropriate resonances (1, 2, 3, *etc*) can be seen separately and assigned to specific sugar residues The $[Gd^{3+}]$ required to broaden resonance 1 in Fig 1 is only 5 2mM, and hence there should be no difficulty in determining the sequence of a tetrasaccharide and probably also a pentasaccharide. Other oligosaccharides for which the sequence was checked by this method were raffinose, which was treated with invertase to release melibiose before the cyanohydrin treatment (see Experimental), maltose, lactose, melibiose, and β-Gal-(1→4)-β-Fru.

TABLE III

T_1 VALUES (SEC) FOR THE GLYCOSIDIC PROTONS[a] OF "α-Glc-(1→4)-α-Glc-(1→4)-GLUCOHEPTONIC ACID" (18% IN D_2O) AT pH 6 AND 20°

[Gd^{3+}] (mM)	Proton 2 (Fig 1)	Proton 1 (Fig 1)
0	0 44	0 61
0 78	0 07 (84%)[b]	0 23 (62%)[b]

[a]At pH 6, the resonance from proton 3 (see Fig 1) has moved upfield due to titration of the adjacent carboxyl group, and is obscured by the envelope of C*H* resonances of the sugar [b]The figures in brackets represent the percentage decrease in T_1 on addition of Gd^{3+}

The second method is based on the measurement of T_1 values for the numbered resonances shown in Fig 1, before and after one addition of Gd^{3+} The T_1 values for the carboxylic acid derived from maltotriose are given in Table III and show that, after addition of Gd^{3+}, the percentage decrease in T_1 of the glycosidic proton nearer to the carboxyl group (proton 2) is considerably greater than that of the other glycosidic proton (proton 1) The percentage decrease falls off sequentially along the chain, as found also with peptides[9]

This method possesses several advantages over the line-broadening technique Firstly, only a small amount of Gd^{3+} is required, this is insufficient to broaden the resonances appreciably and thus there is no problem of coalescence of closely spaced resonances, as may occur in the line-broadening procedure Secondly, only one addition of Gd^{3+} is required, instead of a number of additions Thirdly, in both methods, the effect is mediated along the oligosaccharide chain, and it is likely (by comparison with similar studies on peptide chains[9,10]) that five or even six residues may be distinguished, because the broadening method is less precise, it may not be as useful for longer sequences

As indicated in the Experimental section, the cyanohydrin method can be readily adapted to the milligram level, for maltose and maltobionic acid, spectra were obtained on a continuous-wave 100-MHz spectrometer with a micro-tube[7] of capacity 0 05 ml. With the increased sensitivity of pulsed spectrometers, even smaller amounts of material may be studied by using T_1 methods

^{13}C-N m r spectroscopy There are many papers in which it has been shown that the ^{13}C-n m r spectra of sugars (with broad-band proton decoupling) show well-defined, single resonances for each carbon atom in the molecule[19-21] We have made a preliminary study of the ^{13}C-n m r spectra of maltose, lactose, raffinose, stachyose, gentiobiose, and maltobionic acid The excellent separation of the peaks is a considerable advantage over ^1H-n m r spectroscopy, where the proton peaks are often not resolved For example, with maltotriose, the two glycosidic-proton resonances are not separated at 270 MHz, whereas the corresponding ^{13}C-resonances are clearly separated at the same field strength (see Table I) Two other advantages of ^{13}C-n m r spectroscopy are (*a*) the absence of the solvent peak, and (*b*) the

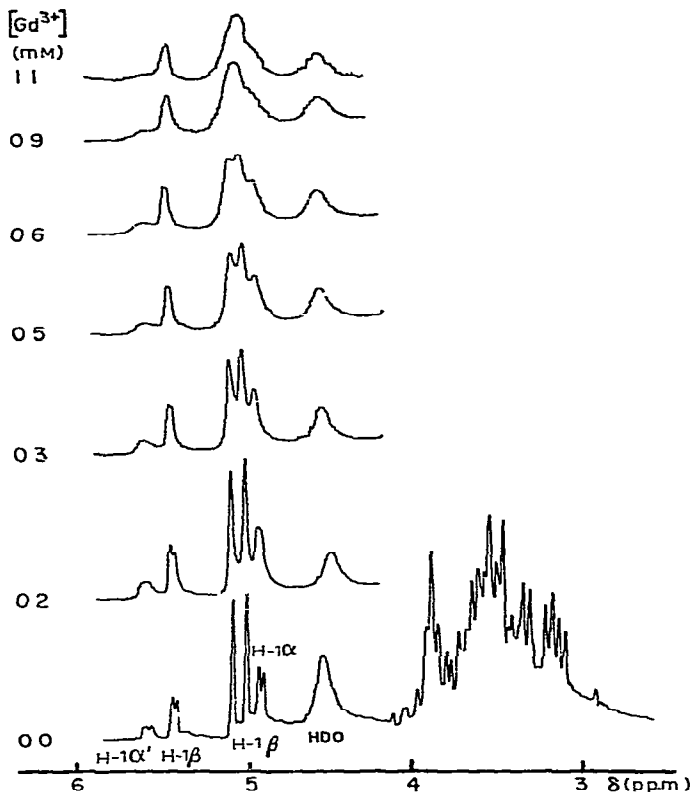
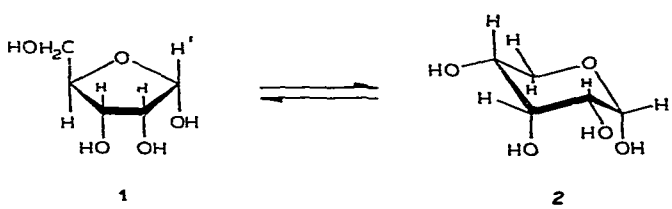


Fig 2 ^{1}H-N m r spectrum at 100 MHz of D-ribose in D_2O at 45° and pH ~4 5 on addition of Gd^{3+} at the concentrations shown The assignment of the marked resonances is read in conjunction with structures 1 and 2



1 2

observation of a downfield resonance from the carboxyl carbon atom, which confirms the effectiveness of the cyanohydrin synthesis

However, the major disadvantage of ^{13}C-n m r. spectroscopy is its low sensitivity[8], which means that ~10 mg of material is needed in order to obtain a good spectrum on a trisaccharide in 5-h accumulation at 67 89 MHz This is very limiting for studies on small amounts of material, and it is clear that ^{13}C-n m r spectroscopy can best be used as a complementary tool to ^{1}H-n m r spectroscopy in the identification of the types of residues and linkages present in the oligosaccharide This will be discussed in a subsequent paper

Alternative binding site for Gd^{3+} It has been shown that the lanthanides will bind to three hydroxyl groups on a monosaccharide residue if (*a*) it forms a six-membered ring and the hydroxyl groups are in an axial–equatorial–axial sequence, or (*b*) it forms a five-membered ring and the hydroxyl groups are aligned in a gauche–gauche sequence[22] Monosaccharides with this type of stereochemistry are ribose, gulose, allose, talose, and lyxose Clearly, the presence of these sugar residues in an oligosaccharide chain, at any position other than at the end where the carboxyl group is inserted, would allow binding of Gd^{3+} at that sugar residue and lead to incorrect determination of the sequence of residues

In order to determine the strength of binding of Gd^{3+} to three such hydroxyl groups, a study was made with D-ribose, which exists as an equilibrium mixture of furanose and pyranose forms, of which the α anomers **1** and **2** have the configuration necessary to bind Gd^{3+} The β anomers, which are also in equilibrium with **1** and **2**, do not have the correct configuration to bind Gd^{3+} Fig 2 shows the ^{1}H-n m r spectrum of D-ribose, with the resonances assigned, in terms of structures **1** and **2**, as given elsewhere[23] On addition of Gd^{3+}, the resonances marked α (the α anomers) broaden more rapidly than those marked β, with that for α-D-ribopyranose broadening more rapidly at 26° (spectra not shown) than that for α-D-ribofuranose At 26°, the broadening for α-D-ribopyranose is complete at $[Gd^{3+}]$ = 0 52mM, compared with 1 1mM in Fig 2 at 45°; hence the binding constant decreases with increase of temperature It is of more importance that resonance 3 is still visible at $[Gd^{3+}]$ = 0 52mM in Fig 1, which shows that the binding of Gd^{3+} to ribose is stronger than that of Gd^{3+} to a half-protonated carboxyl-group introduced at the end of an oligosaccharide

The presence of any of the five sugars mentioned above in an oligosaccharide sequence (except at either end where they would obviate the need for the introduction of a binding site) would present a considerable problem We have not investigated this difficulty further, but suggest that it may be alleviated, although probably not entirely eliminated[24], by the formation of the corresponding methyl ethers *via* the following sequence of steps (*i*) formation of the acid by the cyanohydrin method, (*ii*) permethylation of the oligosaccharide to produce the corresponding methyl ethers and ester, and (*iii*) specific hydrolysis of the ester to the carboxylic acid

Non-reducing sugars Terminal (1↔1)- and (1↔2)-glycosides present difficulties, because the absence of the aldehyde or ketone group prevents the use of the cyanohydrin method to introduce a carboxyl binding-group for Gd^{3+} In the specific case of a terminal β-D-fructofuranose residue, this can be overcome by the use of invertase, which hydrolyses the glycosidic linkage (see Experimental section) In some cases, it has been found that (1↔1)- and (1↔2)-linkages are hydrolysed more rapidly with acid than other linkages[24]. Currently, we are unable to treat this type of sugar

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