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NMR-Studies of the Interaction of Metal Ions with Poly(1,4-hexuronates). IV. Proton Magnetic Resonance Study of Lanthanide Binding to Sodium Methyl α-D-Galactopyranosiduronate in aqueous Solutions

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The interaction of Eu^{3+} and Pr^{3+} with sodium methyl α-D-galactopyranosiduronate in aqueous solutions has been studied by ^1H NMR. The results indicate a large formation constant for 1:3 adducts and that only negligible amounts of 1:2 and 1:1 adducts are present at low molar ratios of lanthanide to substrate. From the LIS-curves at very low ratios of lanthanide to substrate, evidence was obtained for a remarkably high degree of axial symmetry in the 1:3 complex. A lanthanide-binding site has been determined by a computer search for the best fit of calculated and observed LIS assuming only dipolar (pseudocontact) mechanism and an axially symmetrical complex in which the three ligands are magnetically equivalent.

The binding of metal ions to active sites in anionic biopolymers plays an important role in determining their physical and biological properties. Information on binding sites and complex-formation constants is thus of great importance, and we have applied ^1H NMR spectrometry to obtain such information by using paramagnetic lanthanide ions as probes. The ability of these ions to induce chemical shifts in NMR spectra is well established,[1] and spectra which are normally too complex for complete interpretation, such as those of carbohydrates, may become amenable to analysis. A valid analysis of the lanthanide induced shifts (LIS) to provide information on the structure of the complex requires knowledge of the stoichiometry of the complex, the complex-formation constants and, very importantly, also, the symmetry of the complex.[2]

The present ^1H NMR study of sodium methyl α-D-galactopyranosiduronate with $Eu(NO_3)_3$ and $Pr(NO_3)_3$ as shift reagents in D_2O leads to structural assignment for the complexes formed, which predominantly consist of Ln(Me α-D-galactopyranosiduronate)$_3$ (Ln = Eu or Pr). It is possible to visualize the conformation of this complex by molecular models, but more than one conformation seems to be acceptable. A preliminary account has been published previously.[3]

The theoretical background for the structural calculations is given by Reuben [4] and Bleaney.[2,5]

EXPERIMENTAL

Methyl α-D-*galactopyranosiduronic acid methyl ester* (*1*) was synthesized according to Jones and Stacey.[15] The yield of the crude product was 20 %. After recrystallization from methanol-acetone (1:1, v/v) the product had a melting point of 148 °C and a specific rotation $[\alpha]_D^{20} = +127°$ (c 1.0; water).

Sodium methyl α-D-*galactopyranosiduronate* was prepared by hydrolysis of the methyl ester (*1*). The methyl ester (*1*) (50 mg) was dissolved in sodium hydroxide (0.02 N, 25 ml) at room temperature. Aliquots (1 ml) were removed at intervals and titrated with HCl (0.01 N) to follow the progress of the reaction. When the reaction was complete (*ca.* 20 min), excess alkali was removed by passing the sample through a cation-exchange column (Dowex 50W × 8, H^+-form). The eluate was neutralized with dilute sodium hydroxide and the sodium methyl α-D-galactopyranosiduronate was isolated by freeze-drying. The yield was virtually quantitative. The product was used for the NMR studies without further characterization.

NMR spectrometry. Sodium methyl α-D-galactopyranosiduronate (monohydrate) was dissolved in D_2O to give the desired concentration, and *ca.* 0.3 ml of the solution was accurately weighed into the NMR tube. The pH of the solution was checked by means of a Radiometer C-297 capillary glass electrode and adjusted to 5 – 6 by addition of HNO_3 (1.5 N in D_2O). For measuring the initial slopes of the LIS curves, the appropriate lanthanide nitrate was added as a D_2O solution (125 mg/ml) in amounts ranging from 2 to 20 μl. The volume corrections due to these additions were neglected. For higher ratios of lanthanide to substrate, $[Ln_0]/[S_0]$, the nitrates were weighed directly into the NMR tube and dissolved *in situ*. The spectra were recorded on a Varian-A-60A NMR spectrometer at 39 °C using dioxan or sodium 3-(trimethylsilyl)propane sulfonate as an internal reference. Downfield shifts were assigned as positive. All assignments were confirmed by double resonance experiments using a Varian HA-100 NMR spectrometer.

Analysis of data. A computer program was written to carry out an automatic search for the position of the lanthanide ion relative to the substrate which gave the best fit of the observed and the calculated LIS, assuming an axially symmetrical complex and only dipolar mechanism for the LIS. Space coordinates were taken from an X-ray study of methyl α-D-galactopyranosiduronic acid methyl ester.[7] A reasonable starting position for the lanthanide ion was chosen and space coordinates for an auxiliary atom introduced to define the orientation of the principal magnetic axis as the straight line through this atom and the lanthanide. The distances from the lanthanide to the various protons, r_i, and the angles between the vectors $\vec{r_i}$ and the symmetry axis, θ_i, could be calculated. In the case of rapid-ligand exchange, averaged induced dipolar shifts, $\Delta\nu_i$, for the actual substrate protons, H_i, caused by a certain species LnS_n can be calculated according to the formula [4,5]

$$\Delta\nu = \frac{n[LnS_n]}{[S_0]} C \frac{(3\cos^2\theta_i - 1)}{r_i^3} = \frac{n[LnS_n]}{[S_0]} \Delta_{ni} \quad (1)$$

The bound shift, Δ_{ni} is the induced shift for proton i when n substrate molecules are bound to the lanthanide ion. The stoichiometry parameter n, the complex/substrate molar ratio $[LnS_n]/[S_0]$, and the constant C are universal for all protons. The value of C depends on the magnetic properties of the lanthanide ion and the crystal-field coefficients, and is not known. The theory gives only relative, not absolute signs, *i.e.* C can be either positive or negative. Both possibilities have been taken into account here. To avoid introduction of the factors $[LnS_n]$ and C, initial shift ratios relative to those of proton H_1 was used. An agreement factor,[4] ε, was expressed by

$$\varepsilon = \left[\frac{1}{N-1} \sum_{i=2}^{N} \left(\frac{\Delta\nu_{calc}^{(i)}}{\Delta\nu_{calc}^{(1)}} - \frac{\Delta\nu_{obs}^{(i)}}{\Delta\nu_{obs}^{(1)}}\right)^2\right]^{\frac{1}{2}} \quad (2)$$

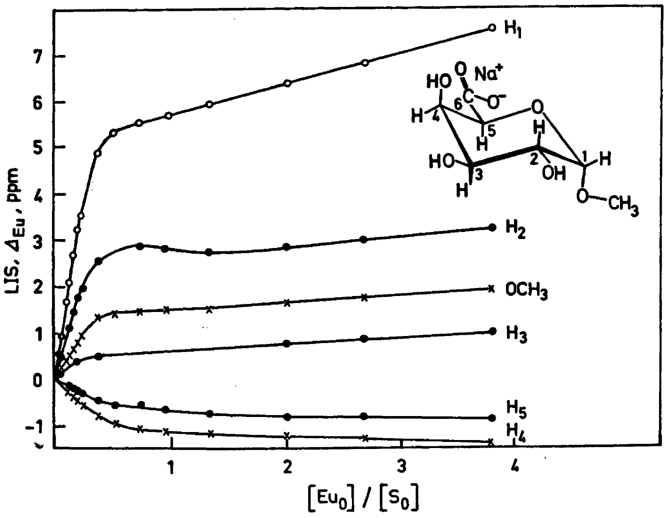

Fig. 1. The induced shifts for the carbon-bound protons of sodium methyl α-D-galactopyranosiduronate in D_2O solution with $Eu(NO_3)_3$ as shift reagent. (Substrate concentration $[S_0] = 0.336$ mol/l).

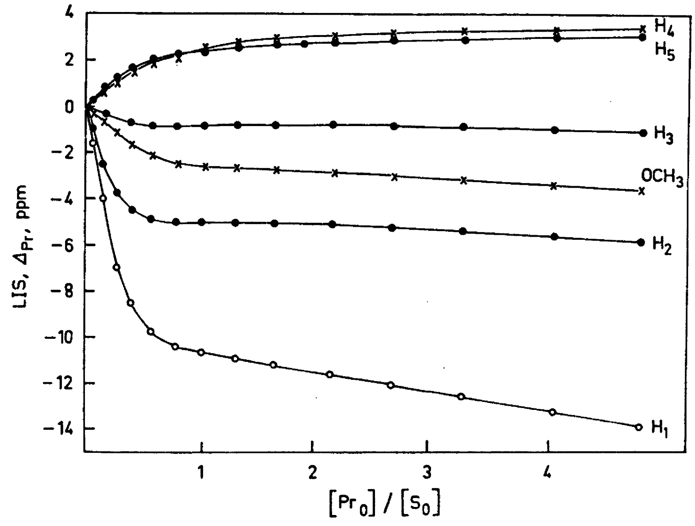

Fig. 2. The induced shifts for the carbon-bound protons of sodium methyl α-D-galactopyranosiduronate in D_2O solution with $Pr(NO_3)_3$ as shift reagent. (Substrate concentration $[S_0] = 0.216$ mol/l.

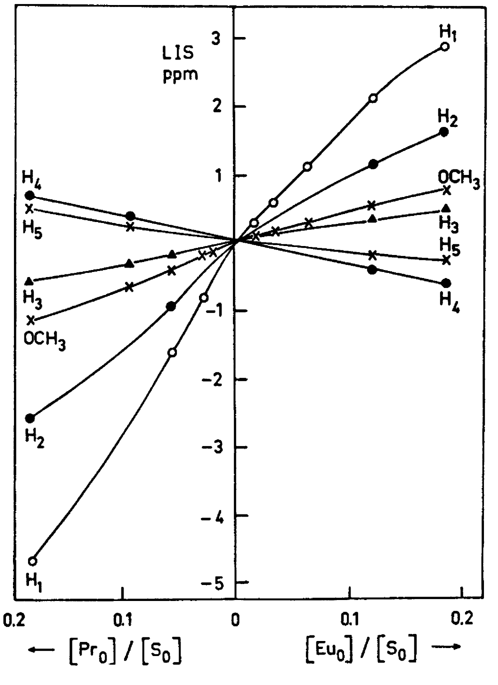

Fig. 3. Same as Figs. 1 and 2, expanded scale. (Substrate concentrations: $[S_0]_{Pr} = 0.216$ mol/l, $[S_0]_{Eu} = 0.336$ mol/l).

where N is the number of protons involved. By changing the x, y, and z coordinates of the lanthanide and the auxiliary atom, respectively, in a stepwise manner with a pre-selected step-size, and selecting the position with the lower ε value, the program searched for a best-fit position defined by a minimum value of ε.

RESULTS

a. Presentation of data. The observed lanthanide-induced shifts (LIS) in ppm for five protons ($H_1 - H_5$) and the CH_3-group in sodium methyl α-D-galactopyranosiduronate in D_2O solutions are shown in Figs. 1, 2, and 3 as a function of the $[Ln]/[S_0]$ ratio with $Eu(NO_3)_3$ and $Pr(NO_3)_3$ as shift reagents. Almost identical curves were obtained with $Pr(ClO_4)_3$ instead of $Pr(NO_3)_3$.

The fact that both upfield and downfield shifts occur clearly demonstrates the angular dependence in the dipolar shift predicted by eqn. (1). This is markedly different from observations with the complexes $Ln(dpm)_3$ and $Ln(fod)_3$ in organic solvents where LIS are almost exclusively in the same direction for all protons in the substrate. Due to overlapping of the resonance peaks, the LIS curves for H_3 and H_2 were difficult to obtain at very low $[Eu_0]/[S_0]$ ratios.

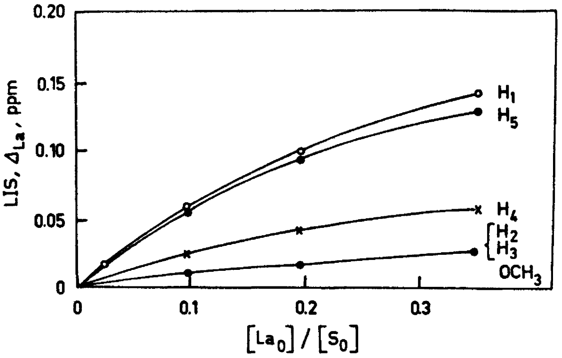

Fig. 4. The induced shifts for the carbon-bound protons of sodium methyl α-D-galactopyranosiduronate in D_2O solution due to complex formation with $La(NO_3)_3$. (Substrate concentration $[S_0] = 0.21$ mol/l).

As was to be expected from theory[5] and earlier experiments,[2] the shifts induced by Pr^{3+} are larger than those induced by Eu^{3+} and of opposite sign as can be seen from Fig. 3. In order to compare the LIS which are due to the paramagnetic nature of the Eu^{3+} and Pr^{3+} ions the LIS must be corrected for the shifts due to electrostatic shielding of the ions.[3] Experiment with the diamagnetic ion La^{3+} therefore was carried out and the results are shown in Fig. 4. From Fig. 4 it is seen that the shift contribution due to complex formation of La^{3+} is largest for H_1. The induced paramagnetic shifts are, as may be seen by comparison with Figs. 1 to 3, completely overriding this effect and the correction is thus not important. For protons H_4 and H_5, on the other hand, this effect is significant and a correction has been applied to the initial slopes given in Table 1. Ratios of Eu^{3+} and Pr^{3+} induced shifts are nearly the same for all protons at low $[Ln_0]/[S_0]$ ratios as appears from the last column of Table 1. This is a characteristic feature for dipolar LIS when the anisotropy of the magnetic susceptibility has axial symmetry provided the structure of the complex is the same for the two lanthanides.[2]

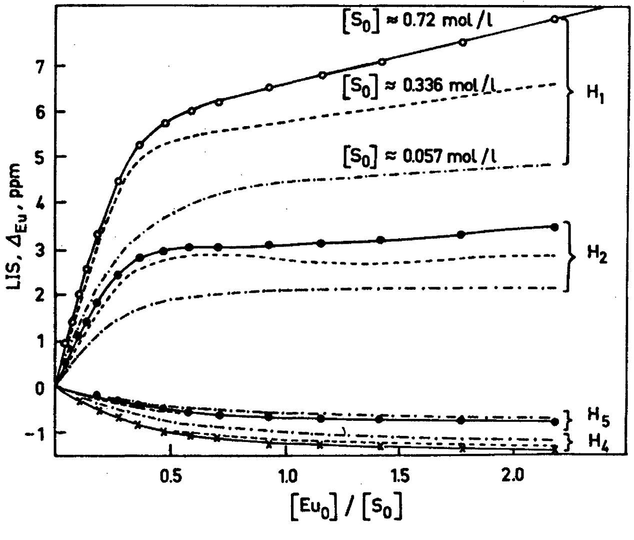

Fig. 5. The induced shifts for the carbon-bound protons of sodium methyl α-D-galactopyranosiduronate in D_2O solution as a function of $[Eu_0]/[S_0]$ for three different substrate concentrations.

b. Stoichiometry. Our first aim was to estimate the formation constants K_1, K_2, and K_3 for the complexes 1:1, 2:1 and 3:1 defined by:

$$\begin{array}{ll} \mathrm{Ln} + \mathrm{S} \rightleftharpoons \mathrm{LnS} & K_1 \\ \mathrm{LnS} + \mathrm{S} \rightleftharpoons \mathrm{LnS_2} & K_2 \\ \mathrm{LnS_2} + \mathrm{S} \rightleftharpoons \mathrm{LnS_3} & K_3 \end{array}$$

and the corresponding bound shifts for proton H_1, Δ_{11}, Δ_{21}, and Δ_{31}.

Even at the highest $[\mathrm{Ln_0}]/[\mathrm{S_0}]$ ratio the LIS are still increasing remarkably linearly (see Figs. 1 and 2) and it is not possible to estimate any asymptotic value for the observed shifts. For a pure 1:1 complex this would give the bound shift Δ_{1i} of the various substrate protons directly. Information on the stoichiometry of the complexes and their bound shifts for the different protons must therefore be sought from the shapes of the curves in Fig. 5, which give the LIS as a function of $[\mathrm{Eu_0}]/[\mathrm{S_0}]$ ratio for three different substrate concentrations, $[\mathrm{S_0}] =$ 0.72, 0.336, and 0.057 mol/l, respectively. The curves have a characteristic knee at molar ratios $[\mathrm{Eu_0}]/[\mathrm{S_0}] \approx 0.3$, and the initial LIS slopes $k = \delta(\Delta\nu_i)/\delta([\mathrm{Eu_0}]/[\mathrm{S_0}]) = n\Delta_{ni}$, increase very slowly at high $[\mathrm{S_0}]$ values. This is characteristic for a system in which 3:1 complexes are formed when the formation constant $K_3 = [\mathrm{LnS_3}]/[\mathrm{S}][\mathrm{LnS_2}]$ is fairly large.[8]

Since the presence of both $\mathrm{LnS_3}$, $\mathrm{LnS_2}$, and LnS has to be considered with their corresponding bound shifts, a theoretical fitting of the LIS-curves requires six-parameter (K_1, K_2, K_3, Δ_{3i}, Δ_{2i}, and Δ_{1i}). By extending the method of Shapiro and Johnston[9] to a six parameter fit almost complete fitting could be obtained for the LIS curve of proton H_1 for the intermediate substrate concentration $[\mathrm{S_0}] = 0.336$ mol/l. The best fit values for the $\mathrm{Eu^{3+}}$-induced bound shifts for 3:1, 2:1, and 1:1 complexes were found to be: $\Delta_{31} = 5.83$ ppm, $\Delta_{21} = -2.67$ ppm and $\Delta_{11} = 40.0$ ppm, respectively. The formation constants giving the best fit were: $K_3 = 350$ $\mathrm{mol^{-1}}$, $K_2 = 160$ $\mathrm{mol^{-1}}$, and $K_1 = 5.5$ $\mathrm{mol^{-1}}$ (standard deviation $\sigma \approx 4$). The ratio K_1:K_2:K_3 was 0.034:1:2.19, whereas binding to identical and independent sites requires 3:1:0.33.[10] This result thus suggests that there is some kind of cooperativity in the binding process.

By comparing the estimated bound shift for H_1 for the 3:1 complex with the one obtained from the initial slope of the LIS curve assuming that the initial shifts are due to the 3:1 complex only (see Table 1, $3\Delta_{31} = 17.4$, *i.e.* $\Delta_{31} = 5.8$ ppm), it is suggested that the 3:1 complex and thus the species $\mathrm{LnS_3}$ is the dominating one in the initial stage of the binding process.

However, the agreement between calculated and observed Δ_{31} is not so good for other substrate concentrations $[\mathrm{S_0}]$. This is probably due to a change in activity coefficients over the large range of salt concentrations used. A full, self consistent analysis, including the activity coefficients (γ) for the lanthanide solutions is necessary to obtain a reliable set of parameters which will fit the LIS curves for all substrate concentrations. It was only intended here to see if the introduction of activity coefficients would improve the fit and a crude correction was employed by letting $[\mathrm{Ln_0}]\mathrm{corr} = \gamma\pm[\mathrm{Ln_0}]$. Using published values[11] for $\gamma\pm$ for $\mathrm{Ln(NO_3)_3}$ a marked improvement was obtained, especially for high $[\mathrm{Ln_0}]/[\mathrm{S_0}]$ ratios. The remarkably good fit obtained without taking activity coefficients into account for the substrate concentration 0.336 mol/l is probably partly due to the fact that in the range of Ln-concentrations used in that experiment, the activity coefficients are relatively independent on the concentration.[11] For the lowest substrate concentration the activity coefficients decrease markedly with increasing Ln-concentration, whereas the opposite is the case for the Ln-concentrations used in the experiments with the highest substrate concentrations. The formation constants given above should therefore not be regarded as true equilibrium constants. The given values for the bound shifts should also be regarded as rough estimates.

Due to the uncertainties introduced by the difficulties in taking activity coefficients properly into account an additional way was sought to find out if initial slopes and slope ratios are purely representative for the 3:1 complex.

Because of rapid ligand exchange between the three species and the solution, the ratios of the LIS ($\Delta\nu_i$) for protons $H_2 - H_5$ (H_i) relative to the LIS of proton H_1 ($\Delta\nu_1$) will in the general case be dependent on the concentration of the three different complexes according to the formula:

$$\frac{\Delta\nu_i}{\Delta\nu_1} = \frac{3\Delta_{3i}[\mathrm{LnS_3}] + 2\Delta_{2i}[\mathrm{LnS_2}] + \Delta_{1i}[\mathrm{LnS}]}{3\Delta_{31}[\mathrm{LnS_3}] + 2\Delta_{21}[\mathrm{LnS_2}] + \Delta_{11}[\mathrm{LnS}]} \quad (3)$$

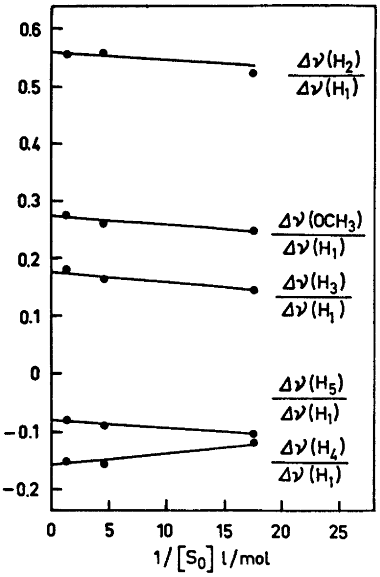

Fig. 6. The induced shift ratios relative to H_1 for the carbon-bound protons of sodium α-D-galactopyranosiduronate in D_2O solution as a function of the reciprocal substrate concentration ($1/[S_0]$).

With the approximate values of the formation constants given above, it is easy to realize that the 3:1 complex must dominate completely over the two other species at infinite concentration of substrate. The initial LIS ratios at different concentration of substrate obtained from Fig. 5 were therefore, plotted against $1/[S_0]$ in Fig. 6. The negligible small variation with substrate concentration shown in Fig. 6 strongly indicates, in agreement with the calculations above, that the initial LIS ratio to a very good approximation can be taken as representative for the 3:1 complex.

c. Structural assignment of the 3:1 complex. It now seems established with a high degree of certainty that the initial slopes of the LIS curves are representative for the 3:1 complex, but a test for the magnetic symmetry of the complex must be carried out before the initial slopes may be used for structural assignment of the 3:1 complex. As mentioned under *a.* the LIS ratio relative to H_1 obtained with $Eu(NO_3)_3$ and $Pr(NO_3)_3$ listed in Table 1, is in fair agreement with each other except for a slight deviation in the values for proton H_3. As mentioned before, initial LIS values are difficult to measure for H_3, and since this shift is rather small its relative value is less accurate. According to Bleaney [2] the data in Table 1, therefore, strongly suggest that the magnetic susceptibility has axial symmetry in the 3:1 complex.

The two sets of relative LIS ratios in Table 1 were used in a computer search for the conformational parameters assuming axial symmetry. Owing to the presumed free rotation of the methyl group around the C_1-O_1 and O_1-CH_3 linkages (*cf.* Fig. 1), a definite set of coordinates can not be assigned to $O-CH_3$ protons. The LIS for the $O-CH_3$ protons were therefore omitted during the search procedure. For both sets of shifts a best-fit position for the lanthanide ion and a best-fit orientation of the principal magnetic axis were obtained, and the data are summarized in Table 2. A step-size of 0.02 Å was used in the computer search procedure. The distance between the Eu^{3+} and Pr^{3+} position is ~0.35 Å, which is in the order

Table 1. Initial slopes in ppm for the LIS of sodium methyl α-D-galactopyranosiduronate with $Eu(NO_3)_3$ and $Pr(NO_3)_3$ as shift reagents. Substrate concentrations: $[S_0]_{Eu} = 0.336$ mol/l, $[S_0]_{Pr} = 0.216$ mol/l. All initial slopes are corrected for shifts due to electrostatic shielding (see Fig. 4).

Proton	Initial LIS slopes $\delta(\Delta\nu)/\delta([Ln_0]/[S_0]) = k$		Relative LIS slopes				k_{Pr}/k_{Eu}
	Eu^{3+}	Pr^{3+}	Eu^{3+} exp.	calc.	Pr^{3+} exp.	calc.	
H_1	17.4	−29.8	1	1	1	1	−1.72
H_2	9.4	−17.2	0.540	0.547	0.578	0.577	−1.82
H_3	2.9	−3.67	0.166	0.155	0.123	0.133	−1.28
H_4	−2.7	4.08	−0.155	−0.160	−0.137	−0.139	−1.49
H_5	−1.47	2.67	−0.084	−0.083	−0.090	−0.090	−1.82
$O-CH_3$	4.47	−7.25	0.257		0.243		−1.62

Table 2. Conformational parameters for the binding of Ln^{3+} in the Ln(methyl α-D-galactopyranosiduronate)$_3$ complexes calculated from the LIS.

Ln^{3+}	Real-space coordinates (Å)						Ln^{3+}-{oxygen / carbon			θ_{H_1}	ε
	Ln^{3+}			Auxiliary atom			distances (Å)			(°)	
	x	y	z	x	y	z	O_4	O_5	C_6		
Eu^{3+}	−2.41	3.75	18.1	−0.57	1.33	16.47	3.01	1.93	2.08	12.47	0.0033
Pr^{3+}	−2.43	3.81	17.7	−0.67	1.49	16.40	2.99	2.00	2.39	12.91	0.0018

of the expected accuracy in positioning the lanthanide ion.[1] The main difference between the positions is their distances from C_6. They are both reasonable from a chemical point of view. The fit to the experimental data is remarkably good in both cases as judged by the root mean square deviations, ε. To illustrate this, calculated initial slopes are given in Table 1. The data are also compatible with a reasonable average position for the $O-CH_3$ protons. Sodium methyl α-D-galactopyranosiduronate with the position of the Ln^{3+} ion and the orientation of the symmetry axis is shown in Fig. 7.

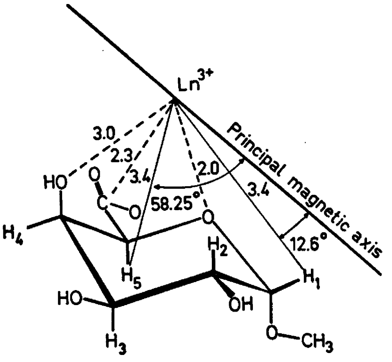

Fig. 7. Proposed binding site for the lanthanide ion and the orientation of the principle magnetic symmetry axis relative to methyl α-D-galactopyranosiduronate.

By using LIS ratios instead of LIS in the search, an ambiquity is introduced since both negative and positive LIS will give a positive ratio with a reference LIS of the same sign. The search for the positions reported above were carried out assuming downfield shifts to be positive with a positive C value [see eqn. (1)] for Eu^{3+} and a negative C value for Pr^{3+}. According to this sign convention the angle factor should be positive for the H_1 proton which shows a large downfield Eu^{3+}-induced shift. Similar calculations were carried out by assuming reversal of the signs of C, and the results are compared with the mean value of the previous ones in Table 3. A best-fit position was obtained also for this sign convention. However, the position is different, especially with reference to the orientation of the principal magnetic axis which is reoriented by approximately 90°. This is expected since the angular factor $(3\cos^2\theta - 1)$ has to change sign. However, with a lanthanide $-O_5$ distance of only 1.57 Å, this position is physically less reasonable. The root means square deviation, ε, is also an order of magnitude larger.

Table 3. Comparison of conformational parameters obtained for the Pr(methyl α-D-galactopyranosiduronate)$_3$ complex according to the sign conventions applied; downfield shifts positive.

Sign conv.	Ln^{3+}			Auxiliary atom			Ln^{3+} − {oxygen / carbon			θ_{H_1}	ε
							distances (Å)			(°)	
	x	y	z	x	y	z	O_4	O_5	C_6		
C(Eu) < 0	−2.57	2.45	17.2	−1.03	5.02	19.44	3.74	1.57	3.15	115.8	0.012
C(Eu) > 0	−2.41	3.83	17.8	−0.63	1.43	16.41	2.97	1.99	2.26	12.6	0.0016

Table 4. Ln^{3+}-proton distances and θ values for the two alternative sites.

Sign convention		H_1	H_2	H_3	H_4	H_5
C(Eu) < 0	θ_i°	115.8	80.14	66.14	38.19	51.63
	r_i (Å)	2.13	3.27	4.71	4.68	3.45
C(Eu) > 0	θ_i°	12.93	27.76	39.00	66.13	58.25
	r_i (Å)	3.37	3.64	4.89	4.14	3.39

As a check on the binding sites obtained, small additions of $Gd(NO_3)_3$ were used to induce line broadening. To obtain data for all protons, $Eu(NO_3)_3$ was first added ($[Ln_0]/[S_0] = 0.18$) to spread out the signals and then $Gd(NO_3)_3$ was added. A line broadening proportional to r^{-6} is predicted by the theory,[12] and should reflect the differences in lanthanide–proton distances r_i. The observed broadening effects were of the order: $H_1 \approx H_2 \approx H_5 > H_3 > O-CH_3$. The broadening was difficult to estimate for H_4, but it was definitely less than for H_1 and larger than for $O-CH_3$. The most pronounced difference between the two sites, for which lanthanide–proton distances, r_i, and angles, θ_i, are given in Table 4, is the ratio $(r_2/r_1)^6$. The ratio $(r_2/r_1)^6$ was found to be 13 for the negative and 1.6 for the positive sign convention. The broadening experiments predict this ratio close to unity and thus favour the latter site to be the most likely.

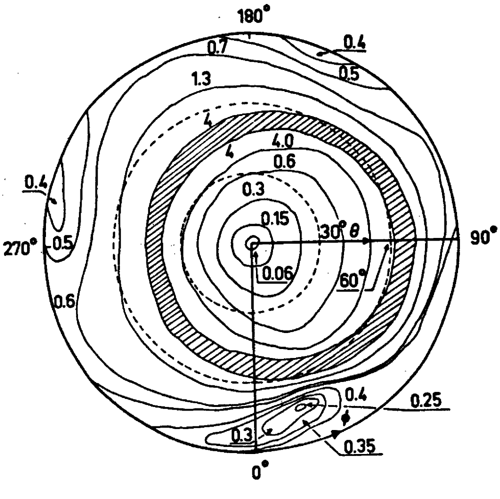

Fig. 8. Contours of the agreement factor ε as a function of the orientation of the principal magnetic axis given by the polar angles θ and ϕ. The centre in the diagram corresponds to the best fit with the axis pointing out of the paper plane.

To investigate whether there exist other orientations of the magnetic axis leading to a minimum value of the agreement factor ε, we have rotated the axis in all orientations, keeping the lanthanide ion position. The result is shown in Fig. 8 as a contour plot of the agreement factor ε. The contours are paths of constant ε values as a function of polar angles θ and ψ defining the axis orientation. The centre in the diagram corresponds to the best fit position with the axis pointing out of the paper plane. In addition to the minimum for the most reasonable site, only one more less defined minimum appeared.

DISCUSSION

The nitrates and perchlorates of Eu^{3+} and Pr^{3+} appear to be well suited as shift reagents for carbohydrates in aqueous solutions. However, Angyal's observations on methyl β-D-hammamelopyranoside [13] suggest that the requirements for structural analysis may not always be fulfilled.

It should be emphasized that a strict pH control is necessary to obtain reliable induced shifts for acidic carbohydrates. The pH curves for the induced shifts of protons H_1 and H_2 of D-galactopyranosiduronic acid published previously [3] clearly demonstrate this point. Owing to the low solubility of the lanthanide hydroxides the safe pH working range is limited to between 5 and 6.

As pointed out by Bleaney *et al.*[2] a number of conditions have to be fulfilled to allow any valid conclusion to be drawn from LIS concerning the conformation of the complexes. Among these, three primary requirements may be formulated: (i) The conformation of the substrate must be retained in the complexed state, (ii) LIS of all protons must be of dipolar origin only, and (iii) it must be possible to measure or evaluate a quantity proportional to the bound shift for one particular stoichiometry of the complex.

(i) The ^{1}H NMR spectrum of sodium methyl α-D-galactopyranosiduronate after addition of $Eu(NO_3)_3$ to a molar ratio of 3.6 has been published previously.[3] The coupling constants were all in reasonable agreement with those observed for the free substrate, thus suggesting that the $^4C_1$ conformation is retained in the lanthanide complexed state.

(ii) Contact shifts have their origin in movement of unpaired electron spin density from the metal cation to the ligand protons by covalent bond formation and are independent of the factor $(3\cos^2\theta - 1)$. This interaction rapidly decreases with increasing Ln-proton distance through bonds and may possibly be significant only for protons H_4 and H_5 according to the lanthanide position shown in Fig. 7. However, since contact shift contributions by Eu^{3+} and Pr^{3+} differ markedly [4,14] in magnitude, and since the LIS for H_4 and H_5 fit the symmetrical scheme for the dipolar mechanism, a significant contact shift contribution seems [5] very unlikely.

(iii) For systems in which complexes of higher than 1:1 stoichiometry is formed, the observed LIS will in the general case be an average over all species according to eqn. (3). Bearing in mind that only the formation constants K_n are universal for all protons in the substrate, while the bound shift Δ_n generally are different, it turns out that even a system where $n = 2$ may be undetermined if no additional information is available. In our case the LIS data strongly indicate a large formation constant for 3:1 complex, and that only small and negligible amounts of 2:1 and 1:1 complexes are present at low $[Ln_0]/[S_0]$ ratios.

In the computer search a set of space coordinates was calculated giving the best fit to the observed shift data without considering the question of more than one substrate molecule participating in the complex formation. The question therefore remains, whether a complex conformation with three magnetically equivalent carbohydrate molecules is physically possible. This is necessary for correlating the results to any physically reasonable complex.

The proposal of complex structures based on the present data is of course hypothetical. However, it is obviously possible to accomodate two sugar rings with the lanthanide ion as an inversion centre, as shown previously.[3] In order to be equivalent to the others, the third sugar moiety must be in a position that can be reached by a hypothetical rotation of the first or the second unit around the axial symmetry axis until no intermolecular interactions occur. Another possibility seems to be that the third unit is located in a position approaching the one corresponding to the negative sign convention in such a way that the shift contributions from two of the ligands cancel. Scaled molecular models indicate that these positions may be possible. If this is not the case it seems unlikely that the analysis should lead to a chemically reasonable site with an almost perfect fit to the observed data.

The induced shifts by lanthanide salts are smaller than those of the β-diketones (*e.g.* $Ln(dpm)_3$ and $Ln(fod)_3$) [15] for which the constant C is about 1000 ppm Å^3. In our case the value of C is only 120 ppm Å^3 or 360 ppm Å^3 if two ligands counteract. This may suggest that the position corresponding to the negative sign convention is more probable for the third ligand, but the dependence of C on solvents and ligands is complex, and more data are needed to settle this question.

The 3:1 stoichiometry leads to a solvation number 9 for the Ln^{3+} ions which is expected [16] in aqueous solutions. Since the LIS curves are similar with perchlorate and nitrate, and the HDO signal is unaffected by both shift- and broadening reagents, it is not likely that any NO_3-anions or water molecules are present in the first solvation sphere.

The interaction between the Ln^{3+} ions and the ligands is mainly of electrostatic nature. It is difficult to realize any amount of LnS_3 species exceeding the statistical value for identical and independent sites, without any formation of interligand hydrogen bonds which may stabilize the 3:1 complex.

Preliminary studies on the β-anomer, at present under way in our laboratories, show different results. The LIS data can be completely fitted by assuming only 1:1 and 2:1 complexes and the formation constants were found to be

$K_1 = 26$ M^{-1}, $K_2 = 35$ M^{-1}, and $K_3 \approx 0$, thus indicating a much weaker coordinating power in the case of the 3:1 complex. The induced shifts for the substrate protons are also entirely different in accordance with different stoichiometry and structure of the complex. Provided that the lanthanide position relative to the substrate is the same as in the α-anomer molecular model studies show that due to steric hindrance caused by the equatorial methoxy group, 3:1 stoichiometry is impossible or difficult for the β-anomer.

We hope that a full analysis of the LIS data for the β-anomer will shed some more light on the structural problems.

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