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ANALYSIS OF SOME PARTLY AND FULLY ESTERIFIED OLIGOGALACTOPYRANURONIC ACIDS BY P.M.R. SPECTROMETRY AT 220 MHz

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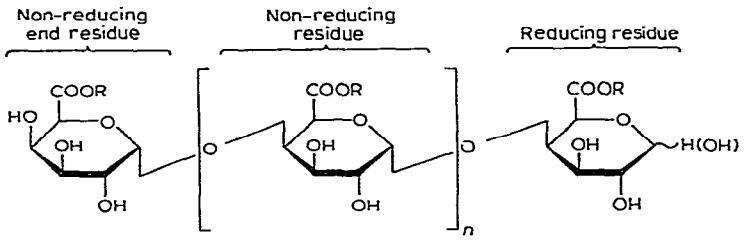
ABSTRACT

The p.m.r. spectra of mono-, di-, tri-, tetra-, and penta-galactopyranuronic acids (**1–5**), the corresponding fully esterified methyl esters (**6–10**), the partly esterified di- (**11**) and tri-galactopyranuronic acids (**12, 13**), and the unsaturated di-, tri-, and tetra-galactopyranuronic acids (**14–16**) were measured on solutions in D_2O at 220 MHz at a pH of 1 and 6. Observation of doublets (J 4 Hz) in the range δ 4.90–5.05 p.p.m. indicates the site of esterification in the non-reducing or reducing sugar residue. Esterification of the sugar residue at the non-reducing end can be deduced from both the presence of a methyl resonance peak at δ 3.80 and the indifference of the signal at δ 4.35 (H-4) to the change in pH. The δ values and coupling constants confirm that all the D-galacturonic acid residues have the *C1* conformation and are α-(1→4)-linked. In the unsaturated oligogalactopyranuronic acids, the double bond is located between C-4 and C-5 of the sugar unit at the non-reducing end. The 4-deoxyhex-4-enopyranosyluronic acid residue occurs in the $^2H_1$(D) conformation. Compound **11** was identified as *O*-(α-D-galactopyranosyluronic acid)-(1→4)-(methyl α,β-D-galactopyranuronate). Compounds **12** and **13** each consisted of a mixture of the three possible isomers; preference for the site of esterification decreases in the order reducing sugar unit, non-reducing sugar unit, sugar unit at the non-reducing end.

INTRODUCTION

As part of an investigation into the characterization and mechanism of action of pectic acid and pectin-degrading enzymes[1], it was necessary to characterize the homologous series of saturated mono-, di-, tri-, tetra-, and penta-galactopyranuronic (Gal*p*UA) acids (**1–5**), the corresponding, fully esterified methyl esters (**6–10**), the partly esterified di- (**11**) and tri-Gal*p*UA acids (**12, 13**), and the unsaturated di-, tri-, and tetra-Gal*p*UA acids (**14–16**). The saturated and unsaturated Gal*p*UA acids were obtained by enzymic degradation of purified pectic acid and fractionated by ion-

exchange chromatography. The corresponding fully and partly esterified methyl esters were synthesized and purified by preparative paper chromatography[1].



2–5 R = H, n = 0–3
7–10 R = Me, n = 0–3
11–13 R = H/Me, n = 0–1

In particular, the sequential analysis of the partly esterified Gal*p*UA acids **11–13** is important as substitution in the carboxyl group adjacent to the glycosidic linkages may affect the pattern of action of the degrading enzymes. The Gal*p*UA acids **1–5** and their corresponding fully esterified methyl esters (**6–10**) can be characterized and identified by descending paper chromatography[1]. As this is not possible for the partly esterified Gal*p*UA acids **11–13**, they need to be further characterized, *e.g.*, by p.m.r. spectrometry.

Few data have been published on the characterization of oligo-uronic acids and their derivatives by p.m.r. spectrometry. Rees and Wight[2] have shown that di-, tri-, and tetra-Gal*p*UA acids in solution in D_2O have the *C1* conformation, and that the D-galactopyranuronic residues are α-(1→4)-linked. In addition, they completely assigned the resonance signals in the p.m.r. spectrum of D-galactopyranuronic acid and of methyl β-D-galactopyranosiduronic acid. For the assignment of H-5, they dissociated the carboxyl group by changing the pH. This effect was also used by Perlin *et al.*[3,4] for the interpretation of the p.m.r. spectra of methyl α- and β-D-glucopyranosiduronic acid and of methyl α- and β-D-idopyranosiduronic acid. They found that the signal for H-5 was shifted ~0.3 p.p.m. downfield when solutions of the salts in D_2O were acidified. The p.m.r. data given by Izumi[5] for α- and β-D-galactopyranuronic acid in solution in D_2O are fully consistent with those of Rees and Wight[2].

P.m.r. data of unsaturated diuronic acids were given by Perlin *et al.*[6] and Hirano[7], and the double bond was shown to be located in the non-reducing sugar residue between C-4 and C-5. Derivatives of unsaturated monouronic acids have been described[8–10].

Together with the above-mentioned p.m.r. data, consideration of both the signal areas and the splitting patterns should enable the partly esterified, saturated di- and tri-galactopyranuronic acids **11–13** to be characterized. The influence of pH on the chemical shift of H-5 should be particularly useful in the determination of the sequence of esterified carboxyl groups in the galactopyranosyl units.

In order to maximise the resolution, the p.m.r. spectra were recorded at 220 MHz. The spectrum of methyl (methyl α-D-galactopyranosid)uronate (**17**) was used as a reference.

RESULTS AND DISCUSSION

For the p.m.r. spectra of an equilibrium mixture of α- and β-D-galactopyranuronic acid (**1**), its corresponding methyl ester **6**, and of methyl (methyl α-D-galactopyranosid)uronate (**17**) in D_2O at 220 MHz, complete assignments were possible on a first-order basis. The chemical shifts and coupling constants of **1** and **6** (pH 1 and 6) and of **17** (pH 6) are summarized in Table I. From the magnitude of $J_{1,2}$, $J_{2,3}$, and $J_{4,5}$, we conclude that **1**, **6**, and **17** have the *C1* conformation[2,5].

TABLE I

P.M.R. DATA[a] FOR COMPOUNDS **1**, **6**, AND **17** IN D_2O[b]

Compound[c]	*Chemical shifts (δ, p.p.m.)*						*Coupling constants (Hz)*			
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*Other protons*	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
1α	5.31 (5.30)	3.80 (3.81)	3.93 (3.91)	4.32 (4.26)	4.72 (4.41)	—	3.8	10.0	3.5	1.5
1β	4.60 (4.58)	3.50 (3.49)	3.71 (3.70)	4.26 (4.20)	4.40 (4.07)	—	8.0	10.0	3.5	1.5
6α	5.31 (5.32)	3.80 (3.81)	3.93 (3.94)	4.32 (4.33)	4.77 (4.79)	3.80 (3.805 or 3.809)	3.8	10.0	3.5	1.5
6β	4.60 (4.61)	3.50 (3.51)	3.71 (3.73)	4.26 (4.27)	4.46 (4.47)	3.80 (3.809 or 3.805)	8.0	10.0	3.5	1.5
17	(4.90)	(3.83)	(3.90)	(4.32)	(4.64)	(3.41; 3.81)	3.5	10.5	3.0	1.5

[a]At pH 1 and 6 (in parentheses). [b]Concentration, 0.67 M. [c]α and β indicate *eq* and *ax* orientation, respectively, of H-1 of the reducing residue.

As found by others[3,4] for glucuronic and iduronic acids, a downfield shift of 0.3 p.p.m. for H-5 of galactopyranuronic acid was observed when the pH was changed from 6 to 1. In addition, a small but significant downfield shift of 0.06 p.p.m. for H-4 was seen when the D_2O solution of sodium galactopyranuronate was acidified. The chemical shifts of H-4 and H-5 of methyl α,β-D-galactopyranuronate were not influenced by the pH. The influence of pH on the signals for H-4 and H-5 thus enables D-galactopyranosyluronic acid and methyl D-galactopyranuronate residues to be distinguished in the partly esterified di- and tri-uronic acids **11–13**, and consequently their sequence can be determined.

The p.m.r. spectra of di-, tri-, tetra-, and penta-Gal*p*UA (**2–5**) and their corresponding fully esterified methyl esters **7–10** at pH 1 can be subdivided into three characteristic regions: (*1*) δ 3.45–4.15 associated with H-2 and H-3 of the reducing and non-reducing sugar residues. In the esters **7–10**, these signals are partly overlapped by the methyl ester peaks; (*2*) δ 4.15–4.90 associated with H-4 of the reducing

and non-reducing sugar residues, H-5 (α and β anomer) of the reducing sugar, and axial (*ax*) H-1; (*3*) δ 4.90–5.40 associated with H-5 of the non-reducing sugar residues and equatorial (*eq*) H-1.

The p.m.r. spectra of the unsaturated di-, tri-, and tetra-Gal*p*UA (**14–16**) at pH 1 are similar, notable differences being due to the olefinic protons (H′-4 of **14**, H″-4 of **15**, and H‴-4 of **16**) and the protons adjacent to the double bond.

Assignment of δ values and first-order coupling constants to compounds **2–5**, **7–10**, and **14–16** are recorded in Tables II–VII, and are discussed in the following sections. The analysis of the partly esterified acids **11–13** is treated separately.

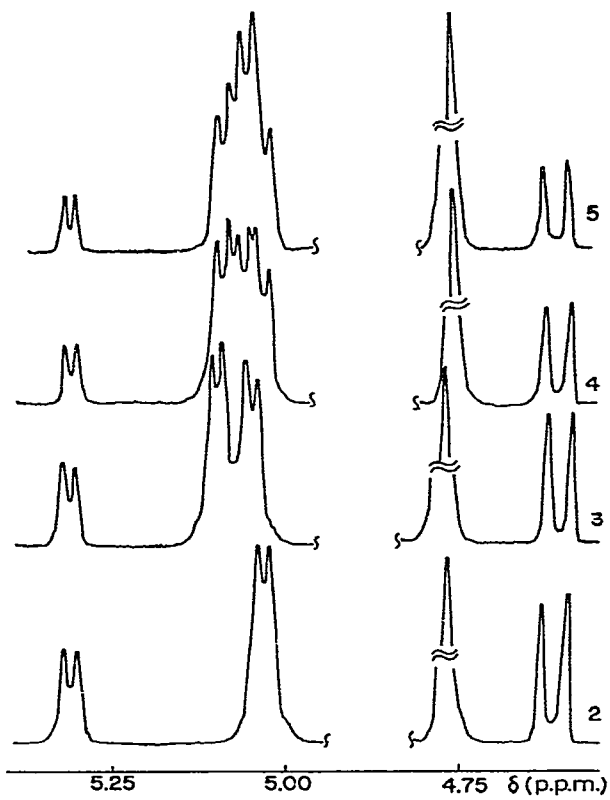


Fig. 1. 220-MHz p.m.r. spectra of **2–5** in the low-field region in D_2O solution at pH 6.

Oligogalactopyranuronic acids **2–5**. — The spectra of **2–5** at pH 6 (see Fig. 1) could be completely analyzed in the low-field region. The doublets at δ 5.32 and 4.61, with spacings of 3.5 and 8.0 Hz, were assigned to *eq* and *ax* H-1 of the reducing sugar residue (*cf.* the assignments of H-1 of **1** and **6** in Table I). These signals showed small, but significant, downfield shifts of 0.05–0.08 when the pH of the solution was changed from 6 to 1 (see Table II). The remaining doublets (δ 5.00–5.15) are attributed to H-1

of the non-reducing sugar residues. The doublet at highest field (δ 5.04) is assigned to H-1 of the sugar residue at the non-reducing end, namely, H″-1 of **3**, H‴-1 of **4**, and H⁗-1 of **5**. The resonance peak at lowest field (δ 5.10) is tentatively assigned to H-1 of the non-reducing sugar adjacent to the reducing sugar residue, namely H′-1 of **3**, **4**, and **5**.

These assignments are supported by the interpretation of the spectra of **15** and **16** in the low-field region. In each compound, the double bond is located in the sugar residue at the non-reducing end (see below) and consequently influences the δ values and coupling constants of the protons in this sugar unit. Therefore, it is reasonable to assign the doublets at δ 5.15 (J 2 Hz) to H″-1 of **15**, and δ 5.10 (J 4 Hz) to H′-1 of **15** at pH 1 (Tables VI and VII).

From the chemical shift values of H-1 of the non-reducing sugar residues of **2**–**5**, which show an up-field shift of 0.21–0.32 p.p.m. with respect to the δ value of the *eq* H-1 of the reducing sugar, and the magnitude of their coupling constants (~4 Hz, Table III), we conclude that the D-galacturonic acid residues are α-(1→x)-linked. H-5 of each non-reducing sugar residue of **2**–**5** can be differentiated at pH 1 (Table II), but not at pH 6 where they resonate at δ 4.77 (Fig. 1). For the non-reducing sugar residues at pH 1, H-5 resonates at a considerably lower field (δ 5.00–5.15) than the

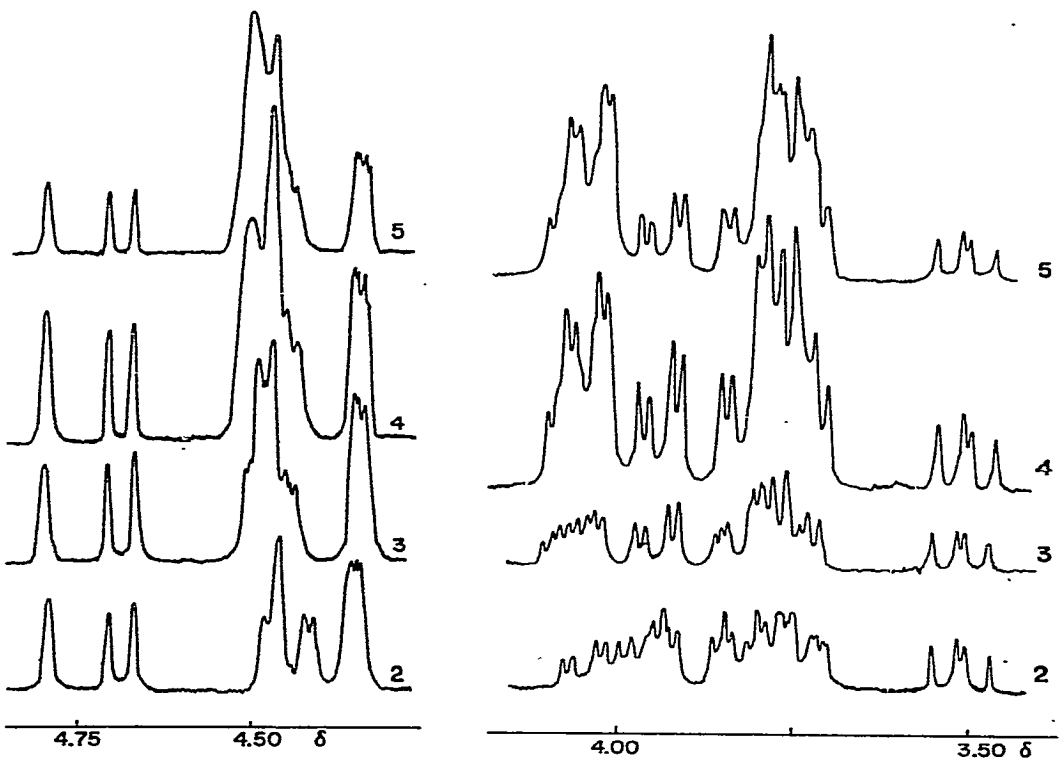


Fig. 2. The 220-MHz spectra (δ 4.15–4.90 and 3.40–4.15) of **2**–**5** in D_2O at pH 1.

TABLE II

CHEMICAL SHIFTS (δ) OF THE OLIGOGALACTOPYRANURONIC ACIDS (2–5) IN D_2O SOLUTION AT pH 1 AND 6 (IN PARENTHESES) FOR

*Compound**	*Chemical shifts*									
	Reducing sugar residue					*Non-reducing sugar residue (NRS)*				
						NRS'				
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*
2α	5.37 (5.32)	3.84[a]	4.05[a]	4.48 (4.42)	4.79 (4.42)	5.09 (5.04)	3.75[a]	3.97[a]	4.36 (4.27)	5.11 (4.78)
2β	4.69 (4.61)	3.52	3.83[a]	4.42 (4.37)	4.46 (4.06)	5.08 (5.04)	3.74[a]	3.95[a]		
3α	5.38 (5.32)	3.84[a]	c ([d])	4.49 ([f])	4.80 (4.45)	g (5.11)	b	c ([d])	4.48 ([f])	5.13[a] (4.76)
3β	4.69 (4.61)	3.52	b	4.44 ([f])	4.47 (4.06)					
4α	5.37 (5.32)	3.84[a]	c ([d])	e ([f])	4.80 ([f])	g (5.09)[a]	b	c ([d])	e ([f])	5.13[a] (4.77)
4β	4.69 (4.61)	3.51	b	4.44 ([f])	4.47 (4.06)					
5α	5.37 (5.32)	3.83[a]	c ([d])	e ([f])	4.79 ([f])	g (5.09)[a]	b	c ([d])	e ([f])	5.13[a] (4.77)
5β	4.68 (4.62)	3.51	b	4.44 ([f])	4.46 (4.06)					

*α and β indicate *eq* and *ax* orientation, respectively, of H-1 of the reducing residue. [a]Tentative assignment. Overlapping signals: [b]3.67–3.88; [c]3.99–4.14; [d]3.96–4.11; [e]4.40–4.55; [f]4.32–4.47; [g]5.00–5.15.

corresponding proton (*cf.* δ value of H-5 of the α anomers of **1** and **6**, Table I) of the reducing sugar residues at the same pH. The deshielding of H-5 of the non-reducing sugar residues may be caused by the carboxyl group of the adjacent sugar residues. Even H-5 of the reducing sugar residue experiences a small deshielding effect (*cf.* the δ values of H-5 of **1** and **6**, Table I, with those of **2–5** and **7–10**, Tables II and IV) of ~0.06 p.p.m; this is probably caused by the carboxyl group of the adjacent, non-reducing residue. Therefore, the highest δ value (5.09) is tentatively assigned to H″-5 of **3**, H‴-5 of **4**, and H⁗-5 of **5**. Definitive assignment, however, would require a study of the spectra of **3–5** that had been specifically deuterated at C-5 in the non-reducing sugar residues. The downfield shift of 0.32–0.41 p.p.m. when the pH is changed from 6 to 1 is characteristic of H-5 in each of the oligogalactopyranuronic acid residues.

Fig. 2 shows the spectra of **2–5** between δ 3.00–4.90 at pH 1. Due to the occurrence of both the α and β anomers of the reducing sugar, it is possible to assign unequivocally the signals in this region of the spectra. On account of the downfield shift of ~0.40 p.p.m. when the pH is changed from 6 to 1, the signals at δ 4.79 and 4.46 are assigned to H-5 of the α and β anomers, respectively, of the reducing sugar residue. The signals for H-4 of the α and β anomer of the reducing sugar of **2** are readily recognized at δ 4.48 and 4.42, respectively. Thus, the multiplet at δ 4.35 in the

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NRS″					NRS‴					NRS⁗				
H-1	H-2	H-3	H-4	H-5	H-1	H-2	H-3	H-4	H-5	H-1	H-2	H-3	H-4	H-5
5.07 (5.06)	3.76[a]	3.95 (3.92)	4.35 (4.27)	5.10[a] (4.76)										
g (5.06)[a]	b	c ([d])	e ([f])	5.11[a] (4.77)	5.06 (5.04)	3.75[a]	3.95 (3.91)	4.35 (4.26)	5.09[a] (4.77)					
g (5.06)[a]	b	c ([d])	e ([f])	5.11[a] (4.77)	g (5.06)[a]	b	c ([d])	e ([f])	5.11[a] (4.77)	5.05[a] (5.04)[a]	3.73[a]	3.94 (3.91)	4.34 (4.25)	5.09[a] (4.77)

spectrum of **2** can be attributed to H-4 of the non-reducing end sugar. Both the coupling constants ($J_{3,4}$ 3.5, $J_{4,5}$ 1.5 Hz) and the δ value correspond well with those of H-4 of compound **1α** (see Table I).

The downfield shift of 0.13 p.p.m. of the signal for H-4 of the reducing sugar with respect to that of H-4 of the non-reducing end sugar strongly indicates that, in **2**, the D-galacturonic acid residues are α-(1→4)-linked. Comparison of the peak area between δ 4.40 and 4.55 in respect of **2–5** shows an extra proton signal for each successive compound, indicating that each H-4, other than that of the end sugar (at δ 4.35), resonates in this region.

Thus, we conclude that all the D-galacturonic acid residues are α-(1→4)-linked. It is interesting to note that each H-4 experiences a downfield shift of ~0.08 p.p.m. when the pH of the solution is changed from 6 to 1.

The two well-resolved multiplets in the high-field region of the spectra of **2–5**, at δ 3.51 and 3.95 (see Fig. 2), are assigned to H-2 of the β anomer of the reducing sugar residue by comparison with the δ value of the corresponding proton in **1β** (Table I) and to H-3 of the non-reducing end sugar, respectively. The latter assignment has been confirmed by the result of a spin-decoupling experiment in which H-4 of the non-reducing end sugar (δ 4.35) was irradiated.

TABLE III

COUPLING CONSTANTS FOR THE OLIGOGALACTOPYRANURONIC ACIDS (2–5)

Compound[a]	*Coupling constants*[b] *(Hz)*																			
	Reducing sugar				*Non-reducing sugar residue (NRS)*															
					NRS'				*NRS''*				*NRS'''*				*NRS''''*			
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
2α	3.5	10.5	3.5	≤1.5	3.8	10.5	3.5	1.5												
2β	8.0	10.0	3.5	≤1.5																
3α	3.8	~10	~3	≤2	3.5	~10	~3	≤2	3.8	10.5	3.0	1.5								
3β	8.0	10.0		≤2																
4α	3.5	~10	3.5	≤2	~4	~10	~3	≤2	~4	~10	~3	≤2	3.5	10.5	3.5	1.5				
4β	8.0	10		≤2																
5α	3.5	~10	~3	≤2	~4	~10	~3	≤2	~4	~10	~3	≤2	~4		~3	≤2	3.5	10.5	3.0	≤2
5β	8.0	10		≤2																

[a]As in Table II. [b]Accuracy of ±0.2 Hz.

In the remaining multiplets, spacings of ~3 and ~10 Hz can be recognized. By correlation with the chemical shifts and coupling constants for D-galactopyranuronic acid (**1**, Table I) and methyl (methyl α-D-galactopyranosid)uronate (**17**), the signals between δ 3.99 and 4.14, and between δ 3.67 and 3.88, can be attributed to H-3 and H-2 of the non-reducing sugar residues.

The magnitudes of $J_{1,2}$, $J_{2,3}$, $J_{3,4}$, and $J_{4,5}$ (see Table III), particularly $J_{2,3}$ (10 Hz), lead to the conclusion that each D-galacturonic acid residue has the *Cl* conformation[2]. The relative intensities of the signals for the anomeric proton of the reducing sugar residues of **2–5** indicate that, in D_2O, the β anomer is favoured over the α anomer (α:β = 2:3).

Methyl oligogalactopyranuronates **7–10**. — As can be seen from the δ values in Table IV, the spectra of **7–10** are not influenced by the pH. For each non-reducing sugar residue, H-5 can therefore be differentiated at pH 6, as is shown in Fig. 3 (*cf.* Fig. 1). The low-field region of the spectra at pH 6 (Fig. 3) also reveals the most striking difference between **7–10** and **2–5**: H-1 of each glycosidic linkage of the esters

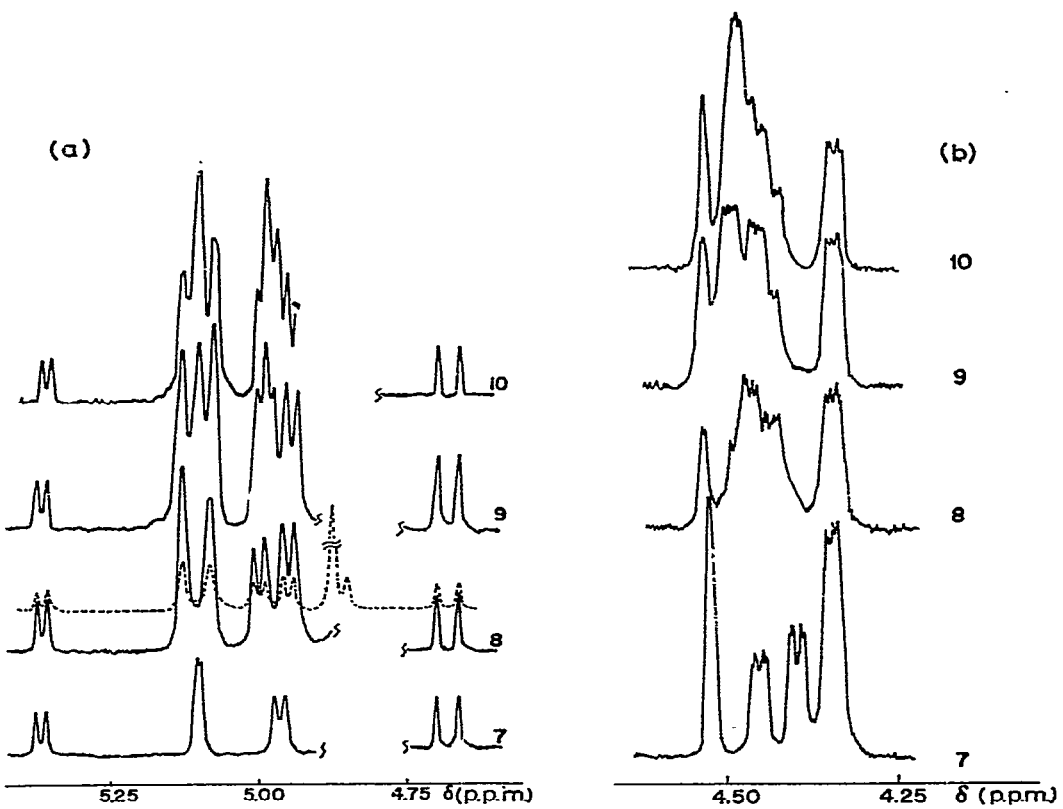

Fig. 3. The 220-MHz spectra of the methyl oligogalactopyranuronates (**7–10**) in D_2O at pH 6: (*a*) at low field [the dotted spectrum (**8**) is recorded with a lower amplification in order to show the signal for H-5 of the α anomer of the reducing sugar at δ 4.84]; (*b*) at δ 4.40–4.51.

TABLE IV

CHEMICAL SHIFTS OF THE METHYL OLIGOGALACTOPYRANURONATES (7–10) IN D_2O SOLUTION AT pH 1 AND 6 (IN PARENTHESES) FOR

*Compound**	*Chemical shifts (δ)*									
	Reducing sugar residue					*Non-reducing sugar residue (NRS)*				
						NRS'				
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*
7α	5.36 (5.36)	~3.81[d]	4.03	4.44 (4.44)	4.83[c] (4.84)[c]	4.96 (4.96)	3.71[b]	3.92	4.34 (4.34)	5.10 (5.10)
7β	4.68 (4.68)	3.49	3.94	4.39 (4.39)	4.52 (4.51)					
8α	5.36 (5.36)	[d]	[e]	4.48	4.84[c] (4.85)	4.99[b] (4.99)	[d]	[e]	4.46	5.13[b] (5.13)
8β	4.68 (4.68)	3.49	[d]	4.43	4.52 (4.53)					
9α	5.36 (5.36)	[d]	[e]	[f]	4.83[c] (4.85)	4.99[b] (4.99)	[d]	[e]	4.49[b]	5.13[b] (5.13)
9β	4.68 (4.68)	3.49	[d]	4.43	4.53 (4.52)					
10α	5.37 (5.36)	[d]	[e]	[f]	4.85[c]	5.01[b] (5.01)	[d]	[e]	4.49[b]	5.14[b] (5.14)
10β	4.69 (4.69)	3.50	[d]	4.43	4.54 (4.54)					

*α and β, as in Table I. [a]Not specifically assigned; numbers in parentheses are relative peak areas. [b]Tentative assignment. [c]Signal obscured under HOD signal. [d]Signal obscured under CO_2Me signals, $\delta = 3.75$–3.85. [e]Overlapping signals, δ 3.95–4.25. [f]Overlapping signals, $\delta = 4.40$–4.51.

resonates 0.11–0.13 p.p.m. upfield with respect to the corresponding protons of the oligogalactopyranuronic acids (*cf.* Tables II and IV). This shielding effect may be due to the COOMe groups of the adjacent sugar residues.

It can also be argued that this shielding effect is caused by a different spatial orientation of the ester carbonyl with respect to the acid carbonyl function in this sugar residue. Interpretation of the spectra of **11** and **12** (see below) confirmed unequivocally that the effect was brought about by the ester group of the sugar residue substituted at HO-1 of the non-reducing sugar. The signals at highest field (δ 4.95) and at lowest field (δ 5.00) are assigned to H-1 of each non-reducing end sugar (H″-1 of **8**, H‴-1 of **9**, and H⁗-1 of **10**) and the non-reducing sugar adjacent to the reducing sugar (H′-1 of **8**, **9**, and **10**), respectively.

Confirmatory evidence for the assignment of the doublet at lowest field (δ 5.00) to H-1 of the non-reducing sugar can be found in the spectra of partly saponified compounds **8** and **9** (Fig. 4, **8a** and **9a**) in the low-field region at pH 6. The signals at δ 5.31 and 4.62 indicate that the methyl ester group of the reducing sugar unit is completely saponified in **9a** and partly so in **8a**. The partial saponification in **8a** is substantiated by the presence of the peaks at δ 5.38 and 4.69. Comparison of the

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Chemical shifts (δ)															Other[a] signals
NRS″					NRS‴					NRS′″″					
H-1	H-2	H-3	H-4	H-5	H-1	H-2	H-3	H-4	H-5	H-1	H-2	H-3	H-4	H-5	
															3.800:(1) 3.823(1)
4.95[b] (4.95)	3.72[b]	3.92	4.33 (4.34)	5.08[b] (5.08)											3.80(1), 3.82 3.83(1) (1)
4.98[b] (4.98)	d	e	4.45[b]	5.10[b] (5.10)	4.94[b] (4.94)	3.71[b]	3.90	4.33 (4.33)	5.07[b] (5.08)						3.80(1); 3.82(3)
5.00[b] (5.00)	d	e	4.49[b]	5.11[b] (5.11)	5.00[b] (5.00)	d	e	4.45[b]	5.11[b] (5.11)	4.96[b] (4.96)	3.72[b]	3.93	4.35 (4.35)	5.09[b] (5.09)	3.80(1); 3.82(4)

spectra of **8a** and **9a** with those of **8** and **9** (Fig. 3) clearly shows that the peak at δ 5.00 is lower in intensity for **8a** and not present at all for **9a**. Thus, this signal can be attributed to H-1 adjacent to the reducing sugar residue (H′-1 of **8** and **9**) and consequently the peak at highest field in **8** (δ 4.95) to H-1 of the non-reducing end sugar (H″-1 of **8**). It is reasonable to assume that the signals at δ 4.95 and 4.98 in **9** correspond to H‴-1 and H″-1, respectively. These assignments again confirm the assumption that H-1 of the glycosidic linkage is influenced by the ester group of the sugar residue substituted at HO-1 of the non-reducing sugar.

The resonance signals of H-4 of each non-reducing end sugar (δ 4.35) and H-4 of both the α anomer (δ 4.44) and the β anomer (δ 4.39) of the reducing sugar unit can be easily recognized in the spectra of **7**–**10** (Fig. 3). The signal at δ 4.35 is assigned to H-5 of the β anomer of the reducing sugar. For the α anomer of the reducing sugar, H-5 resonates at δ 4.84 (in **7**, **9**, and **10**, this signal is obscured by the HDO signal).

The signals in the high-field region (δ 3.71–3.86) are partly overlapped by the methyl ester signals. It is interesting to note that one methyl ester signal (δ 3.80) can be well distinguished from the other methyl ester signals (Table IV), and assigned to the non-reducing end sugar by comparison with the spectrum of **11**, as shown later.

TABLE V

COUPLING CONSTANTS OF THE METHYL OLIGOGALACTOPYRANURONATES (7–10)

Compound[a]	Coupling constants (Hz)[a]																			
	Reducing sugar				Non-reducing sugar residue (NRS)															
					NRS′				NRS″				NRS‴				NRS⁗			
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
7α	3.5	10.5	3.0	≤2	3.5	10.5	3.5	1.5												
7β	8.0	10.0	3.0	≤2																
8α	3.5		~3	≤2	4.0	~10	~3	≤2	4.0	10.5	3.5	1.5								
8β	8.0	10.0	~3	≤2																
9α	4.0	~10	~3	≤2	~4		~3	≤2	~4		~3	≤2	4.0	10.5	3.5	≤2				
9β	8.0	10.0		≤2																
10α	4.0	~10		≤2	~4	~10		≤2	~4	~10		≤2	~4			≤2	4.0	10.5	3.5	1.5
10β	8.0	10.0		≤2																

[a] As in Table IV. Accuracy of ± 0.2 Hz.

TABLE VI

CHEMICAL SHIFTS OF THE UNSATURATED OLIGOGALACTOPYRANURONIC ACIDS IN D_2O SOLUTION AT pH 1 AND 5 (IN PARENTHESES) FOR MUTAROTATION MIXTURES

Compound[a]	*Chemical shifts* (δ)																			
	Reducing sugar residue					*Non-reducing sugar residue (NRS)*														
						NRS'					*NRS''*					*NRS'''*				
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*
14α	5.36 (5.31)	[b] (3.76)	4.02 (4.01)	4.65 (4.63)	4.81 (4.50)	5.20 (5.11)	6 (3.73)	4.42 (4.30)	6.04 (5.80)	—										
14β	4.67 (4.60)	3.46 (3.46)	[b] (3.79)	4.58 (4.52)	4.48 (4.09)			4.38 (4.27)												
15α	5.37 (5.33)	[c]	[d]	4.48 (4.45)	4.83[f] (4.45)	5.10 (5.11)	[c]	[d]	4.63 (4.60)	5.07 ([f])	5.15 (5.11)	[c]		4.37 (4.29)	6.03 (5.82)	—				
15β	4.67 (4.62)	3.52	3.84[a]	4.42 (4.39)	4.34 (4.09)															
16α	5.35 (5.32)	[c]	[d]	4.48 (4.43)	[f] (4.43)	[e] (5.09)[a]	[c]	[d]	4.48 (4.43)	[e] ([f])	[e] (5.05)[a]	[c]		[d]	4.63 (4.58)	[e] ([f])	5.15 (5.09)	[c]	4.36 (4.28)	6.07 (5.79)	—
16β	4.67 (4.61)	3.51	3.83[a]	4.43 (4.38)	4.39 (4.08)															

[a] α and β, as in Table II. [a] Tentative assignment. Overlapping signals: [b] 3.71–3.86; [c] 3.62–3.92; [d] 3.92–4.12; [e] 5.00–5.12. [f] Obscured under HDO signal.

TABLE VII

COUPLING CONSTANTS OF THE UNSATURATED OLIGOGALACTOPYRANURONIC ACIDS (14–16)

*Compound**	*Coupling constants (Hz)*[a]															
	Reducing sugar				*Non-reducing sugar residue*											
					NRS'				*NRS''*				*NRS'''*			
	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	$J_{4,5}$
14α	3.5	10.5	3.5	≤2	2.0	7.0	3.5									
14β	8.0	10.0	3.5	≤2		7.0	3.5									
15α	4.0	~10	~3	≤2	~4			≤5	2.0	7.0	3.5	—				
15β	8.0	10.0	3.0	≤2												
16α	4.0	~10	~3	≤2		~10	~3		~4	~10		≤5	2.0	7.0	3.5	—
16β	8.0	10.0	3.5	≤2												

*As in Table VI. [a]Accuracy of ±0.2 Hz.

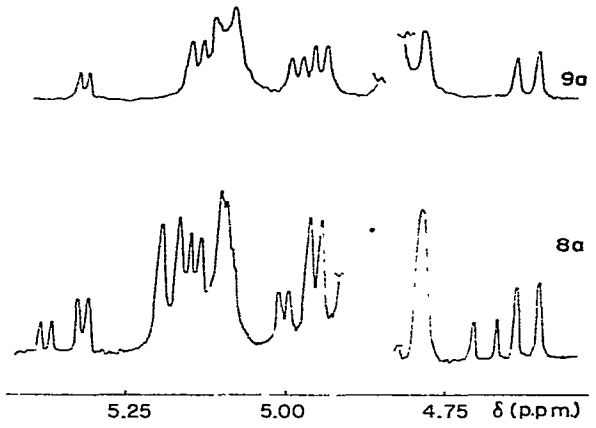


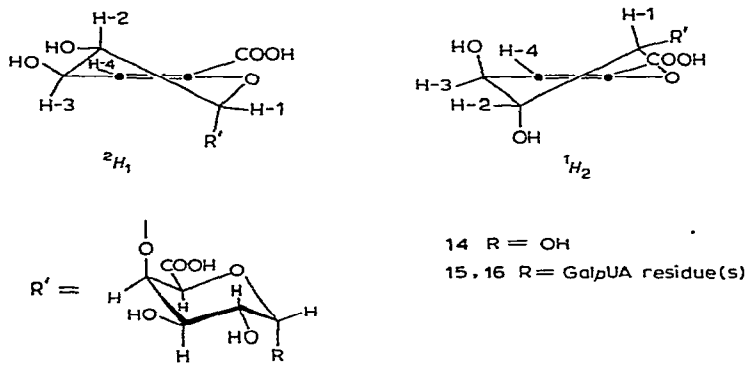
Fig. 4. The p.m.r. spectra (δ 4.55–5.40) of partly saponified **9** (**9a**) and **8** (**8a**) at 220 MHz in D_2O at pH 6.

From the δ values of H-1 and H-4 of the non-reducing sugar residues presented in Table IV and the coupling constants (Table V) of **7–10**, we conclude that each methyl D-galacturonate residue is α-(1→4)-linked and has the *C1* conformation.

Oligogalactopyranuronic acids **14–16.** — Comparison of the spectra of unsaturated digalactopyranuronic acid **14** at pH 1 and 6 with those of the corresponding saturated acid **2** reveals that the signal for H′-5, δ 5.11 (pH 1), is absent. Thus, the double bond is located between C-4 and C-5 of the sugar unit at the non-reducing end. Parallel comparisons show that this is also true for **15** and **16**. The vinylic (H′-4) proton of **14** can easily be recognized at δ 6.04 (J 3 Hz) at pH 1. This assignment is supported by the downfield shift of 0.24 p.p.m. when the pH is changed from 6 to 1 (Table VII). Two signals (δ 4.42 and 4.38), both with spacings at 3.0 and 7.0 Hz, were observed for H′-3 and assigned to the α and β anomer, respectively. H′-1 resonates at δ 5.20 (J 2 Hz) at pH 1. The assignments of the signals at δ 3.95 p.p.m. (J 3, 10 Hz) and δ 4.35 (J 3.0, 1.5 Hz) to H-3 and H-4 of the sugar residues at the non-reducing end in **2–5** (Table II) was confirmed by the absence of these signals in the spectra of the unsaturated sugar residues at the non-reducing end of **15** and **16**.

From the δ values of H-1 and H-4 of the non-reducing sugars (Table VII) and the $J_{1,2}$ coupling constants (Table VI), we conclude that each D-galacturonic acid and 4-deoxy-D-hex-4-enopyranuronic acid residue in **14–16** is α-(1→4)-linked. The magnitude (10 Hz) of the $J_{2,3}$ coupling constants of the D-galacturonic acid residues indicates that they have the *C1* conformation. The 4-deoxyhex-4-enopyranuronic acid residue can occur in two half-chair ($^1H_2$ or $^2H_1$) conformations[8]. The coupling constants $J_{1,2}$ 2.0, $J_{2,3}$ 7.0, and $J_{3,4}$ 3.5 Hz indicate that the $^2H_1$(D) conformation is the most probable for the 4-deoxyhex-4-enopyranosyluronic acid residue.

Partly esterified methyl oligogalactopyranuronates **11–13.** — Comparison of the spectra of monomethyl digalactopyranuronate **11** at pH 1 and 6 (see Fig. 5) clearly shows that the resonances of the reducing sugar residue, and in particular those for



H-1 (δ 5.38 and 4.69), H-5 (δ 4.52), and H-4 (δ 4.49 and 4.43), are not influenced by the pH. The resonances at δ 5.11 and 4.35, attributed to H'-5 and H'-4 of the sugar unit at the non-reducing end, however, experience downfield shifts at 0.35 and 0.07 p.p.m., respectively. Thus, we conclude that **11** is esterified exclusively at the reducing-end sugar. Furthermore, the doublet at δ 4.96 clearly shows that H'-1 of the glycosidic linkage experiences an upfield shift of 0.12 p.p.m. due to the ester group of the reducing sugar residue, as observed for H'-1 of **7** (*cf.* Tables II and IV). The resonance of the methyl ester at δ 3.82 confirms the previous assignment of the peak at δ 3.80 in the spectrum of **7** to the methyl ester of the sugar residue at the non-reducing end.

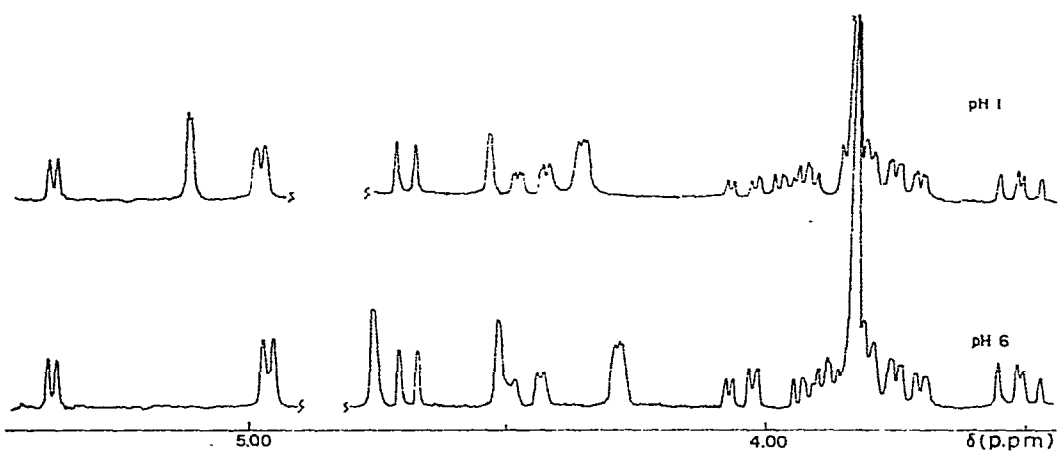


Fig. 5. The p.m.r. spectrum of *O*-(α-D-galactopyranosyluronic acid)-(1→4)-*O*-(methyl α,β-D-galactopyranuronate) (**11**) at 220 MHz in D_2O at pH 1 and 6.

Examination of the spectra of monomethyl trigalactopyranuronate **12** (Fig. 6) at pH 1 and 6 indicates that all three possible isomers of **12** are present, namely, **12a**, **12b**, and **12c** in which, respectively, the reducing sugar residue, the non-reducing sugar residue, and the sugar residue at the non-reducing end is esterified. First, the two signals at δ 5.00 and 4.95 of unequal intensities indicate the presence of **12a** and

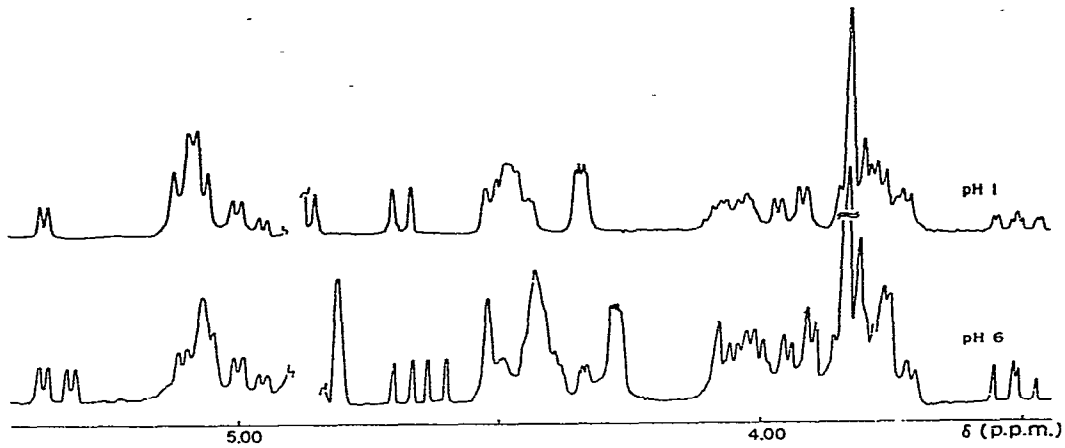


Fig. 6. The 220-MHz spectra of **12** in D_2O at pH 1 and 6.

12b; the presence of **12c** is substantiated by the small signal of δ 4.35 for H″-4 at pH 6 and by the resonance at δ 3.80 of the methyl ester of the non-reducing end sugar.

From the relative intensities (2:2:3:3:2:8) of the signals at δ 5.38 (αH-1 of **12a**), 5.32 (αH-1 of **12b** and **12c**), 4.69 (βH-1 of **12a**), 4.62 (βH-1 of **12b** and **12c**), 4.35 (H″-4 of **12c**), and 4.28 (H″-4 of **12a** and **12b**), the composition of **12** is estimated to be **12a** 50%, **12b** 30%, and **12c** 20%. The proportions of **12a** and **12b** correspond well with the relative intensities of the signals at δ 5.00 and 4.95, and consequently confirm the assignment of the peak at highest field to H″-1. Evidently, the shielding of the anomeric protons of the glycosidic linkage is caused by the ester group of the sugar residue substituted at HO-1 of the non-reducing sugar.

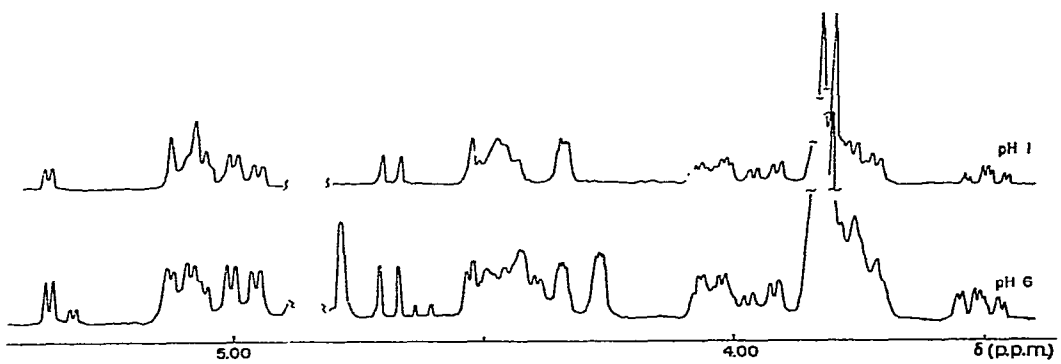


Fig. 7. The 220-MHz spectra of **13** in D_2O at pH 1 and 6.

In the spectrum of the dimethyl trigalactopyranuronate **13**, the presence of peaks of unequal intensities at δ 5.00 and 4.95 (Fig. 7), and the resonances at δ 5.37 (1), 5.32 (0.2), 4.69 (1.5), 4.62 (0.3), 4.35 (1.4), and 4.28 (1.6), it is concluded that **13**

consists of the three possible isomers **13a**, **13b**, and **13c** in which, respectively, the reducing and non-reducing sugar residues, the sugar residues at the reducing and non-reducing ends, and both the non-reducing sugar residues are esterified.

The resonances at δ 5.37 and 4.69 can be attributed to H-1 of the reducing sugar residue of **13a** and **13b**, those at δ 5.32 and 4.62 to H-1 of the reducing sugar residue of **13c**, that at δ 4.35 to H"-4 of the sugar residue at the non-reducing end of **13b** and **13c**, and that at δ 4.28 to H"-4 of the sugar residue at the non-reducing end of **13a**. The composition of **13** was estimated to be **13a** 53%, **13b** 30%, and **13c** 17%, from the relative intensities.

EXPERIMENTAL

Solutions for p.m.r.-spectral examination at pH 1 and 6 were prepared as follows. Samples, which had been subjected to a preliminary deuterium exchange by repeated treatment with fresh D_2O, were dissolved in D_2O, and the pH was adjusted with either DCl or NaOD. Sodium 2,2,3,3-tetradeuterio-3-(trimethylsilyl)propionate was used as the internal standard.

A Varian 220 MHz spectrometer at the Laboratory for Toegepast Natuurwetenschappelijk Onderzoek (TNO), Delft (The Netherlands) was used for measuring the spectra (probe temperature 16°).

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