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APPLICATION OF P.M.R. SPECTROSCOPY IN THE SEQUENCING OF OLIGOSACCHARIDES CONTAINING 1-CARBOXYETHYLIDENE SUBSTITUENTS*

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ABSTRACT

1-Carboxyethylidene acetals (formed by pyruvic acid) are labile to acid. Consequently, when an aqueous solution of an acidic oligosaccharide is examined by p.m.r. spectroscopy at 95°, the acetal is progressively hydrolyzed. The spectrum then reflects the presence of two compounds, one with, and one without, an acetal substituent, and this causes certain signals to be twinned***. The protons affected may be not only on the acetalated sugar unit but also on neighboring residues. Analysis of these spectral changes aids in the sequencing of the oligosaccharide. The potential of the method is illustrated by a study of the acetalated, acidic hexasaccharide obtained from the capsular polysaccharide of *Klebsiella* serotype K46 by action of ϕ46 bacteriophage endoglucanase.

INTRODUCTION

The mutarotational equilibrium existing in an aqueous solution of a reducing oligosaccharide influences the n.m.r. signals (especially those of anomeric protons) of adjacent residues, a phenomenon first observed by Tsui and co-workers[1] and subsequently employed as an aid in sequencing oligosaccharides[2,3]. This influence may be noticed on residues located several units from the reducing end, the distance depending on the spectral dispersion of the spectrometer used[4].

In like manner, the presence or absence of a (1-carboxyethylidene) group (pyruvic acid acetal) influences n.m.r. signals not only of the sugar residue that is acetalated but also of neighboring units. We now report such observations and show how they may aid in the sequencing of an oligosaccharide and, by extension, that of the corresponding polysaccharide.

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***The word "twin" is deliberately chosen, in order to avoid confusion with "doublet". The character of each signal, *i.e.*, singlet, doublet, multiplet, *etc.*, is maintained, but is accompanied by a twin having a small difference in chemical shift.

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RESULTS AND DISCUSSION

Previous results[5] promoted the utility of bacteriophage glycanases for obtaining oligosaccharides, especially those containing such labile groups as acetals. Application of these phage techniques to the capsular polysaccharide isolated[6] from *Klebsiella* serotype K46 yielded two oligosaccharides, **P1** and **P2**. The former corresponded to the repeating unit of the polysaccharide, and the latter, to two such units. In both, the ratios of mannose:galactose:glucose:glucuronic acid were 2:2:1:1, galactose was at the reducing end, and (1-carboxyethylidene) groups were present. Methylation of the original polysaccharide[6], and of **P1** and **P2**, demonstrated that (*a*) **P1** is linear and the single D-glucose unit is terminal and nonreducing, (*b*) the (1-carboxyethylidene) group is linked to O-4 and O-6 of a 3-linked D-mannosyl residue, and (*c*) the terminal D-galactose residue of **P1** is linked to O-3 of the 4-linked glucuronic acid in **P2**. The rest of the methylation data agreed with those reported for the oligosaccharide[6].

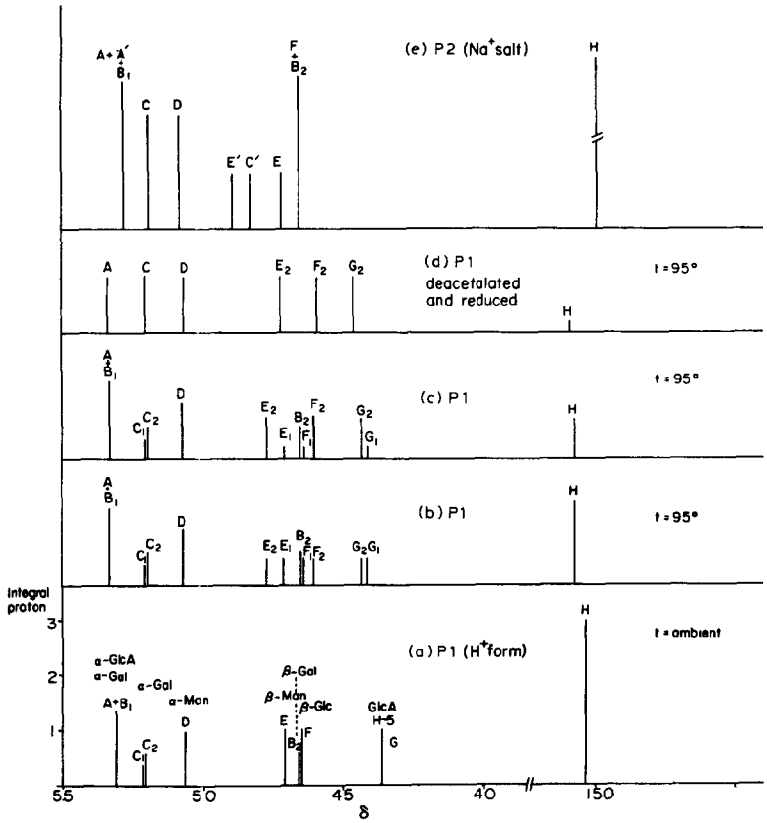


Fig. 1. ^{1}H-N.m.r. spectra (400 MHz) of **P1** and **P2**. [Key: (a) **P1** (H^+ form) at ambient temperature, (b) **P1** at 95°, (c) **P1** after further heating at 95°, (d) deacetalated **P1**-alditol, and (e) **P2** as Na^+ salt at 95°.]

With these analytical results as a background, it was now considered informative to examine the p.m.r. spectra of **P1** and **P2**, recorded under different conditions. The numerical data are assembled in Table III, and the different spectra are compared in Fig. 1, where the height of each vertical line is proportional to the proton integral.

The spectrum of **P1**, recorded at ambient temperature (see Fig. 1a), confirms the hexasaccharide nature of this oligomer, and the two singlets at δ 5.06 (D) and δ 4.71 (E) may be assigned to H-1 of an α- and a β-linked D-mannopyranosyl unit, respectively. The resonances at δ 5.31 (B_1, 0.4 H, coincident with A) and at δ 4.66 (B_2, 0.6 H) are typical of those due to the α/β equilibrium of a terminal hexopyranose residue, known here to be D-galactose.

The twin signals C_1 and C_2 are present in the same ratio as that of the anomeric protons of the reducing residue, and are thus attributable to the unit adjacent to the reducing end. This is confirmed by the spectrum shown in Fig. 1d, where the galactose unit has been reduced to galactitol, causing a single signal, C, having an integral of unity. The signals C_1 and C_2 could be due either to a glucose or a galactose unit, but as the sole glucosyl group is known to be terminal, this possibility is precluded. A partial structure, α-Gal$\rightarrow$Gal (J) may be written. The signals are unlikely to be those of the glucuronic acid residue for a reason explained later. From this one spectrum, Fig. 1a, it is not possible to assign the signals labelled A and F, and the monosaccharide units unaccounted for are those of glucuronic acid and glucose. The signal G at δ 4.36, having a large coupling constant, is due to H-5 of the glucuronic acid, and the alteration of the signal in subsequent spectra is a valuable aid in the sequencing.

Fig. 1b depicts the spectrum, obtained at 95°, of **P1**, under conditions where ~50% of the (1-carboxyethylidene) groups had been hydrolyzed, as judged by the diminution in the integral at δ 1.57. Neither the elevated temperature nor the partial removal of the acetal groups appears to influence the α/β equilibrium or the associated twinning of C_1 and C_2. In contrast, each of the signals E, F, and G now appears as two peaks having equal integrals, consistent with the fact that there are now present two oligosaccharides, *i.e.*, those with and without the acetal group, in approximately equal amounts. When the spectrum of **P1** was recorded on the same sample (as that in Fig. 1b) that had been held at 95° for a further period of time, such that about two-thirds of the acetal groups had been hydrolyzed, the spectrum (see Fig. 1c) demonstrated that there was a proportional change in the ratios of the pairs of peaks for E, F, and G.

It is known from the methylation data that the acetal is linked to O-4 and O-6 of a D-mannopyranosyl residue which, from Fig. 1b, is shown to be β-linked, as signal E, rather than D, becomes twinned. The units represented by signals F and G must be neighbors of this mannosyl residue and, as the d.p. of **P1** is known, the signals must result from two different units.

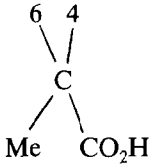
As F corresponds to an anomeric proton, and G to H-5 of glucuronic acid, it is reasonable to write a partial structure, namely,

(F) (E) (G)
β-Hexosyl-(1→3)-β-Man-(1→4)-GlcA. (K)

The alternative sequence, with the hexose and glucuronic acid units interchanged, is improbable on the spectroscopic evidence, and is definitely excluded by the methylation data.

The α-D-mannopyranosyl unit (D) must link partial structures J and K, and, as the reducing and nonreducing termini are known, it follows that **P1** may be represented by the following formula.

β-Glc-(1→3)-β-Man-(1→4)-α-GlcA-(1→3)-α-Man-(1→3)-α-Gal-(1→3)-Gal



The logical sequel to the work leading to the spectrum in Fig. 1c was to continue the heating until virtually all of the acetal had been hydrolyzed. This state is represented by Fig. 1d, where, in addition, **P1** had been reduced with sodium borohydride to the alditol. As was to be expected, each anomeric proton now gave a single signal whose integral was unity.

The enzymic cleavage of the galactosyl bonds in the K46 polysaccharide, to yield **P1**, destroys the information concerning the anomeric configuration of this linkage in the polymer. This problem may be resolved by examination of the ^{1}H-n.m.r. spectrum of the dimer **P2**, where it is known, from the methylation data, that the reducing galactopyranose residue of **P1** is joined to the glucuronic acid residue of the second repeating unit. On this occasion, **P2** was used as the sodium salt, in order to avoid a multiplicity of peaks, and the n.m.r. spectrum was obtained at 95° (see Fig. 1e and Table III). Comparison with the spectrum of **P1** shows the spectra to be similar, but with one major and one minor difference. Most significant is the appearance of a new signal (C′) at δ 4.83 ($J_{1,2}$ 7 Hz), demonstrating that the D-galactopyranosyl group is β-linked in **P2** and, thus, in the polymer. This C′ signal is flanked by two singlets (E and E′) having δ 4.89 and 4.72 ($J_{1,2}$ zero). These signals correspond to the two β-D-mannopyranosyl residues (carrying the acetal substituents), as, in the dimeric unit **P2**, they are in different environments. One is linked to a glucosyluronic acid residue that carries a single substituent, whereas the other has two. The glucuronic acid itself does not reflect these differences, as the anomeric protons of both units have essentially the same chemical shift, δ ~5.30. It may also be noted that, because the spectrum of **P2** was recorded for the sodium salt, not the free acid, the H-5 signal of glucuronic acid has shifted upfield, and is lost in the envelope of ring protons.

The twinning of signal C has been explained by the α/β mutarotational

equilibrium, and of signals E and F, by the progressive removal of the (1-carboxyethylidene) group. These three correspond to anomeric protons, but it is less clear why H-5 of glucuronic acid (signal G) should be similarly affected. Inspection of a Dreiding molecular model showed that H-5 is close to the ring-oxygen atom of the mannosyl residue carrying the acetal substituent, and it is plausible that the electronegativity of this atom may be influenced by the acetal group. An alternative explanation[4] is that, as the acetal is removed, the free hydroxyl group generated on C-6 of the mannosyl residue may interact with H-5 of the acid residue.

CONCLUSION

The sum of these experiments permitted us to write structures for **P1, P2**, and the K46 polysaccharide. Although these structures cannot be deduced *ab initio* from the n.m.r.-spectral data alone, it is clear that this spectroscopic technique is a valuable tool in the sequencing of oligosaccharides, especially when combined with the use of a bacterial virus for their preparation. An alternative approach to the determination of the structure of polysaccharides is thus initially to examine in detail the oligosaccharide(s) corresponding to the repeating unit, and multiples thereof. There are several experimental advantages to working with material of low molecular weight, as opposed to polymeric material, and the use of bacterial viruses to obtain such oligomers merits wider application.

EXPERIMENTAL

General methods. — The instrumentation used for the measurement of optical rotation, infrared and ^{13}C-n.m.r. spectroscopy, g.l.c., and g.l.c.–m.s. has been described[6]. ^1H-N.m.r. spectra were recorded with a Bruker WH-400 FT instrument. Samples dissolved in D_2O were "exchanged" and freeze-dried, three or four times in 99.7% D_2O. Spectra were recorded for solutions in 99.7% D_2O, with acetone [δ 2.23, measured against aqueous sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS)] as the internal standard. Spectra were recorded both at ambient temperature, with simultaneous irradiation of the HOD peak, and at 95°, without irradiation.

Descending paper-chromatography was performed on Whatman No. 1 paper, with the following solvent systems (v/v): (*A*) 2:1:1 1-butanol–acetic acid–water, (*B*) 8:2:1 ethyl acetate–pyridine–water, and (*C*) 18:3:1:4 ethyl acetate–acetic acid–formic acid–water. Chromatograms were developed with a silver nitrate reagent.

Analytical g.l.c. separations were performed in a stainless-steel column (Column *A*; 1.8 m × 3 mm) packed with 3% of OV-225 on Gas Chrom Q (100–120 mesh). Nitrogen was the carrier gas at a flow rate of 20 $mL.min^{-1}$.

Gel-permeation chromatography was performed in a column (80 × 2.5 cm) of Bio-Gel P-4. The column was eluted with 500:5:2 water–pyridine–acetic acid at a flow rate of ~8 $mL.h^{-1}$. Fractions (2–3 mL) were collected, freeze-dried, and weighed, and the elution profile was constructed.

Propagation of bacteriophage 46 (ϕ46). — ϕ46 was isolated from sewage, and purified by successive replating of a single plaque on its host strain[7]. Propagation of ϕ46 in broth was continued until 1 L of lysate containing 10^{13} PFU had been obtained. The phage solution was concentrated somewhat, dialyzed against running tap-water for 2 d, and the dialyzate concentrated to 150 mL.

Depolymerization of polysaccharide, and isolation and purification of the oligosaccharides. — *Klebsiella* capsular polysaccharide K46 (0.83 g) in water (150 mL) was added to the phage solution (150 mL), chloroform (3 mL) was added to prevent bacterial growth, and the mixture was kept for 30 h at 37°, concentrated, and dialyzed against distilled water (3 × 1 L). The dialyzates were combined, and evaporated to dryness, the residue was dissolved in water (50 mL), and the solution stirred with Amberlite IR-120 (H^+) resin. The treatment with resin was repeated until a colorless solution was obtained; on freeze-drying, this afforded oligosac-

TABLE I

DETERMINATION OF THE SUGAR RATIOS, DEGREE OF POLYMERIZATION, AND REDUCING TERMINUS OF **P1** AND **P2**

Peracetylated derivative	*Mole %*[a]			
	P1[b]	**P1**[c]	**P2**[b]	**P2**[c]
Mannononitrile	34.0	34.0	38.0	34.2
Glucononitrile	21.8	33.8	20.6	33.6
Galactononitrile	22.0	32.2	30.9	32.2
Galactitol	22.2		10 5	

[a]Determined on a column of OV-225 at 210°. [b]Reduced with sodium borohydride, followed by hydrolysis, and treatment with hydroxylamine hydrochloride in pyridine, and finally acetic anhydride. [c]Methanolysis, followed by reduction, prior to conversion into the peracetylated aldononitriles.

TABLE II

METHYLATION ANALYSIS OF **P1**, REDUCED DEPYRUVALATED **P1**, AND **P2**

Methylated sugars[a] *(as alditol acetates)*	*Mole %*[b]		
	P1	**P1** *depyruvalated, reduced*	**P2**
1,2,4,5,6-Gal		19.0	
2,3,4,6-Glc	17.9	21.1	17.8
2,4,6-Man	16.0	37.8	16.1
2,4,6-Gal	32.3	22.1	33.0
2,3-Glc	15.9		8.1
2-Man	17.9		16.8
2-Glc			8.2

[a]1,2,4,5,6-Gal = 3-*O*-acetyl-1,2,4,5,6-penta-*O*-methylgalactitol, *etc.* [b]Determined on a column of OV-225 at 190°.

TABLE III

^{1}H-N.M R DATA (400 MHz) FOR **P1**, REDUCED DEPYRUVALATED **P1**, AND **P2**

Compound	*Δ*[a]		$J_{1,2}$[b] *(Hz)*	*Integral*			*Assignmment*[c]	
	ambient	*95°*		*ambient*	*95°*			
				(a)	*(b)*	*(c)*		
P1 (H^+ form)	5.31	5.33	3	1.4	1.4	1.4	4-GlcA-α 3-Gal-α-OH	[A] [B_1]
	5.20	5.19	3	0.6	0.6	0.6	3-Gal-α	[C_2]
	5.21	5.19		0.4	0.4	0.4		[C_1]
	5.06	5.07	s	1	1	1	3-Man-α	[D]
	4.71	4.71	s	1	0.5	0.25	3-Man-β (6 4) C (Me, CO_2H)	[E_1] +
		4.77	s		0.5	0.75	3-Man-β	[E_2]
	4.66	4.65	8	0.6	0.6	0.6	3-Gal-β-OH	[B_2]
	4.65	4.60	8	1	0.5	0.75	Glc-β	[F_2]
		4.64			0.5	0.25		+ [F_1]
	4.36	4.41	10 ($J_{4,5}$)	1	0.5	0.25	H-5 of 4-GlcA	[G_1]
		4.43			0.5	0.75		+ [G_2]
	1.53	1.57		3	1.5	0.75	Me-C-CO_2H	[H]
Reduced, depyruvalated **P1**		5.34	3		1		4-GlcA-α	[A]
		5.21	3		1		3-Gal-α	[C]
		5.07			1		3-Man-α	[D]
		4.72			1		3-Man-β	[E_2]
		4.59	8		1		Glc-β	[F_2]
		4.48	10 ($J_{4,5}$)		1		H-5 of 4-GlcA	[G_2]
		1.59			0.2		Me-C-CO_2H	[H]

TABLE III *(continued)*

Compound	*Δ*[a]		$J_{1,2}$[b] *(Hz)*	*Integral*			*Assignmment*[c]	
	ambient	*95°*		*ambient*	*95°*			
				(a)	*(b)*	*(c)*		
							4-GlcA—(β)	[A]
P2 (Na⁺ form)	5.30	5.29	3	2.3	2.3		3,4-GlcA—(α)	[A']
		5.26	3				3-Gal—(α)—OH	[B_1]
	5.19	5.19	3	2.0	2.0		3-Gal—(α)	[C]
	5.06	5.08	s	2.0	2.0		3-Man—(α)	[D]
	4.85	4.89	s	1.0	1.0		3-Man(β)—3,4-GlcA(α)— (6 4 C; Me, CO_2H)	[E']
	—[d]	4.83	7	—[d]	1.0		3-Gal—(β)	[C']
	4.69	4.72	s	1.0	1.0		3-Man(β)—4-GlcA(α)— (6 4 C; Me, CO_2H)	[E]
	4.65	4.65	8	2.7	2.7		Glc—(β) (×2)	[F]
							3-Gal—(β)—OH	[B_2]
	1.53	1.49	s	3.0	6.0		Me—C(—CO_2H)—	
	1.52		s	3.0				

[a]Chemical shift relative to internal acetone: δ 2.23, downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate (D.S.S.). [b]s = singlet. [c]For example, 4-GlcA—(α) = anomeric proton on a 4-substituted D-GlcA residue that is α-linked. [d]Not observed; coincides with residual-HOD peak.

charides (650 mg). A portion of the oligomers (450 mg) was separated on a column (80 × 2.4 cm) of Bio-Gel P-4 with 500:5:2 water–pyridine–acetic acid as the eluant. Two fractions, **P1** (190 mg) and **P2** (150 mg) were collected; the rest, **P3**, corresponded to higher oligomers, and was not further investigated.

Structure determination of **P1** *and* **P2**. — *(a) Specific rotation.* **P1** had $[\alpha]_D$ +69° (*c* 1.3, water); calc.[8] +72°. **P2** had $[\alpha]_D$ +80° (*c* 0.5, water); calc.[8] +64°.

(b) Determination of the sugar ratios, reducing terminus, and d.p. A sample (10 mg) of each oligosaccharide was reduced with sodium borohydride, and the

isolated oligosaccharide-alditols were hydrolyzed with 2M trifluoroacetic acid (TFA). After removal of the excess of TFA, a 5% solution (0.5 mL) of hydroxylamine hydrochloride in pyridine was added to each sample, which was then heated for 15 min on a steam-bath. Acetic anhydride (0.5 mL) was added to the cooled solution, which was now heated for 1 h on a steam-bath. G.l.c. of the mixture of peracetylated aldononitriles and peracetylated alditols was performed on column *A* at 210°. In a separate experiment, a sample of each oligosaccharide was boiled with 3% HCl in anhydrous methanol for 16 h under reflux. The methyl esters obtained were reduced with sodium borohydride in anhydrous methanol, followed by hydrolysis with TFA, and conversion into the peracetylated aldonitriles (PAAN) according to the method of McGinnis[9]. The PAAN derivatives were analyzed by g.l.c. in column *A*. The results are shown in Table I.

(c) Methylation analysis. A sample (10 mg) of each oligosaccharide was methylated by the Hakomori procedure. Reduction with $LiAlH_4$, hydrolysis, reduction with sodium borohydride, and acetylation afforded mixtures of partially methylated alditol acetates which were separated, and identified (see Table II) by g.l.c. and g.l.c.–m.s.

Reduced depyruvalated **P1**. — Depyruvalation of **P1** (50 mg) was achieved by heating the free-acid form of the oligosaccharide in water (1 mL) for 1 h at 95°. The solution was diluted, treated with sodium borohydride for 1 h, passed through Amberlite IR-120 (H^+) resin, and the boric acid removed as borate by distillation with methanol. The ^{1}H-n.m.r.-spectral data for the product are shown in Table III.

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REFERENCES

1 T. Usui, M. Yokoyama, N. Yamaoka, K. Matsuda, and K. Tuzimura, *Carbohydr. Res.*, 33 (1974) 105–116.
2 J. F. G. Vliegenthart, H. van Halbeek, and L. Dorland, *Pure Appl. Chem.*, 53 (1981) 45–47.
3 H. Björndal, B. Lindberg, and S. Svensson, *Carbohydr. Res.*, 5 (1967) 433–440.
4 K. Bock, personal communication.
5 G. G. S. Dutton, K. L. Mackie, and A. V. Savage, *Carbohydr. Res.*, 84 (1980) 161–170.
6 K. Okutani and G. G. S. Dutton, *Carbohydr. Res.*, 86 (1980) 259–271.
7 H. Thurow, H. Niemann, and S. Stirm, *Carbohydr. Res.*, 41 (1975) 257–271.
8 C. S. Hudson, *J. Am. Chem. Soc.*, 31 (1909) 66–86.
9 G. D. McGinnis, *Carbohydr. Res.*, 108 (1982) 284–292.