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Identification by n.m.r. spectroscopy of oligosaccharides obtained by treatment of the hairy regions of apple pectin with rhamnogalacturonase*

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ABSTRACT

2D-N.m.r. methods have been used to determine the composition of a mixture of oligosaccharides obtained by enzymic degradation of the modified hairy (ramified) regions of apple pectin with a new rhamnogalacturonase. The structures of the oligosaccharides were based on the unit α-Rha*p*-(1→4)-α-GalA-(1→2)-α-Rha*p*-(1→4)-GalA. A β-Gal*p* unit was 4-linked to approximately half of the terminal Rha*p* residues and to half of the (1→2)-linked Rha*p* residues. The sample contained a mixture of a tetrasaccharide, two pentasaccharides, and one hexasaccharide.

INTRODUCTION

Powerful new 2D-n.m.r. techniques which greatly assist analysis of the structure of oligosaccharides are now available, including homonuclear Hartmann–Hahn[1] (HOHAHA) and rotating-frame n.O.e. spectroscopy[2] (ROESY), and ^{13}C–^{1}H shift-correlation using heteronuclear multiple quantum coherence[3] (HMQC). Early applications of these methods[4–6] involved compounds of known structure. We now report the use of 1D- and 2D-n.m.r. methods to determine the structures of several closely related oligosaccharides present in a mixture.

The mixture of oligosaccharides was obtained[8] by enzymic degradation of the hairy (ramified) regions isolated from pectic polysaccharides of apple. The overall sugar composition was known, but there was no reliable information on the size of the oligomers.

The accompanying paper[7] describes the isolation from apple juice of the pectic polysaccharides referred to as the modified hairy region (MHR). The fraction examined here was produced[8] by enzymic degradation of MHR using a new rhamnogalacturonase (RGase). Identification of the degradation product(s) was required in relation to the determination of the structure of MHR and the specificity of the enzyme.

MHR was rich in arabinose (55 mol%) and galacturonic acid (21%), and rhamnose, xylose, and galactose were also present in reasonable proportions[7]. The

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rhamnose/galacturonic acid ratio was particularly high for this type of polysaccharide. MHR was resistant to most technical enzyme preparations and to pectolytic, hemicellulolytic, and cellulolytic enzymes. The availability of the new RGase made it possible to obtain oligosaccharide fractions suitable for study by 2D-n.m.r. spectroscopy.

EXPERIMENTAL

The mixture of oligosaccharides studied was that designated fraction D (from MHR-HCl) in the preceding paper[8].

N.m.r. experiments. — The ^{13}C- (100.4 MHz) and ^{1}H-n.m.r. spectra (399.65 MHz) were obtained with a JEOL GX-400 spectrometer on a solution of fraction D in D_2O (15 mg/0.5 mL; pD 4.7) in a 5-mm tube at 24°. The chemical shifts for the methyl group of internal acetone were taken to be 2.217 (^{1}H) and 31.07 p.p.m. (^{13}C) with respect to the signals for Me_4Si.

COSY, ROESY, and HOHAHA experiments. — All 2D homonuclear correlation experiments were performed[9] in the phase-sensitive mode. A 2048 (t_2) × 512 (t_1) × 2 data matrix was used with spectral widths of 2.5 × 2.5 kHz, which gave digital resolutions of 2.44 and 4.88 Hz in f_2 and f_1, respectively. A double quantum filter[10] was used in the COSY experiment so that all signals could be phased to the pure absorption mode. In the ROESY experiment, the spin-locking (mixing) period was preceded and followed by 90° pulses[11]. A continuous spin-locking field of 2.5 kHz was applied during the mixing time of 200 ms. The HOHAHA experiment[2] involved a repeated MLEV-17 sequence during the mixing time (110 ms), and an r.f. field strength of 6.25 kHz. The carrier frequency was placed at 3.3 p.p.m.

^{13}C–^{1}H Shift-correlation experiments. — These experiments were performed using both the conventional sequence (with ^{13}C detection) and the HMQC method (which has ^{1}H detection, and therefore high sensitivity). The HMQC experiment included a BIRD sequence and was as described[3] except that ^{13}C decoupling was not available in the acquisition period and the data were processed in the absolute-value mode. A 2048 (t_2, ^{1}H) × 128 (t_1, ^{13}C) data matrix was used, with spectral widths of 2000 (^{1}H) × 5556 Hz (^{13}C) which, after zero-filling in t_1, gave digital resolutions of 1.95 (^{1}H) and 21.7 Hz (^{13}C). Delay times were 0.7 s between scans (measured from the start of the acquisition period) and 0.4 s from the end of the BIRD sequence to the first pulse of the HMQC sequence. A conventional ^{13}C-{^{1}H} dual probe was used and the 90° pulse lengths were 8 (^{13}C) and 16 μs (^{1}H).

The resolution in the ^{13}C dimension in the HMQC experiment was not sufficient for an assignment of all of the ^{13}C signals in the region 60–85 p.p.m., so that a conventional phase-sensitive ^{13}C–^{1}H shift-correlation experiment[12] was performed. The data matrix was 4096 (t_2, ^{13}C) × 64 (t_1, ^{1}H) × 2 points, giving digital resolutions of 2.7 Hz for ^{13}C and 3.9 Hz for ^{1}H after zero-filling. The HMQC experiment was carried out with only 96 scans per t_1 value, whereas the ^{13}C–^{1}H shift-correlation with ^{13}C detection required 2000 scans per t_1 value in order to obtain an adequate signal-to-noise ratio.

RESULTS AND DISCUSSION

1D-N.m.r. (^1H and ^{13}C) spectra. — The sugar composition[8] of fraction D was close to 2 rhamnose: 2 galacturonic acid: 1 galactose, and the n.m.r. data showed that the residues were pyranoid. The absolute configurations were assumed to be the same as those invariably found[13] in polysaccharides from plant cell walls, *i.e.*, L-Rha, D-GalA, and D-Gal. The ^{1}H-n.m.r. spectrum of fraction D is shown in Fig. 1. The region for anomeric protons can be divided into two parts, namely, 5.0–5.3 p.p.m. which contains 7 d with J values in the range 1.5–4 Hz, and 4.5–4.7 p.p.m. which contains 3 d, each with a coupling constant of 8 Hz, together with a fourth signal (4.67 p.p.m.) for which the structure was not clear. Resonances for H-1α are expected to be in the first region and those of H-1β in the second region. Other signals which can be assigned readily occurred in the range 1.22–1.32 p.p.m. and arose from H-6,6,6 of the Rha units. Although there appeared to be 2 t in this region, H-6,6,6 gave a dm which, actually, must be 4 d. This finding indicates the presence of four different Rha units in fraction D.

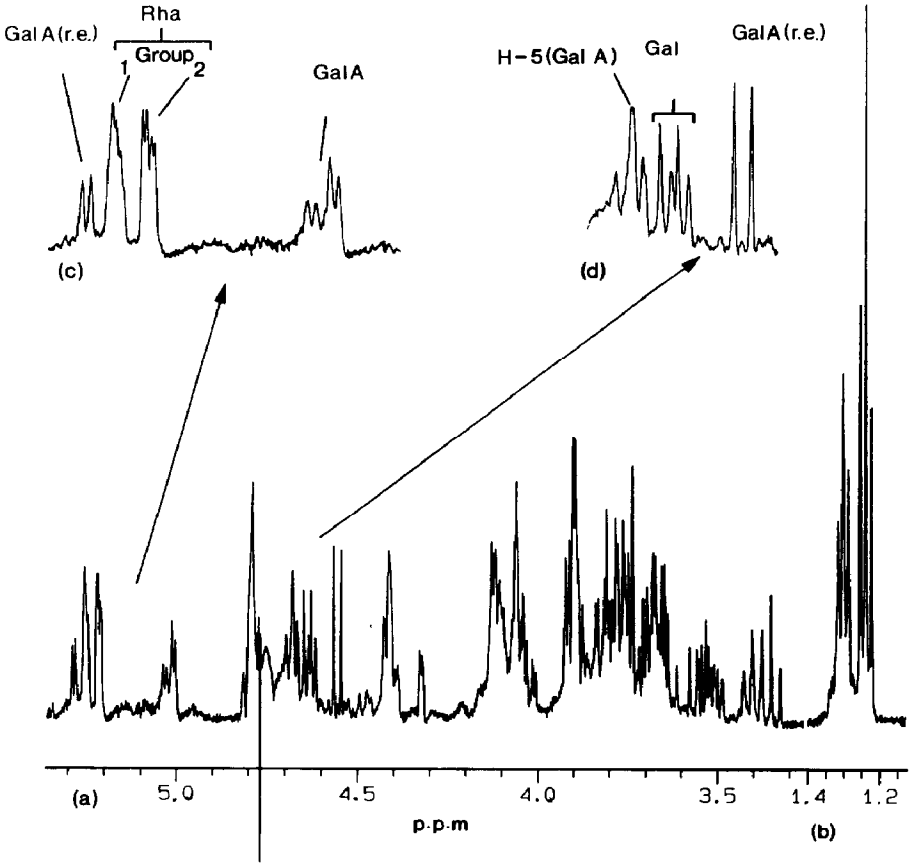

Fig. 1. (a) ^{1}H-N.m.r. spectrum (400 MHz) of fraction D, (b) H-6 (Rha) region, (c) H-1α region, and (d) H-1β region.

The ^{13}C-n.m.r. spectrum of fraction D (Fig. 2) contains four peaks in the region 17–18 p.p.m. (C-6 of the Rha units) and confirms that four different Rha units were present, but the unequal intensities of the lines suggest that there was a mixture of compounds in the fraction. Two lines at 61.73 and 61.76 p.p.m. (assigned to C-6 of Gal not 6-linked) indicate that there were two different Gal units. The region (92–105 p.p.m.) for C-1 resonances contains nine lines, although broadening of two of these lines (at 98.2 and 98.6 p.p.m.) could arise from overlap of resonances with similar chemical shifts. A comparison of the chemical shifts for the C-1 resonances of fraction D with literature data for galactose, rhamnose[14,15], and galacturonic acid[16] suggested the preliminary assignments in Table I. If fraction D contained a single compound, the number of C-1 resonances would indicate it to have been an octamer, but the integrated ^{13}C-n.m.r. spectrum showed that this was not the situation. If the reducing GalA residue is taken as a single unit, then, there are also one α-GalA unit, two α-Rha*p* units with different linkages, and one β-Gal*p* unit. Thus, fraction D contains a mixture of oligosaccharides each of which is smaller than an octasaccharide. In order to confirm the assignments in Table I and reveal further details of the structure of the oligosaccharides in fraction D, 2D-n.m.r. spectroscopy was used.

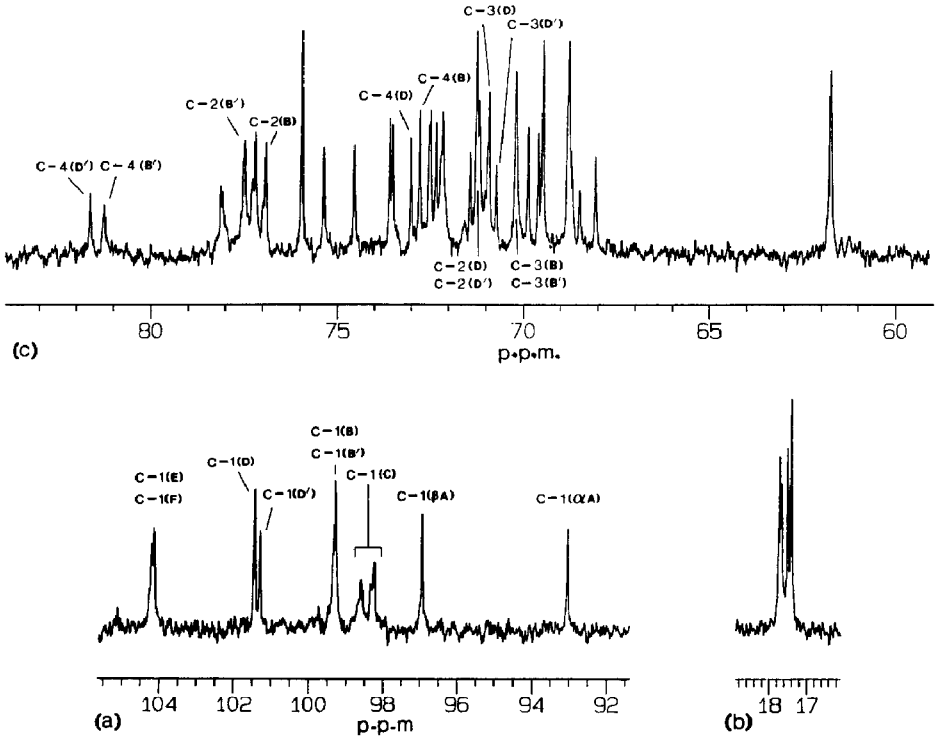

Fig. 2. ^{13}C-N.m.r. spectrum (100 MHz) of fraction D: (a) C-1 region, (b) C-6 (Rha) region, and (c) C-2/6 region, excluding GalA C-6. The labelling scheme is shown in Fig. 6.

TABLE I

^{13}C-N.m.r. data for the anomeric region of fraction D

Unit	*Chemical shift (p.p.m.)*[a]	*Integral*
β-Gal*p*	104.21, 104.14	1.9
α-Rha*p*	101.45, 101.29	2.1
α-Rha*p*	99.32	2.3
α-GalA	98.57, 98.23	2.3
β-GalA (r.e.)[b]	96.94	1.1
α-GalA (r.e.)[b]	93.04	1.0

[a] ±0.01 p.p.m. [b] r.e. = Reducing end.

COSY experiments. — The COSY spectrum of fraction D is shown in Fig. 3. The coupling networks beginning with the H-1 resonances at 5.28 and 4.55 p.p.m. were discerned readily. The chemical shifts of the resonances in these networks accord with those found for the α and β reducing end units in (1→4)-linked oligomers of α-galacturonic acid[17,18]. The *J* values, obtained from the fine structure of the cross-peaks, were also those expected[17] for galacturonic acid residues. The ^{1}H chemical shifts for the resonances of these reducing end units are presented in Table II, together with other data. Taken with the assignments of the ^{13}C-n.m.r. spectrum, the ^{1}H data confirm that the reducing moiety in each oligosaccharide is GalA.

A second type of GalA unit was identified from the COSY spectrum. A coupling network began with the 2 d for H-1 at 5.04 and 5.01 p.p.m., but separate signals could not be observed from the fine structure of the cross-peaks for H-2 or for any of the succeeding protons in the ring. The chemical shifts and *J* values of the resonances of the protons in this network were similar to those obtained[17,18] for the central unit of a (1→4)-linked α-galacturonic acid trimer, indicating that fraction D contained (1→4)-linked α-GalA in addition to the reducing end unit. Thus, the signal at 4.67 p.p.m. did not arise from H-1 but from H-5 of the (1→4)-linked α-GalA residue. The observation of 2 d for H-1, but identical chemical shifts for the H-2 resonance, *etc.*, suggested the presence of two types of (1→4)-linked α-GalA unit that differed only in the nature of the sugar linked to O-1. The 2 d at 4.63 and 4.62 p.p.m. (each with *J* 7.8 Hz) can be assigned to H-1 of β-Gal units. It was not possible to obtain the chemical shifts of the resonances of H-5 and H-6 in the Gal residues from the COSY spectrum, as the coupling network could only be traced as far as H-4. The δ values for the resonances of H-5 and H-6 in Table II were obtained from the ^{13}C/^{1}H correlation spectrum (see below). In common with the (1→4)-linked GalA residues, two signals were observed from H-1 of the Gal units, but only one signal for each of H-2, H-3, and H-4, which indicates again that there were two Gal units that differed only in the sugar unit attached at position 1.

Assignment of the ^{1}H resonances for the Rha units was more complex but provided the key to the determination of the oligosaccharide structure. The H-1 resonances of the Rha units fall into two groups, with equal integrated intensities, centred at 5.25 (group 1) and 5.21 p.p.m. (group 2). Each group contains 2 d with $J \sim 1.5$

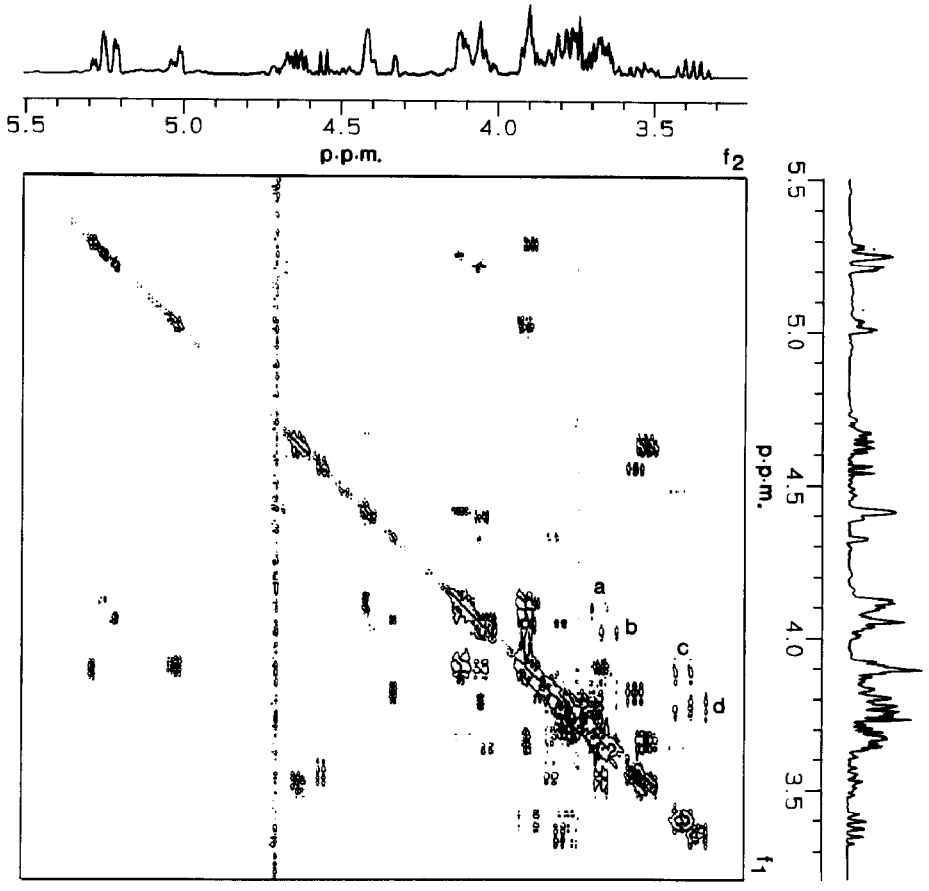

Fig. 3. COSY spectrum of fraction D. Cross-peaks relating H-3 and H-4 for the various Rha units are labelled a,c (group 1) and b,d (group 2).

TABLE II

Chemical shifts[a] (p.p.m.) for the resonances of fraction D

Unit		*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*
GalA (r.e.)[b]	αA	5.28	3.89	4.05	4.39	4.43	
	βA	4.55	3.55	3.82	4.32	4.05	
α-Rha*p*[c]	B[d]	5.26	4.11	3.87	3.40	3.78	1.24
	B′	5.25	4.12	4.09	3.67	3.85	1.31
α-GalA	C	5.04,5.01	3.91	4.11	4.41	4.67	
α-Rha*p*[e]	D[f]	5.22	4.05	3.79	3.35	3.76	1.23
	D′	5.21	4.07	4.02	3.64	3.84	1.29
β-Gal*p*	E,F	4.63,4.62	3.51	3.66	3.90	3.68	3.74[g]

[a] ±0.01 p.p.m. [b] r.e. = Reducing end. [c] Group 1 (see text). [d] B, 2-linked; B′, 2,4-linked. [e] Group 2. [f] D, terminal; D′, 4-linked. [g] H-6,6′.

Hz. The H-6 resonances also gave two pairs of d centred at 1.23 and 1.30 p.p.m. Observation of H-1/H-2 cross-peaks in the COSY spectrum allowed allocation of the chemical shifts of the H-2 resonances to groups 1 (4.11 p.p.m.) and 2 (4.06 p.p.m.), but further tracing of the connectivity network revealed that there were two quite different chemical shifts for the H-3 and H-4 resonances within both groups 1 and 2 (Table II). The chemical shifts of the H-3 (f_1 axis) and H-4 (f_2 axis) resonances may be obtained from the H-3/H-4 cross-peaks labelled a–d in Fig. 3. Cross-peaks a,c originated from H-1 (group 1) and b,d from H-1 (group 2). The difference in the chemical shifts of the H-4 resonances within group 1 (or group 2) can be attributed to the presence or absence of a sugar unit 4-linked to Rha. The provisional assignments in Table I can be extended by suggesting that the four types of Rha unit present are terminal, 2- and 4-linked, and 2,4-linked. However, since the difference in chemical shifts of the H-3 resonances is almost as great as that for the H-4 resonances, the linkage may be at O-3 rather than at O-4. The effects of the formation of glycosidic linkages on the chemical shifts of the resonances of the protons at, or close to, the linkage site have been documented in studies of disaccharides[19,20]. Of particular relevance is the finding[20] that the chemical shifts of the H-3 and H-4 resonances in the L-Rha residue of *β*-D-Glc*p*-(1→4)-*α*-L-Rha*p*-OMe are shifted downfield by ~0.2 p.p.m. in comparison with the values for the corresponding protons in *α*-L-Rha-OMe. The observation of effects of similar magnitude for fraction D supports the proposal that some of the Rha units are 4-linked.

HOHAHA and ROESY experiments. — It was not possible to complete the assignment of the Rha resonances from the COSY spectrum because the cross-peaks that involved H-5 overlapped. This is a common problem which requires the use of additional 2D techniques such as RELAY, multiple RELAY, or HOHAHA spectroscopy[21]. The HOHAHA experiment is particularly useful since magnetisation may be transferred between all the protons of a coupled network. Where none of the *J* values are close to zero, this network will include all the protons of a given sugar unit. Therefore, in the 2D experiment, a cross-section (row or column) taken through the chemical shift of a particular proton resonance in the sugar unit provides a correlation with the chemical shifts of all other resonances in the unit. Although the full 2D spectrum may be extremely complex, cross-sections can usually be taken through the positions of isolated resonances, *e.g.*, in the regions for anomeric or methyl protons.

Figure 4 shows a part of the 2D HOHAHA spectrum of fraction D, in which the methyl signals are correlated with the other resonances of the Rha units. The correlation of the methyl signals to the H-1 region is seen clearly and one downfield Me d belonged to a group 1 Rha unit, whereas the other was associated with group 2. The same inference was true for the upfield 2 d. The complete ^{1}H assignments for the Rha units, obtained from the COSY and HOHAHA experiments, are given in Table II.

The problem of determining the sequence of sugar residues in an oligosaccharide by n.m.r. methods has been approached in several ways. Although the delayed-COSY experiment has been used[22] to detect the small couplings between anomeric and aglyconic protons, the method is not generally applicable. Indirect evidence for the location of glycosylation sites may be obtainable from a comparison of ^{1}H chemical shifts for the

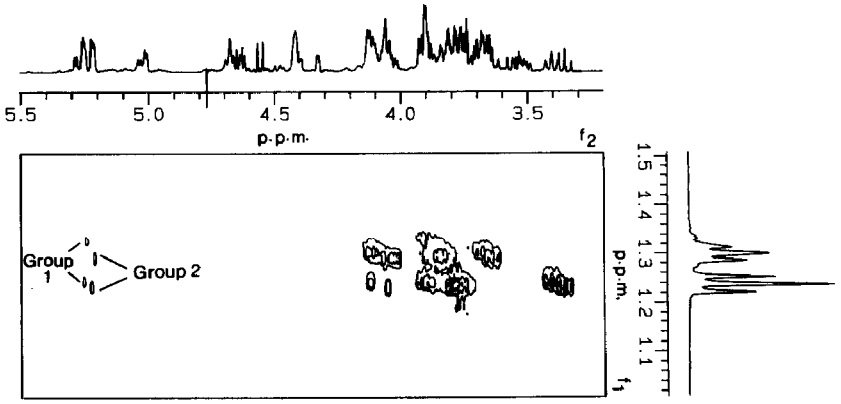

Fig. 4. Partial HOHAHA spectrum of fraction D, showing correlations between H-6 (Rha) and H-1/5 (Rha).

resonances of the oligosaccharide with those for the component monosaccharides[19,20], but the sequence can be determined more readily from n.O.e. data[23], ^{13}C glycosylation shifts[24], or by detection of long-range ^{1}H–^{13}C couplings across the glycosidic linkage[6].

Theoretical and experimental studies[25,26] of the conformations of oligosaccharides have shown that, for most types of glycosidic linkage, the internuclear distance for anomeric and aglyconic protons is in the range 2.5–3.0 Å at the minimum-energy conformation. An inter-residue n.O.e. should be observable for two protons separated by such a short distance, and observation of the n.O.e. can be used to establish which residues are linked together and the linkage positions. In addition, short-range contacts ($\sim$2.5 Å) between protons within the same ring give rise to intra-residue n.O.e.s. For example, in a β-Gal*p* residue, n.O.e.s. are observable from H-1 to H-2,3,5. In NOESY and ROESY spectra, cross-peaks that arise from inter-residue n.O.e.s. can be distinguished from intra-residue cross-peaks if all of the resonances have been assigned previously and there is no overlap of peaks. A correct assignment of all the inter-residue cross-peaks that involve anomeric protons suffices for a complete determination of the oligosaccharide sequence. However, a cross-peak may arise from the close approach of an anomeric proton to a proton in another residue which is not the aglyconic proton. Correctly interpreted, the existence of such a cross-peak may provide information on the overall conformation of the oligosaccharide but, if misinterpreted, it may indicate a linkage where none exists. Therefore, it is advisable to supplement the evidence from NOESY (or ROESY) experiments with ^{13}C-n.m.r. data.

The rate of tumbling of oligosaccharides in aqueous solution is such that the condition $\omega_L\tau_C \sim 1$ is frequently met, where ω_L is the spectrometer operating frequency (rad/s) and τ_C is the correlation time for molecular re-orientation. Under these circumstances, the n.O.e. values are small, irrespective of the internuclear distances, and it may not be possible to use the NOESY technique in order to determine the sequence. The ROESY experiment[1] was developed to overcome the problem of making n.O.e. mea-

surements on molecules that have unfavourable rates of tumbling. The principles of the experiment[1] and its application to oligosaccharides[5] have been discussed elsewhere.

Both NOESY and ROESY experiments were carried out on fraction D. Some of the cross-peaks in the ROESY spectrum (Fig. 5) were also observed in the NOESY spectrum, but the signal-to-noise ratio of the latter was considerably worse for the same total acquisition time. The assignment of resonances to individual sugar units (Table II) permitted identification of intra- and inter-ring cross-peaks in the ROESY spectrum. The only cross-peaks which involved H-1 of the reducing-end GalA unit arose from intra-ring n.O.e.s, but inter-ring cross-peaks involving H-1 of all of the other sugar units were detected, and these are indicated in Fig. 5 and summarised in Table III. Cross-peaks b–e arose from interactions across glycosidic linkages and show that the backbone of the oligosaccharide has an alternating structure of rhamnose and galacturonic acid units, namely, α-Rha*p*-(1→4)-α-GalA-(1→2)-α-Rha*p*-(1→4)-GalA. The group 1 units are the 2-linked Rha residues. The central α-GalA-(1→2)-α-Rha*p* linkage brings H-1 of the GalA and Rha units into close proximity, giving rise to cross-peak a. The group 2 resonances arise from terminal Rha units. A comparison of the intensities of the signals in the anomeric region for the reducing-end GalA unit and the remaining Rha and GalA units makes it clear that the basic structure must be a tetrasaccharide.

The remaining problem is to establish the positions of the linkages of the galactose residues. A cross-peak (f) was detected in the ROESY spectrum, which could possibly connect H-1 of β-Gal*p* with H-4 of certain Rha*p* units (3.64–3.67 p.p.m.). However, intra-ring n.O.e.s. are also expected between H-1 and H-3,5 of β-Gal*p*, and H-3,5 of β-Gal*p* have chemical shifts of ~3.67 p.p.m. Therefore, the ROESY method does not provide definitive evidence for the position of the linkage of the β-Gal*p* unit.

In addition to the ROESY cross-peaks listed in Table III, which were all negative (*i.e.*, opposite in sign to the diagonal), a large number of positive cross-peaks were detected in the region 3.3–4.1 p.p.m. Such cross-peaks arise from coherent magnetisation transfer through the coupled network, as in the HOHAHA experiment. The intensity of these peaks precluded observation of any n.O.e. effects in this region. In the region for anomeric protons, however, cross-peaks that arise *via* the Hartmann–Hahn mechanism are not observed, provided that the carrier frequency is set to one side of the main spectral region. Therefore, the ROESY experiment should be a generally useful method for the determination of the structures of oligosaccharides.

$^{13}C/^{1}H$ Shift-correlation experiments. — A full assignment of the ^{13}C-n.m.r. spectrum of fraction D was undertaken, using C/H shift correlation methods, in order to confirm the structure proposed above and to establish conclusively the positions of the linkages of the Gal residues. In view of the small amount of sample available, an initial attempt to assign the spectrum was made using the high-sensitivity HMQC sequence[3]. The spectrometer did not have the capability for applying ^{13}C broad-band decoupling during the ^{1}H acquisition period, so that the resonances in the ^{1}H dimension appeared at the position of the ^{13}C satellite signals. This method of operation has the advantage that $^{1}J_{C,H}$ values are obtained readily. For example, $J_{C\text{-}1,H\text{-}1}$ of both types of Rha residue was 170 Hz, confirming that they were α.

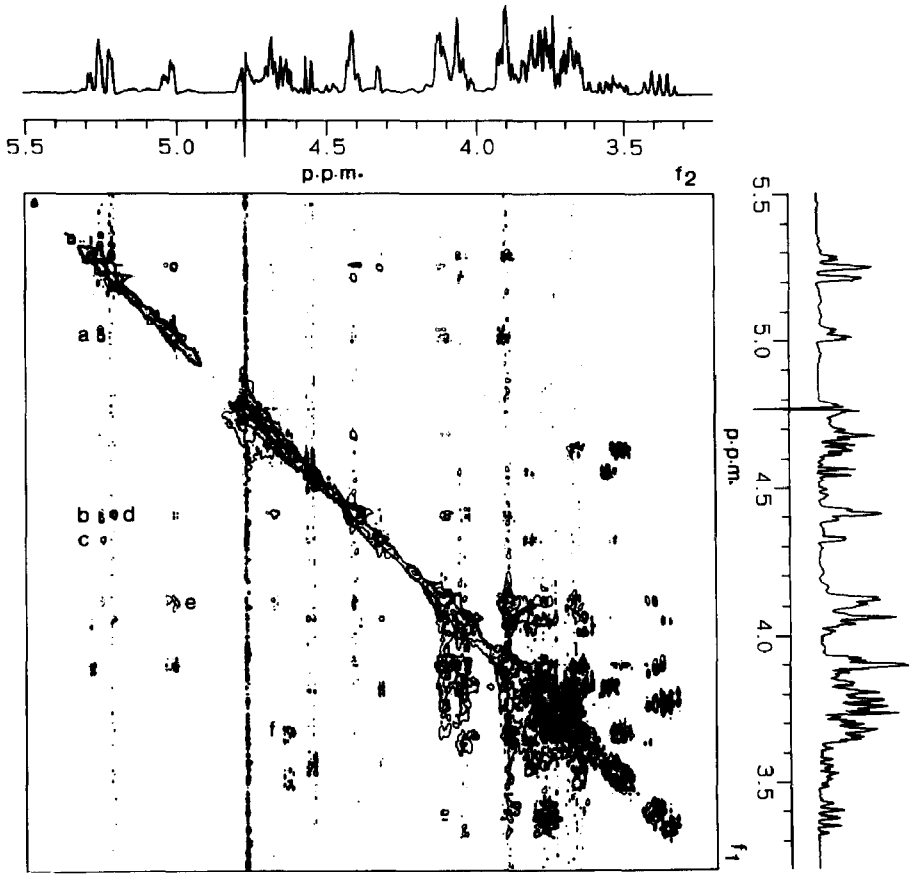

Fig. 5. ROESY spectrum of fraction D. Positive and negative cross-peaks are not distinguished. Peaks a–f (all negative) are identified in Table III. Peaks in the lower right corner (3.3–4.2 p.p.m.) are all positive.

TABLE III

Inter-residue cross-peaks identified in the ROESY spectrum of fraction D

Cross-peak label	*Location of the anomeric proton*	*Connected proton*
a	α-Rha*p* (group 1)	H-1 α-GalA
b	α-Rha*p* (group 1)	H-4 α-GalA (r.e.)[a]
c	α-Rha*p* (group 1)	H-4 β-GalA (r.e.)[a]
d	α-Rha*p* (group 2)	H-4 α-GalA
e	α-GalA	H-2 α-Rha*p* (group 1)
f	β-Gal*p*	H-4 α-Rha*p* (group 1,2)[b]?

[a] r.e. = Reducing end. [b] δ 3.65.

The HMQC experiment confirmed the assignments of the C-1 resonances in Table I, but the lack of ^{13}C decoupling was a handicap in assigning the heavily crowded region (60–85 p.p.m.). Although many correlations were detected, it was not possible to assign the spectrum in full because of the added complexity caused by $^{1}J_{C,H}$ couplings and because of the poor digital resolution in the ^{13}C dimension. The assignments were completed by obtaining a conventional (^{13}C detected) C/H correlation spectrum in which (neglecting zero-filling) the ^{13}C digital resolution was improved by a factor of 16. Consideration of the full assignments of the chemical shifts of the ^{13}C resonances, given in Table IV, proves that the oligosaccharides in fraction D had the structures shown in Fig. 6. The intensities of the signals show that not all of the Rha units were linked to Gal, so that fraction D consisted of a mixture of the basic Rha/GalA tetrasaccharide plus two pentasaccharides and one hexasaccharide that contained one and two Gal units, respectively.

The chemical shifts for the ^{13}C resonances of ring A accorded with those determined[18] for the reducing end unit in several oligomers of (1→4)-linked α-galacturonic acid, the largest difference being found for C-3α,βA which was 1.4 p.p.m. higher than in the oligomers. The C-4α,βA resonances were split into two lines of equal intensity

TABLE IV

Chemical shifts[a] (p.p.m.) of the ^{13}C resonances for fraction D

Unit	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
GalA (r.e.)[b]						
αA	93.04	68.76	70.94	78.10, 78.16	71.40	~175.5
βA	96.94	72.29	74.52	77.55, 77.50	75.35	174.8
α-Rha*p*						
B[c]	99.32	76.86	70.17	72.73	69.88	17.39[f]
B′	99.32	77.44	70.17	81.18	68.47	17.66[g]
α-GalA						
C	98.23, 98.57	68.76	71.14	77.19	72.14	~175.5
α-Rha*p*						
D[d]	101.45	71.25	70.87	72.99	69.60	17.48
D′	101.29	71.22	70.70	81.59	68.10	17.71
β-Gal*p*						
E,F[e]	104.14	72.45	73.49	69.45	75.94	61.73
	104.21	72.50	73.57	"	"	61.76

[a] ±0.01 p.p.m. [b] r.e. = Reducing end. [c] B, 2-linked; B′, 2,4-linked. [d] D, terminal; D′, 4-linked. [e] Rings E and F are not specifically assigned. [f] Assignments for B,D may need to be reversed. [g] Assignments for B′,D′ may need to be reversed.

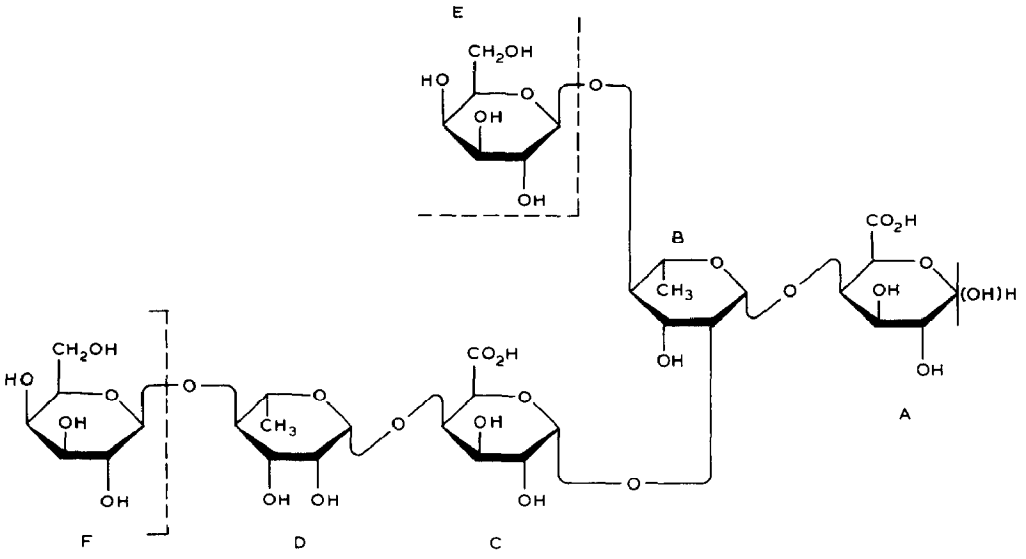

Fig. 6. Structures of the oligomers identified in fraction D. Molecules with zero, one, and two Gal units were present.

because only half of the neighbouring Rha units were substituted with a Gal unit, and there was a long-range effect on the chemical shifts. The chemical shifts of the ^{13}C resonances in oligosaccharides and polysaccharides can be predicted from data for the appropriate monosaccharides and the substituent shifts produced by the immediate neighbours[24]. The present work illustrates the small, but measurable, influences which more remote residues can have on the chemical shift. The ^{13}C-n.m.r. data for ring C may also be compared with those for the (1→4)-linked α-GalA units in oligomers of galacturonic acid. The resonance of C-3C was 1.4 p.p.m. higher, but the shifts of C-1C and C-4C resonances were 1.5 p.p.m. lower, than for the equivalent carbons in the oligomer. Presumably, these changes reflect configurational[20] and conformational[27] effects caused by replacement of two neighbouring α-D-GalA residues by two α-L-Rha*p* residues. The resonances of ring C were rather broad, probably because their chemical shifts are slightly different in each of the four molecules (see below).

The data in Table IV allow the four different types of Rha unit to be identified as 2-linked (ring B), 2,4-linked (B′), terminal (D), and 4-linked (D′). The resonances at 101.45 and 101.29 p.p.m. were assigned to C-1D and C-1D′, respectively, whereas the C-1B and C-1B′ resonances overlapped at 99.3 p.p.m. The relative intensity of the ^{13}C signals of the first pair was 1.4:1, and this gives the ratio of terminal Rha (ring D) to Gal-substituted Rha (D′) units. A resolution-enhanced ^{13}C-n.m.r. spectrum revealed that the resonance at 99.3 p.p.m. was not a single peak, so that measurements of signal intensities were not possible.

The ^{13}C glycosylation shifts showed conclusively that the Gal residues were

4-linked to the Rha units, and not 3-linked, the possible alternative suggested by ^{1}H-n.m.r. data. There was a downfield shift of 8.6 p.p.m. for both the C-4D′ and C-4B′ resonances as compared with those of C-4D and C-4B. By contrast, the shifts of the C-3 resonances hardly changed. Linkage of the group 1 Rha residues (B,B′) at O-2 was also demonstrated by the downfield shifts of ~6 p.p.m. observed for the resonances of C-2B,B′ in comparison with those for C-2D,D′. Two sets of ^{13}C resonances were found for C-1,2,3,6 of Gal that corresponded to the two possible locations (E,F) for these residues. However, the resonances could not be assigned specifically. The chemical shifts were fully consistent with those expected for terminal *β*-Gal*p* residues.

Evidence that all four oligosaccharides might be present came from detailed examination of the C-1C resonance. Resolution enhancement split each of the resonances at 98.23 and 98.57 p.p.m. into two components, suggesting four possible environments for C-1C. It seems reasonable to infer that the larger splitting (0.34 p.p.m.) reflects the presence or absence of ring E, whereas the smaller splitting (0.03 p.p.m.) reflects the more remote influence of ring F. Precise quantification of the oligosaccharides was difficult because of overlap in the ^{1}H-n.m.r. spectrum and poor signal-to-noise ratio in the ^{13}C-n.m.r. spectrum. However, it was estimated that < 50% of the Rha units were substituted with Gal residues.

Methylation analysis (using the methods described in Part I[7]) confirmed the conclusions of the n.m.r. study concerning linkage positions, but gave somewhat higher values for the degree of substitution of the Gal. Thus, the distribution of rhamnosyl units was terminal (18%), 4-linked (31%), 2-linked (21%), and 2,4-linked (30%). Of the galactose residues, 75% were terminal with the remainder 3- (4%), 4- (16%), and 6-linked (5%). The longer chains were not detectable in the n.m.r. spectra.

Structures similar to those determined for the oligosaccharides of fraction D have been described for rhamnogalacturonan I, isolated from sycamore cells[28,29]. The backbone of this polysaccharide had the same alternating sequence of Rha and GalA residues and possessed a variety of side chains 4-linked to ~50% of the Rha residues.

Elucidation of the structures of the oligosaccharides in Fraction D is an important contribution to the model[8] for the modified hairy regions. The isolation, in reasonable quantity, of these oligosaccharides with the same basic tetrasaccharide unit suggests that the complex hairy regions from apple pectin may be more regular than has been assumed hitherto. It is proposed that the hairy regions are composed, in part, of different repeating units.

REFERENCES

1 A. Bax and D. G. Davis, *J. Magn. Reson.*, 65 (1985) 355–360.
2 A. Bax and D. G. Davis, *J. Magn. Reson.*, 63 (1985) 207–213.
3 A. Bax and S. Subramanian, *J. Magn. Reson.*, 67 (1986) 565–569.
4 S. W. Homans, R. A. Dwek, J. Boyd, N. Soffe, and T. W. Rademacher, *Proc. Natl. Acad. Sci. U.S.A.*, 84 (1987) 1202–1205.
5 J. Breg, D. Romijn, J. F. G. Vliegenthart, G. Strecker, and J. Montreuil, *Carbohydr. Res.*, 183 (1988) 19–34.

6 L. Lerner and A. Bax, *Carbohydr. Res.*, 166 (1987) 35–46.
7 H. A. Schols, M. A. Posthumus, and A. G. J. Voragen, *Carbohydr. Res.*, 206 (1990) 117–129
8 H. A. Schols, C. C. J. M. Geraeds, M. F. Searle-van Leeuwen, F. J. M. Kormelink, and A. G. J. Voragen, *Carbohydr. Res.*, 206 (1990) 105–115
9 D. J. States, R. A. Haberkorn, and D. J. Ruben, *J. Magn. Reson.*, 48 (1982) 286–292.
10 M. Rance, O. W. Sørensen, G. Bodenhausen, G. Wagner, R. R. Ernst, and K. Wüthrich, *Biochem. Biophys. Res. Commun.*, 117 (1983) 479–485.
11 C. Griesinger and R. R. Ernst, *J. Magn. Reson.*, 75 (1987) 261–275.
12 M. Ohuchi, M. Hosono, K. Furihata, and H. Seto, *J. Magn. Reson.*, 72 (1987) 279–297.
13 M. McNeil, A. G. Darvill, S. C. Fry, and P. Albersheim, *Annu. Rev. Biochem.*, 53 (1984) 625–663.
14 K. Bock, C. Pedersen, and H. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 42 (1984) 193–225.
15 J. H. Bradbury and G. A. Jenkins, *Carbohydr. Res.*, 126 (1984) 125–156.
16 M. Rinaudo, G. Ravanat, and M. Vincendon, *Makromol. Chem.*, 181 (1980) 1059–1070.
17 S. B. Tjan, A. G. J. Voragen, and W. Pilnik, *Carbohydr. Res.*, 34 (1974) 15–23.
18 C. Chouly, I. J. Colquhoun, and G. A. De Ruiter, unpublished results.
19 A. De Bruyn, M. Anteunis, and G. Verhegge, *Bull. Soc. Chim. Belg.*, 84 (1975) 721–734.
20 I. Backman, B. Erbing, P.-E. Jansson, and L. Kenne, *J. Chem. Soc., Perkin Trans. 1*, (1988) 889–898.
21 F. Inagaki, D. Kohda, C. Kodama, and A. Suzuki, *FEBS Lett.*, 212 (1987) 91–97.
22 G. Massiot, C. Lavaud, L. Le Men-Oliver, G. Van Binst, S. F. Miller, and H. M. Fales, *J. Chem. Soc., Perkin Trans. 1*, (1988) 3071–3079.
23 T. A. W. Koerner, J. H. Prestegard, P. C. Demou, and R. K. Yu, *Biochemistry*, 22 (1983) 2687–2690.
24 P. -E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.*, 168 (1987) 67–77.
25 H. Thøgersen, R. U. Lemieux, K. Bock, and B. Meyer, *Can. J. Chem.*, 60 (1982) 44–57.
26 K. Bock, *Pure Appl. Chem.*, 55 (1983) 605–622.
27 K. Bock, A. Brignole, and B. W. Sigurskjold, *J. Chem. Soc., Perkin Trans. 2*, (1986) 1711–1713,
28 M. McNeil, A. G. Darvill, and P. Albersheim, *Plant Physiol.*, 70 (1982) 1586–1591.
29 J. M. Lau, M. McNeil, A. G. Darvill, and P. Albersheim, *Carbohydr. Res.*, 137 (1985) 111–125.