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Isolation and characterisation of a partially methylated galacto–glucurono–xylo-glycan, a unique polysaccharide from the red seaweed *Apophloea lyallii*

Derek K. Watt[1,*], Shaun A. O'Neill, Ann E. Percy[1], Donald J. Brasch

Department of Chemistry, University of Otago, P.O. Box 56, Dunedin, New Zealand

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Abstract

Polysaccharides of the red seaweed *Apophloea lyallii* were extracted with hot water in a yield of 63.6% w/w of the dry seaweed. The polysaccharides were non-sulphated and were composed of various proportions of D-xylose, 2-*O*-methyl D-galactose, D-glucuronic acid, D- and L-galactose, 3-*O*-methyl galactose, and D-glucose. The aldobiuronic acid 3-*O*-(β-D-glucopyranosyluronic acid)-D-xylopyranose was isolated from the acid hydrolysate of the polysaccharides. The polysaccharides were fractionated by DEAE-sephadex ion exchange chromatography, and two of the fractions obtained were characterised by neutral sugar analyses, linkage analyses, and NMR spectroscopy. A major fraction obtained was shown to be largely composed of a unique (2-*O*-methyl galacto)–glucurono–xylo-glycan with a repeating unit represented by [α-D-(2-*O*-Me-Gal*p*)-(1 → 4)-β-D-GlcA*p*-(1 → 3)-α-D-Xyl*p*-(1 → 4)-]$_n$. The structure of this new glycan was explored by beta-elimination analyses. A minor fraction obtained by ion exchange chromatography contained (1 → 4)-linked xylose, (1 → 4)-linked galactose, (1 → 4)-linked 3-*O*-methyl galactose, (1,3,4)-linked galactose, and terminal uronic acid in a ratio of approximately 1:0.3:0.5:0.4:0.4.

Keywords: *Apophloea lyallii*; Red seaweed; Galacto–glucurono–xylo-glycan

1. Introduction

Many structural studies have been performed on the polysaccharides of the Rhodophyta (red algae). These studies have shown that polysaccharides occur at three main sites within the algae and fulfil three main functional roles. These are the relatively insoluble cell wall materials which provide structural strength, the intercellular mucilages which act as a 'cement' and a protective layer between the cells and intracellular polysaccharides which are food storage reserves.

The cell walls generally contain a primitive form of cellulose with mannan or xylan present as the 'α-cellulose' (Painter, 1983). By far the majority of the hot water soluble intercellular mucilages from red seaweeds studied to date are composed of sulphated galactans (agars and carrageenans). These contain D- and L-galactose residues which are often sulphated or in the 3,6-anhydro form, and sometimes partially methylated. Other mucilages from red seaweeds include sulphated mannans, and xylomannans (Usov, 1992) and β-D-xylans which are comprised of a mixture of (1 → 3) and (1 → 4) linkages (Painter, 1983).

Uronic acid containing galactans from red seaweeds are rare, two examples are those from *Anatheca dentata* (Nunn, Parolis, & Russell, 1971) and *Dilsea edulis* (Barry and McCormick, 1957). Both of these polysaccharides also contain xylose. Xylose is also found in other galactans from red seaweeds including a sulphated galactan from *Corallina officinalis* in which it is present at a level of 23% (Simpsoil & Turvey, 1965). Some galactans have been reported to contain residues such as 4-*O*-methyl L-galactose, 2-*O*-methyl D-xylose, and 2-*O*-methyl 3,6-anhydro-L-galactose (Usov, 1992). A sulphated xylogalactan from *C. officinalis* (Cases, Stortz, & Cerezo, 1992) contained approximately 20% xylose, 20% 2-*O*-methyl galactose, and 50% galactose, with fractions obtained by ion exchange chromatography containing up to 41% 2-*O*-methyl galactose.

In the present work we report for the first time the characterisation of the polysaccharides from the red seaweed *Apophloea lyallii*. *A. lyallii* is endemic to New Zealand, it is particularly profuse around the Fiordland coastline, and is

* Corresponding author. Tel.: +64-4-569-0000; fax: +64-4-566-6004.
E-mail address: d.watt@irl.cri.nz (D.K. Watt).
[1] Present address: Industrial Research Ltd, P.O. Box 31-310, Lower Hutt, New Zealand.

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unusual among seaweeds in that it spends a large amount of time out of the water. It grows near the high tide mark in the midlittoral zone. Apart from *A. lyallii*, the only other member of the genus is *A. sinclairii* which is endemic to the northern half of the North Island, New Zealand.

2. Experimental

2.1. Materials

All reagents were AR grade or redistilled. Glucose oxidase from *Aspergillus niger* was obtained from Serva Fine Biochemicals. Catalase, and glucose dehydrogenase from *Bacillus megaterium* were obtained from the Sigma Chemical Company.

2.2. General methods

Gas chromatography (GC) was performed on a Hewlett Packard 5890 gas chromatograph equipped with a splitter and flame ionisation detector (FID). Gas chromatography–mass spectrometry (GCMS) was performed using a Perkin Elmer 8420 G.C. fitted to a Perkin Elmer Ion trap detector (ITD). Helium was used as the carrier and make-up gas. Alkylations were analysed using a SGE-BP1-0.25 (12 m) or a SGE-BP10-0.25 (25 m) column, chiral glycoside analyses was performed with a SGE-BP10-0.25 (25 m) column, alditol acetate analyses was performed with a SGE-BP-1-0.25 (12 m) column.

2.3. NMR spectroscopy

^{1}H and ^{13}C-NMR were recorded on a Varian VXR 300 MHz spectrometer, using a 5 mm internal diameter tube. The spectrometer was field frequency locked to the deuterium resonance of the solvent and internal referencing was used. The spectra were indirectly referenced to TMS, with dioxane or methanol being used as a references (^{13}C-NMR methanol, 25 °C, δ50.165; 75 °C, δ50.232, dioxane, 75 °C, δ67.816; ^{1}H-NMR methanol, 75 °C, δ3.357). In the ^{1}H-NMR of fraction (e), an acquisition time of 10 s was used, the integrals were determined by the cut and weigh method. A HETCOR proton–carbon correlation spectrum of fraction (e) was obtained at 75 °C.

2.4. Extraction of polysaccharides

Fresh seaweed was collected from Doubtful Sound, New Zealand. It was air dried and milled to a powder in a Wiley mill. The powder (87.4 g dry weight) was extracted by boiling for 3 h in 3 l of distilled water. The mixture was cooled and then filtered to clarity through Whatman #5 filter paper. The insoluble residue was washed with distilled water (500 ml), and the washings filtered and combined with the extract. This extraction procedure was repeated two times, and a fourth extraction was then carried out on the residue by boiling for 8 h in 2 l of distilled water. An aliquot of each extract was concentrated by rotary evaporation, and added dropwise to nine volumes of absolute ethanol. The precipitates were collected by filtration, and dried in vacuo. Deionisation of the first extract (extract #1) was achieved by stirring with an excess of Amberlite IR-120™ (H^+) at 65 °C. The Amberlite was removed by filtration, and the polysaccharide solution was then subjected to two additional treatments with Amberlite IR-120™ (H^+). The resulting solution was concentrated by rotary evaporation, and dialysed versus frequently changed distilled water. The polysaccharide was recovered by precipitation with 90% ethanol, and dried in vacuo.

2.5. Hydrolyses and alditol acetate formation

Polysaccharide (5–10 mg) was hydrolysed with 88% formic acid (1 ml) at 100 °C for 16 h. The formic acid was removed with a stream of filtered air at 50 °C. The product was then hydrolysed with trifluoroacetic acid (1 ml) at 100 °C for 1 h. The TFA was removed with a stream of filtered air at 50 °C. Reduction of the liberated monosaccharides and subsequent acetylation was carried out as described by Albersheim, English, Karr, and Nevins (1967).

2.6. Nitrogen, sulphur, and ash content

Nitrogen, sulphur, and ash contents were determined by the Campbell Micro Analytical Laboratory at the University of Otago.

2.7. Paper chromatography

Preparative paper chromatography was performed on Whatman 3MM, 0.3 mm thick chromatography paper using the descending method. Before use the paper was washed with 10% acetic acid, 0.5 M NH_3, distilled water, and 10% isopropyl alcohol, and then dried. Two solvent systems were used for chromatography: a basic solvent sytem; *n*-butanol/pyridine/water (10:3:3 v/v), and an acidic solvent system; a freshly prepared solution of ethyl acetate/acetic acid/water (9:2:2 v/v). The sugars were detected with *p*-anisidine hydrochloride spray as described by Hough, Jones, and Wadman (1950).

2.8. Chiral glycoside analyses

Chiral glycosides were prepared using R- or S-butanolic HCl using a modification of the method of Gerwig, Kamerling, and Vliegenthart (1978): removal of the butanolic HCl was achieved by co-evaporation with tertiary butyl alcohol using a stream of filtered air at 25 °C. The products were trimethylsilated, and analysed by GC and GCMS.

2.9. DEAE-sephadex™ chromatography

De-ionised extract #1 from *A. lyallii* (0.525 g) was dissolved in the running buffer (0.04 M potassium phosphate buffer, pH 6.0 and NaN_3, 0.02%), and fractionated

on a jacketted glass column (4.5 cm × 66 cm, bed volume 466 ml) of DEAE-sephadex™ (A-50-120). The column temperature was 40 °C, and the initial head of eluant was 1.2 m. The head was gradually decreased during the run to maintain the flow rate at approximately 1 ml min^{-1}. The polysaccharide was eluted over 5.5 days using a linear gradient of 0–1.1 M NaCl (8 l). An additional gradient of 1.1–2 M NaCl (2 l), followed by elution with 2 M NaCl (1 l), failed to elute further carbohydrate material. The column eluate was monitored for carbohydrates using the phenol–sulphuric method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). Fractions were pooled as indicated in Fig. 2. The pooled fractions were dialysed versus 0.05 M HCl at 3 °C, then exhaustively versus distilled water at 3 °C and then freeze-dried.

2.10. Carboxyl reduction

The method of Taylor, Shively, and Conrad (1976) was used for the reduction of the unfractionated extract #1.

2.11. Glucose oxidase oxidation

To the dry hydrolysate of the carboxyl reduced polysaccharide (5 mg), a known amount of inositol and 1 ml of a solution containing glucose oxidase and catalase was added (glucose oxidase 47 units ml^{-1}, catalase 25 μg ml^{-1}, phosphate buffer, 0.12 M, pH 7). The solutions were mixed gently for 2 h and incubated for 22 h in open vials at room temperature. The enzyme was precipitated by the addition of four volumes of ethanol, and pelletted by centrifugation. The solutions were transferred to clean vials, concentrated to dryness, and reduced and acetylated using the method of Blakeney, Harris, Henry, and Stone (1983) for subsequent analyses by gas chromatography.

2.12. DIONEX ion chromatography

DIONEX ion chromatography (DIONEX IC) was used solely for the determination of the absolute configuration of xylose as described in Section 2.13. It was carried out using a DIONEX DX300 (DIONEX, CA) equipped with a DIONEX Carbopac PA1 column (4 mm × 250 mm). Samples (10 μl) were injected using a Shimadzu SIL-10Ai auto-injector. The eluate (1 ml min^{-1}) was monitored using a Pulsed Amperometric Detector (DIONEX). The column was eluted as follows; the first 40 min, isocratic, H_2O followed by 100 mM NaOH containing 100 mM NaOAc (isocratic) for 3 min followed by 100 mM NaOH (isocratic) for 6 min. A suppressor of 0.5 M NaOH (1.5 ml min^{-1}) was used for post column ionisation for PAD detection.

2.13. Glucose dehydrogenase oxidation of D-xylose (Price & Burling, 1994)

De-ionised polysaccharide (extract 1, 155 mg) was hydrolysed in 2 M TFA at 121 °C for 1 h after which the TFA was removed by rotary evaporation. The resulting syrup was dissolved in a minimum of water and applied to a 2.5 cm × 40 cm column of Daianion UBK 555 ion exchange resin (Mitsubishi Kasei) in the Ca^{2+} form. The column was eluted with water, and eluant was monitored by DIONEX IC. The fractions containing xylose were pooled. The concentration of xylose in the resulting solution was 0.43 mg ml^{-1} as determined by DIONEX IC. To 0.1 ml of this solution, a solution of nicotinamide adenine dinucleotide (0.0075 M in 0.2 M phosphate buffer pH 6.0 and 0.38 M NaCl, 0.4 ml), and a solution of glucose dehydrogenase (600 units ml^{-1} in 0.2 M phosphate buffer pH 6.0 and 0.38 M NaCl, 0.1 ml) were added. The resulting solution was incubated at 37 °C for 5 h whereupon the reaction was terminated by heating at 100 °C for 3 min. Analyses of the reaction mixture by DIONEX IC failed to detect any residual xylose in the reaction mixture. Treatment of a solution of D-xylose (1 mg ml^{-1}) in the same way gave the same result and treatment of a solution of L-xylose (1 mg ml^{-1}) confirmed that L-xylose was not affected by this process.

2.14. Alkylations

Alkylations were performed by the Hakamori method using the adaptation described by Conrad and Sandford (1966). Sodium dimsyl was used as the ionising reagent and methyl iodide or ethyl iodide was used as the alkylating agent. Alkylated polysaccharides were hydrolysed with 88% formic acid at 100 °C for 2 h, followed by 2 M TFA for 4 h at 100 °C. Evaporations were carried out using filtered air at 25 °C. Reduction with $NaBH_4$ was carried out in the presence of 1 M NH_4OH, the reaction was terminated by the addition of Amberlite IR-120™ (H^+) resin. Boric acid was removed from the products by co-evaporation with methanol (rotary evaporation, 30 °C), mannitol was added as an internal standard, and the products acetylated with acetic anhydride and pyridine (1:1, v/v) at 100 °C for 3 h. Excess acetylating reagents were removed by co-distillation with toluene (rotary evaporation, 30 °C). Finally the products were dissolved in THF for GC and GCMS analyses.

2.15. Syntheses of 2-O-methyl D-galactose and methyl 2-O-methyl galactosides

2-*O*-methyl galactose was prepared by methylation of 3,4-*O*-isopropylidene-D-galactosan which was prepared using literature methods (Dey, 1967; Gent, Gigg, & Penglis, 1976). Methylation was carried out with NaH and methyl iodide in dry THF. The resulting 2-*O*-methyl isopropylidene-galactosan was heated at reflux in aqueous HCl (McCreath, Smith, & Cox, 1939) to give 2-*O*-methyl D-galactose which was crystallised from ethanol and hexanes. The ^{13}C-NMR is reported in Table 2.

Two preparations of methyl glycosides greatly differing in furanoside content were made from 2-*O*-methyl galactose: (1) 2-*O*-methyl galactose was heated with methanolic HCl (2 M) at 80 °C for 24 h in a sealed vial. The methanol

Table 1
Yield and neutral monosaccharide composition of successive hot water extractions of dried *A. lyallii* (expressed as molar ratios; 2-*O*-MG, 2-*O*-methyl D-galactose; 3-*O*-MG, 3-*O*-methyl galactose)

Extract no.	Yield[a] (% w/w)	Xyl	2-*O*-MG	3-*O*-MG	Gal	Glc	Ash (% w/w)
1	38.6	34.6	34.6	4.00	21.2	5.74	6.13
2	9.0	35.8	20.4	7.21	34.4	2.07	4.55
3	6.6	33.5	14.3	7.99	41.9	2.33	3.74
4	9.4	30.04	11.7	8.14	46.46	3.61	3.18

[a] Expressed as % w/w, dried extract/dried seaweed.

was diluted with water, and the Cl^- removed with Amberlite IR-45™ (OH^-) resin. The product was dried and used directly for NMR spectroscopy. (2) 2-*O*-methyl galactose was heated at reflux in methanolic HCl (0.006 M) over non-indicating Drierite (anhydrous $CaSO_4$, eight mesh), for 5 h. Water (two volumes), was added, and the product was deionised by treatment with Amberlite IR-120™ (H^+) resin and then Amberlite IR-45™ (OH^-) resin, dried, and used directly for NMR spectroscopy.

3. Results and discussion

3.1. Extraction

Water soluble polysaccharides from *A. lyallii* were extracted with water at 100 °C for 3 h. Four successive extractions of the dried seaweed yielded a total of 63.6% w/w of polysaccharide (Table 1). These extracts contained an average of 5.2% ash. The nitrogen content of the first three extracts was 1.1, 0.3, and 1.0%, respectively. This was taken as an indication of the protein content in the extracts. No sulphur was detected by microanalyses in any of the extracts and therefore the polysaccharides extracted were not sulphated. The neutral sugar composition was determined for the four successive hot-water extracts (Table 1). The 2-*O*-methyl D-galactose content decreased from 34.6% of the neutral sugars in the first extract to 11.7% in the final extract. The levels of 3-*O*-methyl hexose and galactose both increased with successive extractions.

Table 2
^{13}C-NMR chemical shifts of 2-*O*-methyl D-galactose and methyl 2-*O*-methyl D-galactosides

	C-1	C-2	C-3	C-4	C-5	C-6	1-*O*-Me	2-*O*-Me
2-*O*-methyl D-galactose								
α-Pyranose	90.8	78.9	69.9	70.5	71.5	62.5		58.7
β-Pyranose	97.6	83.0	73.7	70.0	76.3	62.2		61.5
α-Furanose	94.8	86.3	74.2	82.1	73.1	63.7		59.0
β-Furanose	100.8	92.4	75.7	82.9	71.7	64.0		58.8
Methyl 2-*O*-methyl D-galactosides								
α-Pyranoside	97.9	78.7	70.1	70.4	71.8	62.5	56.0	58.9
β-Pyranoside	104.9	81.8	73.6	70.0	70.3	62.2	58.3	61.4
α-Furanoside	101.8	86.2	74.7	82.7	74.0	63.4	56.3	59.2
β-Furanoside	107.4	91.9	76.1	83.6	71.9	64.0	55.9	58.6

3.2. Monosaccharide characterisation

Component sugars of the extracts of *A. lyallii* were characterised after isolation by preparative paper chromatography. Neutral sugars were isolated using the basic solvent system and acidic sugars were isolated using the acidic solvent system. In order to characterise the 2-*O*-methyl galactose component, authentic 2-*O*-methyl galactose was synthesised using routine methods. Detailed ^{13}C-NMR data for 2-*O*-methyl galactose is reported here (Table 2). Comparison of the relative intensities of the ^{13}C-NMR signals as they changed with mutarotation allowed signals to be assigned to the different ring forms. The spectra were then assigned by comparison with galactose (Bock & Pederson, 1983). The anomeric chemical shifts of methyl glycosides more closely approximate the anomeric chemical shifts of polysaccharides than those of reducing sugars and in order to aid in the assignment of the ^{13}C-NMR spectra of the *A. lyallii* polysaccharides, the NMR spectra the methyl glycosides of 2-*O*-methyl galactose were obtained. By using different concentrations of HCl in anhydrous methanol, the ratios of the different glycosidic products were varied, and this allowed signals to be assigned to the different ring forms. The individual signals were then assigned by comparison with the methyl glycosides of galactose (Bock & Pedersen, 1983; Table 2). The ^{13}C-NMR spectra of the 2-*O*-methyl galactose isolated from *A. lyallii* was identical to that of the synthetic compound, and both compounds co-eluted on GC as alditol acetates. GC of the TMS ethers of the R and S butyl glycosides of synthetic 2-*O*-methyl D-galactose and the R butyl glycoside of that from *A. lyallii* revealed that the later was the D-sugar. The xylose from *A. lyallii* was identified by R_f and colour on paper chromatography and by R_t on GC (alditol acetates). In addition the ^{13}C-NMR of the monosaccharide isolated by paper chromatography was identical to that of authentic xylose. Oxidation of the xylose with glucose dehydrogenase (Price & Burling, 1994) showed the xylose had the D-configuration. The glucuronic acid from *A. lyallii* had identical R_f on paper chromatography with authentic glucuronic acid, and the ^{13}C-NMR of the monosaccharide isolated by paper chromatography was identical to that of authentic glucuronic acid. Reduction of the polysaccharide (extract #1) with carbodiimide result in a large increase in the amount of glucose in the acid hydrolysate of the polysaccharide as observed by alditol acetate analyses. Treatment

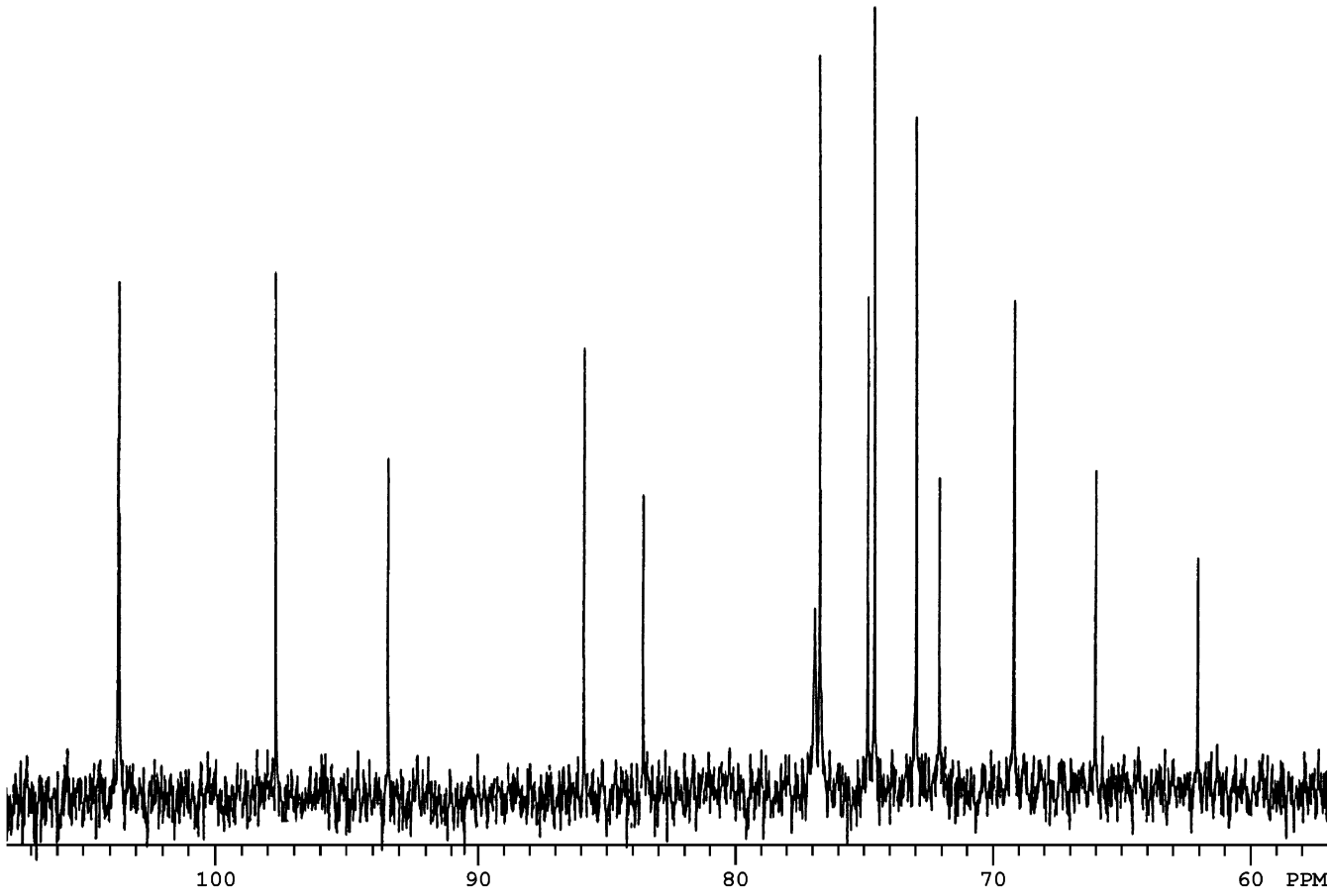

Fig. 1. ^{13}C-NMR spectra of the aldobiuronic acid from *A. lyallii* (pH 7.7).

Table 3
The ^{13}C-NMR spectra of the aldobioronic acid from *A. lyallii* and comparison with similar compounds

	C-1	C-2	C-3	C-4	C-5	C-6
Aldobiuronic acid, pH 7.7						
β-D-GlcA	103.8[a]	74.6	76.7	73.0	76.9	Not resolved
α-D-Xyl	93.4	72.1	83.6	69.3	62.1	
β-D-Xyl	97.7	74.9	85.9	69.3	66.0	
Aldobiuronic acid, pH 1.5						
β-D-GlcA	103.9[a]	74.3	76.4	72.5	75.4	173.6
α-D-Xyl	93.4	72.1	83.5	69.2[b]	62.1	
β-D-Xyl	97.7	74.9	85.8	69.1[b]	66.0	
Methyl β-D-glucopyranosiduronic acid (Bock & Pedersen, 1983)						
	104.3	73.8	76.5	72.3	75.6	Not resolved
Methyl α-D-glucopyranosiduronic acid (Bock & Pedersen, 1983)						
	100.7	71.9	73.8	72.5	71.9	Not resolved
β-D-Xyl-(1 → 3)-D-Xyl, reducing residue (Bradbury & Jenkins, 1984)						
α-Xyl	93.3	72.1	82.9	68.9	62.1	
β-Xyl	97.6	74.9	85.3	68.9	65.5	
β-D-Xyl-(1 → 4)-D-Xyl, reducing residue (Gast, Atalla, & McKelvey, 1980)						
α-Xyl	92.8	72.3[b]	71.9[b]	77.5	59.8	
β-Xyl	97.3	74.9	74.9	77.3	63.9	

[a] This was a double peak (Δ0.05 ppm).
[b] These assignments may be interchanged.

of the hydrolysate of the reduced polysaccharide with glucose oxidase resulted in the complete elimination of the glucose showing that both the original glucose in the polysaccharide and that which originated from the glucuronic acid had the D-configuration. The galactose was identified after isolation by paper chromatography by ^{13}C-NMR and by GC of the alditol acetate. GC of the TMS ethers of the chiral butyl glycosides of the galactose isolated from the *A. lyallii* extract #1 showed that galactose is present in both the D and L forms. The ratio of D/L in extract #1 was 1.3:1. A 3-(or 4)-*O*-methyl hexose was shown to be present in the hydrolysate of the *A. lyallii* by GCMS of the alditol acetates (*m/z* values *43*, 73, *85*, *87*, 99, 111, 127, *129*, 149, 159, 171, 189, 201). Ethylation analyses of the extract showed the *O*-methyl group to be on the third carbon. Methylation analyses of a preparation containing this component gave 2,3,6-tri-*O*-methyl galactose as the only 3-*O*-methylated hexose derivative present, and therefore this component was taken to be 3-*O*-methyl galactose.

3.3. Structural determination of the aldobiuronic acid from A. lyalli

An aldobiuronic acid was isolated from the acid hydrolysate of hot water extract 1 by paper chromatography. It

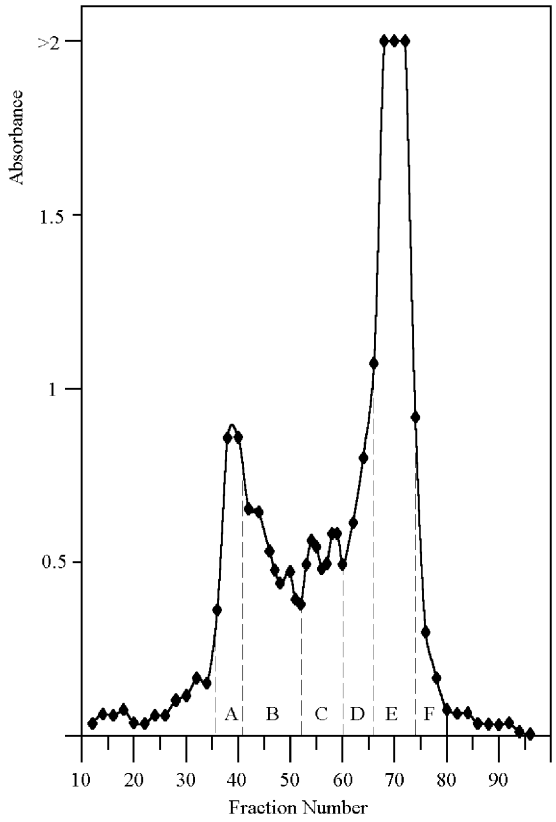

Fig. 2. Fractionation of de-ionised hot water extract #1 from *A.lyallii* by DEAE-sephadex™ ion exchange chromatography.

was found to be a major component of the hydrolysate and gave an intense red colour upon development of the paper chromatograph. Methanolyses of the sodium borohydride-reduced disaccharide followed by TMS derivatisation and GCMS, gave products which co-eluted, and gave identical mass spectra with the TMS ethers of authentic xylitol and the methyl esters of the methyl glycosides of glucuronic acid. From this it was apparent that the disaccharide was composed of glucuronic acid and xylose, and that xylose was in the reducing position. D-glucuronic acid was the only uronic acid detected in the hydrolysate of the hot water extract of *A. lyallii* when examined by paper chromatography, and it is concluded that the uronic acid was D-glucuronic acid. The ^{13}C-NMR of the aldobiuronic acid was obtained both at neutral (Fig. 1) and at low pH. Comparison of the spectra with that of methyl β-D-glucopyranosiduronic acid (Bock & Pedersen, 1983) and the reducing xylose residues in the disaccharides β-D-Xyl-(1 → 3)-D-Xyl (Bradbury & Jenkins, 1984; Table 3) and β-D-Xyl-(1 → 4)-D-Xyl (Gast, Atalla, & McKelvey, 1980) clearly indicated that the xylose was (1 → 3) linked. As methylation analyses of the hot water extract #1 showed the presence of only (1 → 3), and (1 → 4)-linked xylose, only these linkages were considered in this analyses. The closeness of the xylosyl ^{13}C-NMR signals in the aldobiuronic acid to those of the reducing residue in β-D-Xyl-(1 → 3)-D-Xyl is remarkable, and presumably is due to the similarity of the arrangement of atoms around the glycosidic linkage between the two residues in the disaccharides. By comparison with methyl β-D-glucopyranosyluronic acid (Table 3; Bock & Pedersen, 1983) it is apparent that the glucuronic acid in the aldobiuronic acid has the β-anomeric configuration and that the aldobiuronic acid from *A. lyallii* is 3-*O*-(β-D-glucopyranosyluronic acid)-D-xylopyranose. It is worth noting that this aldobiuronic acid has been synthesised by chemical methods (Bishop, 1953), but to the authors' knowledge has not previously been isolated from a natural source.

Table 4
Yield of fractions, and neutral monosaccharide composition (expressed as mol%) of fractions of de-ionised hot water extract of *A. lyallii* obtained by ion-exchange chromatography

Fraction	Yield (%)[a]	Xyl	2-*O*-Me-Gal	3-*O*-Me-Gal	Gal	Glc
A	6.7	43.5	0.3	20.4	33.6	2.2
B	7.2	40.6	3.2	15.7	38.0	2.6
C	7.0	32.4	9.3	8.9	46.6	2.9
D	6.5	32.4	21.7	5.3	35.1	5.5
E	60.0	39.0	48.8	0.7	8.1	3.4
F	2.3	32.9	33.1	4.0	24.1	5.9

[a] Yield of each fraction as a percentage w/w of material applied to the column.

3.4. De-ionisation and fractionation of A.lyallii polysaccharides

Attempts to per-*O*-methylate the non-deionised polysaccharide extracts were not successful, and only partial methylation was achieved in one step. This was due to insolubility in DMSO. It was found that de-ionisation with a strongly acidic ion-exchanger gave a product which was soluble in DMSO and which per-*O*-methylated in one step. In addition, attempts at fractionating the non-deionised polysaccharides by selective precipitation with cetyltrimethylammonium bromide were not successful, and fractions obtained were similar to each other in composition (data not shown). However, fractionation of the de-ionised polysaccharides by anion-exchange chromatography was successful (Fig. 2). It should be noted that the de-ionisation procedure may have resulted in some depolymerisation, even although no reducing end-groups were detected by ^{1}H and ^{13}C-NMR spectroscopy. Analyses of the fractions obtained by anion-exchange chromatography showed that at least two types of polysaccharide were present (Table 4). The neutral sugars present in fraction (a) were mainly xylose, 3-*O*-methyl galactose, galactose. Whereas the major fraction, fraction (e), contained mainly xylose and 2-*O*-methyl galactose. Other fractions were not so homogenous, and contained various amounts of xylose, 2-*O*-methyl galactose, 3-*O*-methyl galactose and galactose. A high galactose content in fraction (c) when compared to adjacent fractions seems to indicate additional heterogeneity within the polysaccharides.

Table 5
Alkylations of unfractionated and fractionated polysaccharides from the hot water extract of *A. lyallii* (expressed as molar ratios)

Preparation	Unfractionated		Fraction A	Fraction E
Akylation type	Ethylation	Ethylation	Methylation	Methylation
Reducing agent	None	$LiAlH_4$	$LiAlD_4$	None
Deduced linkage				
Xyl (1,3)	2.66	3.04	–	1
Xyl (1,4)	1.00	1.00	1.00	–
2-*O*-Me-Gal (1,4)	3.31	3.30	–[a]	2.3[a]
Gal (1,4)	0.51	0.53	0.28[a]	0.4[a]
3-*O*-Me-Gal (1,4)	0.37	0.43	0.46[a]	–[a]
Gal (1,3,4)	0.83	0.71	0.40	0.02
terminal uronic acid	–	0.43	0.43	–
terminal hexose	–	–	0.05	–
GlcA (1,4)	–	3.19	–	–

[a] These values were calculated using the methylation analyses data and the neutral sugar composition.

3.5. Ethylation and methylation studies on the fractionated and unfractionated polysaccharides

Methylation analyses of fraction (e) (Table 5) showed the presence of large amounts of 1,3-linked xylose and 1,4-linked 2-*O*-methyl galactose. The presence of 1,4-linked glucuronic acid in this fraction will be shown later by NMR analyses. The ethylation studies on the unfractionated polysaccharide showed the presence of only two types of acidic residue, a terminal acidic residue, and a 1,4-linked glucuronic acid residue. As there are only very low levels of branch points in fraction (e), the glucuronic acid attached to the xylose in this fraction cannot be a terminal uronic acid, but must be the 1,4-linked glucuronic acid.

3.6. Base degradation of per-O-methylated fraction (e)

In order to provide additional information on the structure of fraction (e), the method of β-elimination described by Aspinall and Rosell (1977) was used. The methyl uronate residues in a polysaccharide carry a good leaving group at position 4 if they are linked through C-4 to another sugar or to a methyl group (Lindberg, Lonngren, & Thompson, 1973). On treatment with molar sodium dimsyl in DMSO the uronate residues undergo beta-elimination, and the resulting unsaturated residues undergo further degradation and are lost from the polysaccharide (Aspinall and Rosell, 1977). In the present study the methylated fraction (e) was subjected to this treatment, and ethyl iodide was used to label the point of attachment of the glucuronic acid. The ratios of the resulting sugars are shown in Table 6. The presence a small amount of 1,4-linked pentose in the base degradation products was puzzling considering that there was no 1,4-linked xylose found in the methylated polysaccharide prior to base degradation. The mass spectrum of the 3-*O*-ethylated xylose was entirely consistent with the spectrum of the corresponding 3-*O*-methylated analogue (a 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylpentitol) (Jansson, Keene, Liedgren, Lindberg, & Lonngren, 1976). This analysis confirms that the glucuronic acid in fraction (e) was linked (1 → 3) to xylosyl residues, as the presence of the aldobiuronic acid 3-*O*-(β-D-glucopyranosyluronic acid)-D-xylopyranose in the hydrolysate of the unfractionated polysaccharides suggests. Complete elimination of the acid residue was not achieved in one step, however, no galactose was labelled by an ethyl ether group. In addition from the very simple ^{13}C-NMR spectra of fraction (e) (Fig. 5) it is not likely that xylose is incorporated in two different positions in the polysaccharide to any major extent.

Table 6
Base degradation analyses of methylated fraction (e)

Residue	Molar ratio
3-*O*-ethyl labelled Xyl[a]	0.71
Xyl (1,3)	1.00
Pentose (1,4)	0.22
2-*O*-Me-Gal (1,4) and Gal (1,4)	1.06
Gal (1,3,4)	0.14

[a] Identified by the mass spectra *m/z*: *43*, *59*, 74, *87*, 101, *115*, *117*, 175.

3.7. Methylation and ethylation of fraction (a)

Fraction (a) contained 1,4-linked xylose, 1,4-linked galactose, 1,4-linked 3-*O*-methyl galactose, 1,3,4-linked galactose, and a terminal uronic acid (Table 5). A small amount of a terminal hexose was detected in the methylation analyses, but not the ethylation analyses. The uronic acid is assumed to be glucuronic acid, as only glucuronic acid was detected in the hydrolysate of the unfractionated polysaccharides by paper chromatography. It is not clear whether fraction (a) represents a single type of polysaccharide or a mixture of polysaccharides, and further work needs to be done to ascertain this.

3.8. Nuclear magnetic resonance of A. lyallii polysaccharides

Fraction (a) was only slightly soluble in D_2O and the ^{13}C-NMR of was obtained by aquiring a large number of transients. The proton NMR of this fraction revealed no signals probably due to low T_2 values. The methylation analyses of this fraction revealed five different linkages were present, and the ^{13}C-NMR spectra had five peaks in the anomeric region (Fig. 3). However, assignment of the ^{13}C-NMR spectra is not possible with the present data. ^{13}C-NMR analyses of the unfractionated polysaccharides from *A. lyallii* (Fig. 4) showed that it contained three major monosaccharide residues and had a very regular structure. Although the polysaccharide(s) present in fraction (a) were at a level of about 20% (calculated from the $LiAlH_4$ reduced ethylation of the unfractionated polysaccharides), the ^{13}C-NMR of the mixed polysaccharides (Fig. 4) showed little sign of their presence. This can be taken as a cautionary note with respect to

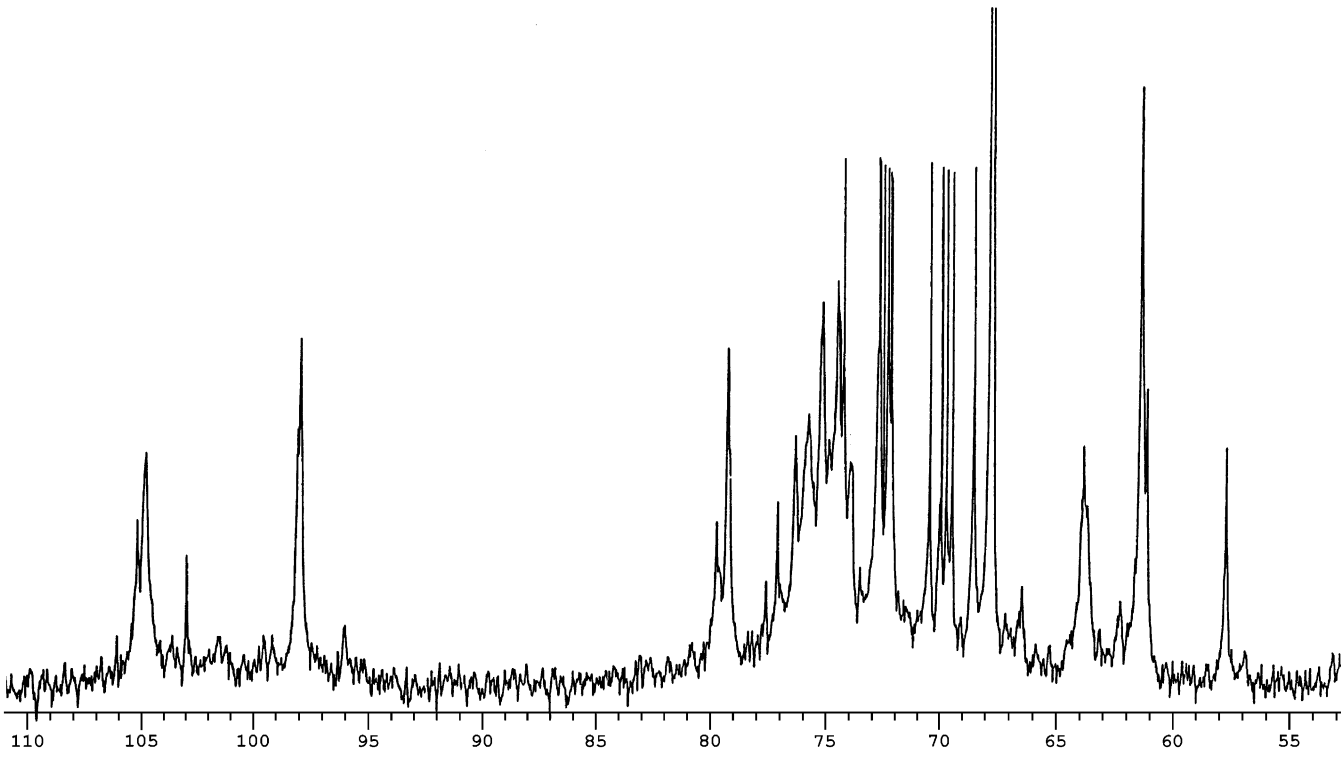

Fig. 3. ^{13}C-NMR spectra of fraction (a) (+ dioxane, pH 2).

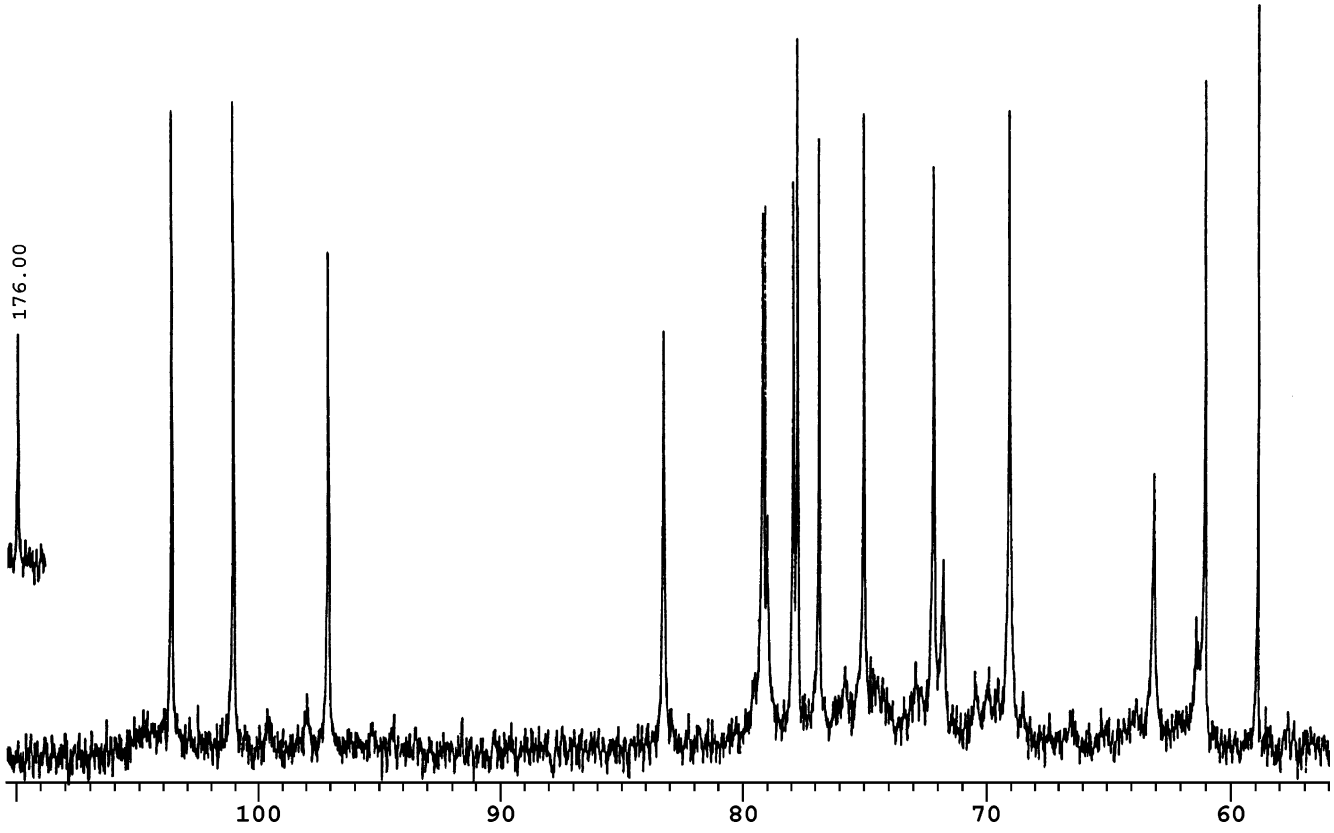

Fig. 4. ^{13}C-NMR spectra of the unfractionated polysaccharides of *A. lyallii* (extract #1, pH 7.8).

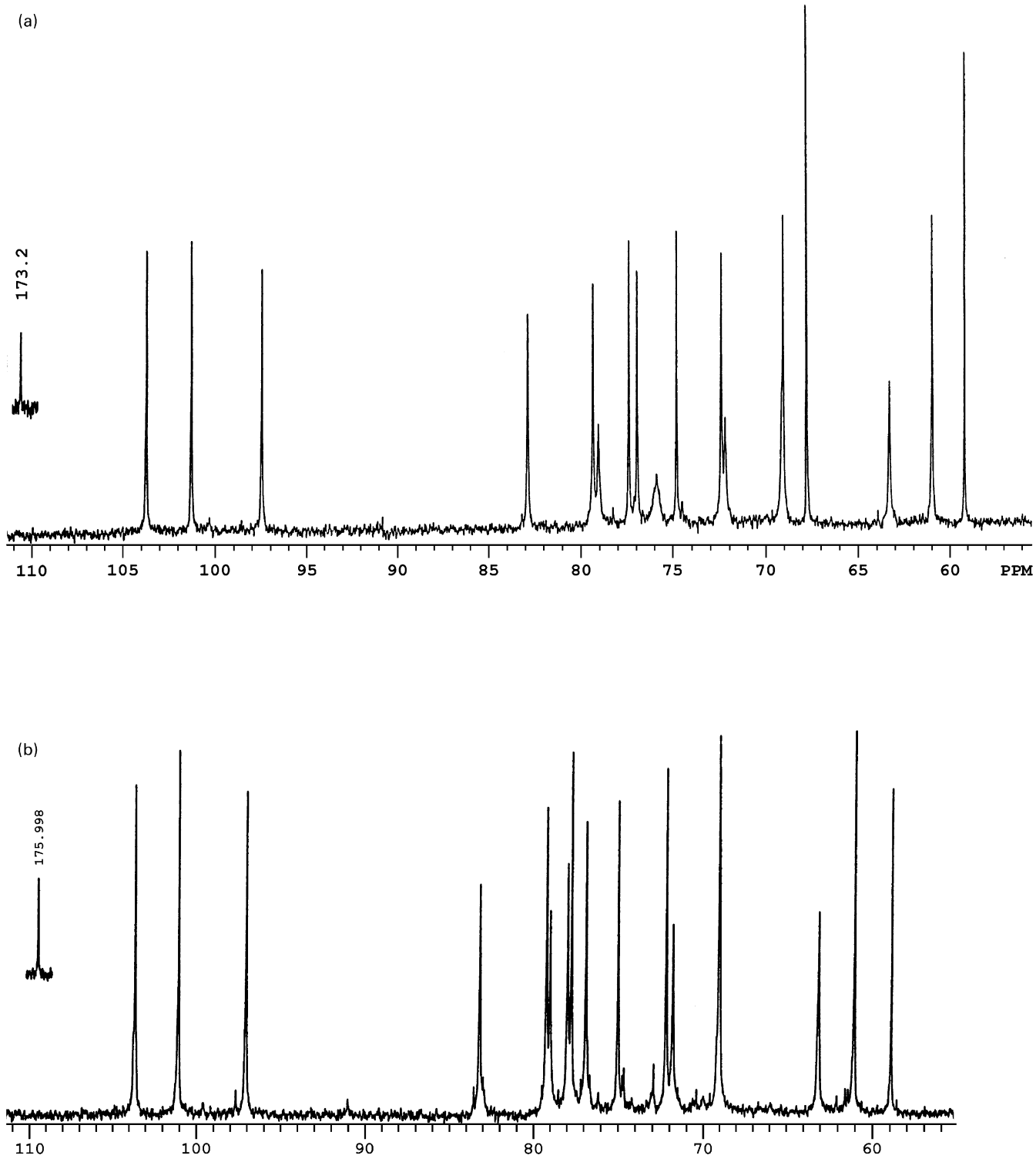

Fig. 5. (a) ^{13}C-NMR spectra of fraction (e) (+ dioxane, pH 2.2); (b) ^{13}C-NMR spectra of fraction (e) (pH 7.8).

relying solely on NMR data for the analyses of polysaccharide mucilages.

The ^{13}C-NMR of the largest fraction obtained by ion-exchange chromatography, fraction (e) (Fig. 5a and b), gave a NMR spectra similar to that of the non-fractionated polysaccharides. This fraction contains mainly 1,4-linked-D-2-*O*-methyl galactose, 1,3-linked-D-xylose, and 1,4-linked-glucuronic acid. The glucuronic acid is linked directly to the xylose as determined by the presence of the aldobiuronic acid β-D-GlcA-(1 → 3)-α-D-Xyl, and the base degradation data. Given that the three sugars all have the gluco-configuration at C-2, the proton NMR (Fig. 6, Table 7)

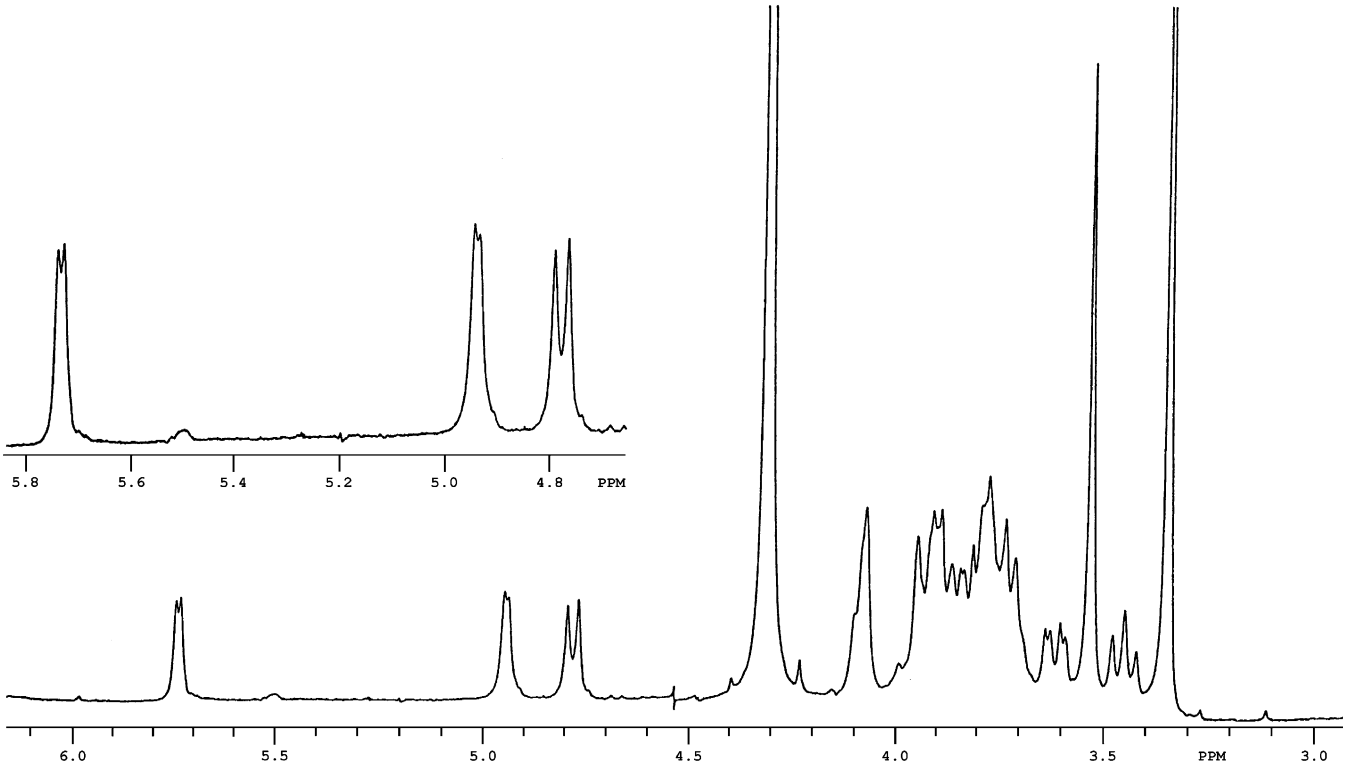

Fig. 6. ^{1}H-NMR spectra of fraction (e) (+ methanol, pH 2.2).

shows the presence of only one β-linkage, therefore the xylose and 2-*O*-methyl galactose residues must have the α-configuration. Given the presence of the disaccharide β-D-GlcA-(1 → 3)-α-D-Xyl, and the ^{13}C-NMR spectra showing only 18 signals (Table 8), there are only two possibilities for the position of the 2-*O*-methyl galactose in the polymer: either the 2-*O*-methyl galactose exists in blocks of monosaccharide repeating units which are linked to blocks of aldobiuronic acid repeating units in a polysaccharide represented by {[α-D-(2-*O*-Me-Gal)-(1 → 4)]$_n$-[β-D-GlcA-(1 → 3)-α-Xyl-(1 → 4)-]$_n$}. Or the 2-*O*-methyl galactose exists in a trisaccharide repeating unit of [α-D-[2-*O*-Me-Gal]-(1 → 4)-β-D-GlcA-(1 → 3)-α-Xyl-(1 → 4)-]$_n$.

To distinguish between these two possibilities the ^{13}C-NMR spectra must first be assigned. To assign the spectra, the α, β and δ effects of glycosylation on the ^{13}C-NMR spectra of similar sugars with similar linkages are applied to the sugars present. The α, β and δ effects of the 1-*O*-methylation of α-xylopyranose (methyl α-xylopyranoside compared with α-xylopyranose) (Bradbury & Jenkins, 1984) were used to predict the α, β and δ effects on the xylose chemical shifts of linking the aldobiuronic acid to another sugar residue through the xylose C-1. For the C-6 of the glucuronic acid residue the chemical shift of the C-6 of α-GlcA (Bock & Pedersen, 1984) was used, as the signal

Table 7
Anomeric ^{1}H-NMR data for fraction (e)

Residue	H-1 (ppm)	Relative area	$J_{1,2}$ (Hz)	Conformation
GlcA*p*	4.78	1.0	7.8	β
Xyl*p*	4.95	1.0	3.0	α
2-*O*-Me-Gal*p*	5.74	0.9	3.0	α

Table 8
Calculated chemical shifts for [α-D-[2-*O*-Me-Gal]-(1 → 4)-β-D-GlcA-(1 → 3)-α-D-Xyl-(1 → 4)-]$_n$, and observed ^{13}C chemical shifts (ppm) for fraction (e), and for the carboxyl reduced unfractionated polysaccharides

Residue	C-1	C-2	C-3	C-4	C-5	C-6	OMe
Calculated chemical shifts for [α-D-[2-*O*-Me-Gal]-(1 → 4)-β-D-GlcA-(1 → 3)-α-Xyl-(1 → 4)-]$_n$ at pH 7							
2-*O*-Me-Gal	97.9	78.7	68.5	79.9	70.1	62.5	59.9
GlcA	103.8	74.5	77.2	79.8	77.4	176.9	
Xyl	101.2	71.9	84.0	69.3	62.2		
Observed ^{13}C chemical shifts (ppm) of fraction (e) at pH 7.8							
2-*O*-Me-Gal	97.1	79.1	69.1	79.3	71.9[a]	61.1	59.0
GlcA	103.7	75.1	76.9	77.8	78.0	176.0	
Xyl	101.1	72.3[a]	83.3	69.1	63.2		
Observed ^{13}C chemical shifts (ppm) of fraction (e) at pH 2.2							
2-*O*-Me-Gal	97.5	79.0	69.0	79.3	72.1[a]	60.9	59.2
GlcA	103.7	74.8	76.9	77.4	75.9	173.2	
Xyl	101.3	72.3[a]	82.9	69.0	63.3		
Observed ^{13}C chemical shifts (ppm) of carboxyl-reduced unfractionated *A. lyallii* polysaccharides (small signals observed due to incomplete reduction are not reported)							
2-*O*-Me-Gal	98.1	79.5	69.2	79.5	73.0[a]	61.6	59.7
Glc	103.7	75.0	76.9	77.6	76.0	62.2	
Xyl	101.2	72.3[a]	83.0	69.2	63.3		

[a] May be interchanged.

from the C-6 of the aldobiuronic acid was not resolved at neutral pH. The α and β effects of 4-*O*-glycosylation on the signals from the C-4, C-5, and C-3 of α-Gal*p*(1-CH_3) were determined using the spectra of α-Gal*p*-(1 → 4)-α-Gal*p*-(1-CH_3) (Bradbury & Jenkins, 1984), and applied to the C-4, C-5, and C-3 signals from α-2-*O*-Me-Gal*p*(1-CH_3) to predict the chemical shifts of a (1 → 4)-linked 2-*O*-methyl D-galactose residue. Similarly the α and β effects of 4-*O*-glycosylation on the signals from β-Gluc(1-CH_3) were determined using the spectra of α-Gluc(1 → 4)-β-Gluc(1-CH_3) and applied to the C-4, C-3, and C-5 of the aldobiuronic acid's glucuronic acid residue to approximate the effect on the NMR spectra of linking the 2-*O*-methyl D-galactose to the C-4 of the glucuronic acid (although due to the presence of the 2-*O*-methyl group the predicted chemical shifts may not be highly accurate). Table 8 reports the results of this analysis. Fig. 5a and b show the ^{13}C-NMR at different pH. Some small signals due to reducing residues are present in the NMR obtained at pH 7.8. These were due to the polysaccharide being held for about 24 h at 75 °C and pH 2 in order to obtain a ^{13}C-NMR and a HETCOR spectra. The two signals at 69.0 (pH = 2.2) and 69.1 (pH = 7.8) were not resolved in these two NMR spectra but they have been resolved in another ^{13}C-NMR spectrum of this fraction (spectra not shown). With the spectra assigned it is possible to discuss the effects of changing the pH, or reducing the glucuronic acid to glucose.

With a change of pH from 7.8 to 2.2 the anomeric signal from the 2-*O*-methyl D-galactose residue moved upfield 0.4 ppm and the *O*-methyl signal moved upfield 0.2 ppm. The effect on the anomeric signal of 2-*O*-methyl galactose was equivalent to the effect on the C-4 of the glucuronic acid residue. Upon reduction with carbodiimide, the anomeric signal from the 2-*O*-methyl D-galactose residue moved upfield 1 ppm, the *O*-methyl signal moved upfield 0.7 ppm, and the C-6 signal moved upfield 0.7 when compared to the non-reduced polysaccharide at pH 7. These results indicate that the 2-*O*-methyl D-galactose is adjacent to the glucuronic acid residue. An alternative explanation for these observed effects is that the reduction of the glucuronic acid or the change in pH both resulted in a change of environment for the 2-*O*-methyl galactose residue even though it is distant from the glucuronic acid residue. This seems to be an unlikely proposition, particularly as the chemical shifts of the xylose residue and those from the C-1, C-2, and C-3 of the glucuronic acid did not change significantly upon reduction, or a change in pH. It is concluded that the polysaccharide is predominantly of the structure [α-D-[2-*O*-Me-Gal*p*]-(1 → 4)-β-D-Glc*A*p-(1 → 3)-α-Xyl*p*- (1 → 4)-]$_n$.

The proton NMR was used to determine the relative amounts of the three major sugars in fraction (e). The relative ratio of glucuronic acid/xylose/2-*O*-methyl galactose was 1.0:1.0:0.9 (Table 7). The slightly smaller amount of 2-*O*-methyl galactose in the polysaccharide than xylose or glucuronic acid may indicate some type of irregularity. It is possible that the non-methylated galactose which was present at a level of about 17% of the methylated galactose was part of the same polysaccharide. However, the non-methylated galactose present in fraction (e) did not appear in the ^{13}C-NMR spectra. This may indicate that it is a component of a more rigid polysaccharide with high T_2 values.

4. Conclusions

Further research is needed in order to ascertain the structures of all of the polysaccharides of *A. lyallii*, and to determine the finer points of the structure of the 2-*O*-methyl galactose containing polysaccharide. However, it is clear from this work that its predominant structure is [α-D-[2-*O*-Me-Gal*p*]-(1 → 4)-β-D-Glc*A*p-(1 → 3)-α-D-Xyl*p*-(1 → 4)-]$_n$. This is a new polysaccharide, which according to convention can be labelled a galacto–glucurono–xylo-glycan.

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