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STRUCTURAL STUDIES OF THE WATER-SOLUBLE FUCAN FROM *Lessonia nigrescens*[*]

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ABSTRACT

Partial hydrolysis of the water-soluble "fucan" from *Lessonia nigrescens* gave monosaccharides, neutral and acidic oligosaccharides, a soluble polymer, and an insoluble residue. The neutral oligosaccharides, consisting solely of galactose, were separated into six fractions. The eight acidic oligosaccharides were inseparable mixtures, of which two were shown to contain glucuronic acid and fucose and the remainder to contain all the monosaccharides present in the original polysaccharide. The soluble polymer, which comprised 20% of the starting material and had a molecular weight of ~8000, had an acid stability and n.m.r. spectrum indicative of a linear polymer of ~44 alternating units of glucuronic acid and mannose.

INTRODUCTION

In an earlier publication[1], the carbohydrates of the brown seaweed *Lessonia nigrescens* harvested in Chile were described. The "fucans" consisting of fucose, galactose, mannose, xylose, glucuronic acid, and half ester sulphate severally extracted from the stipes + haptera and from the fronds were essentially similar. The "fucan"[***] extracted with hot water from one batch of powdered whole weed was therefore subjected to structural study.

RESULTS AND DISCUSSION

The composition of the "fucan" (2.6% of the dry weed) and the molar proportions of the monosaccharides in a formic acid-hydrolysate are given in Table I. Ninhydrin detected a number of amino acids, including serine, alanine, and

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[*] Carbohydrates of Brown Seaweeds, Part VII. For Part VI, see ref. 1. A brief account of this work was given at the meeting of the Royal Society of Chemistry Carbohydrate Group at Birkbeck College, London, in September 1981.

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[***] The term "fucan" connotes a glucuronomannogalactofucan.

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TABLE I

COMPOSITION OF "FUCAN"[a]

Carbohydrate	*Sulphate*	*Uronic acid*	*Protein*	*Moisture*	*Ash*
60%	9% (15%)	26% (43.3%)	11.4%	10%	12%

[a]Values are calculated on the weight of the "fucan", except those in brackets which are calculated on carbohydrate content

APPROXIMATE, RELATIVE MOLAR PROPORTION OF MONOSACCHARIDES IN A FORMIC ACID HYDROLYSATE

Monosaccharide	*G.l.c. on OV-225*		*G.l.c. on SE-30 (capillary)*	
	Molar prop.	*(%)*	*Molar prop.*	*(%)*
Fucose	6.0	(45)	7.0	(49)
Xylose	1.0	(7)	1.0	(7)
Mannose	2 9	(21)	2.5	(18)
Galactose	3.5	(27)	3.6	(26)

TABLE II

PROPERTIES OF GALACTO-OLIGOSACCHARIDES

Fraction	*Weight (mg)*[a]	R_{Glc}			*$[\alpha]_D^{25}$ in water (degrees)*	*Approximate d.p.*[3]
		A[b]	B	C		
1	15.3	0.05	0.04	0.04	+69	7
2	14.8	0.10	0.09	0.10	+37	5
3	17.8	0.15	0.12	0.18	+127	4
4	27.2	0.29	0.27	0.24	+54	3
5	14.7	0.42	0.40	0.43	+66	3
6	27.7	0.82	0.76	0.77	+45	2

[a]Absorbance read from a standard graph obtained using D-galactose. [b]*A*, 1-Butanol–ethanol–water (40:11:19); *B*, ethyl acetate–acetic acid–formic acid–water (18:3:1:4); *C*, 1-butanol–pyridine–water (6:4:3).

glycine, in a 12M-HCl hydrolysate, thus indicating that the nitrogen found in this extract was derived from protein.

Elution from a column[2] of Sepharose 4B indicated the "fucan" to be highly polydisperse with M_r 500,000 for the greater part of the material.

Hydrolysis of the "fucan" (8 g; 4.8 g of carbohydrate) with 0.25M oxalic acid for 3 h at 100° gave the highest yield of mono- and oligo-saccharides (3 g), together with a solid water-soluble polymer (1.2 g, 960 mg of carbohydrate) and an insoluble residue (163 mg). Elution of the mixture of mono- and oligo-saccharides from an anion-exchange resin with water gave neutral material (1.8 g of carbohydrate) and acidic material (with formic acid; 0.63 g of carbohydrate). From the neutral material, six galacto-oligosaccharide fractions were separated, each of which gave a distinct spot in p.c. (Table II) and was dextrorotatory (Table II).

TABLE III

METHYLATION ANALYSIS OF GALACTO-OLIGOSACCHARIDES

Fraction[a]	O-*Methylgalactose*	*Linkages*
1	2,3,4,6-tetra	(1→3), (1→6), (1→2)
2	2,4,6-tri	
3	2,3,4-tri	
4	3,4,6-tri	
5	2,3,4,6-tetra	(1→3), (1→6)
6	2,4,6-tri	
	2,3,4-tri	

[a] See Table II.

Fractions 1–6 were methylated and then hydrolysed, and the products were characterised as their alditol acetates by g.l.c. and g.l.c.–m.s. From these data, the linkages between residues in the oligosaccharides were deduced (Table III). Fractions 4 and 6 were mixtures and it is likely that the other fractions were also mixtures. Although these results do not reveal linkage sequences, they show that the "fucan" contains segments having seven or more galactosyl residues[3] with (1→2), (1→3), and/or (1→6) linkages.

The mass spectra of the alditol acetates derived from fractions 1 and 4 also contained peaks with *m/z* 59, 89, and 97, characteristic[4] of 1,4-di-*O*-acetyl-2,3,5,6-tetra-*O*-methylhexitol, thus indicating the presence of galactofuranose residues in the polymer.

From the action of α- and β-D-galactosidases on fractions 1–3, evidence for both α and β linkages in 1 and 2, and β linkages in 3, was obtained. This was confirmed for fractions 2 and 3 by ^{1}H-n.m.r. spectroscopy, which also indicated a small proportion of α linkages in fraction 3, but β linkages preponderated in both.

Earlier publications on polysaccharides extracted by acid from the brown seaweeds *Sargassum linifolium*[5] and *Padina pavonia*[6] reported the presence of the same five monosaccharide components as in the present "fucan", as well as glucose from the latter weed. The proportion of fucose was much lower and that of mannose higher than those found in the present "fucan". Similar partial-hydrolysis studies[5] of the *Sargassum* polysaccharide gave sulphated galactose oligosaccharides which, in contrast to the present oligosaccharides, were laevorotatory. Additional partial-hydrolysis studies of the *Sargassum*[5] and the *Padina*[6] polysaccharides gave laevorotatory neutral oligosaccharides which contained various proportions of galactose, xylose, and fucose, together with glucose from the *Padina* polysaccharide. In the present study, no evidence for such oligosaccharides was found.

Characterisation of the acidic oligosaccharides. — P.c. of the fractions eluted with 0.5M, M, and 2M formic acid in anion-exchange chromatography revealed similar patterns of oligosaccharides with a higher proportion of slower moving materials in the M and 2M fractions. Further resolution into eight fractions (7–14) was achieved by p.c.[R_{Glc} (solvent *B* of Table II) 0.03, 0.18, 0.27, 0.37, 0.54, 0.67, 0.84, and 1.0] of the material (130 mg of carbohydrate) eluted with 0.5M formic acid. Hydrolysis of each of these fractions, except 11 and 12, gave the five monosaccharide constituents of the polymer (*i.e.*, fucose, galactose, mannose, xylose, and glucuronic acid) in various proportions, indicating that they were complex mixtures of oligosaccharides. Fractions 11 and 12 contained glucuronic acid and fucose and were a tri- and di-saccharide, respectively.

The Egyptian workers[5,6] also isolated complex acidic oligosaccharides, each containing at least four monosaccharides, from partial acid hydrolysates of their polysaccharides and found complete characterisation to be impossible. They did not isolate any oligosaccharides containing only fucose and glucuronic acid.

Characterisation of the water-soluble and insoluble polymeric materials separated from the partial acid hydrolysate. — The soluble polymeric material (1.2 g) contained ~80% of carbohydrate (20% of the initial polysaccharide) and 12% of protein, gave 5.3% of ash, and had $[\alpha]_D^{25}$ −13°. P.c. of a hydrolysate revealed glucuronic acid and mannose. The molecular weight was estimated to be ~8000 by elution[2] of a sample from Sepharose 4B. Carboxyl-reduction of the glucuronic acid residues followed by methylation gave chloroform-soluble and water-soluble methylated material. G.l.c.–m.s. of the alditol acetates of the products of hydrolysis of these two fractions revealed two main peaks corresponding in retention time and fragmentation pattern to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methylglucitol and 1,2,5-tri-*O*-acetyl-3,4,6-tri-*O*-methylmannitol, thereby indicating that some of the glucuronic acid and mannose residues in the "fucan" were (1→4)- and (1→2)-linked, respectively. The water-soluble fraction also gave a peak corresponding to 1,2,3,4,5,6-hexa-*O*-acetylhexitol, indicating incomplete methylation. A

TABLE IV

^{13}C-N M.R CHEMICAL-SHIFT DATA FOR THE SOLUBLE RESIDUE AFTER PARTIAL HYDROLYSIS

Atom	*References[7,8] shifts*	*Observed shifts with tentative assignment*
C-6 of β-D-Glc*p*A		175.4
C-1 of β-D-Glc*p*A	104.3	104.4
C-1 of α-D-Man*p*	101.9	101.3
C-4 of 4-*O*-β-D-Glc*p*A	80.3	80.6
C-2 of 2-*O*-α-D-Man*p*	76.6	75.9
C-3 of β-D-Glc*p*A	76.5	75.5
C-5 of β-D-Glc*p*A	75.6	
C-2 of β-D-Glc*p*A	73.8	
C-5 of α-D-Man*p*	73.7	72 4
C-3 of α-D-Man*p*	71.8	
C-4 of α-D-Man*p*	68.0	69.7
C-6 of α-D-Man*p*	62.1	63.4

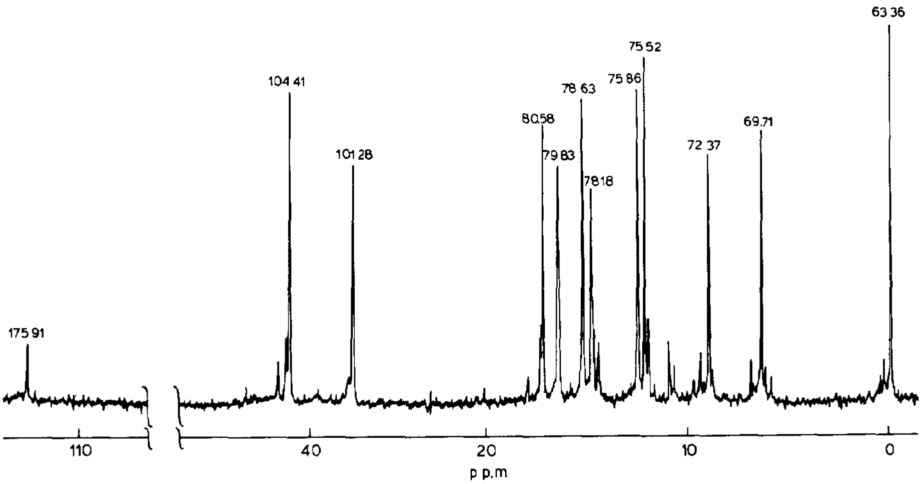

Fig. 1. ^{13}C-N.m.r. spectrum (100 MHz) of the water-soluble polymeric material separated from the partial acid hydrolysate. Chemical shifts are in p.p.m. from that of external 3-(trimethylsilyl)propanesulphonate.

glucuronomannan structure with alternating units of the two monosaccharides is supported by the ^{1}H- and ^{13}C-n.m.r. data. Clear features of the ^{1}H-n.m.r. spectra included (*a*) a single peak ($J \leqslant 5$ Hz) at δ 5.39 indicative of an equatorial H-1 and, hence, an α-D- or β-L-pyranose residue; (*b*) a doublet at δ 4.47 (J 7.8 Hz), the intensity of which (1 H) was similar to that of the peak at δ 5.39, indicative of an axial H-1,2 as in a β-D-glucopyranosyl residue in the $^4C_1$ conformation; (*c*) a peak at δ 4.15 (1 H) with a splitting of ~3 Hz corresponding to H-3 of a mannopyranosyl residue; (*d*) a quartet at δ 3.39 (1 H) with splittings of 8 and 9 Hz indicative of an axial proton coupled to two vicinal axial protons, *e.g.*, H-3 of a glucopyranosyl residue or H-4 of a mannopyranosyl residue in the $^4C_1$ conformations. These data suggested the presence of substituted β-D-glucosyluronic acid and α-D-mannosyl residues in the ratio 1:1.

The ^{13}C-n.m.r. spectrum (Fig. 1) showed 12 major peaks (Table IV), together with 17 minor peaks generally of intensity of <10%; the ^{13}C chemical shifts expected[7,8] for β-D-Glc*p*A and α-D-Man*p* units are also shown in Table IV, but the three peaks observed at 78.18, 78.63, and 79.83 p.p.m. could not be assigned. Furanosyl residues are unlikely since the material was isolated after acid hydrolysis, but *O*-substitution is a possibility.

The insoluble residue contained 10% of carbohydrate and 10% of protein, and gave 24% of ash. Of the carbohydrate, 30% was uronic acid. P.c. and g.l.c. of a hydrolysate revealed glucuronic acid, mannose, and glucose, with traces of fucose, xylose, and galactose.

Evidence for the presence of a backbone chain of D-glucosyluronic acid and D-mannosyl residues for the acid-extracted "fucan" of *Sargassum linifolium* has been advanced[5].

The present study of the partial hydrolysis of the *Lessonia nigrescens* "fucan" shows that, in addition to a glucuronomannan, there are up to seven adjacent galactose residues, some of the glucuronic acid residues are linked to fucose, and the majority of the fucose and xylose residues are on the periphery of the macromolecules.

The finding that the soluble and insoluble polymeric materials isolated after partial hydrolysis both contain protein and carbohydrate supports the previous suggestion[1] that protein and polysaccharide are closely associated in the seaweed and difficult to separate.

EXPERIMENTAL

The general methods have been described previously[9,10].

The seaweed *Lessonia nigrescens* was harvested in Chile and supplied as the dried weed by Alginate Industries Ltd. The powdered weed, fronds, stipes and haptera (770 g), after treatment with formaldehyde[11], was extracted with aqueous 2% $CaCl_2$ (4 × 2 L) for 5 h at 70°. After centrifugation, the extract was concentrated, dialysed, and freeze-dried, to give a white powder (20.3 g, "fucan").

Characterisation of the "fucan". — The analysis data for carbohydrate, uronic acid[12], sulphate, protein, ash, and moisture are shown in Table I. Samples were hydrolysed with formic acid[9] and with 12M HCl, and the hydrolysates were analysed by p.c. (solvents *B* and *C*) with detection by sprays *1*, *4*, and D-glucose and D-galactose oxidase[13] for the former, and ninhydrin for the latter. The "fucan" (150 mg of carbohydrate) was hydrolysed with formic acid[9] and the hydrolysate fractionated (solvent *C*) on Whatman 3MM paper into neutral and acidic components. The monosaccharides in the neutral fraction were converted into the alditol acetates and analysed by g.l.c. on an SE-30 WCOT column (25 m × 0.35 mm) operating at 165°, and on a 3% OV-225 column (3 m × 5 mm) operating at 205°.

An aliquot of the "fucan" (2 mg/mL) was layered on to a column (16.5 ×1.3 cm) of Sepharose 4B and eluted with M KCl. Fractions (2 mL) were analysed for carbohydrate.

Partial hydrolysis of the "fucan". — The "fucan" (8 g) was hydrolysed with 0.25M oxalic acid (300 mL) for 3 h at 100°. The hydrolysate was dialysed against deionised water (10 × 500 mL). The combined solutions outside the dialysis sac were concentrated to 500 mL, neutralised with $CaCO_3$, centrifuged, and concentrated, and the carbohydrate content of the residue was determined by the phenol–sulphuric acid method[9]. The absorbances were read from a graph of a mixture containing L-fucose–D-glucuronic acid–D-galactose–D-xylose–D-mannose in the ratios of 5:2:1.5:1:0.5.

The non-dialysable material, which consisted of soluble and insoluble material, was centrifuged, and the residue and supernatant solution were freeze-dried.

The dialysed fragments were eluted from a column of Zerolit FFIP SRA69 resin. Elution with water gave the neutral material, and the acidic oligosaccharides were eluted stepwise with 0.5M, M, and 2M formic acid. The carbohydrate contents of the fractions were determined. The neutral fraction was concentrated (to ~20 mL). Formic acid was removed from the acidic fractions by repeated distillation with methanol.

(a) Neutral oligosaccharides. The neutral fraction was applied to sheets of Whatman No. 17 paper and eluted with solvent *C* for 22 h, by which time the monosaccharides had travelled beyond the bottom of the paper. Areas corresponding to the individual oligosaccharides were excised and eluted with water, and the eluates were passed through a millipore filter (0.45 μm) and analysed for carbohydrate content. Each oligosaccharide was analysed by p.c. (solvents *A–C*), and its $[\alpha]_D$ value and d.p. were measured[3]. An aliquot of each oligosaccharide was hydrolysed with M H_2SO_4 (0.5 mL) in an atmosphere of CO_2 for 4 h at 100° and the cooled solution was neutralised[14] with 5% dioctylamine in chloroform. Aliquots of the hydrolysates were analysed by p.c. (solvents *A–C*). The remainder of the hydrolysates were reduced with $NaBH_4$. The derived alditols were acetylated and analysed[10] by g.l.c.–m.s. Each neutral oligosaccharide (~2 mg) was treated[15] with methyl iodide (0.2 mL) and dry silver oxide (0.2 mg) in *N,N*-dimethylformamide (0.2 mL). The methylated materials were hydrolysed, and the methylated sugars were converted into their alditol acetates and analysed by g.l.c. on a column of 3% of OV-225 and by g.l.c.–m.s.

Neutral oligosaccharide fractions 1–3 were each treated with α- and β-D-galactosidases[16], melibiose and lactose being used as standards. After incubation for 30 min, each solution was subjected to t.l.c. on silica gel F 1500 (Schleicher and Schüll), using ethyl acetate–pyridine–acetic acid–water (6:3:1:1) and detection with 4-aminobenzoic acid at 110–115° for 3 min. Solutions of fractions 2 and 3 in D_2O were subjected to 400-MHz ^{1}H-n.m.r. spectroscopy at 70° [external 3-(trimethylsilyl)propanesulphonate] and at 23° using the HOD signal as a reference.

(b) Acidic oligosaccharides. Portions of each acidic fraction were subjected to ionophoresis[17] at pH 6.8 and 10, and to p.c. [solvents *A–C* and 1-butanol–acetic acid–water (50:12:25), multiple elutions]. The best separation of oligosaccharides was obtained with the 0.5M formic acid fraction, using solvent *B*. The remainder of the material (~130 mg of carbohydrate) eluted with 0.5M formic acid was fractionated on Whatman 3MM paper (2 × 20-h developments, solvent *B*). Eight fractions were obtained.

(c) Soluble and insoluble polymeric materials. Each of these materials was analysed for carbohydrate, uronic acid, protein, and ash contents. Aliquots of soluble (30.7 mg) and insoluble (68.6 mg) materials were hydrolysed with 90% formic acid[9], and the products were analysed by p.c. (solvents *B* and *C*, spray *1*) and by g.l.c. of the derived alditol acetates. The molecular weight of the soluble residue was estimated by elution from a column of Sepharose 4B, as for the original "fucan".

The glucuronic acid residues in the soluble polymer (20 mg) were carboxyl-reduced[18], and the neutral product was methylated by the Hakomori method[19] as modified by Björndal and Lindberg[20]. The methylated material was dialysed against deionised water and then extracted with chloroform (2 × 150 mL). The organic and aqueous extracts were concentrated to dryness and each residue was hydrolysed with 2M trifluoroacetic acid in a sealed tube for 2 h at 120°. The methylated alditol acetates derived from the two hydrolysates were analysed by g.l.c.–m.s.

The soluble polymer was thrice shaken with D_2O and freeze-dried after each treatment; portions (30 and 60 mg, respectively) were then subjected to 400-MHz ^{1}H-n.m.r. spectroscopy at room temperature (external 4,4-dimethyl-4-silapentane-1-sulphonic acid) and 100-MHz ^{13}C-n.m.r. spectroscopy at 70° [external 3-(trimethylsilyl)propanesulphonate].

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