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Sulfated polysaccharides from the red seaweed *Georgiella confluens*

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Abstract

The water-soluble polysaccharides from *Georgiella confluens*, collected in Antarctica, were fractionated with cetrimide. The complexed material was subjected to fractional solubilization in solutions of increasing sodium chloride concentration. The initially extracted polysaccharide and the major fraction isolated, soluble in 0.5 M NaCl, were studied. These are sulfated xylogalactans naturally rich in methylated sugar residues, comprising of 3,6-anhydro-2-*O*-methyl-L-galactose, 2-*O*-methyl-L-galactose and 6-*O*-methyl-D-galactose. Structural analysis was carried out by methylation, ethylation, desulfation–ethylation, desulfation–methylation, Smith degradation, ^{13}C NMR spectroscopy and determination of the absolute configuration of monosaccharides by gas chromatography of diastereomeric derivatives produced by reductive amination. The results indicated the presence of an agaran backbone with an unusual substitution pattern: sulfation mainly at the 3-position of the α-L-galactose units and the presence of xylose side chains at the 4-position of the β-D-galactose residues.

Keywords: Polysaccharides, red algal; Polysaccharides, sulfated; Galactans, sulfated; Agarans; Structural analysis

1. Introduction

The major, matrix-phase polysaccharides extracted from marine red macroalgae are sulfated galactans. These galactans essentially consist of linear chains of alternating 3-linked β-D-galactopyranosyl and 4-linked α-galactopyranosyl units, which are classified either as carrageenans if the 4-linked residue is in the D configuration or agarans if the 4-linked residue is in the L configuration. This regular backbone is usually masked by different O-linked groups, particularly methyl ether, sulfate ester, pyruvate acetal or β-D-xylopyranosyl residues. Some of the α-galactopyranosyl units may also occur in the 3,6-anhydro form.[1,2] Agars are typically low in sulfate ester substitution, but those from numerous sources are rich in methyl ether or pyruvate acetal substitution. Conversely, carrageenans are comparatively rich in sulfate ester substitution but poor in methyl ether substitution, and rarely contain significant levels of pyruvate acetal substitution.[3]

Previous reports on polysaccharides from algae belonging to the Ceramiaceae indicated that *Campylaephora hypnaeoides*,[4] *Carpoblepharis flaccida*,[5] and *Euptilota formosissima*[6] biosynthesized sulfated agarans that could be converted to agarose by base treatment. At the same time, a high degree of methylation was observed in *E. formosissima*,[6] *Ceramium boydenii*,[7] and *Neoptilota asplenioides*[8] where 6-*O*-methyl-D-galactose units were found. Besides the highly methylated agarose, *N. asplenioides* also contained 3,6-anhydro-2-*O*-methylgalactose. Interesting results were obtained for *Ceramium rubrum*,[9] which yielded an agaran with 6-*O*-methyl-D-galactose, 2-*O*-methyl-L-galactose, 3,6-anhydro-L-galactose and 3,6-anhydro-2-*O*-methyl-L-galactose units. In this mucilage, a proportion of the anhydro sugar was replaced by non-sulfated L-galactose residues, with only about one in five of these units being 6-sulfated. The remainder of the sulfate was distributed on the D-galactose, mainly at the 6-position but with smaller amounts at the 2- and 4-positions.

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Table 1
Fractionation of the polysaccharides from *G. confluens*

Fraction	Redissolution concentration (M NaCl)	Yield [a] (%)
1 [b]		11.0
2	0.5	60.8
3	1.0	25.2
4	2.0	1.8
5	3.0	0.4
6	4.0	0.3
7 [c]	4.0	0.5

[a] Yields are given as percentages of the recovered (79.2% of CP).
[b] Soluble in aqueous cetrimide solution.
[c] Insoluble in 4.0 M NaCl.

Herein we report the structural analysis of polysaccharides extracted from the red seaweed, *Georgiella confluens*, which belong to the same family.

2. Results and discussion

G. confluens (Reinsch) Kylin[10] was collected during the summer of 1993 in the Jubany Base (Antarctica). The seaweed was extracted with water at room temperature, and the crude polysaccharide that was obtained (yield, 16.7%) was purified by redissolution in water to give CP (yield, 71.9% of the crude polysaccharide). Fractionation was carried out with cetrimide in aqueous solution, which precipitated the sulfated polysaccharides, and the insoluble complexes were subjected to fractional solubilization in solutions of increasing sodium chloride concentration. Table 1 shows the redissolution concentration and yields of the seven fractions isolated. The two major fractions, fractions 2 and 3, were further redissolved in sodium chloride solutions of the corresponding concentration to ensure exhaustive counterion exchange, giving fractions F2′ and F3′, respectively. Table 2 shows the molecular weight, sulfate content and monosaccharide composition of CP, F2′ and F3′. A significant amount of methylated monosaccharides was detected in these products (31–38%). The D-:L-galactose ratio and the absolute configuration of 6-*O*-methyl-D-galactose were determined by hydrolysis of the samples and derivatization of the monosaccharides with chiral 1-amino-2-propanol to the diastereomeric 1-deoxy-1-(2-hydroxypropylamino)alditols that were acetylated and further analyzed by GLC; for assignment of the configuration of 2-*O*-methyl-L-galactose, chiral 1-phenylethylamine was used.[11] Since hydrolysis previous to the reductive amination procedure prevents the determination of absolute configurations of 3,6-anhydrogalactose and 3,6-anhydro-2-*O*-methylgalactose units, they were assigned as belonging to the L-series from the ^{13}C NMR spectra (see below). As a result, the D-:L-unit ratios in the three native polysaccharides were consistent with an agaran backbone. This fact was further confirmed by ^{13}C NMR spectroscopy.

Fraction F2′ was submitted to ion-exchange chromatography on a DEAE Sephadex A-25 column, eluted with a stepwise increase in the NaCl concentration. The elution pattern is given in Fig. 1, while Table 3 shows yield, sulfate content, and monosaccharide composition

Table 2
Composition and molecular weight of CP, F2′, their desulfated and Smith-degraded derivatives

Sample	Sulfate (% $NaSO_3$)	Molecular weight	Monosaccharide composition (mol%)					
			Xyl	2-Me L-Gal	6-Me D-Gal	Gal	L-AnGal	2-Me L-AnGal
CP								
Native	12.6	13,500	13	7	19	47 (1.0) [a]	2	12
Desulfated	4.5	3200	13	6	23	46	3	9
Smith-degraded	8.7	2700	1	8	25	64	1	1
F2′								
Native	12.6	19,600	11	8	18	53 (1.2)	3	7
Desulfated	4.6	3400	11	8	22	52	1	6
Smith-degraded	12.0	4600	2	9	25	53	2	9
F3′								
Native	16.3	17,800	9	6	22	51 (1.5)	3	9

[a] Figures in parentheses indicate the D-:L-galactose ratio.

Table 3
Yield, sulfate content, and monosaccharide composition of the fractions obtained by ion-exchange chromatography of F2′ on DEAE Sephadex A-25

Fraction	NaCl (M)	Yield [a] (%)	Sulfate (% $NaSO_3$)	Monosaccharide composition (mol%)							
				Xyl	2-Me L-Gal	6-Me D-Gal	Gal	L-AnGal	2-Me L-AnGal	Man	Glc
F2′ [b]			12.6	11	8	21	45	2	7	2	5
F2′D1 [c]	0.0	14.5	4.1	15	6	12	51		3	2	8
F2′D2 [c]	0.3	4.4	12.7	12	5	16	50	2	6	3	5
F2′D3 [c]	0.3	3.4	19.0	11	4	13	52	2	4	5	7
F2′D4	0.3	2.2	19.0	15		12	54	2	5	7	5
F2′D5	0.5	34.1	14.2	13	5	24	37	2	14	2	3
F2′D6	0.5	10.3	14.7	14	5	25	35	2	14	2	2
F2′D7	0.7	15.2	14.7	10	6	22	48	2	8	2	2
F2′D8	0.7	12.6	17.5	12	6	23	43	3	8	3	2
F2′D9	1.0	3.2	19.8	21	5	14	31	4	7	13	5

[a] Percentages of the recovered (84.1%).
[b] Included for comparison.
[c] 3-Me Gal was detected in fractions F2′D1, F2′D2, and F2′D3 (3, 2, and 2% respectively).

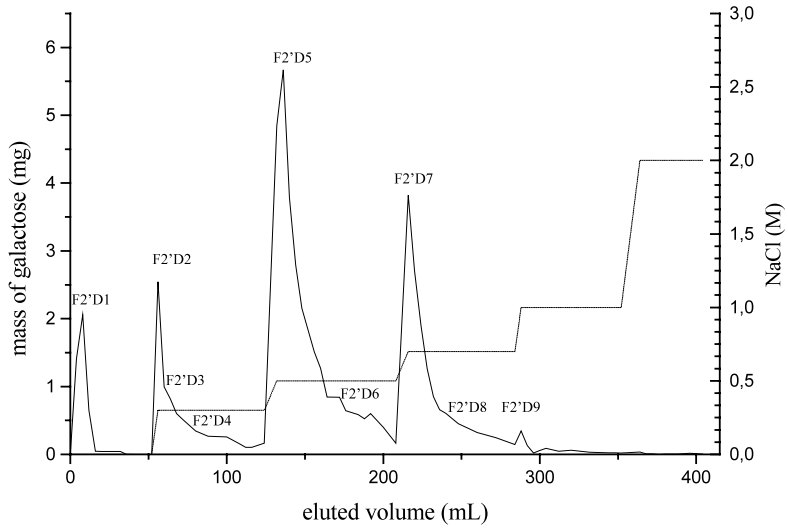


Fig. 1. Elution pattern of the ion-exchange chromatography of F2′.

Table 4
Methylation analysis (mol%) of native and desulfated CP; and native, desulfated and Smith-degraded F2′

	CP		F2′		
	Native	Desulfated	Native	Desulfated	Smith-degraded
2,3,4-Me_3 Xyl	4	5	8	8	
2,3,4,6-Me_4 Gal	3		5	7	
2,4,6-Me_3 Gal	26	30	23	33	33
2,3,6-Me_3 Gal	5	24	11	27	8
2-Me AnGal	4	10	6	5	8
2,6-Me_2 Gal	45 [a]	17 [b]	29 [c]	12 [d]	33 [e]
3,6-Me_2 Gal	2	4	2	4	
2,3-Me_2 Gal			4	2	2
2,4-Me_2 Gal	2	2	4	1	3
6-Me Gal	5	5	4	1	1
2-Me Gal	4	3	4		5

[a] 2,6-Di-*O*-methyl-D-:L-galactose ratio, 20:25.
[b] 2,6-Di-*O*-methyl-D-:L-galactose ratio, 12:5.
[c] 2,6-Di-*O*-methyl-D-:L-galactose ratio, 14:15.
[d] 2,6-Di-*O*-methyl-D-:L-galactose ratio, 10:2.
[e] 2,6-Di-*O*-methyl-D-:L-galactose ratio, 10:23.

of the fractions. Analysis of the isolated fractions revealed similar monosaccharide composition among them and the original product, with fractionation being based mainly on sulfate content. Small quantitative differences in 3,6-anhydro-2-*O*-methylgalactose and xylose contents led to the separation of fractions eluted with 0.5 (F2′D5 and F2′D6) and 0.7 M NaCl (F2′D7) with similar sulfate contents. It is noteworthy that fractions F2′D5 and F2′D6, accounting for 44.4% of the recovered, eluted at the same concentration of NaCl used to release F2′ from its cetrimide complex. Two minor fractions eluted with 0.3 M NaCl, F2′D3 and F2′D4, and exhibited higher sulfation levels than expected considering those of F2′D2 and F2′D5. Even though the reason for this is unclear, it can be seen that the monosaccharide composition of the three fractions eluted at 0.3 M NaCl showed an increase in galactose and manose content and a decrease in methylated galactose units, which might induce a conformational change on the backbone that may lead to the observed elution order. Discrepancy between the peak area of the chromatogram and the total recovered for these three fractions may arise from mass losses originated in subdivision of the sample.

Fractions F2′D1, F2′D5 and F2′D7 were studied by ^{13}C NMR spectroscopy, which provided similar spectra to that obtained with CP and F2′ (see below).

The elution profile of the ion-exchange chromatogra-

phy of F2′ suggests an unimodal distribution function, which is consistent with a polydisperse sample that is homogeneous in composition.[12] Then, structural complexity outlined should be intrinsic for the polysaccharide and not arising from contaminants.

It may be concluded that the previous fractionation procedure applied on CP, using cetrimide, was efficient and yielded an homogeneous, polydisperse F2′, which was further subjected to structural analysis. Native extracted polysaccharide, CP, was subjected to study at the same time.

Linkage analysis.—Due to the high percentage of methylated monosaccharides present in CP and in F2′, the samples were subjected to both methylation (Table 4) and ethylation (Table 5) analyses. Alkylation procedures gave compatible results indicating that: (a) xylose was present as single stubs; (b) 6-*O*-methyl-D-galactose units were 3-linked and two-thirds unsubstituted; (c) 3,6-anhydro-2-*O*-methyl-L-galactose units were 4-linked; (d) 2-*O*-methyl-L-galactose units were 4-linked and unsubstituted; and (e) the presence of 2,6-di-*O*-alkylgalactose could be ascribed either to 4-substituted, 3-linked D-residues or 3-substituted, 4-linked L-residues. The linkage pattern that gave rise to 2,6-di-*O*-ethylgalactose was determined as follows. Permethylated CP and F2′ were hydrolyzed and further subjected to the reductive amination procedure above mentioned, using 1-phenylethylamine as the chiral reagent[13] (Table 4). Final 2,6-di-*O*-ethyl-D-:L-galactose ratios were calculated taking into account that 2,6-di-*O*-methyl-D-galactose in the permethylated product would result in 2,6-di-*O*-ethyl-D-galactose and 2-*O*-ethyl-6-*O*-methyl-D-galactose in the perethylated one. At the same time, absolute configurations of monomethylated units were assigned. Association of these results led to the linkage pattern shown in Table 6 for CP and in Table 7 for F2′.

A general agreement between methylation and ethylation analyses can be observed, which indicated that the detected monomethylated units (for example, 2-*O*-methyl-L-galactitol pentaacetate derived from disubstituted, 4-linked L-galactose, Table 4) were not due to underalkylation. Besides, the fact that 3,6-anhydrogalactose content did not increase after methylation (by comparison of results in Tables 4 and 5 with those of the native samples), together with the substitution pattern proposed in Tables 6 and 7, is consistent with the absence of 3,6-anhydrogalactose precursors. It is also important to point out that the simultaneous presence of sulfate ester groups and xylose single stubs made impossible the straightforward assignment of the structure only by alkylation analysis.

Desulfation.—In order to determine unequivocally the glycosidic linkages and the substitution pattern in the backbone, it was necessary to carry out a desulfation–ethylation/desulfation–methylation analysis on the polysaccharides. Desulfation by acid methanolysis may give deep depolymerization of galactans with labile 3,6-anhydrogalactose units[2] and thus preclude its application on the samples. Therefore, the first approach was solvolytic desulfation, which involves heating the pyridinium salt of the sulfated polysaccharide in dimethyl sulfoxide–pyridine in the presence of

Table 5
Ethylation analysis (mol%) of native, desulfated and Smith-degraded CP; and native, desulfated and Smith-degraded F2′

	CP			F2′		
	Native	Desulfated	Smith-degraded	Native	Desulfated	Smith-degraded
2,3,4-Xyl	7	7		8	7	
2,3,4,6-Gal	2			1	3	2
2,4,6-Gal	8	16	17	10	14	15
2,4,VI-Gal [a]	16	16	16	14	15	15
2,3,6-Gal		13	4	5	13	4
II,3,6-Gal	3	5	3	5	5	4
II-AnGal	7	3	5	8	6	6
2,6-Gal	39	20	37	28	18	27
2,VI-Gal	5	5	3	3	5	5
3,6-Gal	2	5	1	3	4	2
2,4-Gal	1			4	5	6
6-Gal	4	2	2	4	2	2
VI-Gal	3	4		2	1	
2-Gal	3	4	5	5		
AnGal					2	4

[a] 2,4-Di-*O*-ethyl-6-*O*-methylgalactose.

Table 6
Linkage analysis (mol%) of native, desulfated and Smith-degraded CP

Deduced linkage and position of substitution	Native [a]	Desulfated [b]	Smith-degraded [a,c]
→3) D-Gal (1→			
Unsubstituted	8	15	17
4	14	8	8
6	1	2	
2,4	4	2	2
→3) 6-Me D-Gal (1→			
Unsubstituted	16	15	16
4	5	4	3
2,4	3	3	
→4) L-Gal (1→			
Unsubstituted		17	4
2	2	4	1
3	25	5	29
3,6	3	3	5
→4) 2-Me L-Gal (1→	3	7	3
→4) 2-Me L-AnGal (1→	7	10	5
T-Xyl [d]	7	5	
T-Gal [d]	2		

[a] Based on ethylation data.
[b] Based on methylation data.
[c] Out of a total of 93%.
[d] Terminal xylose and galactose.

Table 7
Linkage analysis (mol%) of native, desulfated and Smith-degraded F2′

Deduced linkage and position of substitution	Native [a]	Desulfated [b]	Smith-degraded [a,c]
→3) D-Gal (1→			
Unsubstituted	10	16	15
4	11	7	4
6	4	1	6
2,4	4	1	2
→3) 6-Me D-Gal (1→			
Unsubstituted	14	17	15
4	3	3	5
2,4	2		
→4) L-Gal (1→			
Unsubstituted	5	20	4
2	3	4	2
3	17	2	23
6		2	
3,6	5		
→4) 2-Me L-Gal (1→	5	7	4
→4) L-AnGal (1→		2	4
→4) 2-Me L-AnGal (1→	8	5	6
T-Xyl [d]	8	8	
T-Gal [d]	1	7	2

[a] Based on ethylation data.
[b] Based on methylation data.
[c] Out of a total of 92%.
[d] Terminal xylose and galactose.

methanol and/or water.[14–17] However, no significant elimination of sulfate was observed for the several conditions assayed (data not shown).

Taking into account the desulfation of sulfated monosaccharides mediated by silylating agents and the non-selectivity reported for chlorotrimethylsilane,[18] this reagent was tested on sulfated galactans. CP was treated with chlorotrimethylsilane in anhydrous pyridine at 100 °C for 8 h, and the desulfated derivative (sulfate content, 4.5% as $NaSO_3$) was obtained in 74.9% yield. For F2′ the yield was 56.1% and the sulfate content was 4.6%. Thus, for both samples the sulfate elimination was of 64%. It may be observed in Table 2 that the monosaccharide compositions of the original and desulfated products are similar. Desulfated CP and desulfated F2′ were subjected to methylation (Table 4) and ethylation (Table 5) analyses. In both desulfated derivatives a considerable increase of unsubstituted, 4-linked galactose, together with a concomitant significant decrease of 4-substituted, 3-linked D- and/or 3-substituted, 4-linked L-galactose were observed. Besides, a minor increase of unsubstituted, 3-linked galactose was detected. Both desulfated samples exhibited significant differences between alkylation results for two residues. In both cases, percentages of 2,3,6-tri-*O*-methylgalactose units were higher (24 for desulfated CP and 27 for desulfated F2′) than the calculated values for the sum of 2,3,6-tri-*O*-ethyl- and 3,6-di-*O*-ethyl-2-*O*-methylgalactose (18 for both). Simultaneously, lower percentages of 2,6-di-*O*-methylgalactose (17 and 12, for desulfated CP and desulfated F2′) than those expected for the sum of 2,6-di-*O*-ethyl- and 2-*O*-ethyl-6-*O*-methylgalactose (25 and 23, respectively) were obtained. Since selective desulfation is unlikely to occur during methylation, this fact would suggest selective underethylation at the 3-position of 4-linked L-galactose units, possibly related to conformational effects on less-charged, galactan chains. This suggestion is supported by the similar selectivity order reported for further benzylation of methyl 2,6-di-*O*-benzylgalactopyranosides, where the hydroxyl group at C-3 was less reactive than that at C-4 for the two anomers.[19]

Another portion of the permethylated products was hydrolyzed and subjected to reductive amination with 1-phenylethylamine to complete the resulting linkage pattern shown in Tables 6 and 7.

All the results up to this moment were consistent with an agaran backbone highly sulfated at the 3-position of 4-linked L-galactose units and with minor sulfation at the 4-position of 3-linked D-galactose residues.

Smith degradation.—In order to confirm the point of attachment of the xylose side chains, CP and F2′ were

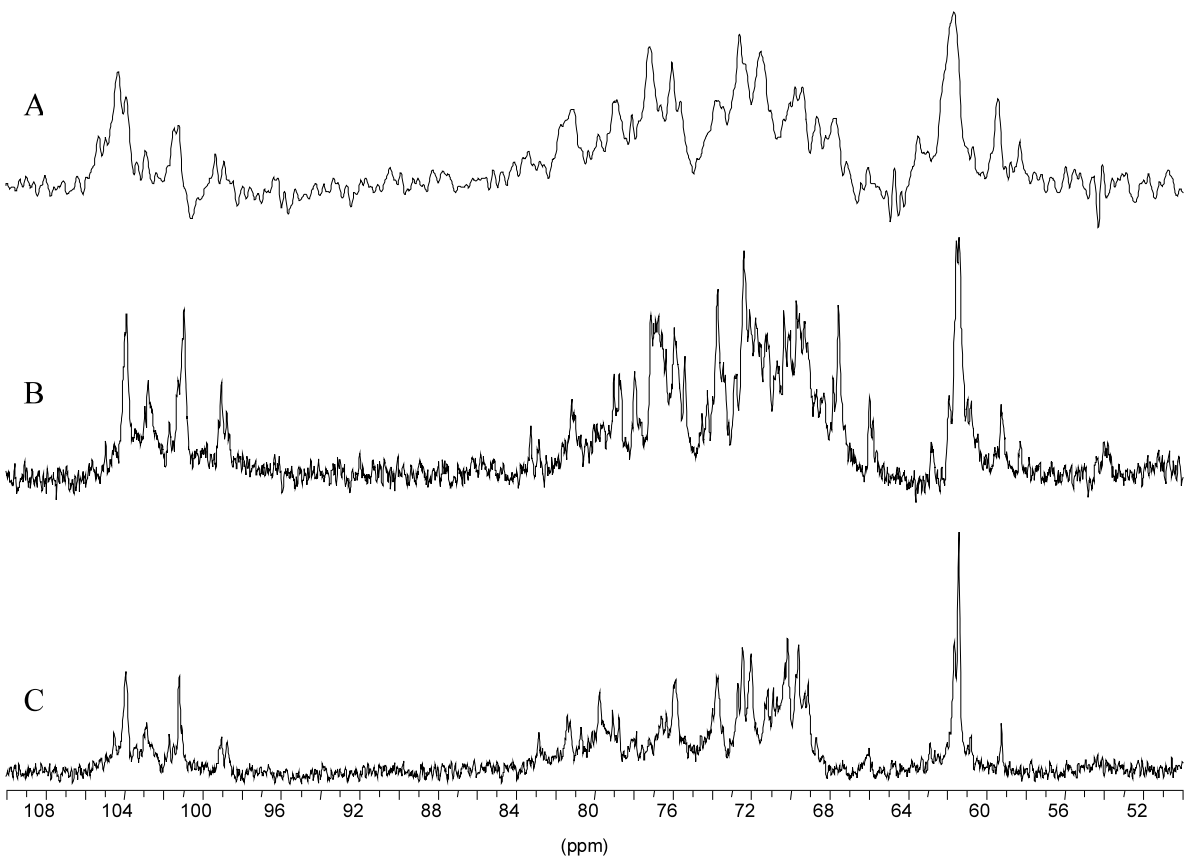


Fig. 2. Proton-decoupled ^{13}C NMR spectrum of (A) Smith degraded; (B) native; and (C) desulfated F2′.

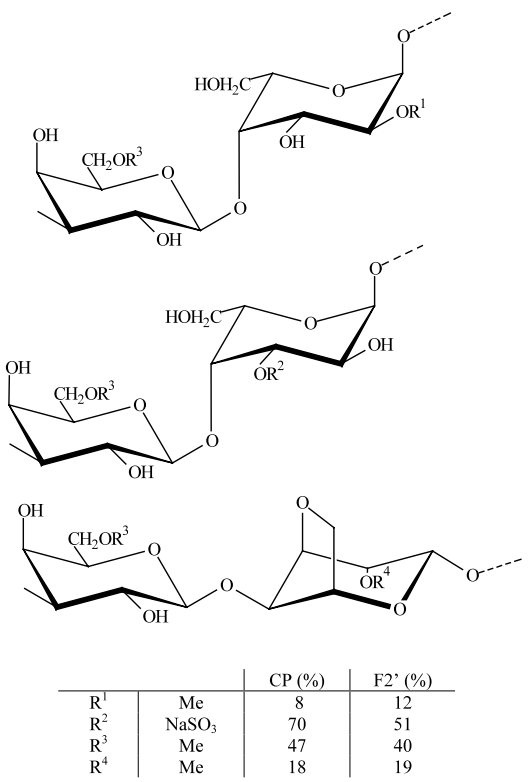
Table 8
Assignments (in ppm) of the ^{13}C NMR spectra of CP, F2′ and their desulfated derivatives

Unit	C-1	C-2	C-3	C-4	C-5	C-6	OMe
G	102.9	70.4	82.9	68.6	75.5	61.4	
G6M	102.9	70.4	82.9	69.1	73.8	72.1	59.2
LAG2M	98.7	79.1	78.7	77.9	75.9	69.6	59.3
G	103.9	70.1	81.3	69.1	75.9	61.6	
G6M	103.9	70.1	81.2	69.1	73.8	72.1	59.2
LG	101.3	69.6	71.3	79.0	72.1	61.4	
LG2M	99.0	79.1	69.7	79.7	72.7	61.4	59.2
LG3S	101.1	67.6–67.8 [a]	79.0	77.1	71.6	61.4	
Xyl	101.7	73.8	76.5	70.4	66.0		

[a] Depending on substitution of the residue and its neighbors.

subjected to Smith degradation,[20] giving Smith-degraded CP and Smith-degraded F2′ in 46.4 and 64.4% yield, respectively (Table 2). A loss of 3,6-anhydro-2-*O*-methylgalactose units and of sulfate groups was detected in CP but not in F2′.

Both Smith-degraded products were ethylated and methylated. As undermethylation was observed for Smith-degraded CP (data not shown), reductive amination was not applied, and the 4-substituted, 3-linked D-galactose:3-substituted, 4-linked L-galactose ratio was calculated on the basis of a 1:1 ratio for the D-:L-residues (Table 6). Reductive amination with chiral 1-phenylethylamine was carried out on permethylated Smith-degraded F2′ (Tables 4 and 7).



		CP (%)	F2' (%)
R^1	Me	8	12
R^2	$NaSO_3$	70	51
R^3	Me	47	40
R^4	Me	18	19

Fig. 3. Dominant proposed structures for the polysaccharides from *G. confluens* (percentages are based on substitution at indicated positions of α- or β-galactose residues).

The major change in the linkage analysis of the polysaccharides following Smith degradation was the significant increase of unsubstituted, 3-linked D-galactose. This could have arisen only from the loss of xylose side chains attached at O-4 of the 4-substituted, 3-linked D-galactose units. This observation is consistent with the linkage pattern deduced for desulfated samples, where the main substituted residues are 4-substituted, 3-linked D-galactose (Tables 6 and 7).

From all the results outlined above, it may be concluded that the major structural features of the highly methylated agarans from *G. confluens* are sulfation mainly at the 3-position of α-L-galactose, with a lesser amount at the 4-position of β-D-galactose, and the presence of xylose single stubs at the 4-position of β-D-galactose.

^{13}C NMR spectroscopy.—The ^{13}C NMR ^{1}H-decoupled spectra of CP and F2′ were very complex (Fig. 2), which is in accord with the complex substitution pattern obtained by linkage analysis. Direct interpretation of the spectra would have been impossible if complete chemical analyses of the samples were not previously achieved. On the contrary, important residues were assigned based on the knowledge of the complex substitution pattern present in these polysaccharides.

The observed resonances are consistent with an alternating structure of the agaran type, while the absence of any anomeric signals below 98 ppm indicated the lack of 4-linked D-galactose residues.

Distorsionless enhancement by the polarization-transfer (DEPT 135) pulse technique[21] was applied on F2′ to assist the assignment of the methylene carbons. Two signal groups, at 65–66 and 60.5–62 ppm, gave inverted peaks which corresponded to C-5 of xylose and C-6 of galactose residues, respectively.

From alkylation results, the diads to be found for these polysaccharides should include pairs formed by any of the following main D-residues: β-D-galactose (G), 6-*O*-methyl-β-D-galactose (G6M), β-D-galactose 4-sulfate (G4S), β-D-galactose 4-xylose (G4X), with another one of the following L-residues: α-L-galactose (LG), α-L-galactose 3-sulfate (LG3S), 2-*O*-methyl-α-L-galactose (LG2M), and 3,6-anhydro-2-*O*-methyl-α-L-galactose (LAG2M). Since resonances corresponding to the diads G4S-LAG[22] or G4S-LAG2M[23] were not observed, they were discarded from the possible linkage pattern. Desulfation simplified the spectra (Fig. 2) and allowed the assignment of some diagnostic signals[23–26] (Table 8), even though it was not possible to establish a unique diad composition for all the observed signal sets. However, the diads G-LAG2M[23] and G6M-LAG2M[24] were clearly identified.

The approach described by Miller and Blunt[27] was applied to calculate the contribution of sulfation at the 3-position, based either on the pair of matrices formed by the signal sets of: (a) methyl α-D-galactopyranoside 3-sulfate; methyl α-D-galactopyranoside[28] or (b) α-L-galactopyranose 3-sulfate; α-L-galactopyranose reported for sulfated α-L-galactans extracted from ascidians.[29] This contribution was added to the set of chemical shifts corresponding to LG in the diad G-LG or G6M-LG to give the 'calculated' chemical shifts for LG3S in these diads: 101.3; 67.6; 79.8; 77.3; 71.8; 61.2 ppm, for C-1–C-6, respectively (it was considered that no significant deviations would be observed for the β-units). The final assignments were made considering that: (a) observed chemical shifts should be close to those expected from calculations; and (b) these peaks should be more intense in spectra of the native products and weaker in those corresponding to the desulfated ones. In this way, it was noted that in the spectra of CP and F2′ there was a main signal at 101.1 ppm with a shoulder at 101.3 ppm, while in the spectra of desulfated CP and desulfated F2′ the intensities were reversed, giving a main signal at 101.3 ppm and a shoulder at 101.1 ppm. Thus, the upfield resonance was assigned to LG3S units and the downfield one to LG units. Two signals, at 67.6 and 67.8 ppm, disappeared on desulfation, and both could be assigned to C-2 of LG3S. Considering 3-substituted L-galactose and the D-galactose residues that could be linked to give a diad, as deduced from alkylation analyses, it seems that G6M-LG3S would be the most probable pair and G-LG3S, G4S-LG3S or G4X-LG3S should be less abundant. Assuming that the sum of the intensities of the signals at 67.6 and 67.8 ppm corresponded to the sum of 3-substituted L-galactose units (obtained from linkage analysis, Tables 6 and 7), the intensity ratio was applied to the total of these units to give an estimated percentage for the two kinds of residues having a different environment, which would give rise to the two different peaks. The molar percentages obtained for the signals at 67.6 ppm were coincident with the contents of unsubstituted 6-*O*-methyl-D-galactose in alkylation results; while the minor ones (corresponding to 67.8 ppm) were similar to the contents of unsubstituted D-galactose, 4-sulfated D-galactose or D-galactose with xylose linked to the 4-position (Tables 6 and 7). Thus, the signal at 67.6 ppm could be assigned to C-2 of LG3S in the diad G6M-LG3S, while the smaller signal at 67.8 ppm could be ascribed to C-2 of LG3S in the diad G-LG3S. The latter assignment is in agreement with evidence of 3-sulfation at α-L-galactose residues linked to β-D-galactose found in polysaccharides isolated from *Gymnogongrus torulosus*, where the signal at 67.8 ppm was observed.[30] The signal at 67.6–67.8 ppm may be considered as diagnostic for this unit, since in this region (68.5–66.5 ppm) there are usually only few signals. The complete carbon assignment of the LG3S unit is in agreement with the one calculated from matricial analysis.

Resonances of LG2M residues are coincident with those previously reported by Usov (with a constant displacement of ~ 0.4 ppm).[26] The ^{13}C NMR spectra of the Smith-degraded samples were also very complex (Fig. 2); unfortunately, it was impossible to detect disappearance of the peaks corresponding to G4X, but the absence of signals at 101.7 and 66.0 ppm was observed. The anomeric signal of β-D-xylopyranosyl residues was assigned to the peak at 101.7 ppm[26] and C-5 was ascribed to the peak at 66.0 ppm,[31] which was present in the spectra of the original and desulfated samples, while absent in those of the Smith-degraded products.

Spectra of F2′D1, F2′D5 and F2′D7, the main fractions isolated from F2′ by anion-exchange chromatography, were recorded. Fractions F2′D5 and F2′D7, with similar sulfate content and monosaccharide composition, gave similar spectra between them and F2′; while F2′D1, less sulfated and with the lowest content of 3,6-anhydrogalactose residues, exhibited two significant differences in the anomeric region. A more intense peak at 101.3 ppm and a lower one at 101.1 ppm were consistent with minor sulfate content, in particular, with less sulfation at the 3-position of the α-L-galactose units. The lack of resonances at 98–99 ppm reflected the low content of 3,6-anhydrogalactose and its 2-*O*-methyl derivative. The overall substitution pattern for these subfractions deduced from their spectra is consistent with the conclusion that F2′ is a polydisperse sample homogeneous in composition.

In conclusion, the polysaccharides isolated from *G. confluens* are highly methylated agarans, with a low 3,6-anhydrogalactose content, bearing xylose single stubs linked at the 4-position of β-D-galactose and sulfated mainly at the 3-position of α-L-galactose, with a lesser amount of sulfation at the 4-position of β-D-

galactose. Even though it was not possible to determine the residues linked to 4-substituted, 3-linked β-D-galactose, the other dominant proposed structures for this polysaccharides are shown in Fig. 3. Some evidence of the presence of 4-linked α-L-galactose 3-sulfate[26,32–36] residues and xylose single stubs linked to the 4-position of β-D-galactose[37,38] have been found; however, this is the first report on a polysaccharide exhibiting both unusual features in an agaran backbone.

3. Experimental

Extractions.—*G. confluens* was collected in Antarctica at the Jubany Base in January 1993 and dried in the open under strong winds. A voucher specimen (35,806) has been deposited in the herbarium of the Museo de Ciencias Naturales Bernardino Rivadavia (Buenos Aires, Argentina).

The seaweed (54 g) contained epiphytic diatoms which were eliminated by washing with 70% EtOH (3 × 1 L, during 1 h) and then under a strong stream of running tap water on a sieve (for a very short period of time). This treatment led to a material almost free of diatoms, as seen under a microscope.

The dried sample (28 g) was extracted with water (1.0 L) with mechanical stirring for 20 h at rt. The residue was removed by centrifugation, and the supernatant was dialyzed, concentrated, and freeze-dried. The residue was extracted (× 5); the crude products were pooled (4.7 g; yield 16.7%). A portion of the water-soluble polysaccharides (1.3 g) was dissolved in water (400 mL), and the solution was centrifuged, concentrated, filtered, and freeze-dried (CP, 935 mg).

Fractionation with cetrimide.—To a solution of CP (903 mg) in water (175 mL), a 10% (w/v) aq solution of cetrimide (9 mL) was added slowly with stirring, until no further complex formation occurred; stirring was continued for an additional 7 h. The precipitated complex was removed by centrifugation and suspended in 0.5 M NaCl (60 mL), and the stirring was continued overnight. The precipitate was centrifuged off, and the supernatant was extracted with 1-pentanol (3 × 30 mL), dialyzed, concentrated, and freeze-dried. The precipitate was subjected to successive similar procedures so that the concentration of NaCl was increased, in a first step, in 0.5 M, and then in 1.0 M each time (Table 1). The upper limit of NaCl concentration was 4.0 M, the residual precipitate was suspended in water, the suspension was dialyzed and freeze-dried.

The supernatant from the cetrimide precipitation was dialyzed, concentrated, and freeze-dried.

Fractions F2′ and F3′ were obtained from fractions 2 (386.3 mg) and 3 (121.3 mg) by redissolution in 0.5 (500 mL) and 1.0 M (200 mL) NaCl, respectively. These solutions were re-extracted with 1-pentanol, dialyzed, concentrated, and freeze-dried as described above for the original samples; yields: F2′, 310.1 mg, and F3′, 80.2 mg.

General methods.—Carbohydrate content was analyzed by the phenol–H_2SO_4 method[39] without previous hydrolysis of the polysaccharide. Sulfate was measured using the turbidimetric method of Dogdson and Price.[40] Molecular weights were calculated by the determination of reducing end-groups using the colorimetric method of Park and Johnson.[41] Unless otherwise stated, dialyses were carried out with tubing with a molecular weight cutoff of 3500 daltons.

Reductive hydrolysis of the samples and acetylation of the sugar mixtures was performed as described in Ref. 42. GLC was carried out on a Hewlett–Packard 5890A gas chromatograph equipped with flame-ionization detector and fitted with a fused-silica capillary column (0.25 mm i.d. × 30 m) WCOT-coated with a 0.20 μm film of SP-2330. Chromatography was performed: (a) from 160 to 210 °C at 2°C min^{-1}, then at 5°C min^{-1} from 210 to 240 °C, followed by a 30-min hold for alditol acetates and methylated alditol acetates; (b) with an initial 2-min hold at 180 °C, then at 1 °C min^{-1} to 210 °C, and from 210 to 230 °C at 2 °C min^{-1} followed by a 30-min hold, for methylated and ethylated alditol acetates. N_2 was used as the carrier gas at a flow rate of 1 mL min^{-1}, and the split ratio was 80:1. The injector and detector temperature was 240 °C. Conversion of GLC areas to molar basis was calculated for the partially methylated, partially ethylated, and partially ethylated methylated alditol acetates according to the effective carbon response theory;[43] for 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol a value of 0.64 was used.[42]

GLC–MS of the methylated and ethylated alditol acetates was carried out on a Shimadzu GCMS-QP5050A gas chromatograph/mass spectrometer working at 70 eV. Chromatography was performed on the SP-2330 capillary column using the programme temperature (a). The He total flow rate was 7 mL min^{-1}, the injector temperature 240 °C, and the split ratio 11:1. Mass spectra were recorded over a mass range 30–500 amu.

Samples were hydrolyzed with 2 M TFA at 121 °C for 2 h previous to the reductive amination which was carried out using (*S*)-1-amino-2-propanol or (*S*)-1-phenylethylamine, and the acetylated derivatives were analyzed by GLC and GLC–MS on an Ultra 2 column under the previously described conditions.[11,13]

For ^{13}C NMR spectroscopy, samples (10–20 mg) were dissolved in 1:1 water–D_2O (0.5 mL) and a 5-mm tube was used. The 75-MHz ^{13}C NMR ^{1}H-decoupled spectra of native CP and F2′ and their desulfated and Smith-degraded derivatives (except for Smith-degraded F2′) were recorded at 80 °C on a Bruker DPX 300 spectrometer using a spectral width of 9.1 kHz, 90°

pulse (5.5 μs), an acquisition time of 0.7 s, and a relaxation delay of 4.5 μs for 39,000–100,000 scans. The 50-MHz ^{13}C NMR ^{1}H-decoupled spectrum of Smith-degraded F2′ was recorded at rt on a Bruker AC 200 spectrometer using a spectral width of 11.1 kHz, 45° pulse (4.4 μs), an acquisition time of 0.4 s, and no relaxation delay for 298,000 scans. The 125-MHz ^{13}C NMR ^{1}H-decoupled spectra of F2′, F2′D1, F2′D4, and F2′D5 were recorded at rt on a Bruker AM 500 spectrometer using a spectral width of 29.4 kHz, 51.4° pulse, an acquisition time of 0.56 s, and a relaxation delay of 0.6 s, for 28,200–42,400 scans. Methylene carbons of F2′ were assigned by a DEPT 135 experiment.[21] This spectrum was recorded at 125 MHz and rt, on the Bruker AM 500 spectrometer using a spectral width of 26.3 kHz, an acquisition time of 0.56 s and a relaxation delay of 0.6 s for 5376 scans. In all cases, signals were referenced to internal Me_2SO at 39.6 ppm at 80 °C and at 39.5 ppm at rt.

Fractionation of F2′.—A column (1.0 × 10 cm) was filled with 630 mg of DEAE Sephadex A-25 (Cl^-), which had been previously stabilized with water and boiled for 2 h. F2′ (135 mg) was dissolved in water, which was used as the first eluant, then increasing concentrations of NaCl were applied; the upper concentration was 2 M. Fractions of 4 mL were collected, and the aliquots were assayed by the phenol–H_2SO_4 method,[39] using a galactose solution as reference for the carbohydrate content. After obtaining blank readings, the eluant was replaced by another with higher NaCl concentration. The eluting solution was fractionated as follows: F2′D1, from 0 to 16 mL; F2′D2, from 52 to 60 mL; F2′D3, from 60 to 80 mL; F2′D4, from 80 to 128 mL; F2′D5, from 128 to 164 mL; F2′D6, from 164 to 208 mL; F2′D7, from 212 to 236 mL; F2′D8, from 236 to 284 mL; F2′D9, from 284 to 360 mL.

Desulfation.—CP (34.3 mg) and F2′ (32.0 mg) were converted into their pyridinium salts (CP, 20.9 mg; F2′, 29.7 mg) and desulfated by treatment with chlorotrimethylsilane in anhyd pyridine at 100 °C for 8 h. After careful addition of water, the solutions were dialyzed against water, 0.1 M NaCl, and again water; further freeze-drying yielded desulfated CP (15.0 mg) and desulfated F2′ (16.3 mg). The application of this desulfation technique to other sulfated galactans will be described elsewhere.

Ethylation analysis.—CP (5.6 mg), desulfated CP (3.2 mg), Smith-degraded CP (6.4 mg), F2′ (8.8 mg), desulfated F2′ (10.3 mg) and Smith-degraded F2′ (7.1 mg) were converted into their corresponding triethylammonium salts and ethylated as described in Ref. 44. The ethylated derivatives were recovered by dialysis (molecular weight cutoff 1000 for desulfated or Smith-degraded derivatives) and freeze-drying. Yields: CP, 4.4 mg; desulfated CP, 2.6 mg; Smith-degraded CP, 2.5 mg; F2′, 6.9 mg; desulfated F2′, 6.4 mg; Smith-degraded F2′, 7.3 mg. In order to achieve complete alkylation, ethylated F2′ (4.3 mg) and Smith-degraded F2′ (4.5 mg) were subjected to a second ethylation procedure. Yields: F2′, 4.7 mg; Smith-degraded F2′, 2.4 mg.

Methylation analysis.—CP (4.4 mg), desulfated CP (8.1 mg), F2′ (9.5 mg), desulfated F2′ (4.2 mg) and Smith-degraded F2′ (6.8 mg) were converted into their corresponding triethylammonium salts and methylated by the Hakomori procedure (sodium methylsulfinyl-methanide–iodomethane)[45] modified by Stevenson and Furneaux.[42] The methylated derivatives were recovered by dialysis and freeze-drying. Yields: CP, 2.8 mg; desulfated CP, 6.4 mg; F2′, 7.5 mg; desulfated F2′, 2.0 mg; Smith-degraded F2′, 5.5 mg.

Smith degradation.—A solution of $NaIO_4$ (0.02 M, 20 mL) was added to a solution of the sulfated xylogalactan (CP, 51 mg; F2′, 49.7 mg, dissolved in 20 mL) and then stirred in the dark at rt until consumption of oxidant ceased (monitored by reading the absorbance at 223 and 305 nm). Excess of $NaIO_4$ was destroyed with ethylene glycol (0.5 mL), and the reaction mixture was dialyzed (molecular weight cutoff 1000), concentrated to about 10 mL and reduced overnight with excess of $NaBH_4$. The solution was acidified with HOAc, dialyzed with the same type of tubing and freeze-dried to give the oxidized and reduced polysaccharide (CP, 36.4 mg; F2′, 38.3 mg).[26] These products (CP, 32.4 mg; fraction 2′, 38.3 mg) were mixed with 2 mL of an aq solution of 4-methylmorpholine–borane (80 mg mL^{-1}) and 4 mL of 1.5% HOAc, and hydrolyzed at 100 °C for 2 h. After evaporation to dryness and redissolution, the residue was dialyzed with tubing of the same cutoff and freeze-dried to yield Smith-degraded CP (23.7 mg) and Smith-degraded F2′ (32.0 mg).

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