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Unusual structures in the polysaccharides from the red seaweed *Pterocladiella capillacea* (Gelidiaceae, Gelidiales)

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Abstract

Sequential extraction of tetrasporic *Pterocladiella capillacea* with water at room temperature and then at 50 °C led to the isolation of two products that were each fractionated with cetrimide to give a soluble fraction and a precipitate. The precipitates were then subjected to fractional solubilization in solutions of increasing sodium chloride concentration. The whole treatment yielded two major fractions in each case, one soluble in the cetrimide medium and the other soluble in 0.5 M NaCl, which were further fractionated by anion-exchange chromatography. Structural analysis, carried out by methylation, desulfation–methylation, ^{13}C NMR spectroscopy and determination of the absolute configuration of the 2,6-di-*O*-methylgalactose units in the permethylated products, indicated the presence of xylogalactans, with low content of 3,6-anhydrogalactose and low molecular weight. These polysaccharides varied in the level of xylopyranosyl and sulfate substitution, primarily on the 6-position of the 3-linked β-D-galactopyranosyl and on the 3-position of the 4-linked α-galactopyranosyl units. Moreover, herein we report, for the first time, the presence of 3-substituted, 4-linked D-galactopyranosyl residues in an alga belonging to the Gelidiales.

Keywords: Polysaccharide, red algal; Galactans, sulfated; Agarans; Galactans, D-galactose blocks; *Pterocladiella capillacea*; Gelidiales

1. Introduction

Red algal galactans generally consist of an alternating structure of 3-linked β-D-galactopyranose and 4-linked 3,6-anhydro-α-galactopyranose or α-galactopyranose. These galactans are classified in two groups: (i) carrageenans in which the 4-linked residue has the D-configuration; and (ii) agarans in which it occurs as the L-enantiomer.[1,2] Agaran substitution consists of sulfate ester, pyruvate acetal, methyl ether and single branching sugar residues such as 4-*O*-methyl-α-L-galactopyranose and β-D-xylopyranose. Carrageenans are considered to have low levels of methyl ether and pyruvate acetal substituents and high levels of sulfate ester groups.[3,4]

While carrageenans and agarans are respectively obtained from carrageenophytes and agarophytes, polysaccharides containing both agaran- and carrageenan-type backbones have been isolated from several red seaweeds belonging to the old order Cryptonemiales[5–8] (now some families have been forwarded to the order Gigartinales and the others to the order Halymeniales[9]). These polysaccharides have more complex structures than agarans and carrageenans, and they have not been fully characterized.

Although there are reports of the coexistence of agaran and carrageenan sequences in polysaccharides obtained from diverse carrageenophytes,[10–15] in the case of polysaccharides obtained from agarophytes, evidence of that coexistence was only found in *Digenea simplex*[16] and *Rhodomela larix*[17] belonging to the order Ceramiales.

Fractionation of an agar from *D. simplex* by ion-exchange chromatography led to the isolation of a sulfated galactan, which afforded, by further partial methanolysis, β-D-galactopyranosyl-(1→4)-3,6-anhy-

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dro-L-galactose dimethylacetal and β-D-galactopyranosyl-(1→4)-3,6-anhydro-D-galactose dimethylacetal, disaccharides that are characteristic of agaran and carrageenan structures, respectively.

Similar results were obtained after partial hydrolysis of a partial methanolysis-resistant moiety of the main polysaccharide fraction from *R. larix*, which gave sets of disaccharides and trisaccharides with an alternating structure of 3-linked β-D-galactopyranosyl and 4-linked α-L- or α-D-galactopyranosyl residues.

Thus far, there have been no reports of the presence of 4-linked D-galactose units in the polysaccharides from algae belonging to the Gelidiales. Herein we report the occurrence of 3-substituted, 4-linked D-galactose units in the polysaccharides from *Pterocladiella capillacea*.

2. Results and discussion

Our previous studies[18–20] on the polysaccharides from *P. capillacea* revealed that the major polysaccharide biosynthesized by this alga was agarose which represented ~19% on dry seaweed (~46% of the total polysaccharides extracted).

P. capillacea, which was carefully hand-sorted and examined in order to avoid contamination with endo- and/or exo-epiphytes (see Section 3), was newly subjected to sequential extraction with water at room temperature and 50 °C. The procedure gave two products, RTP and 50P, in total yields of 2.9 and 1.8% on dry seaweed, respectively. RTP and 50P were treated with cetrimide, and the precipitates were subjected to fractional solubilization in solutions of increasing sodium chloride concentration. As was previously reported,[18,19] two major fractions were obtained from each product: one soluble in cetrimide (RTCS and 50CS), and the other soluble in 0.5 M NaCl (RTCI and 50CI) (Table 1). The four fractions were further fractionated on DEAE Sephadex A-25 (Cl^-), eluting with water and solutions of increasing sodium chloride concentration (Figs. 1–3).

Evidence of the presence of 3-substituted, 4-linked D-galactose units in the polysaccharide system of *P. capillacea*, which are described below, were only found after fractionation of RTCI, RTCS and 50CI

2.1. Ion-exchange chromatography of RTCI

Chromatography of RTCI yielded two major fractions, one eluted with 0.5 M NaCl (RTCI05), and the other eluted with 0.7 M NaCl (RTCI07). Table 2 shows the yield and analyses of these fractions. Noteworthy is their low molecular weight determined by end-group analysis.

The D:L galactose molar ratio of RTCI05 and RTCI07 was 1.8:1.0 and 3.7:1.0, respectively; taking into account the 3,6-anhydro-L-galactose content (4% for both fractions) the total 'D:L' molar ratio was 1.6:1.0 for RTCI05 and 2.9:1.0 for RTCI07. These results indicated the presence of an excess of D-units in both fractions and suggested a deviation from the alternating D/L-structure characteristic for agarans.

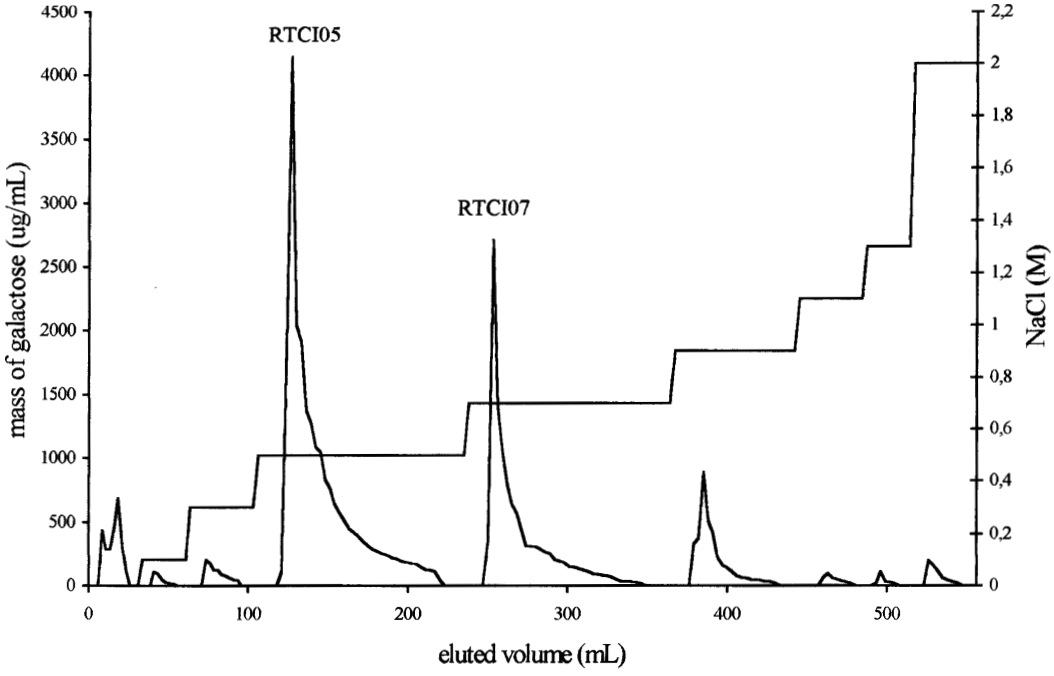

Fig. 1. Elution pattern of the ion-exchange chromatography of RTCI.

Table 1
Yields and analyses of the fractions obtained after treatment of RTP and 50P with cetrimide followed by fractional solubilization of the complexed material in sodium chloride solutions

Fraction	Range of redissolution (M NaCl)	Yield [a] (%)	Gal:AnGal:Xyl:sulfate (molar ratio)	Monosaccharide composition (mol%)						
				Gal	L-AnGal	3-Gal [b]	Xyl	Glc	Rha	Man
RTCS		41.2	1.00:0.22:0.14:0.17	58	13	3	8	6	8	4
RTCI	0–0.5	19.3	1.00:0.19:0.34:0.50	53	10	1	18	6	9	3
50CS		40.0	1.00:0.46:0.24:0.16	46	21	1	11	7	10	4
50CI	0–0.5	37.3	1.00:0.07:0.24:0.35	74	5	1	18	2		

[a] Yields are given as percentages of the recovered (RTP, 59.5% and 50P, 42.7%).
[b] 3-*O*-Methylgalactose determined after hydrolysis of the sample and further derivatization of the monosaccharides to the aldononitrile acetates.

Table 2
Yields and analyses of the fractions obtained by ion-exchange chromatography on DEAE Sephadex A-25 of RTCI, RTCS and 50CI

Fraction	Range of elution (M NaCl)	Yield [a] (%)	$[\alpha]_D$ (°)	Gal:AnGal:Xyl:sulfate (molar ratio)	Molecular weight	Monosaccharide composition (mol%)						
						D-Gal	L-Gal	3-Gal	L-AnGal	Xyl	Glc	Man
RTCI05	0.3–0.5	40.8	−45.7	1.00:0.06:0.25:0.21	3800	44	24	1	4	17	7	3
RTCI07	0.5–0.7	23.4	−6.2	1.00:0.06:0.23:0.29	6200	52	14	2	4	15	11	2
RTCS05 [b]	0.3–0.5	25.7	−19.6	1.00:0.11:0.52:0.06	7100	30	16	3	5	24	7	5
50CI05	0.3–0.5	46.0	−33.5	1.00:0.22:0.49:0.30	4600	31	22	5	12	26	4	
50CI07	0.5–0.7	34.7	+14.0	1.00:0.06:0.32:0.31	4500	58	11	1	4	22	4	

[a] Yields are given as percentages of the recovered (RTCI, 99.0%; 50CI, 99.0%; RTCS, 87.0%).
[b] Rhamnose (6%) and fucose (4%), were also detected.

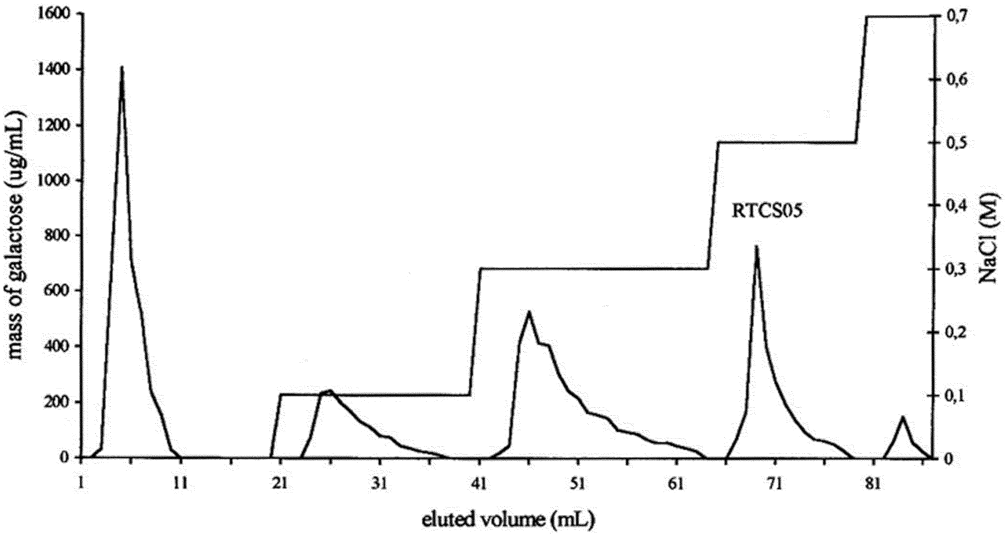

Fig. 2. Elution pattern of the ion-exchange chromatography of RTCS.

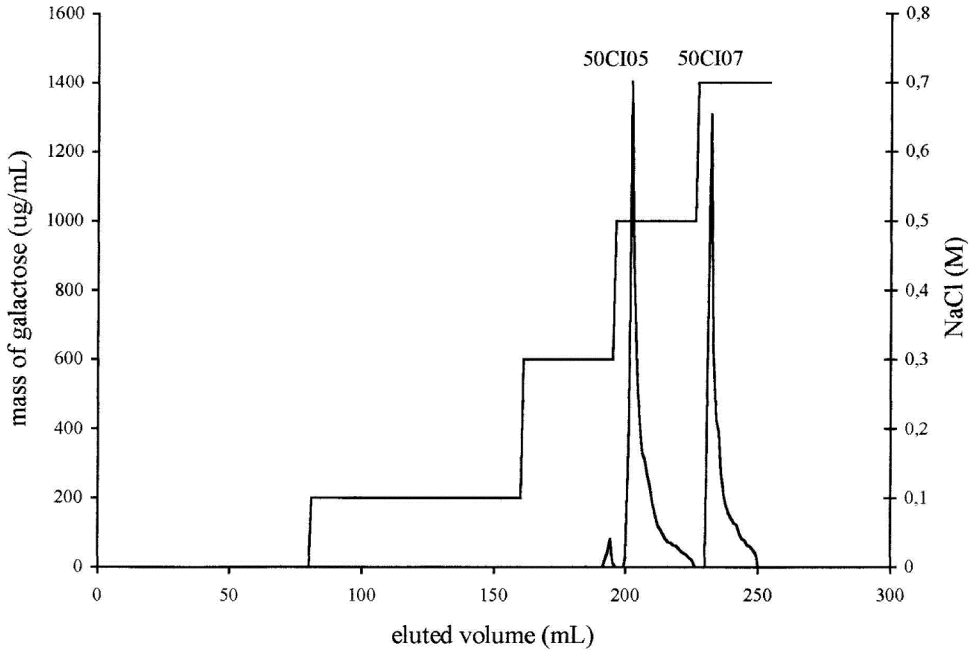

Fig. 3. Elution pattern of the ion-exchange chromatography of 50CI.

In order to establish the origin of the excess of D-units, RTCI05 and RTCI07 were subjected to methylation analysis, determination of the absolute configuration of the methylated monosaccharides, and ^{13}C NMR spectroscopy of the native samples.

Methylations were carried out according to the Hakomori[21] procedure modified by Stevenson and Furneaux.[22] Permethylated RTCI05 and RTCI07 were hydrolyzed, and the configuration of the 2,6-di-*O*-methylgalactose residues was assigned by applying the reductive amination procedure using (*S*)-1-phenylethylamine as the chiral reagent.[23]

The methylation patterns of both fractions were essentially the same, and the origin of most of the methylated monosaccharides detected was clear and according with that expected for a highly substituted alternating structure of 3-linked β-D-galactopyranosyl and 4-linked α-galactopyranosyl units (Table 3). However, the 2,6-di-*O*-methyl-D- and -L-galactose contents were different in both fractions: RTCI05 showed a lower amount of the D-isomer and a higher amount of the L-isomer than RTCI07. The origin of 2,6-di-*O*-methyl-D-galactose was ambiguous because it could be ascribed to 3-substituted, 4-linked D-galactose or 4-substituted, 3-linked D-galactose units. In both cases, the

excess of D-galactose units determined from the corresponding permethylated fractions (D:L-galactose molar ratio: RTCI05, 2.5:1.0; RTCI07, 4.1:1.0), was consistent with that determined for the native polysaccharides.

At this point, it was necessary to establish: (a) the presence of either 3-substituted, 4-linked D-galactose or 4-substituted, 3-linked D-galactose units and their substituent; (b) the substituent on C-6 of the 3-linked β-D-galactose units and on C-3 of the 4-linked α-L-galactose. Considering the similar contents of xylose and sulfate in both fractions (Table 2), substitution by one or the other would be equally probable.

RTCI05 was subjected to desulfation–methylation analysis and further determination of the absolute configuration of 2,6-di-*O*-methylgalactose. Desulfation was carried out by treatment of RTCI05 with chlorotrimethylsilane in anhydrous pyridine at 100 °C for 8 h,[24] and the desulfated derivative was then methylated.

The decrease in 2,6-di-*O*-methyl-D-galactose and the concomitant increase in 2,3,6-tri-*O*-methylgalactose in the desulfated fraction compared to the native polysaccharide (Table 3), indicated that a considerable proportion of the 2,6-di-*O*-methyl-D-galactose detected arose from 4-linked D-galactose 3-sulfate units. However, a minor amount of 3-linked D-galactose 4-sulfate residues should be present, in accord with the increase of the 2,4,6-tri-*O*-methylgalactose content in the permethylated product after the desulfation treatment.

On the other hand, as no significant change in the 2,6-di-*O*-methyl-L-galactose and 2,4-di-*O*-methylgalactose content was observed, xylose should be the substituent on C-3 of the 4-linked L-galactose units and on C-6 of the 3-linked D-galactose units. This result was in agreement with the observation in the ^{13}C NMR spectrum (not shown) of signals corresponding to β-D-xylose single stubs linked to C-6 of the 3-linked β-D-galactose units (see later for assignment).[25]

According with the literature[9] no departure from the α-(1→3)-, β-(1→4)-alternating structure has been proved up to the moment for red seaweed galactans. Within this context the 2,6-di-*O*-methyl-D-galactose units, detected in the methylation analysis, were distributed between the 3- and 4-linked residues to reflect an equimolar proportion of these residues. From this followed that the structure of RTCI05 was in agreement with the classical pattern in which ~36% of the α-units belonged to the D-series (Table 4).

In the case of RTCI07, considering the (1→3)-, (1→4)-alternating structure, almost all of the 2,6-di-*O*-methyl-D-galactose detected in the methylation analysis (Table 3) should derive from 3-substituted, 4-linked α-D-galactose units (Table 4). In addition, an important amount of the 3-linked galactose units were substituted on C-6. Considering the similar sulfate and xylose content of this fraction (Table 2), substitution by one or the other would be equally possible.

Unfortunately, the ^{13}C NMR spectrum (not shown) was noisy, and the only signals which were clearly observed were those corresponding to β-D-xylose single stubs linked to C-6 of the 3-linked β-D-galactose (see later for assignments). However, since xylose is mainly the substituent on C-6 of the 3-linked β-D-galactose and is probably also the substituent on C-3 of the 4-linked α-L-galactose (as in the case of RTCI05), then the major substituent of the 4-linked D-galactose should be sulfate, as in the other fraction.

Table 3
Methylation analysis (mol%) of RTCI05, desulfated RTCI05, RTCI07, RTCS05, 50CI05 and 50CI07 [a]

Monosaccharide	RTCI05	Desulfated RTCI05	RTCI07	RTCS05	50CI05	50CI07
2,3,4-Xyl [b]	11	8	11	7	11	12
2,3-Xyl				3		
2,3,4,6-Gal	3	5	2	3	10	3
2-AnGal	4	6	3	5	3	7
2,4,6-Gal	16	23	17	24	17	14
2,3,6-Gal	4	17	4	4	3	3
2,6-D-Gal	19	5	27	14	15	37
2,6-L-Gal	11	9	6	12	21	7
2,3-Gal	3		3			
2,4-Gal	18	18	19	14	4	13
6-L-Gal	4	5	2	8	9	
2-D-Gal	5	3	4	3	3	2
2-L-Gal	2	1	2	3	4	2

[a] No methylated derivatives of glucose were detected.
[b] 2,3,4-Tri-*O*-methylxylose.

Table 4
Linkage analysis (mol%) of RTCI05, desulfated RTCI05, RTCI07, RTCS05 and 50CI07

Deduced linkage and position of substitution	RTCI05	Desulfated RTCI05	RTCI07	RTCS05	50CI07
→3) D-Gal (1→					
Unsubstituted	16	23	17	24	14
4	3		3	2	12
6	18	18	19	14	13
4,6	5	3	4	3	2
→4) L-Gal (1→					
Unsubstituted	4	6	4	4	3
3	11	9	6	12	7
6	3		3		
2,3	4	5	2	8	
3,6	2	1	2	3	2
→4) D-Gal (1→					
Unsubstituted		11			
3	16	5	24	12	25
→4) L-AnGal (1→	4	6	3	5	7
T-Xyl [a]	11	8	11	7	12
→4) D-Xyl (1→				3	
T-Gal [a]	3	5	2	3	3

[a] Terminal xylose and galactose.

2.2. Ion-exchange chromatography of RTCS

Fractionation of RTCS led to the isolation of RTCS05, eluting with 0.5 M NaCl. Table 2 shows the yield and analyses of this fraction. Its D:L galactose molar ratio was 1.9:1.0 and, taking into account the 3,6-anhydro-L-galactose content (5%), the total 'D:L' molar ratio was 1.4:1.0 This result indicated, as in the case of the fractions described above, that there was an excess of D-galactose residues in RTCS05, suggesting again a deviation from the D/L-structure characteristic for agarans.

In order to determine the origin of the excess of D-units, RTCS05 was investigated by methylation analysis, including determination of the absolute configuration of the methylated monosaccharides, and ^{13}C NMR spectroscopy of the native fraction.

The methylation pattern of RTCS05 (Table 3) indicated that the percentage of xylose side-chains (7%), could have been underestimated in this study, considering that the percentage of xylose detected in the monosaccharide analysis of the native fraction (Table 2) was much higher (24%). Similar discrepancies have been observed previously and attributed to the volatility of the acetylated 2,3,4-tri-*O*-methylxylitol that is lost, in part, during the sample workup for GLC and GLC–MS analysis.[14,26]

The origin of most of the methylated monosaccharides was clear and in accord with that expected for a permethylated, highly substituted agaran but, as in the case of RTCI05 and RTCI07, a high percentage of 2,6-di-*O*-methyl-D-galactose residues was detected (14%). As was mentioned above, these residues could be ascribed either to 3-substituted, 4-linked D-galactose or 4-substituted, 3-linked D-galactose units, but taking into account the classical alternating structure, almost all the 2,6-di-*O*-methyl-D-galactose should originate from 4-linked D-galactose units substituted on C-3.

The 125-MHz, ^{1}H-decoupled ^{13}C NMR spectrum of this fraction (Fig. 4) showed a signal at 105.3 ppm. The chemical shift of this signal is coincident with that reported for the C-1 of 3-linked β-D-galactose units belonging to the β-D-galactose→α-D-galactose diad in which the α-D-galactose units were substituted on C-3.[27] In the range expected for the resonance of the 4-linked α-D-units (91–96 ppm),[28] a signal at 95.6 ppm was observed.

Considering the methylation pattern and the xylose and sulfate content (galactose:xylose:sulfate molar ratio, 1.00:0.46:0.06) of this fraction, it was assumed that xylose was the main substituent on C-3 of the 4-linked α-D- and α-L-galactose units, and on C-6 of the 3-linked β-D-galactose units.

Substitution with β-D-xylose single stubs on C-6 of 3-linked β-D-galactose units was confirmed by the observation, in the ^{13}C NMR spectrum, of the signals corresponding to C-1–C-5 of these units, at 104.6; 74.0; 76.6; 70.3 and 66.1 ppm, respectively (Fig. 4).[25] Besides,

signals at 103.2; 71.5; 73.5; 69.7; 76.6 and 62.0 were assigned to C-1–C-6, respectively, of β-D-galactose non-reducing terminal residues linked to 4-linked 3,6-anhydro-α-L-galactose.[29]

Moreover, signals corresponding to C-1–C-6 of 3-linked β-D-galactose units in the 3-linked β-D-galactose → 3-substituted, 4-linked α-L-galactose diad, were also observed at 103.9; 70.3; 81.2; 69.7; 76.0 and 61.5 ppm, respectively.[30] Signals at 101.8 and 101.6 ppm were assigned to the C-1 of the 3-substituted, 4-linked α-L-galactose and the β-D-xylose residues, respectively.[31]

Thus, the results were consistent with the linkage pattern shown in Table 4, with ~30% of the 4-linked α-galactose units belonging to the D-series.

2.3. Ion-exchange chromatography of 50CI

Chromatography on DEAE Sephadex A-25 (Cl^-) yielded, as in the case of RTCI, two major fractions, one eluted with 0.5 M NaCl (50CI05), and the other eluted with 0.7 M NaCl (50CI07). Table 2 shows the yield and analyses of these fractions.

Both fractions were subjected to methylation analysis; Table 3 shows the carbohydrate composition of the permethylated products.

The contents of 3,6-anhydro-2-*O*-methylgalactose and 2,4-di-*O*-methylgalactose in permethylated 50CI05 were considerably lower than those expected from the composition of the native polysaccharide (Table 2). This decrease could be attributed to the loss of fragments during the dialysis step of the methylation procedure. Accordingly, in the ^{13}C NMR spectrum of 50CI05 (Fig. 5, Table 5), the resonances due to the agarobiose diad[32] were clearly observed together with the peaks corresponding to β-D-xylose linked to C-6 of the 3-linked β-D-galactose units; in addition, signals corresponding to C-5 and C-6 of the latter residues were also observed.[28] Table 5 also shows the assignment of the 3-linked β-D-galactose and 4-linked α-L-galactose residues that are conforming a β-D-galactose → α-L-galactose diad.[30]

The 2,6-di-*O*-methyl-L-galactose detected in the methylation analysis derived from 4-linked α-L-galactose units substituted on C-3. The resonance of the C-2 of the 4-linked α-L-galactose 3-sulfate residues occurs at 67.9 ppm[24] and, at this chemical shift, only a small signal was observed indicating that the α-L-galactose units were mainly substituted with xylose, and signals at 101.7 and 101.5 ppm were assigned to the C-1 of the 3-substituted, 4-linked α-L-galactose and the β-D-xylose residues, respectively.[31]

As the total 'D:L' molar ratio of 50CI05 was in agreement with an agaran backbone, the 2,6-di-*O*-methyl-D-galactose units should derive mainly from 4-substituted, 3-linked β-D-galactose units. Taking into account the important levels of substitution with xylose on C-6 of the 3-linked β-D-galactose and on C-3 of the 4-linked α-L-galactose units, substitution with sulfate would be more likely.

In the spectrum of 50CI05, a broad signal at 96.9 ppm was clearly observed, and this chemical shift was not only coincident with that reported for the C-1 of the terminal reducing β-galactose,[29] but also with that observed in the spectrum of RTCI05, which contained 4-linked α-D-galactose 3-sulfate units. It is possible that the C-1 resonance of these latter units also occurs at 96.9 ppm. Overlapping of signals is in agreement with

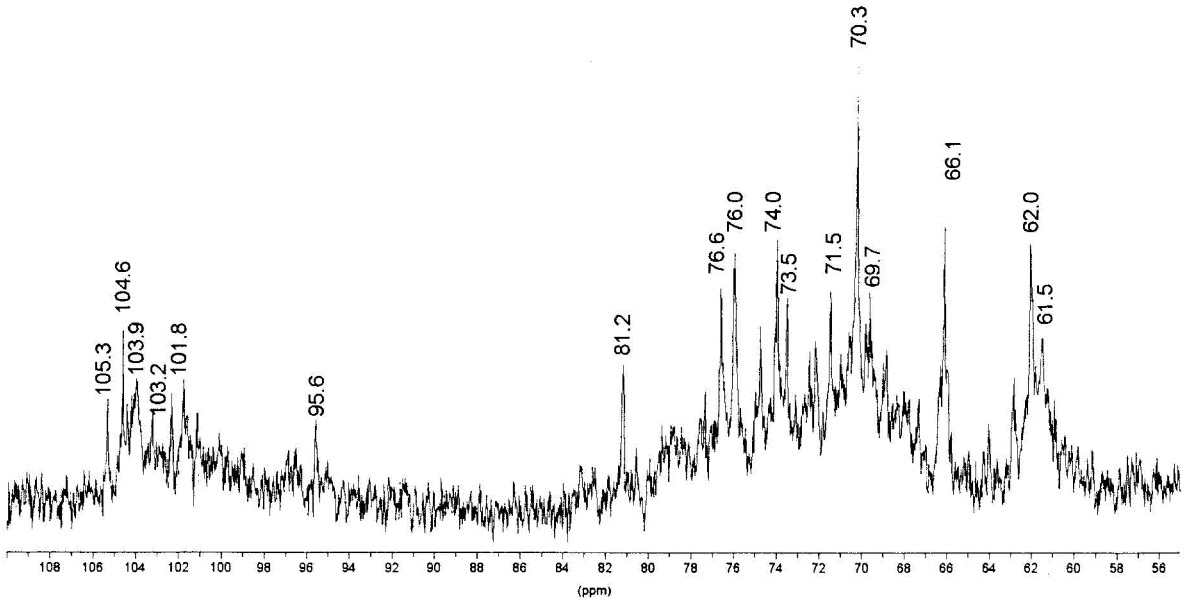

Fig. 4. ^{13}C NMR spectrum of RTCS05.

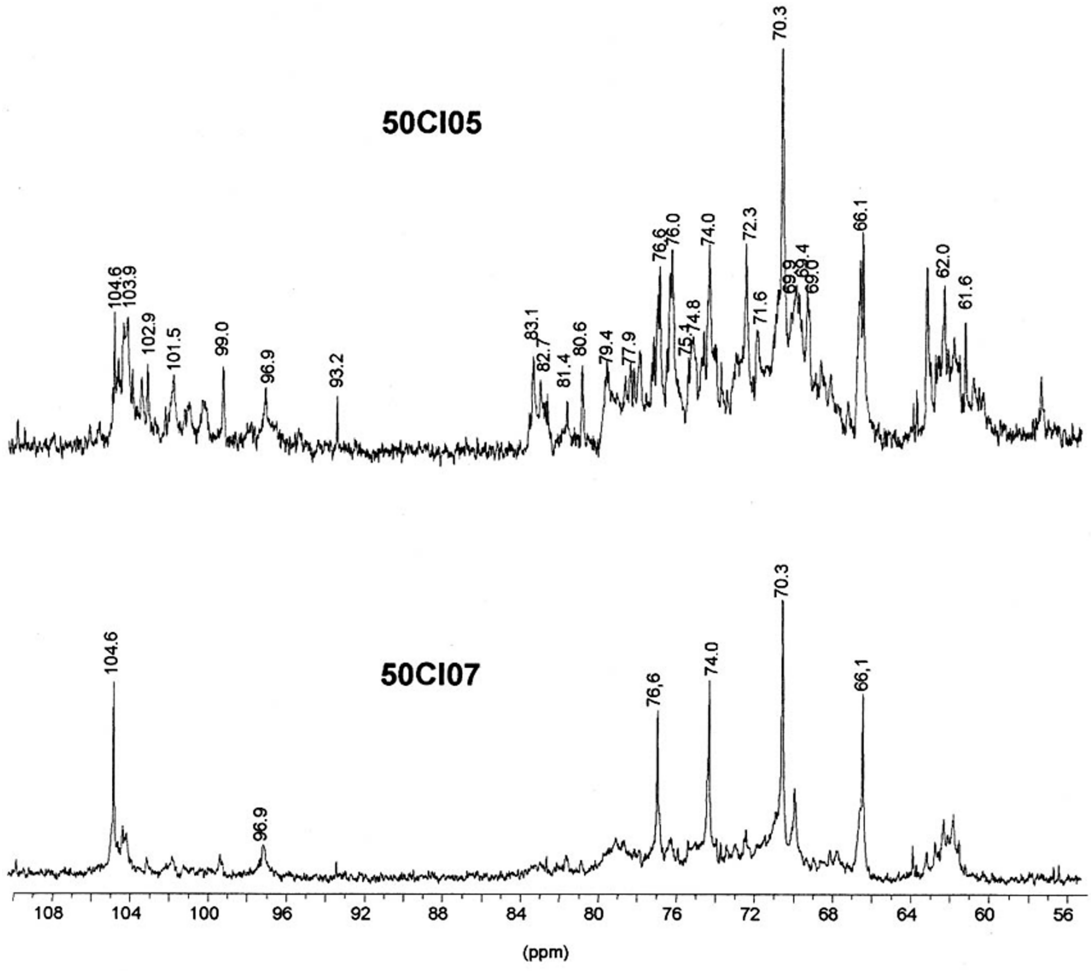

Fig. 5. ^{13}C NMR spectra of 50CI05 and 50CI07.

the width of this peak (Fig. 5). Thus, at least a minor proportion of 4-linked α-D-galactose 3-sulfate residues would be present. It is noteworthy that Sen Sr. and co-workers reported recently[33] the ^{13}C NMR spectrum of a sulfated polysaccharide fraction from *Grateulopia indica* Boergensen in which an important signal at 96.8 ppm was found. Although this signal was not assigned, it was suggested that it could arise from C-1 of 4-linked α-D-galactose in a carrageenan-type structure. The fact that this signal disappeared in the spectrum of the desulfated fraction was strongly indicative that this unit was sulfated. This result supports our assignment of the signal at 96.9 ppm to the C-1 of 4-linked α-D-galactose 3-sulfate residues.

Structural analysis of 50CI07 was particularly relevant since the great excess of D-galactose units detected clearly showed the presence of D-galactose blocks in the polysaccharide system from *P. capillacea*. The D:L-galactose molar ratio (5.3:1.0) of the native fraction was consistent with its positive optical rotation,[15,34,35] (Table 2) and was in agreement with the value (5.7:1.0) determined by the reductive amination procedure on the permethylated sample.

Presuming that the α-(1→3)-, β-(1→4)-alternating structure was maintained, ~68% of the 2,6-di-*O*-methyl-D-galactose units would derive from 3-substituted, 4-linked D-galactose units (Tables 3 and 4). Moreover, according with the methylation pattern, substitution was mainly on C-6 of the 3-linked β-D-galactose and on C-3 of the 4-linked D- and L-galactose residues. The ^{13}C NMR spectrum of 50CI07 (Fig. 5) showed, besides the resonances corresponding to β-D-xylose single stubs linked to C-6 of the 3-linked β-D-galactose units, a broad signal at 96.9 ppm and, as in the case of the spectra of RTCI05 and 50CI05. This signal could include the anomeric resonance of α-D-galactose 3-sulfate units.

The substitution pattern was not completely deduced but, considering that the contents of xylose and sulfate were similar and that the 3-linked β-D-galactose residues were mainly substituted on C-6-with xylose, a considerable number of the 4-linked α-D-galactose units would be substituted on C-3 with sulfate.

In conclusion, in the polysaccharides from *P. capillacea* that were studied—all of them of relatively low molecular weight—in addition to the agaran sequences with a high degree of substitution with β-D-xylose single stubs on C-6 of the 3-linked β-D-galactose residues and on C-3 of the α-L-galactose units, 3-substituted, 4-linked D-galactose residues were detected. These unusual units would be substituted with sulfate in RTCI05, RTCI07, 50CI05 and 50CI07, and with xylose in RTCS05.

This is the first time that evidences of the presence of 4-linked D-galactose units in polysaccharides from an alga belonging to Gelidiales are reported. It is important to point out that, in view of the high proportion of agarose in the polysaccharide system of this alga, it would not have been possible to detect these unusual units without the sequential extraction, followed by the exhaustive fractionation of the crude products. Moreover, the use of new methodologies, such as the reductive amination procedure on permethylated polysaccharides, was of great value for investigating this type of structure.

3. Experimental

3.1. Materials

P. capillacea was collected in La Paloma (Departamento de Rocha, Uruguay), dried in the open, carefully hand sorted and examined in order to avoid contamination with epiphytes and endophytes. Thalli of the seaweed were washed with filtered seawater and analyzed for epiphytic and epizoic contaminants in a Nikon AFX-II macroscope (Nikon, Japan). Anatomical observation was made based on dry material rehydrated in seawater. For checking the absence of any kind of endophytes, cross sections of the thallus were obtained manually with a single-edge razor blade at different distances from the apical zone. Photomicrographs were taken on a Zeiss Axioplan microscope (Zeiss, Germany). In the cases where the epiphytes were abundant, the algal material was discarded.

A voucher specimen (B.A. 40.463) has been deposited in the herbarium of the Museo de Ciencias Naturales Bernardino Rivadavia (Buenos Aires, Argentina).

The tetrasporic plants were washed with water (1 L) containing NaOCl (1 mL of a solution with maximum available chlorine of 6%) and dried before extraction.

3.2. Extractions

Dried, milled plant material (66 g) was extracted with H_2O (3 L), containing 3 mL of the NaOCl solution with mechanical stirring for 4 h at room temperature (rt). The residue was removed by centrifugation, and the supernatant was dialyzed (molecular weight cutoff of 3500 Da), concentrated and freeze-dried; the residue was extracted ($\times 4$) with H_2O. The crude products were pooled (2.5 g) and dissolved in H_2O (500 mL), leaving an insoluble residue that was centrifuged off, suspended in H_2O and freeze-dried (0.6 g). The supernatant was concentrated and freeze-dried (RTP, 1.9 g; 2.9% on dry seaweed, 7.0% on the total polysaccharides extracted).

Table 5
Assignments (in ppm) of the ^{13}C NMR spectrum of 50CI05

Residue [a]	C-1	C-2	C-3	C-4	C-5	C-6
Diad						
G	102.9	70.6	82.7	69.4	75.9	62.0
LA	99.0	70.3	80.6	77.9	76.0	69.9
G	103.9/104.0	70.3	81.4	69.4	75.9	61.6
L	101.7	69.7	71.6	79.4	72.3	61.6
G6X	103.9/104.0	70.3	81.4	69.4	74.8	69.6
L	101.7	69.7	71.6	79.4	72.3	61.6
Terminal units						
X [b]	104.6	74.0	76.6	70.3	66.1	
Gnr [c]	103.2	71.6	73.7	69.6	76.5	61.8
Grβ [d]	96.9	71.6	83.1	69.0	75.1	61.8
Grα [d]	93.2	68.3	79.4	69.9	70.6	62.0

[a] Nomenclature according to Knutsen and co-workers.[2]
[b] β-D-Xyl in G6X.
[c] Non-reducing terminal unit linked to 3,6-anhydro-α-L-galactose.
[d] Reducing terminal unit linked to 3,6-anhydro-α-L-galactose.

The residue of the extraction with H_2O at rt was then treated with H_2O (3 L) containing the NaOCl solution (3 mL) for 4 h at 50 °C. The residue was removed by centrifugation, and the supernatant was dialyzed (molecular weight cutoff of 3500 Da), concentrated and freeze-dried; the extraction procedure was repeated (× 2). The crude products were pooled (1.8 g) and dissolved in H_2O (500 mL), leaving an insoluble residue that was centrifuged off, suspended in H_2O and freeze-dried (0.6 g). The supernatant was concentrated and freeze-dried (50P, 1.2 g; 1.8% on dry seaweed, 4.3% on the total polysaccharides extracted).

When the procedure was repeated at 70 and 90 °C yields (expressed as percentages of the total polysaccharides extracted) of 3.6 and 85.1% were obtained.

3.3. Fractionation with cetrimide

The fractionation with cetrimide (Sigma H 5882) of RTP (1.9 g) and 50P (1.1 g) was carried out as described previously.[18]

3.4. Fractionation on DEAE Sephadex A-25 (Cl^-)

Columns (2.0 × 27 cm) were filled with DEAE Sephadex A-25 (Cl^-) which had been previously stabilized with water and boiled for 2 h. RTCS (268 mg), RTCI (137 mg), 50CS (246 mg) and 50CI (168 mg) were dissolved in H_2O, which was used as the first eluant; then increasing concentrations of NaCl were applied; the upper concentration was 4 M. Fractions of 3 mL were collected, and the aliquots were assayed by the phenol–H_2SO_4 method,[36] 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 polysaccharide solutions were dialyzed, concentrated and freeze-dried.

Yields, expressed as percentages of the total polysaccharides obtained by sequential extraction of the seaweed, were: RTCI05, 0.3%; RTCI07, 0.2%; RTCS05, 0.4%; 50CI05, 0.3%; 50CI07, 0.2%.

3.5. General methods

Carbohydrate content was analyzed by the phenol–H_2SO_4 method without previous hydrolysis of the polysaccharide. Sulfate was analyzed by the method of Refs. 37 and 38, before and after hydrolysis of the sample. Molecular weights were calculated by determination of reducing end-group using the colorimetric method of Park and Johnson.[39] Unless otherwise stated, dialyses were carried out with tubing having a molecular weight cutoff of 1000 Da.

Optical rotations (Na D-line) were measured in a Perkin–Elmer 343 polarimeter, using 0.3–0.5% solutions of the samples in H_2O.

Reductive hydrolysis of the native and permethylated samples and acetylation of the sugar mixtures was performed as described in Ref. 22, but the second step of hydrolysis was at 121 °C for 2 h. The native samples were also hydrolyzed with 2 M TFA at 121 °C for 2 h, and the monosaccharides were further derivatized to the aldononitrile acetates.[40]

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) coated with a 0.20-μm film of SP-2330. Chromatography was performed: (a) with an initial 5-min hold at 200 °C, then at 1.5 °C min^{-1} to 220 °C, followed by a 30-min hold, for the alditol acetates; (b) at 220 °C isothermally for the aldononitrile acetates; and (c) with an initial 5-min hold at 180 °C, then at 1.0 °C min^{-1} to 210 °C, and from 210 to 230 °C at 3.0 °C min^{-1}, followed by a 30-min hold, for the partially methylated 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 alditol acetates according to the effective carbon response theory.[41] For 1,4,5-tri-*O*-acetyl-3,6-anhydro-2-*O*-methylgalactitol a value of 0.64 was used.[22]

GLC–MS of the methylated alditol acetates was carried out on a Shimadzu QP5050A gas chromatograph–mass spectrometer working at 70 eV. Chromatography was performed on the SP-2330 capillary column using the programme temperature (c). 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.

Configuration of the 3,6-anhydrogalactose units was established by the method described in Ref. 42.

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.[23,43]

For ^{13}C NMR spectroscopy, samples (10–20 mg) were dissolved in 1:1 H_2O–D_2O (0.4 mL) and a 5-mm tube was used. The 125-MHz ^1H-decoupled spectra 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 44480–318702 scans. In all cases signals were referenced to internal Me_2SO at 39.8 ppm at rt.[44]

3.6. Desulfation of RTCI05

The sample (7.7 mg) was converted into its pyridinium salt and desulfated by treatment with chlorotrimethylsi-

lane in anhyd Py at 100 °C for 8 h. After careful addition of H_2O, the solution was dialyzed against H_2O, 0.1 M NaCl, and again H_2O; further freeze-drying yielded desulfated RTCI05 (7.6 mg; hygroscopic sample).[24]

3.7. Methylation analysis

RTCI05 (13.8 mg), desulfated RTCI05 (7.6 mg), RTCI07 (9.6 mg), RTCS05 (10.0 mg), 50CI05 (13.1 mg), and 50CI07 (9.8 mg) were each converted into their corresponding triethylammonium salts and methylated by the Hakomori procedure (sodium methylsulfinylmethanide–iodomethane)[21] as modified by Stevenson and Furneaux.[22] The methylated derivatives were recovered by dialysis and subsequent freeze-drying. Yields: RTCI05, 12.2 mg; desulfated RTCI05, 5.2 mg; RTCI07, 7.3 mg; RTCS05, 5.8 mg; 50CI05, 8.4 mg; 50CI07, 6.5 mg.

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