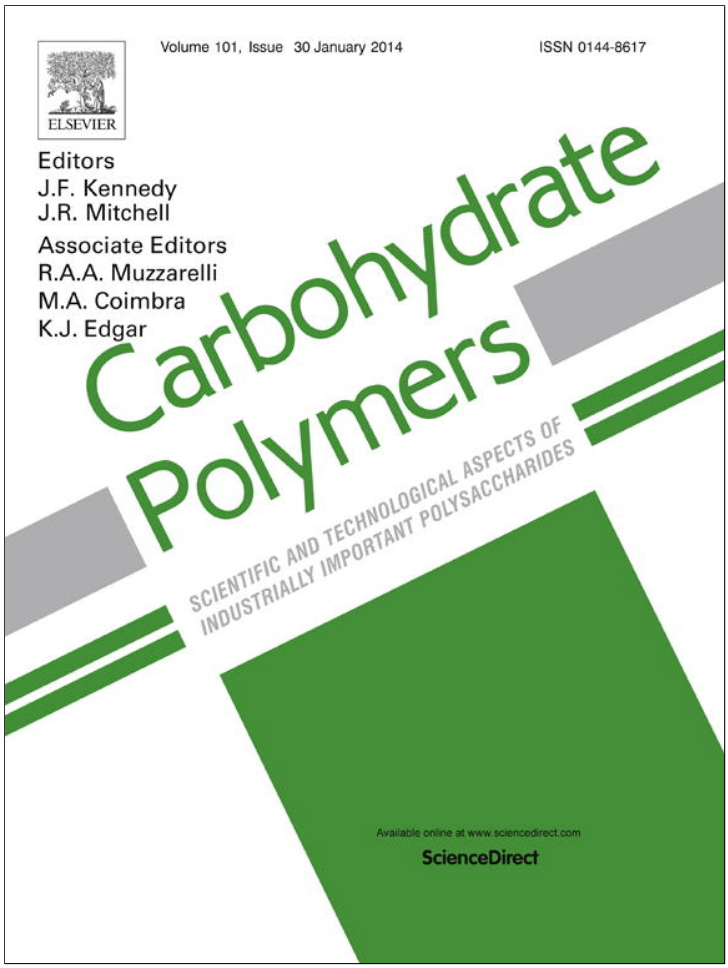

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Structure of highly substituted agarans from the red seaweeds *Laurencia obtusa* and *Laurencia filiformis*

Dilsia J. Canelón[a], Marina Ciancia[b,c,*,1], Alírica I. Suárez[d], Reinaldo S. Compagnone[e], María C. Matulewicz[c,**,1]

[a] *Escuela de Bioanálisis, Facultad de Medicina, Universidad Central de Venezuela, Av. Carlos Raúl Villanueva, Ciudad Universitaria, Los Chaguaramos, 1051 Caracas, Venezuela*
[b] *Cátedra de Química de Biomoléculas, Departamento de Biología Aplicada y Alimentos, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, 1417 Buenos Aires, Argentina*
[c] *Departamento de Química Orgánica – CIHIDECAR (CONICET-UBA), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, Pabellón 2, 1428 Buenos Aires, Argentina*
[d] *Facultad de Farmacia, Universidad Central de Venezuela, Av. Paseo de los Ilustres, Ciudad Universitaria, Los Chaguaramos, 1051 Caracas, Venezuela*
[e] *Escuela de Química, Facultad de Ciencias, Universidad Central de Venezuela, Av. Paseo de los Ilustres, Ciudad Universitaria, Los Chaguaramos, 1450 Caracas, Venezuela*

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ABSTRACT

The water extracts from red seaweeds *Laurencia obtusa* and *Laurencia filiformis* comprise complex sulfated agarans. Those from *L. obtusa* have 3-linked β-D-galactose units in part sulfated on 2-position or methylated on 6-position, while the 4-linked units are mostly 3,6-anhydro-α-L-galactose and α-L-galactose 6-sulfate, some of the latter units are substituted with β-D-xylose on 3-position, precluding alkaline cyclization. The 3-linked β-D-galactose units of the agarans from *L. filiformis* are mostly sulfated on 2-position, but approximately half of these residues also carry the 4,6-*O*-(1-carboxyethylidene) group. The 4-linked 3,6-anhydro-α-L-galactose units are methylated or substituted in part with single stubs of β-D-xylose on 2-position. This is the first time that substitution with xylose of 3,6-anhydro-α-L-galactose is reported. Besides, α-L-galactose 2-sulfate carrying single stubs of β-D-xylose on 3-position was also detected. These galactans have some common structural characteristics with those of other species of this genus, but also others that are specific for these species.

1. Introduction

Agarans are galactans biosynthesized by red seaweeds, constituted by alternating 3-linked β-D-galactose and 4-linked α-L-galactose units and present different degrees of cyclization of the α-L-galactose residues to give 3,6-anhydro-α-L-galactose. The neutral, completely cyclized agaran is agarose and it is industrially obtained mainly from seaweeds of the orders Gracilariales and Gelidiales. Its gelling properties have a wide range of uses. However, agarans usually have a certain degree of substitution with sulfate ester groups, methyl ethers, pyruvate ketals, D-xylose and/or 4-*O*-methyl-L-galactose side chains, and different percentages of 3,6-anhydrogalactose, depending on the source and sample work up (Usov, 2011).

All the representatives of the Ceramiales studied until now biosynthesize agarans with a wide range of substituents as major cell wall matrix polysaccharides (Miller, 1997). Nevertheless, a carrageenan structure, even though in small quantity, was found for the first time in a fraction of the extract from the red seaweed *Rhodomela larix* (Takano, Yakoi, Kamei, Hara, & Hirase, 1999). On the other hand, the sulfated galactan systems from other Rhodomelaceae, i.e. *Bostrychia montagnei* (Duarte, Noseda, Cardoso, Tulio, & Cerezo, 2002), *Polysiphonia nigrescens* (Prado, Ciancia, & Matulewicz, 2008), *Acanthophora spicifera* (Duarte et al., 2004) showed to be composed by agarans with different substitution patterns, and no carrageenan structures were detected in significant quantities, in spite of the fact that extensive fractionation procedures were carried out.

The structures of agarans from some species of the genus *Laurencia* have been studied previously. That of *L. pinnatifida* was constituted by 3-linked β-D-galactose units partially sulfated on the 2-position and partially methylated on the 6-position and 4-linked α-L-galactose units, partially sulfated on 6-position and

* Corresponding author at: Cátedra de Química de Biomoléculas, Departamento de Biología Aplicada y Alimentos, Facultad de Agronomía, Universidad de Buenos Aires, Av. San Martín 4453, 1417 Buenos Aires, Argentina. Tel.: +54 11 4524 8088; fax: +54 11 4524 8088.
** Corresponding author. Tel.: +54 11 4576 3346.
E-mail addresses: ciancia@agro.uba.ar (M. Ciancia), cristina@qo.fcen.uba.ar (M.C. Matulewicz).
[1] Research Member of the National Research Council of Argentina (CONICET).

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partially methylated on the 2-position; these α-units also occurred in the 3,6-anhydro form. In addition, a significant quantity of galacturonic acid was found and it was suggested that it could be forming small chains of 3-linked units attached to the galactan backbone. Xylose was also detected, but it was not possible to determine whether it was part of the main galactan structure, or a separate xylan (Bowker & Turvey, 1968a,b). Later, other species of *Laurencia* were studied and agaran structures with some of the previously described characteristics were found (Miller, 1997; Miller & Blunt, 2000a; Miller, Falshaw, & Furneaux, 1993; Siddhanta et al., 2002; Usov & Elashvili, 1991, 1997; Usov, Ivanova, & Elashvili, 1989; Valiente, Fernandez, Perez, & Marquina, 1993; Villanueva, Romero, Ragasa, & Montaño, 2010). Besides those structural units, the agaran from *L. nipponica* contained also 4-linked α-L-galactose 6-sulfate units with single stubs of β-D-xylose linked to C-3, which precluded the alkaline cyclization of α-units (Usov & Elashvili, 1991) and those from *L. gemmifera*, small quantities of 3-linked β-D-galactose units substituted with pyruvate ketal (Valiente et al., 1993). An agaran with moderate gel strength which did not improve by alkaline treatment was isolated from *L. flexilis*. This product was partially methylated on the 6-position and slightly sulfated on the 4-position of the 3-linked units (Villanueva et al., 2010). Some of these species have been transferred to other genera of the *Laurencia* J.V. Lamouroux complex (Guiry & Guiry, 2013; Nam, 2006, 2007).

Recently, the system of sulfated polysaccharides biosynthetized by *Palisada flagellifera*, seaweed of the *Laurencia* J.V. Lamouroux complex, previously studied as *Chondrophycus flagelliferus*, was investigated in depth (Cardoso, Noseda, Fujii, Zibetti, & Duarte, 2007; Ferreira et al., 2012). The water-soluble extracts from this seaweed were fractionated by potassium chloride precipitation; the insoluble product was a sulfated xylomannan (Cardoso et al., 2007). Highly complex agaran structures were isolated from the soluble fraction, comprising mostly cyclized α-units, partially methylated on the 2-position, however a significant amount of α-L-galactose 6-sulfate units substituted on the 3-position by xylosyl-, galactosyl- and/or 2,3-di-*O*-methyl-galactose units were also found. The β-units were mostly 4,6-pyruvylated and 2-sulfated, or 6-methylated galactoses, but also minor quantities of β-D-galactose units 2- and 6-sulfated, 2,6-disulfated, 6-glycosylated and non-substituted, were present.

In this paper, we report the structure of two species of this genus, *Laurencia obtusa* and *Laurencia filiformis* which were studied in detail, these studies showed that the substitution pattern has some common features for all or at least most the species of this genus, but there are some characteristics unique for each of the species. Also, some new structural features were found.

2. Materials and methods

2.1. Algal samples

Nonfertile plants of the red macroalga *L. obtusa* (Hudson) Lamouroux were collected at a 1 m depth in Punta Varadero, Chichiriviche, Falcón State (10°54′06 N, 68°14′44 O), Venezuela, in March 2006, Nonfertile *L. filiformis* (C. Agardh) Montagne was collected in Paraguaná, Falcón State (12°09′05 N, 70°05′50 O), Venezuela, in February 2008. Both seaweeds were washed with sea water, carefully hand sorted in order to avoid contamination with epiphytes, and identified by Dr. Santiago Gómez from Instituto de Biologia Experimental, Universidad Central de Venezuela and Dr. Mayra García from Fundación Jardín Botanico Tobias Laser de Caracas, Venezuela according to the literature (Rios, 1972; Taylor, 1960).

Voucher specimens of both seaweeds were deposited in the National Herbarium of Venezuela (*L. obtusa*, VEN 290158; *L. filiformis*, VEN 383858).

2.2. Extraction of the polysaccharides

Cleaned vegetative plants were freezed immediately after collection and then freeze dried. The dry material was first extracted with methanol and the residue from the alcohol extraction was sequentially and exhaustively extracted with H_2O (20 g/L) at room temperature and hot-water as previously reported (Estevez, Ciancia, & Cerezo, 2001). Briefly, the residue of the first room temperature extraction was removed by centrifugation and the supernatant was concentrated, dialyzed and freeze-dried, to give LORT1 and LFRT1, from *L. obtusa* and *L. filiformis*, respectively. The residue was extracted once more in the same way and extracts LORT2 and LFRT2 were isolated. The final residue obtained at room temperature was freeze-dried. Then, it was re-suspended in H_2O (20 g/L) and extracted at 90 °C. The extract was treated as described above, to give extracts LO901 and LF901. The residue was extracted once more in the same way, rendering LO902 and LF902.

2.3. General methods

The total sugar content was analyzed by PhOH–H_2SO_4 method (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956) or by the method adapted for insoluble material (Ahmed & Labavitch, 1977). Sulfate was determined turbidimetrically (Dodgson & Price, 1962) except in the case of desulfated samples for which ion chromatography with conductimetric detection was used. The latter samples were hydrolyzed in 2 M CF_3CO_2H at 121 °C for 2 h, evaporated to dryness under nitrogen and redissolved in high purity water from a Milli-Q system. A DIONEX DX-100 ion chromatography system (Sunnyvale, CA, USA) was used with an AS4A column (4× 250 mm), an AMMS-II micromembrane suppressor and a conductivity detector (eluent: 1.8 mM Na_2CO_3/1.7 mM $NaHCO_3$, flow rate: 2 mL min^{-1}). The percentage of pyruvic acid was determined according to Koepsell and Sharpe (1952). The uronic acids content was measured according to Filisetti-Cozzi and Carpita (1991). Number-average molecular weights were determined using the colorimetric method of Park and Johnson (1949). Dialyses were carried out with tubing with molecular weight cutoff of 3500 Da.

Reductive hydrolysis of the native and methylated/ethylated samples and acetylation of the sugar mixtures was performed according to Stevenson and Furneaux (1991) (method A). Alternatively, hydrolysis was carried out in 100% TFA for 1 h at 37 °C, followed by dilution of the acid to 80%, heating at 100 °C for 1 h, and further dilution to 2 M to achieve the regular hydrolysis conditions for insoluble polysaccharides (Morrison, 1988); the sugar mixture was derivatized to the corresponding alditol acetates (method B).

Methylated samples were hydrolyzed with 2 M TFA for 2 h at 120 °C and the partially methylated sugars were converted into the corresponding aldononitrile acetates (Stortz, Matulewicz, & Cerezo, 1982).

GLC of the alditol acetates, as well as those of the partially alkylated alditol and aldononitrile acetates were carried out on a Hewlett Packard 5890A gas-liquid chromatograph (Avondale, PA, USA) equipped with a flame ionization detector and fitted with a fused silica column (0.25 mm i.d. × 30 m) WCOT-coated with a 0.20 μm film of SP-2330 (Supelco, Bellefonte, PA, USA). Chromatography was performed: (a) from 200 °C to 230 °C at 1 °C min^{-1}, followed by a 30-min hold for alditol acetates; (b) from 180 °C to 210 °C at 1 °C min^{-1}, then from 210 °C to 230 °C at 2° min^{-1} followed by a 30-min hold for partially alkylated alditol acetates arising from methylation or ethylation analyses, and methylated aldononitrile 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.

GLC–MS of the methylated/ethylated alditol acetates was performed on a Shimadzu GC-17A gas-liquid chromatograph equipped with the SP-2330 capillary column interfaced to a GCMSQP 5050A mass spectrometer (Kyoto, Japan) working at 70 eV. Chromatography was performed using the programme temperature (b). The total flow rate was 7 ml min^{-1} and the injector temperature was 240 °C. Mass spectra were recorded over a mass range of 30–500 amu.

The D,L-galactose ratio and the configuration of monomethylated galactoses were estimated by the method of Cases, Cerezo, and Stortz (1995) through their diastereomeric acetylated 1-deoxy-1-(2-hydroxypropylamino)alditols; the configuration of 3,6-anhydrogalactose and 3,6-anhydro-2-*O*-methylgalactose were estimated by the method of Navarro and Stortz (2003). The 2,6-di-*O*-methyl-L:2,6-di-*O*-methyl-D-galactose ratio was determined on the permethylated polysaccharide by conversion of the monosaccharides, obtained by hydrolysis of the sample to the diastereomeric acetylated 1-deoxy-1-(1-phenylethylamino)alditols (Errea, Kolender, & Matulewicz, 2001).

2.4. Gel-permeation chromatography

A solution of polysaccharide (3–4 mg) in water (1 mL) was applied to a column (1 cm × 14.5 cm) of Sephadex G-200. The column was eluted with water; fractions of 0.5 mL were collected and analyzed for carbohydrate content by the PhOH–H_2SO_4 reaction. The void (4.5 mL) and total (13.6 mL) volumes of the column were determined using dextran sulfate (average molecular weight 500,000; Sigma D-6001) and galactose, respectively.

2.5. Alkaline treatment

The analytical alkaline treatments were carried out according to Navarro and Stortz (2003).

In the preparative treatment (Matulewicz, Ciancia, Noseda, & Cerezo, 1989), the sample (LORT1 and LO901; 50 mg) was dissolved in H_2O (25 mL), and $NaBH_4$ (3 mg) was added. After 16 h at room temperature, 3 M NaOH (12.5 mL) was added with a further quantity of $NaBH_4$ (3 mg). The solution was heated at 80 °C, and then cooled to room temperature, dialyzed without previous neutralization, concentrated and freeze-dried to give LORT1T (64.2%) and LO901T (79.4%).

2.6. Methylation analysis

The polysaccharide (10 mg) was converted into the corresponding triethylammonium salt (Stevenson & Furneaux, 1991) and methylated according to Ciucanu and Kerek (1984) using finely powdered NaOH as base; the procedure was repeated to ensure permethylation. The methylated derivatives were recovered by dialysis and freeze-drying. Yields of permethylated samples, 70–95%.

2.7. Ethylation analysis

The sample (10 mg) was converted into the corresponding triethylammonium salt (Stevenson & Furneaux, 1991) and was ethylated as described by Cases, Stortz and Cerezo (1994). The ethylated derivatives were recovered by dialysis and freeze-drying. Yields of perethylated samples, 50–60%.

2.8. Desulfation

The reaction was carried out by the microwave-assisted method described by Navarro, Flores, and Stortz (2007). The polysaccharide (20 mg) was converted to the pyridinium salt and dissolved in DMSO (10 mL) containing 2% pyridine. The mixture was heated for 1 min intervals and cooled to 50 °C (6×). It was dialyzed and freeze-dried (yield ~60%, considering the sulfate loss) and methylated as described in Section 2.5. Sulfate content (as $NaSO_3$): LORT1DS, 3.4%; LFRT1DS, 1.8%. The monosaccharide composition of both desulfated derivatives was similar to that of the corresponding native extract.

2.9. NMR spectroscopy

Samples (10–20 mg), previously exchanged with deuterium by repeated solubilization in D_2O and freeze-drying, were dissolved in D_2O (0.5 mL) and 5 mm tubes were used. Spectra were recorded at room temperature on a Bruker Avance II 500 spectrometer (Karlsruhe, Germany). For ^1H NMR experiments the parameters were: a spectral width of 6.25 kHz, a 76° pulse angle, an acquisition time of 3 s, a relaxation delay of 3 s, for 32 scans. For 125 MHz proton decoupled ^{13}C NMR experiments the parameters were: a spectral width of 29.4 kHz, a 51.4° pulse angle, an acquisition time of 0.56 s, a relaxation delay of 0.6 s, for 25,000 scans. In all the cases, signals were referenced to internal acetone at 2.21 ppm for ^1H NMR and 31.1 ppm for ^{13}C NMR experiments, respectively.

Pulse sequences for ^1H–^1H COSY and ^1H–^{13}C HMQC techniques were supplied by the spectrometer manufacturer; spectra were recorded at room temperature and were obtained at a base frequency of 500 MHz for ^1H and 125 MHz for ^{13}C.

3. Results and discussion

The residues of the methanolic extraction of both seaweeds were extracted twice with water at room temperature to give extracts LORT1 and LORT2 from *L. obtusa*, and LFRT1 and LFRT2 from *L. filiformis*. The residues from these extraction procedures were then extracted twice with hot water, to afford LO901 and LO902 from *L. obtusa*, and LF901 and LF902, from *L. filiformis*. Yields and analyses of the extracts are shown in Table 1. The overall yield of the water extractions was 12.8% and 16.2%, respectively. When the carbohydrate content of the extracts was determined using the standard conditions for the determination of the total carbohydrate content (Dubois et al., 1956) low values were obtained (21–36%), hence, the modified procedure for insoluble material was employed (Ahmed & Labavitch, 1977). In this way, the total carbohydrate content determined varied between 41.6 and 52.5%. Besides, the sulfate content was somewhat higher for the samples from *L. obtusa*. All the samples showed small, but still meaningful amounts of uronic acids: 4–8% for the extracts of *L. obtusa* and 5–7% for those from *L. filiformis*. The difference in pyruvic acid content between the galactans from both seaweeds was noteworthy. Thus, in LFRT1 an important degree of substitution was observed, giving a D-galactose:pyruvic acid molar ratio of 1.00:0.76 while in LORT1 no pyruvylation was detected. The monosaccharide composition of extracts from both seaweeds showed significant differences, but only minor differences were found between extracts of the same seaweed. Enantiomeric analysis of the major monosaccharide components was carried out and results are included in Table 1. Extracts from *L. obtusa* showed major quantities of D- and L-galactose, and 3,6-anhydro-L-galactose; also important quantities of 6-*O*-methylgalactose and xylose were present. Extracts from *L. filiformis* showed the same major components; however, 6.6–8.9% of 3,6-anhydro-2-*O*-methyl-L-galactose was present in these samples as the only methylated

Table 1
Yields and analyses of the crude products obtained from *Laurencia obtusa* and *Laurencia filiformis* after extraction with water at room temperature and at 90 °C.

Extract	Yield[a]	Sulfate	Gal:3,6-AnGal:sulfate[b]	Monosaccharide composition (mol%)[c]								
	(%)	(% $NaSO_3$)	(molar ratio)	D-Gal	L-Gal	L-3,6-AnGal	2-Me L-3,6-AnGal	6-Me D-Gal	Xyl	Glc	Man	Fuc
LORT1[d]	4.0	14.9	1.00:0.18:0.67	47.2	24.7	12.8	tr[e]	6.4	7.7	1.2	tr	tr
LORT2	4.0	14.9	1.00:0.31:0.79	42.6	18.5	18.9	tr	9.3	9.4	1.3	–	–
LO901	3.6	14.5	1.00:0.28:0.92	42.7	18.8	17.0	tr	9.7	8.5	1.9	1.4	tr
LO902	1.2	12.1	1.00:0.40:0.82	32.9	15.5	18.3	1.2	12.7	10.0	2.5	4.0	1.5
LFRT1[d]	2.7	10.5	1.00:0.91:0.95	33.2	5.6	28.5	6.9	–	16.0	5.4	2.5	1.9
LFRT2	3.9	11.7	1.00:0.87:0.95	34.3	7.4	27.3	8.9	–	15.8	2.9	2.0	1.4
LF901	7.8	13.1	1.00:0.88:0.94	35.2	6.5	29.8	7.1	–	14.7	2.1	3.4	1.2
LF902	1.8	10.5	1.00:0.79:0.80	35.0	6.4	26.0	6.6	–	15.9	5.9	2.4	1.8

[a] Given as percentage of the product obtained after methanolic extraction.
[b] In the 3,6-anhydrogalactose (3,6-AnGal) molar ratio the content of 3,6-anhydro-2-*O*-methylgalactose (2-Me 3,6-AnGal) is included.
[c] LO902 contains 1.4% of rhamnose. The absolute configuration of the monosaccharides was determined as indicated in the Experimental.
[d] The content of pyruvic acid was determined for LORT1 (0.2%) and LFRT1 (6.0%).
[e] Percentages lower than 1% were considered as traces (tr).

Table 2
Analyses of alkali-treated samples from *Laurencia obtusa* and *Laurencia filiformis*.

Extract	Sulfate	Gal:3,6-AnGal[a]	Monosaccharide composition (mol%)						
	(as $NaSO_3$)	(molar ratio)	Gal	3,6-AnGal	2-Me 3,6-AnGal	6-Me Gal	Xyl	Glc	Fuc
LORT1Tp[c]	3.7	1.00:0.68	49.7	32.1	1.6	7.2	8.2	1.2	tr[b]
LO901Tp	3.6	1.00:0.77	44.8	34.6	1.5	9.7	7.8	1.6	tr
LFRT1Ta[d]	n.d.[e]	1.00:0.71	42.9	30.6	6.9	–	13.1	4.7	1.8
LF901Ta	n.d.	1.00:0.83	40.5	33.8	8.7	–	14.8	2.2	tr

[a] In the 3,6-anhydrogalactose (3,6-AnGal) molar ratio the content of 3,6-anhydro-2-*O*-methylgalactose (2-Me 3,6-AnGal) is included.
[b] Percentages lower than 1% were considered as traces (tr).
[c] Preparative alkaline treatment.
[d] Analytical alkaline treatment.
[e] n.d. = not determined.

derivative detected. LORT1 and LO901 from *L. obtusa*, and LFRT1 and LF901 from *L. filiformis* were submitted to an alkaline treatment in conditions to cyclize the α-galactose 6-sulfate units to give the 3,6-anhydro-derivative (Rees, 1961). Analysis of the monosaccharide composition of the alkali-modified extracts showed that the molar ratio galactose:3,6-anhydrogalactose changed significantly for samples from *L. obtusa*, while for those of *L. filiformis*, it remained approximately constant, indicating that the latter extracts did not have precursor units. Hence, extracts LORT1 and LO901 were submitted to preparative alkaline treatments, obtaining LORT1Tp and LO901Tp, respectively (Table 2). The low sulfate content found in the modified samples confirmed that an important amount of the sulfate present in the parent samples was in the 6-position of the α-L-galactose units.

Taking into account the similar composition obtained for the four extracts of each seaweed, only extracts LORT1 and LO901 (from *L. obtusa*), LFRT1 and LF901 (from *L. filiformis*) were further studied. Gel-permeation chromatography on Sephadex G-200 showed that they were homogeneous and that those obtained at room temperature had lower number-average molecular weights (LORT1, 37 kDa; LFRT1, 111 kDa) than the extracts isolated with hot water (LO901, 96 kDa; LF901, 167 kDa). Then structural analysis was carried out.

Results from methylation and desulfation–methylation analyses of LORT1 are indicated in Table 3. Half of the β-D-galactose units are not substituted or 6-*O*-methylated (25.6%); the rest of the β-residues is mainly substituted on 2-position (18.1%), but there is also monosubstitution on C-6 (2.7%). On the other hand, similar amounts of cyclized α-L-galactose (12.8%) and α-L-galactose 6-sulfate (14.5%) residues were detected; the presence of minor amounts of 2,6-di-*O*-methylgalactose (3.9%) and 2-*O*-methylgalactose (6.7%), suggested monosubstitution on the 3-position and disubstitution on the 3- and 6-positions, respectively. Desulfation–methylation of LORT1 confirmed the presence of: (a) β-D-galactose 2-sulfate residues; (b) α-L-galactose residues sulfated on the 6-position; and (c) single stubs of xylose linked to the 6-position of the β-units and to the 3-position of the α-units. Methylation analysis of LO901 (Table 3) is very similar to that of LORT1 being the most important difference the increase in the amount of α-L-galactose 6-sulfate units, in agreement with the results of the alkaline treatment.

Analysis of the HMQC spectrum of alkali-treated LORT1 (LORT1T, Table 4) showed three major signals in the anomeric region. The most important signal corresponds to 3,6-anhydro-α-L-galactose units ($\delta_{C/H}$ 98.9/5.07) linked to β-D-galactose (anomeric

Table 3
Composition (mol%) of monosaccharides produced by permethylation and hydrolysis of the agarans from *Laurencia obtusa*.[a]

Monosaccharide	Structural unit[b]	LORT1[c]	LORT1DS	LO901
2,3,4-Me_3 Xyl	Terminal	6.0	2.6	4.6
2-Me AnGal	LA	12.8	11.2	17.0
2,3,4,6-Me_4 Gal	Terminal	2.6	–	3.1
2,4,6-Me_3 Gal	G + G6M	25.6	50.4	26.0
2,3,6-Me_3 Gal	L	1.2	21.4	3.0
2,6-Me_2 Gal[d,e]	L3X	3.9	10.0	18.7
4,6-Me_2 Gal[d]	G2S	18.1	–	
6-Me Gal		1.7	–	2.4
2,3-Me_2 Gal	L6S	14.5	–	20.1
2,4-Me_2 Gal	G6X	2.7	4.4	–
2-Me Gal	L6S, 3X	6.7	tr[f]	5.1
3-Me/4-Me Gal + Gal		4.2	–	tr

[a] The molar percentage of 2-Me AnGal was normalized according to the GLC values shown in Table 1.
[b] Nomenclature of Knutsen, Myslabodski, Larsen, and Usov (1994). G: 3-linked β-D-galactopyranosyl; L: 4-linked α-L-galactopyranosyl; LA: 4-linked 3,6-anhydro-α-L-galactopyranosyl; M: *O*-methyl; P: 4,6-*O*-(1-carboxyethylidene); S: sulfate ester; X: xylopyranosyl.
[c] 1.3% of glucose was detected.
[d] In LORT1 and LORT1DS determined by derivatization to the acetylated aldononitriles. In LO901, the presence of both dimethylated monosaccharides was detected by GLC–MS.
[e] Configuration determined according to Errea et al. (2001).
[f] Percentages lower than 1% were considered as traces (tr).

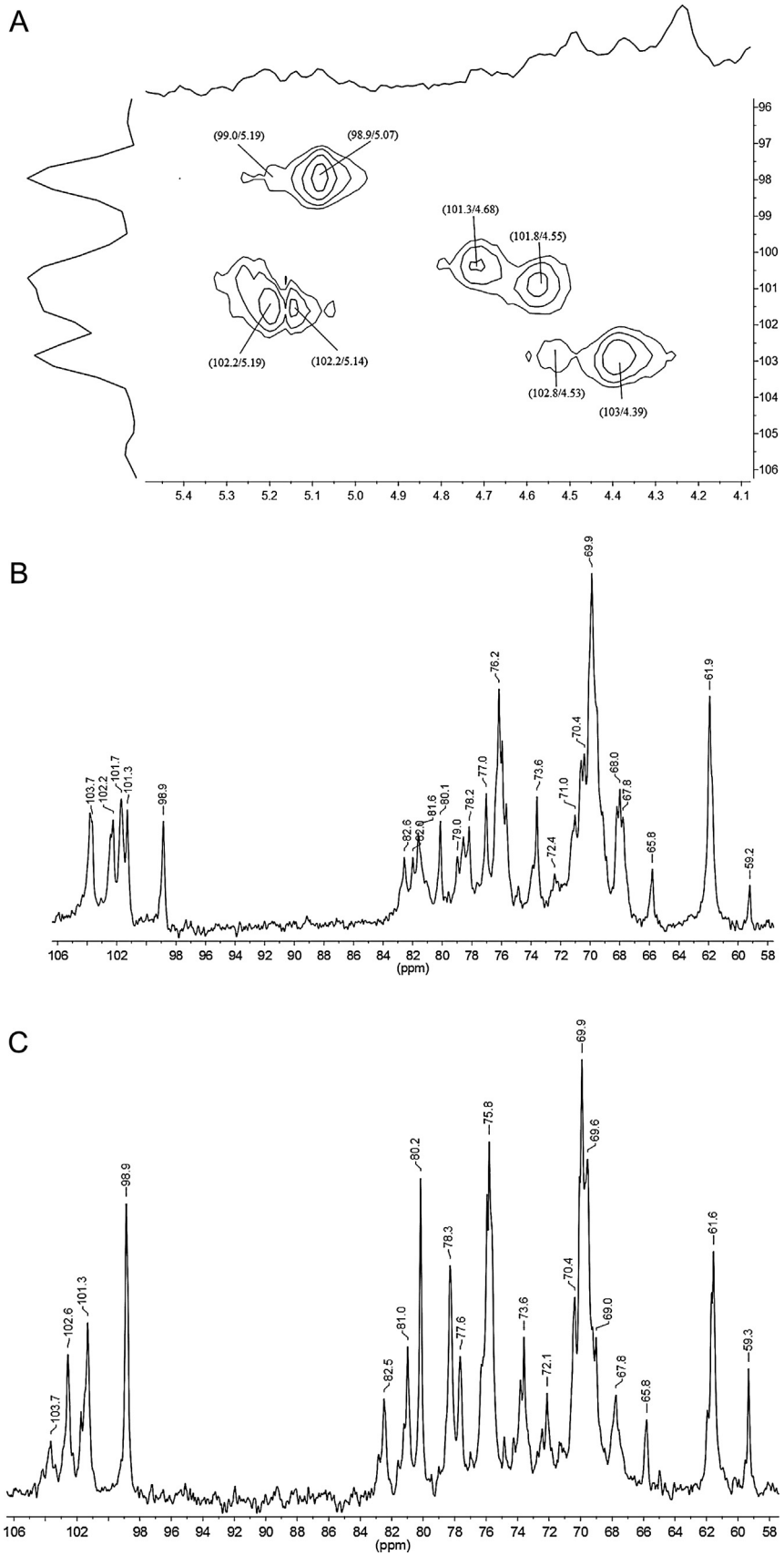

Fig. 1. NMR spectra of LORT1 and LORT1T. (A) Anomeric region of HMQC spectrum of LORT1, (B) ^{13}C NMR spectrum of LORT1, (C) ^{13}C NMR spectrum of LORT1T.

Table 4
NMR signal assignments (ppm) of agarans from *Laurencia obtusa*.[a,b]

Structural unit	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5,5′	C-6/H6,6′
Present in LORT1, LORT1T, and LO901						
G	102.5/4.52	70.4/3.55	82.5/3.73	69.5/4.06	75.5/3.67	61.6/3.68
G6M						72.0/3.62
G2S	101.3/4.68	78.2/4.5778.3/4.26	80.9/3.93	68.9/4.08	75.5/3.67	61.6/3.68
LA	98.9/5.07	69.9–69.5/4.06	80.1/4.47	77.6/4.58	75.8/4.47	69.5/4.06
L3X, 6S	101.4/5.26	67.7/3.98	73.3			68.0/4.22
tX	101.8/4.55	73.5/3.31			65.7/3.24,3.90	
Present only in LORT1 and LO901						
G	103.7/4.39		81.1/3.71			61.8/3.73
L6S	101.9/5.14–5.19	71.3	78.9			68.2/4.24

[a] Nomenclature of Knutsen et al. (1994). See also Table 3.
[b] Assignment of substituents: 59.4/3.34 ppm for methoxyl group on 6-position of G.

signal at $\delta_{C/H}$ 102.5/4.52) or β-D-galactose 2-sulfate (anomeric signal at $\delta_{C/H}$ 101.3/4.68); no other signals were observed in this region indicating that the amount of the minor structures present in this sample is not enough to detect them. The remaining signals are in agreement with those previously reported for these diads (Miller & Blunt, 2000a; Rochas, Lahaye, Yaphe, & Phan Viet, 1986; Usov & Elashvili, 1991; Welti, 1977). A strong signal at 59.3/3.34 ppm corresponds to the methoxyl group on the 6-position of some of the β-D-galactose units (Miller & Blunt, 2000b). The HMQC spectrum of LORT1 is more complex (Fig. 1, Table 4); the signal at $\delta_{C/H}$ 102.5/4.52 is not present, but there are two signals at $\delta_{C/H}$ 103.7/4.39 and 101.9/5.14–5.19 which correspond to β-D-galactose linked to α-L-galactose 6-sulfate (Lahaye, Yaphe & Rochas, 1985). This indicates that in the original sample the β-D-galactose units substituted on the 2-position are mainly linked to cyclized 4-linked residues, but the non-substituted β-D-galactose units are linked to precursor residues. The anomeric signal at $\delta_{C/H}$ 101.8/4.55 would correspond to β-D-xylose units linked to the 3-position of some of the α-L-galactose units of the galactan backbone and that at $\delta_{C/H}$ 101.4/5.26 was assigned to the anomeric peak of α-L-galactose 6-sulfate with single stubs of xylose in the 3-position.

Results from methylation and desulfation–methylation analyses of LFRT1 and methylation analysis of LF901 are shown in Table 5. Agarans from *L. filiformis* are composed by important amounts of highly substituted β-D-galactose units: most of them are 4,6-*O*-(1-carboxy)-ethylidene-β-D-galactose 2-sulfate (20.1%). Besides, β-D-galactose units sulfated on the 2-position are also present in considerable quantities (12.5%). Most of the 4-linked units correspond to non-substituted 3,6-anhydro-α-L-galactose units, however, a significant amount of them are methylated on the 2-position in the native polysaccharide (Table 1) and some are substituted with xylose side chains, in agreement with desulfation–methylation analysis. In addition, methylation of LFRTDS confirmed the presence of single stubs of xylose linked to the 3-position of α-L-galactose 2-sulfate residues.

Although it has been reported (Chiovitti et al., 1997; Ferreira et al., 2012) that presence of pyruvate ketals in the galactan backbone has a negative effect in the release of 3,6-anhydrogalactose units, in this case, this effect was not observed and the amount of the cyclized units determined from reductive hydrolysis, acetylation and GLC of the corresponding alditol acetates was similar to that determined from the ^{1}H NMR spectrum of LFRT1.

NMR spectra of LFRT1 were complex; however, they were partially assigned in agreement with the structural analysis and previously reported data (Fig. 2, Table 6).

Signals due to substitution with pyruvate ketal (*R*-isomer) were found at δ_C 177.0, 101.7 and 26.1 in the ^{13}C NMR spectrum and also at $\delta_{C/H}$ 26.1/1.41 in the HMQC spectrum (Garegg, Lindberg, & Kvarnström, 1979; Gorin, Mazurek, Duarte, Iacomini, & Duarte 1982). The most important signals of the galactan backbone are those corresponding to the anhydrogalactose units. Those of unsubstituted 3,6-anhydro-α-L-galactose were fully assigned (Rochas et al., 1986). Resonances of these units methylated on the 2-position were also found (Lahaye, Yaphe, Phan Viet, & Rochas, 1989), the peak corresponding to the methoxyl group was observed at $\delta_{C/H}$ 59.3/3.43. Besides, this is the first report on the presence of 3,6-anhydro-α-L-galactose substituted at 2-position with single stubs of β-D-xylose and the signal at $\delta_{C/H}$ 97.4/5.21 was tentatively assigned to the anomeric resonance of the α-unit. The anomeric signal at $\delta_{C/H}$ 96.2/5.17 corresponds to α-L-galactose 2-sulfate 3-xylose; signals of the other carbons were also present (Miller et al., 1993).

The most important β-unit is 4,6-*O*-(1-carboxy)-ethylidene-β-D-galactose 2-sulfate, whose signals were identified in the spectra and assigned (Ferreira et al., 2012; Lahaye et al., 1989). The resonances of the remaining β-units were more difficult to identify. The signal at $\delta_{C/H}$ 103.1/4.71 was assigned to 4,6-*O*-(1-carboxy)-ethylidene-β-D-galactose with a free hydroxyl group at 2-position (Lahaye et al., 1989). Signals corresponding to β-D-galactose, β-D-galactose 6-xylose, and β-D-galactose 2-sulfate units were also assigned (Miller & Blunt, 2000a; Usov & Elashvili, 1991).

In addition, the anomeric signal of single stubs of β-D-xylose linked to 3-position of α-L-galactose 2-sulfate was assigned to $\delta_{C/H}$ 99.8/4.90; the low displacement to higher field of the value reported before (Usov & Elashvili, 1991) could be due to the presence of sulfate on the 2-position of the α-L-galactose unit. Another signal at $\delta_{C/H}$ 103.8/4.90 was tentatively assigned to β-D-xylose linked to the 2-position of 3,6-anhydrogalactose. The difference

Table 5
Composition (mol%) of monosaccharides produced by permethylation and hydrolysis of the agarans from *Laurencia filiformis*.[a]

Monosaccharide	Structural units[b]	LFRT1	LFRT1DS	LF901
2,3,4-Me$_3$ Xyl	Terminal	7.2	10.9	5.2
2-Me AnGal[c]	LA + LA2M	29.7	23.4	28.8
2,4,6-Me$_3$ Gal	G	3.6	15.1	4.9
2,3,6-Me$_3$ Gal	L	1.9	5.8	–
2,6-Me$_2$ Gal[d]	L3X	–	5.5	–
4,6-Me$_2$ Gal	G2S	12.5	–	11.1
AnGal	LA2X	5.7	8.0	8.1
6-Me Gal	L2S, 3X	10.0	–	7.8
2,4-Me$_2$ Gal	G6X	4.0	9.3	5.1
2-Me Gal	GP	5.3	20.9	7.8
3-Me Gal/4-Me Gal		–	tr[e]	1.7
Gal	GP, 2S	20.1	1.1	19.5

[a] The molar percentage of 2-Me AnGal + AnGal was normalized according to the GLC values shown in Table 1.
[b] Nomenclature of Knutsen et al. (1994). See also Table 3.
[c] Ethylation analysis indicated that 5.9% was methylated in LFRT1 and 8.1% in LF901.
[d] Configuration determined according to Errea et al. (2001).
[e] Percentages lower than 1% are considered as traces (tr).

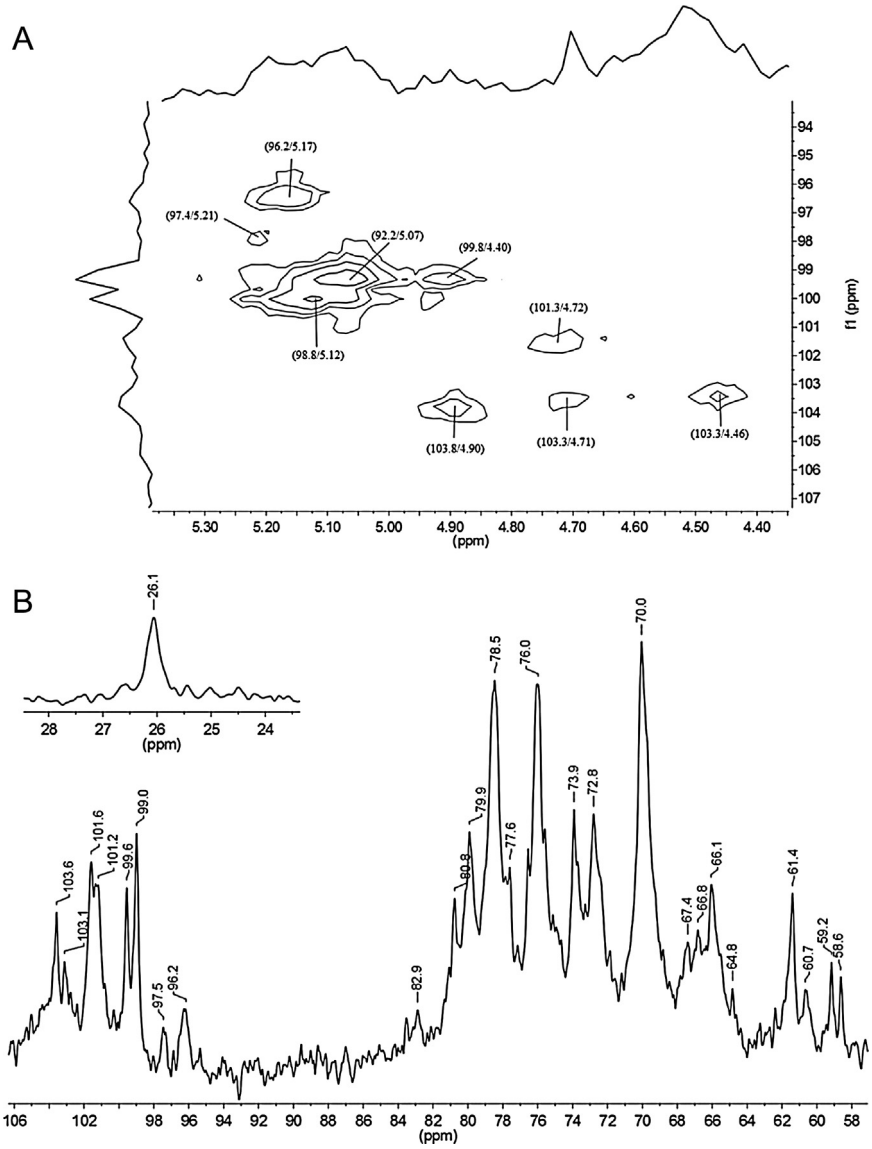

Fig. 2. NMR spectra of LFRT1. (A) Anomeric region of HMQC spectrum of LFRT1, (B) ^{13}C NMR spectrum of LFRT1.

Table 6
NMR signal assignments (ppm) of agarans from *Laurencia filiformis*.[a,b]

Structural unit	C-1/H-1	C-2/H-2	C-3/H-3	C-4/H-4	C-5/H-5,5′	C-6/H6,6′
GP2S	101.3/4.72	78.5/4.52			67.0/3.59	65.8/3.90
GP	103.3/4.71	70.2/3.53				
G/G6X	103.3/4.46					
G2S	101.2/4.60	78.2	80.9			61.5/4.71–4.67
LA	99.2/5.07	69.8/4.04	80.0/4.47	78.1/4.63	75.8/4.50	69.8/4.04–4.00
LA2M	99.8/5.12	78.9/4.23	78.5	77.6/4.37		
LA2X	97.4/5.21		73.7/3.88	76.6/3.36	72.4	61.0/3.55, 3.96
L2S, 3X	96.2/5.17	73.2/3.84	81.1/3.71			61.8/3.73
tX (linked to C-2 of LA)	103.8/4.90				65.9/3.88, 3.21	
tX (linked to C-3 of L2S)	99.8/4.90					

[a] Nomenclature of Knutsen et al. (1994). See also Table 3.
[b] Assignment of substituents: 177.0, 101.7, and 26.1/1.41 ppm for C-1, C-2 and C-3/H-3 of pyruvate ketal (*R*-isomer); 59.3/3.43 ppm for methoxyl group on the 2-position of LA.

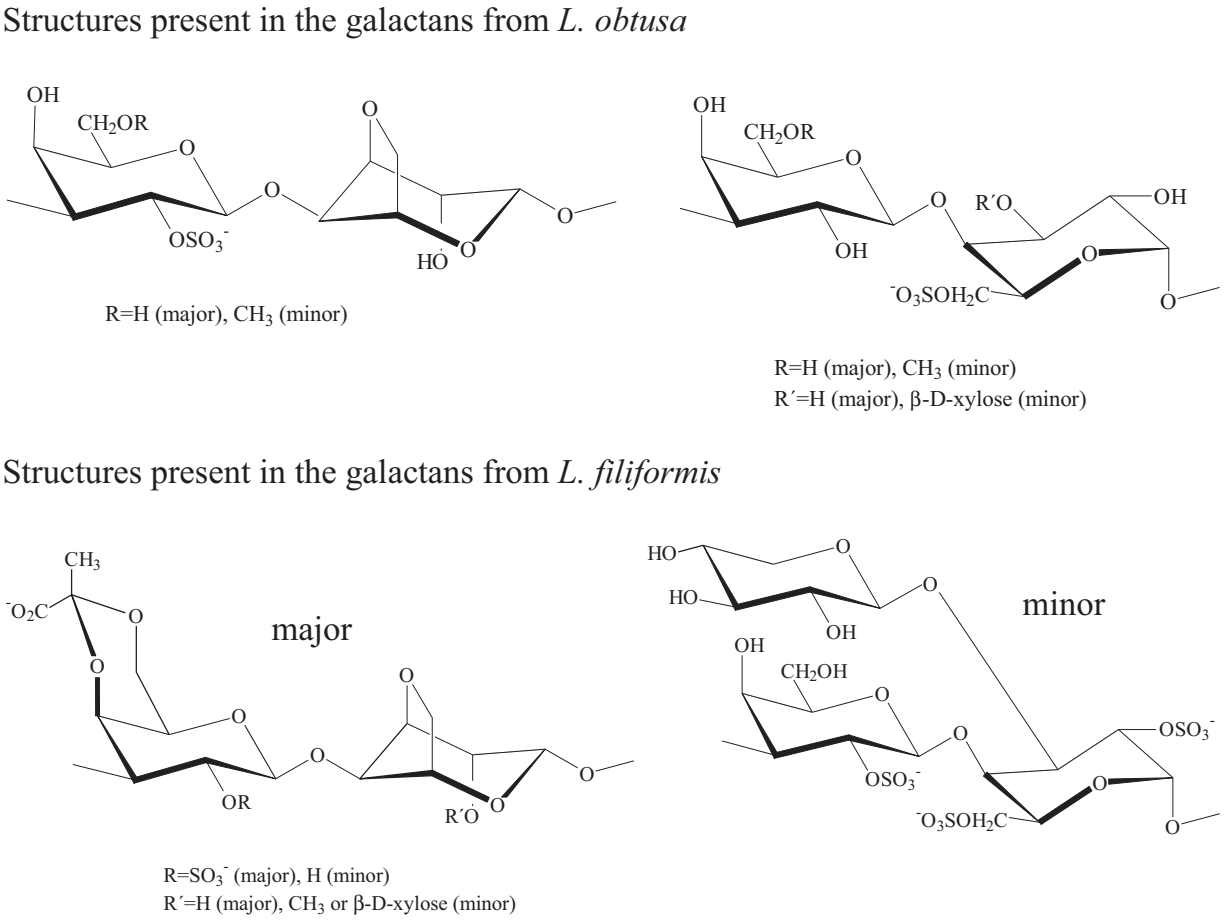

Fig. 3. Diads present in galactans from *Laurencia obtusa* and *L. filiformis*.

between both δ_C could be related to the different environment due to the fact that conformation of the galactose units is $^4C_1$ for the former residue, and $^1C_4$ for the latter.

4. Conclusions

The red seaweeds *L. obtusa* and *L. filiformis* biosynthesize agarans as matrix polysaccharides. These polysaccharides do not form gels, sulfation is not very high, but both species have other substituents in the agaran chain, giving complex substitution patterns.

The major structures present on each of these galactans are shown in Fig. 3. Both species of *Laurencia* have some common structural characteristics, which are also found in other species of this genus, namely: (a) partial sulfation on the 2-position of the β-D-galactose units and (b) single stubs of xylose linked to the 3-position of the α-L-residues. Besides, important differences were found, namely, (a) the presence of precursor units (α-L-galactose 6-sulfate) in galactans from *L. obtusa*, completely absent in those of *L. filiformis*; (b) the presence of important quantities of pyruvate ketals, in the latter seaweed, absent in *L. obtusa*; (c) methoxyl groups are linked to the 6-position of some of the β-D-galactose units in agarans from *L. obtusa*, while in those of *L. filiformis* they are linked to the 2-position of 3,6-anhydro-α-L-galactose residues; and (d) the presence of β-D-xylose side chains possibly linked to 2-position of some of the 3,6-anhydrogalactose residues only in the galactans from *L. filiformis*.

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