J. Phycol. **46,** 507–515 (2010)

DOI: 10.1111/j.1529-8817.2010.00828.x

NOTE

DIFFERENCES IN POLYSACCHARIDE STRUCTURE BETWEEN CALCIFIED AND UNCALCIFIED SEGMENTS IN THE CORALLINE *CALLIARTHRON CHEILOSPORIOIDES* (CORALLINALES, RHODOPHYTA)[1]

Patrick T. Martone[2,3]

Hopkins Marine Station of Stanford University, Pacific Grove, California 93950, USA

Diego A. Navarro,[2,4] *Carlos A. Stortz*[4]

Departamento de Química Orgánica (CIHIDECAR-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria – Pabellón 2, 1428 Buenos Aires, Argentina

and José M. Estevez[4,5]

Laboratorio de Fisiología y Biología Molecular (IFIByNE-CONICET), Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Pabellón 2, Ciudad Universitaria – Buenos Aires C1428EGA, Argentina

The articulated coralline *Calliarthron cheilosporioides* Manza produces segmented fronds composed of calcified segments (intergenicula) separated by uncalcified joints (genicula), which allow fronds to bend and reorient under breaking waves in the wave-swept intertidal zone. Genicula are formed when calcified cells decalcify and restructure to create flexible tissue. The present study has identified important differences in the main agaran disaccharidic repeating units [→ 3)-β-D-Gal*p* (1 → 4)-α-L-Gal*p*(1 →] synthesized by genicular and intergenicular segments. Based on chemical and spectroscopical analyses, we report that genicular cells from *C. cheilosporioides* biosynthesize a highly methoxylated galactan at C-6 position with low levels of branching with xylose side stubs on C-6 of the [→ 3)-β-D-Gal*p* (1 →] units, whereas intergenicular segments produce xylogalactans with high levels of xylose and low levels of 6-*O*-methyl β-D-Gal units. These data suggest that, during genicular development, xylosyl branched, 3-linked β-D-Gal*p* units present in the xylogalactan backbones from intergenicular walls are mostly replaced by 6-*O*-methyl-D-galactose units. We speculate that this structural shift is a consequence of a putative and specific methoxyl transferase that blocks the xylosylation on C-6 of the 3-linked β-D-Gal*p* units. Changes in galactan substitutions may contribute to the distinct mechanical properties of genicula and may lend insight into the calcification process in coralline algae.

Key index words: **agarans; *Calliarthron cheilosporioides*; cell wall; coralline algae; galactans; genicula; intergenicula; red seaweed**

Abbreviation: **HSQC, heteronuclear single quantum coherence**

Articulated coralline algae in the subfamily Corallinoideae (Rhodophyta, Corallinaceae) develop flexible genicula by secondarily decalcifying cells along their calcified thalli (Johansen 1969). Coralline decalcification is a well-regulated process that locally alters specific regions of genicular cell walls creating novel soft tissues from calcified cells. The development of flexible genicula permits calcified thalli to bend under breaking waves and to thrive in wave-swept intertidal habitats (Martone 2006, Martone and Denny 2008). However, the striking transformation from calcified intergenicula to flexible genicula is not well understood. In particular, while past studies have noted differences between intergenicular and genicular segments based on distinct staining patterns for neutral and anionic polysaccharides (Yendo 1904, Johansen 1969), these patterns have never been investigated by chemical methods.

Red algal cell walls are defined by both the wall material immediately adjacent to the plasma membrane and the intercellular matrix between cells. Cell walls in the Florideophyceae are mostly composed of sulfated galactans, (glyco)proteins, and skeletal polysaccharides (e.g., cellulose, mannans, xylans) (Heaney-Kieras et al. 1977, Craigie 1990, Liu and Reith 1996, Liu et al. 1996, Flores et al. 1997,

[1]Received 9 June 2009. Accepted 7 December 2009.
[2]These authors contributed equally to the present study.
[3]Present address: Department of Botany, University of British Columbia, Vancouver, BC, Canada V6T 1Z4.
[4]Research Member of the National Research Council of Argentina (CONICET).
[5]Author for correspondence: e-mail jestevez@fbmc.fcen.uba.ar.

2000, Lechat et al. 2000). Sulfated galactans are the most abundant polymers in red seaweeds, including carrageenans, agarans, and DL-galactan hybrids (Usov 1992, Ciancia et al. 1993, Takano et al. 1993, Miller 1997, Stortz and Cerezo 2000, Estevez et al. 2004, 2008, Zibetti et al. 2005), although other structures have been found in Nemaliales and Palmariales (e.g., sulfated xylomannans, xylans, etc.) (Matulewicz et al. 1992, Kolender et al. 1995, Jerez et al. 1997, Stortz and Cerezo 2000). These cell wall polymers play a central role in algal physiology, contributing to the mechanical stability, hydration, and ionic and osmotic regulation of seaweeds in the marine environment (Kloareg and Quatrano 1988). Furthermore, past studies linked polysaccharide composition to calcium carbonate precipitation, suggesting that these polysaccharides may be involved in thallus calcification (Okazaki et al. 1984, Cabioch and Giraud 1986, Borowitzka and Larkum 1987, Bilan and Usov 2001).

Carrageenans are linear polysaccharides composed of alternating 3-linked β-D-galactose (G) and 4-linked α-D-galactose (DG) units or its 3,6-anhydro derivative (DA) (Fig. 1). Agarans, on the other hand, are diastereomeric sulfated galactans in which the α-galactose (and/or its 3,6-anhydro derivative) units belong to the L-series (LG and LA, respectively; Fig. 1). The regular backbone of these galactans is usually masked by the presence of different *O*-linked groups such as methyl ethers, sulfate esters, pyruvic acid ketals, or side chains of β-D-Xyl*p* or β-D-Gal*p* (Miller 1997, Stortz and Cerezo 2000). Members of the Corallinales primarily biosynthesize agarans with low to intermediate sulfate content (Stortz and Cerezo 2000). They are always substituted with β-D-Xyl*p* units, and often with methoxyl or sulfate groups attached to the C-6 of the β-D-Gal*p* units (G6X, G6M, or G6S, respectively). In addition, α-L-Gal*p* units are substituted with methoxyl or sulfate groups on C-2 (LG2M or LG2S, respectively) and C-3 (LG3M or LG3S, respectively) (Cases et al. 1992, 1994, Takano et al. 1996, Usov and Bilan 1996, 1998, Navarro and Stortz 2002, 2008). Although highly substituted ''xylogalactans'' are the most prevalent agarans in the Corallinales, intermediate quantities of partially sulfated or methoxylated LA units (LA2S and LA2M, respectively), low quantities of G2M, and side stubs of 2,3-di- and 3-*O*-methyl-D-galactose on C-3 and C-6 on LG units have been reported in some articulated genera (Navarro and Stortz 2002, 2008).

Herein, we compare the structure of water-soluble xylogalactans in genicular and intergenicular walls of the articulated coralline alga *C. cheilosporioides*. Until now, investigations of coralline polysaccharides have been carried out on whole thalli, so

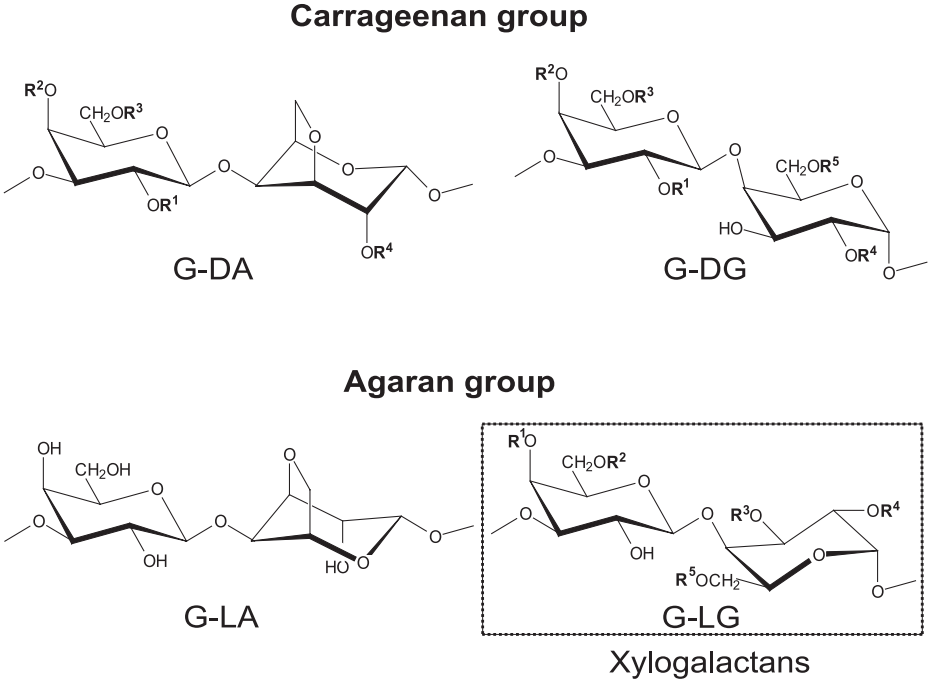

FIG. 1. Chemical structure of the idealized repeating units of sulfated galactans in red seaweeds. Carrageenan group comprises cyclized carrageenans (G-DA) and carrageenan precursor structures (G-DG). Different carragenan families are defined according to the substitutions on: $R^1 = SO_3^-$, $R^2 = R^3 = H$, lambda family. $R^2 = SO_3^-$, $R^1 = R^3 = H$, kappa family. $R^3 = SO_3^-$, $R^1 = R^2 = H$, omega family. $R^1 = R^2 = R^3 = H$, $R^4 = SO_3^-$, H, beta family. The agaran group comprises agarose structure (G-LA) and agarose precursor (G-LG6S), where $R^1 = R^2 = R^3 = R^4 = H$ and $R^5 = SO_3^-$. Agarans in articulated corallinoid algae present the following substitutions: $R^1 = H$, $R^2 =$ Xyl, Me, H; $R^3 =$ Me, H, SO_3^-; $R^4 =$ Me, H, SO_3^-; $R^5 = H$. Because of the high content of G6X units (and *t*-Xyl units), these particular agarans are also named as xylogalactans.

structural differences of xylogalactans present in genicula remained undetected (Cases et al. 1992, 1994, Takano et al. 1996, Usov et al. 1997, Navarro and Stortz 2002, 2008). In this work, we show for the first time that there are significant differences in the substitution pattern of xylogalactan backbones synthesized by genicular and intergenicular segments.

Sample collection. *C. cheilosporioides* Manza fronds were collected from the low-intertidal zone at various wave-exposed sites at Hopkins Marine Station, Pacific Grove, California. Only large branching thalli, ~10 cm in length (corresponding to the large size class described by Martone 2006), were selected for this study. All fronds were carefully inspected and epiphytized, or unhealthy branches were discarded.

Polysaccharide extraction. The extraction procedures were similar to those described elsewhere (Cases et al. 1994, Navarro and Stortz 2002), with few modifications. In addition to analyzing whole thalli, genicular and intergenicular regions were separated using a razor blade and were analyzed individually. Briefly, ball-milled whole thalli (15 g) and intergenicula (3 g) were suspended in water ($50\ mL \cdot g^{-1}$), and HCl (d) was added dropwise (preventing the pH from dropping below 6) with mechanical stirring at 4°C until no more CO_2 evolution was detected. Solutions were neutralized, dialyzed (molecular weight cutoff 6.0–8.0 kDa), and freeze-dried. Isolated genicula (20 mg), intergenicula (250 mg), and whole thalli (350 mg) were extracted with water ($0.4\ mL \cdot mg^{-1}$) by stirring for 24 h at room temperature and 6 h at 90°C. Solutions were cooled and then incubated with α-amylase (Sigma) for 24 h at room temperature to degrade floridean starch, dialyzed to remove salts (molecular weight cutoff 3.5 kDa), and freeze-dried to obtain entire cell walls (CW). Soluble and insoluble cell wall fractions from each CW (18 mg from genicula, 230 mg from intergenicula, and 320 mg from whole plant) were separated by redissolution in water and centrifugation (Hitachi CT1SRE, Tokyo, Japan). Supernatants corresponding to xylogalactan fractions obtained from intergenicula (Intergen), genicula (Gen), and from the whole thalli (Gen + Intergen) represent ~30%–40% w/w of the initial CW. Soluble cell wall fractions were freeze-dried and analyzed separately.

Chemical analysis. Total sugar content was analyzed by phenol-sulfuric acid method (Dubois et al. 1956) without previous hydrolysis of the polysaccharide. Sulfate was determined turbidimetrically (Dodgson and Price 1962). To analyze sulfated (xylo)galactans composition, alditol acetates were obtained by reductive hydrolysis and acetylation of the samples (Stevenson and Furneaux 1991). Inositol was used as an internal standard. For the absolute configuration assignment of the galactose units, Gen and Gen + Intergen fractions were hydrolyzed with TFA 2M, and the acid was eliminated by evaporation. Aliquots were converted to the acetylated aminodeoxyalditols using (S) 1-amino-2-propanol and (S)-α-methylbenzylamine (Cases et al. 1995) and analyzed as stated therein. For the linkage analysis, isolated polysaccharides from Gen + Intergen samples (3–10 mg) were converted into the corresponding triethylammonium salt (Stevenson and Furneaux 1991) and methylated according to Ciucanu and Kerek (1984) using finely powdered NaOH as base. The methylated sample was derivatized to alditol acetates as described elsewhere (Albersheim et al. 1967). Acetylated aminodeoxyalditols, alditol acetates, and partially methylated alditols were analyzed by gas chromatography (GC) as described previously (Navarro and Stortz 2002, 2008).

Percent calcium carbonate and genicula. To determine the $CaCO_3$ proportion of *C. cheilosporioides* thalli, 10 fronds were dried overnight at 50°C and weighed. Fronds were then decalcified thoroughly in 1N HCl at 25°C, dried overnight, and reweighed. Percent of $CaCO_3$ was calculated from weight loss. To measure the mass of the genicula, 20 segments proximal to the first dichotomy were isolated from four fronds. For each frond, segments were weighed intact, and then genicula were removed and weighed separately. Difference in sample weight was used to calculate percent of dry frond mass composed of genicula. Dividing proportion genicula by (1 – proportion $CaCO_3$) yielded the proportion of uncalcified dry mass composed of genicula

LM and TEM. *C. cheilosporioides* fronds were immersed in dilute fixative (1% glutaraldehyde, 1% formaldehyde, 98% filtered seawater) for 24 h and then decalcified as described above. Genicula were excised completely by cutting through decalcified intergenicula. Samples were dehydrated with ethanol and embedded in Spurr's resin. For LM, thin long-sections (4 μm) were cut through genicula using a standard microtome. For TEM, ultrathin sections were stained in uranyl acetate and lead citrate. Images were taken using a JEOL 1230 TEM (JEOL Ltd., Akishima, Tokyo, Japan) at 80 kV.

NMR spectroscopy. Polysaccharides (10–30 mg) were exchanged three times with 99.9% D_2O (0.5 mL), stirred 24 h at room temperature, centrifuged, and freeze-dried. Gen and Gen + Intergen fractions were analyzed by one-dimensional (1-D) and two-dimensional (2-D) NMR carried out with a Varian Unity INOVA 600 MHz spectrometer operating at ^{1}H and ^{13}C frequencies of 599.801 and 150.830 MHz, respectively. Proton decoupled 125 MHz ^{13}C NMR spectra were recorded using a Varian Unity INOVA 500 MHz NMR spectrometer (Palo Alto, CA, USA). Spectra were collected at 25°C, referenced to acetone or methanol, and expressed with respect to tetramethylsilane (TMS), with δ 31.1 ppm or 50.1 ppm, respectively. For ^{1}H

NMR spectroscopy, spectra were recorded at 25°C, with external reference to TMS and internal reference to acetone (δ 2.17 ppm). Pulse sequences for 2-D heteronuclear single quantum coherence (HSQC) followed the spectrometer manufacturers. Data were processed using Sparkly 3.113 for 2-D spectra and MestRe-C Nova for 1-D spectrum.

Results. The articulated red corallinoid *C. cheilosporioides* is characterized by the presence of large genicula separating integenicular segments (Fig. 2, a and b). At the TEM level, the interface between genicula and intergenicula is very clear (Fig. 2, c and d). Intergenicular cells (Fig. 2e) are encased in calcified primary cell walls, whereas genicular cells locally decalcify and develop thick electron-dense primary and secondary walls (Fig. 2f), suggesting important chemical differences as previously suggested (Yendo 1904, Johansen 1969, Martone et al. 2009). In order to detect differences in the water-soluble cell wall polysaccharides in genicular and intergenicular segments from *C. cheilosporioides*, thallus regions were isolated, analyzed separately, and compared to cell wall polymers extracted from whole thalli. Calcium carbonate comprised 84.7 ± 0.4% of the dry weight (w/w) of the thalli and dominates the cell walls from intergenicular regions (Fig. 2, a–e). Overall, genicular segments (Fig. 2, a and f) comprised only 3.3% of the dry weight fronds, but 21.8% of the decalcified dry frond weight.

Galactose, 2- and 6-*O*-methylgalactose, and xylose were the main sugars detected, although molar ratios varied between soluble cell wall polysaccharides obtained from genicula (Gen) and from intergenicula and whole thalli (Intergen and Gen + Intergen, respectively) (Table 1). In Gen, the molar ratio of [galactose + mono-*O*-methylgalactose]: xylose was 1:0.07, while in Intergen and Gen + Intergen, the ratio was 1:0.36 and 1:0.24, respectively. Variable amounts of glucose and mannose were also detected in several fractions as reported previously (Cases et al. 1992, 1994, Usov et al. 1997, Navarro and Stortz 2002), possibly arising from minor uncharacterized cell wall or cytosolic carbohydrates. The D:L galactose ratios were close to unity (~1:0.97 and ~1:1.05 for whole thalli and intergenicular fractions, respectively). Low amounts of L-arabinose were also detected in genicular and intergenicular samples (Table 1). This sugar in the furanosic form is a common side chain of several pectic polysaccharides present in vascular plant cell walls (Cosgrove 2005). In order to assign the absolute configuration of the galactose residues, samples were derivatized with chiral amines (Cases et al. 1995). In the Gen + Intergen fraction, mono-*O*-methylated galactoses were mainly present as 6-*O*-methyl-D-galactose (G6M) and 2-*O*-methyl-L-galactose units (LG2M). In addition, small amounts of 2-*O*-methyl-D-galactose units (G2M) were detected. Monosaccharide analysis (Table 1) showed that

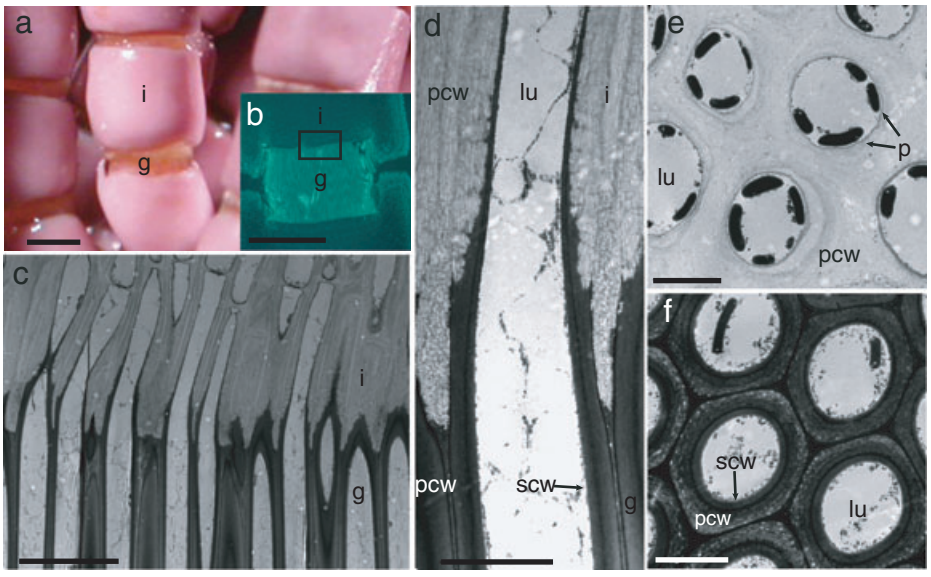

FIG. 2. *Calliarthron cheilosporioides* thallus structure. Thalli are segmented with uncalcified joints, called genicula, separating calcified segments, called intergenicula. (a) General aspect of the articulated frond. (b) Long-section of the thallus showing one geniculum between two intergenicular segments under UV light. Boxed area shows a geniculum-intergeniculum transition zone. A comparable area is magnified under TEM in (c). (c) Longitudinal section of a geniculum-intergeniculum transition zone. Each geniculum is made up of a single tier of elongated cells that connect two adjacent intergenicula. (d) TEM long-section of single cell at transition zone in detail. (e) TEM cross-section of intergeniculum showing low electron-dense primary cell walls. (f) TEM cross-section of a geniculum showing thick electron-dense primary and secondary cell walls. Scale bars: (a) = 200 μm, (b) = 200 μm, (c) = 20 μm, (d) = 5 μm, (e) = 5 μm, (f) = 5 μm. i, intergeniculum; g, geniculum; lu, lumen; p, plastids; pcw, primary cell wall; scw, secondary cell wall.

TABLE 1. Analyses of the galactan sulfates obtained from genicula (Gen), intergenicula (Intergen), and whole thalli (Gen + Intergen) of *Calliarthron cheilosporioides*.

		Monosaccharide composition (mol%)[b]					
(Xylo)galactans[a]	SO_3Na %	D-Gal	L-Gal	2-D-Gal	2-L-Gal	6-D-Gal	D-Xyl
Gen + Intergen	4.9	32.0	25.6	1.7	12.6	8.9	19.2
Gen	5.8	← 63.0 →		nd	3.6	26.5	6.9
Intergen	4.7	27.2	25.2	nd	12.3	8.5	26.8

2-D-Gal = 2-*O*-methyl-D-galactose; nd = not detected.
[a](Xylo)galactans isolated from the whole thalli (Gen + Intergen), genicula (Gen), and intergenicula (Integen).
[b]Percentages are shown after discounting glucose (3.3–7.1 mol%), mannose (2.9–11.4 mol%), and arabinose (3.7–6.0 mol%).

Intergen and Gen + Intergen fractions carry a high proportion of xylose units (~27 and 19 mol%, respectively) and a low proportion of 6-*O*-methyl-D-galactose units (~9 mol% and 8 mol%, respectively). This pattern is typical of xylogalactans previously reported for other corallinoid seaweeds (Cases et al. 1992, 1994, Usov and Bilan 1996, 1998, Navarro and Stortz 2002, 2008). Unlike intergenicular segments, genicular fractions contained ~3.2-fold increased proportions of 6-*O*-methyl-D-galactose (27 mol%) and 3.9-fold decreased proportions of xylose and 2-*O*-methyl-L-galactose (7 mol% and 4 mol%, respectively; Table 1).

Linkage analysis (Table 2) showed a ratio of 3-linked β-D-Gal and 4-linked α-L-Gal of ~1:1.1 in the Gen + Intergen fraction, suggesting a classic agaran backbone structure. The 3-linked β-D-Gal*p* residues were mostly substituted on C-6 (~22 mol%) by single side chains of xylose units (G6X), corresponding to the *t*-xylosyl units (~22 mol%) that were also detected (as 2,3,4-tri-*O*-methylxylose derivative). Both are in agreement with the 19.2 mol% of xylose detected in the monosaccharide analysis for the same fraction (Table 1). Some residues were methoxylated at C-6 based on the initial monosaccharide analysis (~9 mol%, G6M, Table 1) with a low proportion of nonsubstituted galactose units (~2 mol%, G). In most cases, either 4-linked α-L-Gal units were methylated on C-2 (LG2M) or nonsubstituted (LG) based on the compositional analysis (Table 1). Small amounts of putative 4-linked α-L-Gal units may be sulfated either on C-2 (LG2S) or on C-6 (LG6S). Several attempts to achieve a complete methylation of the Gen sample were unsuccessful.

TABLE 2. Linkage analyses of the isolated (xylo)galactans obtained whole thalli (Gen + Intergen).

Deduced linkage[a]	Structural unit[b]	Gen + Intergen[c]
3-linked D-units		
t-Gal	G or LG	4.1
3-Gal	G(6M)	10.4
3,6-Gal	G6X(6S)	22.3
4-linked L-units		
4-Gal	LG(2M)	26.8
3,4-Gal	LG(2M)3S and/or G4S	5.2
2,4-Gal	LG2S	7.5
4,6-Gal	LG(2M)6S	2.0
D-units:L-units		1.0:1.1
t-Xyl	G6X	21.7

Units are expressed as mol%.
[a]3-Gal = 3-linked β-D-galactose; 4-Gal = 4-linked α-L-galactose.
[b]LG2S = 4-linked α-L-galactose substituted on C-2.
[c]Percentages are expressed after discounting glucose, mannose, and arabinose units.

The structure of the xylogalactans obtained after linkage analysis of the Gen + Intergen fraction (Table 1) was corroborated by NMR analysis (Fig. 3 and Table 3). The most conspicuous signals in the ^{13}C NMR spectrum of Gen + Intergen (Fig. 3a) corresponded to the C1-C5 of the β-D-Xyl*p* units (δ_{C1-C5} at 104.3, 73.7, 76.4, 70.0, 65.8) linked to the C-6 of the β-D-Gal*p* (G6X), as typically occurs with xylogalactans from corallines seaweeds (Usov and Bilan 1996, 1998, Navarro and Stortz 2008). In the anomeric zone of the HSQC spectrum, 3-linked β-D-Gal*p* units substituted with 6-*O*-methyl groups (G6M) were assigned to δ_{H1}/δ_{C1} at 4.55/104.1 and to δ_{C6} at 70.5 for the substituted C-6. In addition, some nonsubstituted 3-linked β-D-Gal*p* units (G) were detected (δ_{H1}/δ_{C1} 4.42/103.2). The 4-linked α-L-units were mainly unsubstituted (LG, δ_{H1}/δ_{C1} 5.28/101.3), but some carried 2-*O*-Me groups (LG2M, δ_{H1}/δ_{C1} at 5.49/98.8 and δ_{H2}/δ_{C2} at 3.60/79.1) and some 2-sulfated units (LG2S, δ_{H1}/δ_{C1} at 5.53/99.3 and δ_{H2}/δ_{C2} at 4.49/76.8 for the sulfate ester substitution). Similar structural units were detected in the linkage analysis (Table 2).

Based on the configurational analysis, it was possible to assign by $^{13}C/^{1}H$ NMR most of the 6-*O*-methylgalactose units with D-configuration to δ_{H1}/δ_{C1} 3.38/59.0 (G6M) and 2-*O*-methylgalactose units with L-configuration at δ_{H1}/δ_{C1} 3.44/58.0 (LG2M). Extra minor correlations were detected mainly in Gen + Intergen fraction at δ_{H1}/δ_{C1} 3.42/60.0 and 3.46/60.0. One of them indicates small amounts of 2-*O*-methyl-D-galactose units (G2M, Fig. 3a) also shown to occur by GC analysis. The assignments for *O*-methyl groups in G6M and LG2M units are in concordance with those reported previously (Usov et al. 1997, Navarro and Stortz 2008).

The HSQC spectrum of the Gen fraction (Fig. 3b) showed major correlations corresponding

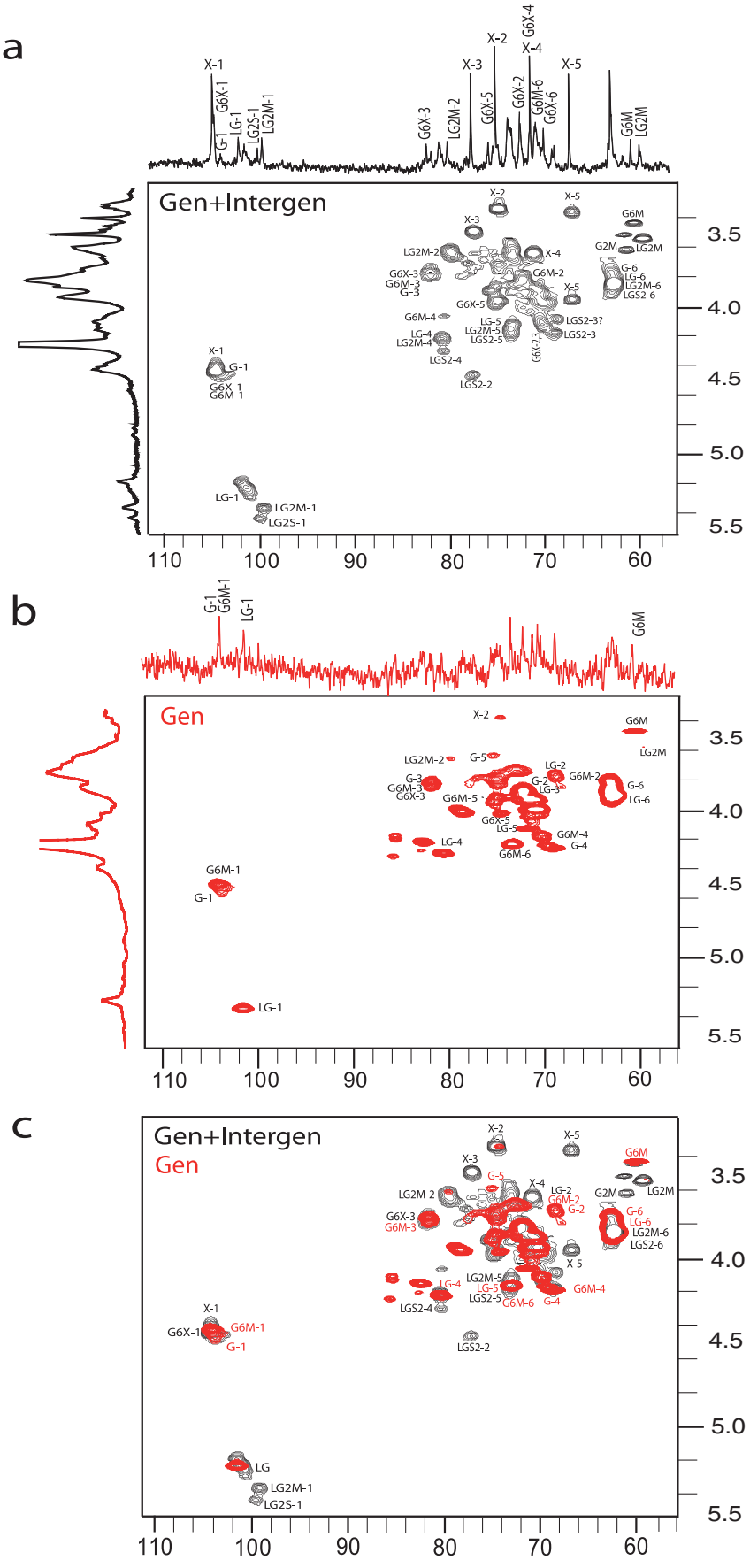

FIG. 3. Heteronuclear single quantum coherence (HSQC) spectra of (xylo)galactans of *Calliarthron cheilosporioides* obtained from whole thallus (a) and Gen fraction isolated only from geniculа (b). Both spectra were superimposed (c) in order to reveal differences between them. (a) The most evident signals in Gen + Intergen HSQC spectra correspond to the β-D-Xyl*p* units (X) linked to the C-6 of the β-D-Gal*p* (G6X). In addition, important correlations were detected for 3-linked β-D-Gal*p* units substituted with 6-*O*-methyl groups and substituted with β-D-Xyl*p* units (G6X). In addition, some nonsubstituted 3-linked β-D-Gal*p* units (G) were detected. On the other hand, intense correlations were assigned to 4-linked α-L-units mainly unsubstituted (LG), to some 2-*O*-Me substituted (LG2M), and for 2-sulfated units (LG2S). (b) Gen HSQC spectra showed major correlations for 3-linked 6-*O*-methyl and nonsubstituted 6-*O*-methyl β-D-Gal*p* (G6M and G, respectively) units together with 4-linked nonsubstituted α-L-Gal*p* residues (LG). Those signals for G6X, LG2M, and LG2S units are still present in some cases, but their intensity is much lower or undetectable in comparison with the HSQC spectra of Gen + Intergen. (c) See Table 3 for assignments of the structural units present in the xylogalactan fractions from Gen + Intergen and Gen

TABLE 3. Chemical shift assignments[a] of the NMR spectra of (xylo)galactans from whole thalli (Gen + Intergen) and genicula (Gen) fractions isolated from *C. cheilosporioides*.

Residue	C-1/H-1	C-2/H2	C-3/H-3	C-4/H-4	C-5/H-5a,b	C-6/H-6a,b
3-linked D-units						
G	103.2/4.42	71.1/3.75	81.2/3.75	69.4/4.10	74.6/3.70	61.4/3.70, 3.85
G6X	104.1	71.1	80.6–81.2	~69.5	74.6	69.4
G6M	104.1/4.55	71.1	80.6–81.2	68.6	73.6	70.5
6-*O*-Me	59.0/3.38					
4-linked L-units						
LG	101.3/5.28	69.4/3.84	70.4/3.92	79.8/4.22	72.4–72.0/4.15	61.6/3.82, 3.95
LG2S	99.3/5.53	76.8/4.49	67.8/4.08	77.0/4.30	71.6/4.18	61.4/3.82, 3.95
LG2M	98.8/5.49	79.1/3.60	69.4/3.95	79.5/4.22	72.4–72.0/4.18	61.4/3.82, 3.95
2-*O*-Me	58.0/3.44					
t-Xyl (G*6X*)	104.3/4.45	73.8/3.37	76.4/3.48	70.0/3.66	65.8/3.77, 4.00	

[a]Chemical shift assignments were based on previous work of Usov et al. (1997) and Navarro and Stortz (2008).

to 3-linked 6-*O*-methyl (G6M, δ_{H1}/δ_{C1} 4.42/104.1) and nonsubstituted 3-linked Gal*p* units (G, δ_{H1}/δ_{C1} 4.42/103.3) with 4-linked nonsubstituted α-L-Gal*p* residues (LG, δ_{H1}/δ_{C1} 5.28/101.3). Signals for G6X, G2M, and G2S units were sometimes present (e.g., *t*-Xyl, δ_{H2}/δ_{C2} 3.4/73.8), but their intensities were reduced or undetectable in comparison with those found in the Gen + Intergen spectra (Fig. 3c). The main structural unit assignments of xylogalactans in Gen + Intergen and Gen fractions are described in Table 3 based on the interpretation of HSQC spectra (Fig. 3).

This study identifies important differences in the agaran structures synthesized by genicular and intergenicular segments in *C. cheilosporioides*. Based on the agaran disaccharidic repeating unit [→ 3)-β-D-Gal*p* (1 → 4)-α-L-Gal*p*(1 →] (Fig. 2), we conclude that genicular cells biosynthesize a highly C-6 methoxylated galactan with relatively low levels of xylose side chains, while intergenicular cells produce ''typical'' xylogalactans with relatively low levels of 6-*O*-methyl groups. These data suggest that, during genicular development, the galactose units substituted at *O*-6 with xylosyl stubs (G6X) in the xylogalactans of intergenicular cell walls are mostly replaced by 6-*O*-methylgalactose units (G6M) in genicular cell walls. In addition, 2-*O*-methyl groups (LG2M) in the intergenicular cell walls may be partly replaced by sulfate groups (LG2S) in genicula, suggesting another difference in the substitution pattern on the galactan backbone (Tables 1–3). Given that genicula comprise a small but significant proportion of the decalcified dry weight of articulated fronds (~22% w/w in *C. cheilosporioides*), polysaccharide characterizations of other corallines based on pooled samples of genicula and intergenicula (Cases et al. 1992, 1994, Usov et al. 1997, Navarro and Stortz 2002, 2008) likely overlooked the presence of low-xylosyl xylogalactans detected in this study. Thus, the galactans synthesized by genicula have intermediate characteristics between those present in the intergenicular segments and the noncyclized agarans found in uncalcified red seaweeds such as representatives of the Ceramiales and Gracilariales (Usov 1992, Takano et al. 1993, Miller 1997, Stortz and Cerezo 2000).

One feasible explanation for the structural difference between genicular and intergenicular xylogalactans is that putative methoxyl transferases block the C-6 of the β-D-Gal*p* units with a methoxy group before the xylosyltransferase adds the xylosyl units to the galactan backbone. Alternatively, xylosyltransferase activity or abundance of these putative enzymes may differ among genicular and intergenicular segments. For example, putative xylosyltransferase may be highly expressed in intergenicular cells but repressed or absent in genicular cells. Previous studies of the articulated coralline *Corallina officinalis* demonstrated lower levels of xylose (~23 → ~7 mol%) and higher levels of 6-*O*-methyl-D-galactose (tr. → ~9–11 mol%) after fractionation (Cases et al. 1992, 1994). If the chemical structure of galactans from genicular cell walls is highly conserved among all articulated corallinoids, these galactan fractions may have arisen primarily (or exclusively) from genicular cell walls. Furthermore, high levels of 6-*O*-methyl groups in the β-D-galactose units (G6M) detected from the articulated coralline *Bossiella orbigniana* (Navarro and Stortz 2002) may reflect morphological similarities between *C. cheilosporioides* and *Bossiella*, which are both characterized by large genicula. Delicate corallines, such as *C. officinalis*, *C. pilulifera*, and *Jania rubens,* have smaller genicula, and, perhaps as a consequence, the quantity of 6-*O*-methylgalactose units is generally <3 mol% of the overall polysaccharide content and is often undetectable (Cases et al. 1992, Takano et al. 1996, Usov et al. 1997, Navarro and Stortz 2008). Further studies of genicula from other articulated corallinoids are needed in order to understand if the agaran structures reported here are generalizable to other seaweeds in this group.

Differences in galactan substitution patterns among genicula and intergenicula likely affect the spacing and overall packaging of adjacent molecules within *C. cheilosporioides* cell walls. Few xylose side chains and increased 6-*O*-methoxylation of xylogalactans may allow or improve polymer compaction

during the expansion and elongation of genicular cell walls. Changes in polysaccharide chemistry have been shown to affect mechanical properties of algal thalli (Toole et al. 2002), and highly methylated agarans may contribute to the distinct biomechanical properties of genicular tissue (Martone 2006, 2007). Correlations between chemical composition and mechanical properties of genicular cell walls are currently being explored.

Differences in galactan substitutions among calcified intergenicula and uncalcified genicula may help clarify the calcification process in coralline algae. Cell wall polysaccharides may influence precipitation of calcium carbonate into coralline cell walls by generating sites for crystal nucleation or by attracting ions electrostatically, although details about this process are not completely understood (Borowitzka 1977, Borowitzka and Larkum 1987, Cabioch and Giraud 1986). Studies have shown that $CaCO_3$ crystals align precisely along an organic matrix (thought to be a protein-polysaccharide complex) within coralline cell walls (Borowitzka and Vesk 1978, Cabioch and Giraud 1986, Borowitzka and Larkum 1987), and it is plausible that the xylose-branched structure of xylogalactans within intergenicular walls helps define sites for calcium carbonate nucleation. Highly methylated galactans produced by genicula may not support carbonate nucleation, preventing mineral deposition after decalcification and maintaining genicular flexibility.

Conclusion. The present study identifies important differences in the xylogalactans of the agaran-type synthesized by genicula and intergenicula in the articulated coralline *C. cheilosporioides*. The data suggest that during genicular development, 6-xylosyl-substituted, 3-linked β-D-Gal*p* units present on the xylogalactan backbones in intergenicular cell walls are mostly replaced by 6-*O*-methyl galactose moieties. We speculate that this structural shift may be a consequence of a putative and specific methoxyl transferase that could block the xylosylation site at C-6 of the 3-linked β-D-Gal*p* units. Differential galactan substitutions may contribute to the distinct mechanical properties of genicula and may lend insight into the calcification process in coralline algae.

The authors acknowledge S. Lynch for the NMR experiments (NMR Facility, Stanford University), and C. Somerville for making available to J. M. E. the infrastructure of Carnegie Institution of Washington (Stanford University). Special thanks to the volunteers at Hopkins Marine Station who helped dissect genicula out of coralline fronds. This work was supported in part by grants from FONCyT (PICT 2006-0983, J. M. E.), CONICET (PIP 5699, C. A. S.), UBA (X-214, C. A. S.), NSF Grant# IOS-0641068 (M. W. Denny) at the Hopkins Marine Station, and the Phycological Society of America (P. T. M.).

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