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The system of xylogalactans from the red seaweed *Jania rubens* (Corallinales, Rhodophyta)

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ABSTRACT

The main acidic polysaccharides from the red seaweed *Jania rubens* share the general characteristics of corallinans (agar-like xylogalactans). After fractionation by ion-exchange chromatography, ten fractions were separated and characterized by sugar composition, other components, methylation, ethylation, desulfation–methylation, and NMR analyses. The main group of fractions carry the agaran disaccharidic repeating unit [→3)-β-D-Gal-(1→4)-α-L-Gal-(1→] substituted mainly on O-6 of the β-D-Gal unit by β-xylosyl side stubs, and less with sulfate or methoxyl groups, and also on O-2 of the α-L-Gal unit with methoxyl or sulfate, or less on O-3 of the same unit with methoxyl groups. These features are somehow common to the four members of the order already studied. However, a sugar uncommon to the order appears in moderate proportions for all the fractions: it is 3,6-anhydro-L-galactose (partly sulfated or methoxylated on O-2) replacing the L-Gal unit. Besides, several other structural features never found in the order (and uncommon in any polysaccharide) appear in some minor fractions: the presence of side stubs of 2,3-di- and 3-*O*-methyl-D-galactose, and also part of the 3-*O*-methyl-L-galactose acting as side stubs. These results show that, although the main features of the corallinean xylogalactans are common to all the species studied, each one has minor characteristics of its own.

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1. Introduction

Sulfated galactans are the main polysaccharides constituting the intercellular matrix and non-fibrillar cell walls of most red seaweeds (Rhodophyta). Their structure can be depicted by linear chains of alternating 3-linked β-galactose units and 4-linked α-galactose residues. The galactose in the residues with the β-configuration always belongs to the D-series, whereas the α-galactose residues may belong to the D- or the L-configuration, giving rise to a classification of the red seaweed galactans as carrageenans (those with α-galactose units of the D-series) and agarans (those with 4-linked α-L-galactose residues).[1,2] This simple scheme can be complicated by the appearance of 4-linked D- and L-galactose units interspersed in the same molecules, and by the masking of these regularly repeating units by substitution with sulfate groups, pyruvic acid ketals, methoxylation, side chains, or the presence of a 3,6-anhydro ring replacing the α-galactose unit.[1,2]

Red seaweeds usually produce large amounts of galactans, and many of them have industrial interest given their gelling and thickening properties. Thus, the products from species belonging to different orders have been studied in detail.[2] However, those belonging to the Corallinales have received less attention, due to their calcareous cover, which diminishes the yield of polysaccharides. Besides a preliminary report,[3] the first detailed study was carried out with the Atlantic species *Corallina officinalis*,[4] where a special type of agaran was found (named 'corallinan') having methoxyl and/or sulfate groups on positions 2 and 3 of the L-galactose residues, and large amounts of β-D-xylosyl or minor ones of sulfate groups attached at position 6 of the D-galactose units.[5] Similar structures, but with special characteristics in each case, were found in the Pacific Ocean species *Corallina pilulifera*,[6,7] *Joculator maximus*,[8] and *Bossiella cretacea*.[9,10] It was also found that these seaweeds produce floridean starch[3,4,6,8,9] and alginate.[6,9] The extraction procedures for corallinean polysaccharides are an important issue, as the calcium carbonate cover[11] has to be removed without affecting the polysaccharide integrity. Each one of the three groups studying these xylogalactans used a different procedure.[4,6,8] A thorough study of the three methods[12] showed that the acid medium gave better yields of xylogalactans, without evidence of degradation in the first extraction, but some losses were noted in further hot-water extractions when traces of acid remained.

Jania rubens (L.) Lamoroux is another member of the order Corallinales present in the Atlantic Coast, usually mixed with *C. officinalis*. Only a preliminary study of its polysaccharides was ever published.[12] Herein, we report the isolation and fractionation of the xylogalactans from *J. rubens*, as well as the structural

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characterization of the fractions using methylation, desulfation, ethylation, and NMR analyses.

2. Results

2.1. Isolation and fractionation

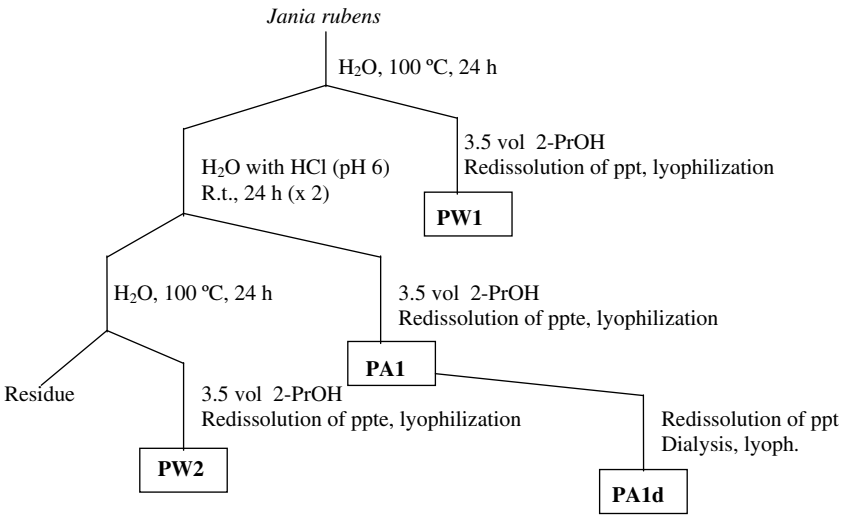
A sample of the milled seaweed *J. rubens* was subjected to the successive extractions steps shown in Scheme 1, yielding three different fractions after purification by redissolution. The analytical characteristics of these three fractions are shown in Table 1. The yields of the products are similar to those encountered in a smaller scale study.[12] The three fractions contain carbohydrates, protein, and sulfate ester. The proportion of uronic acid was very low (<2%) for all fractions. The proportion of protein and starch (as indicated by Glc) is much higher in the fractions extracted with boiling water. However, the three fractions contain similar Gals/Xyl ratios (indicative of the presence of xylogalactans), and D/L-Gals ratios close to 1, suggesting that agaran structures are dominant. The molecular weight of the products containing starch is lower, as expected.[12] Some degradation can also be present in **PW2**, considering the possible presence of traces of acid in the hot-water extraction.[12] Units of galactose methylated in three out of the four available positions are present in the three fractions: those methoxylated in position 2 belong to the L-series, whereas those methoxylated in position 6 belong to the D-series, as occurs with other similar polysaccharides.[1] However, both enantiomers of 3-*O*-methylgalactose (3-MeGal) were encountered. The L-enantiomer was already found in *C. officinalis* and *C. pilulifera*,[5–7] but the D-enantiomer is encountered for the first time, in low proportions in the three fractions. Another sugar found for the first time in the corallinans is 3,6-anhydrogalactose (3,6-AnGal).[12] As expected for an agaran, in **PA1** it belongs to the L-series. However, the presence of both enantiomers in the hot-water extractions may suggest the occurrence of a carrageenan or hybrid-like product in these fractions. Besides the already mentioned contamination with starch and protein, and the presence of 3,6-An-D-Gal in **PW1** and **PW2**, the composition of the xylogalactans in the three fractions is very similar (Table 1). Thus, further studies were carried out with **PA1**, which contains the largest amount of xylogalactans. **PA1** was purified by redissolution, dialysis, and lyophilization. In this way, the product **PA1d** appeared enriched in carbohydrates (Table 1). Small amounts of two new sugars were encountered in **PA1d**: the 2-*O*-methyl ether of 3,6-An-L-Gal and 2,3-di-*O*-methyl-D-galactose (2,3-diMeGal).

Table 1
Yields and analyses of the products extracted from *Jania rubens*

	PW1	PA1	PW2	PA1d
Yield (%)	1.4	1.0	0.5	0.3
Carbohydrates (%)	71	40	50	80
Protein (%)	15	5	19	6
Sulfate (% $NaSO_3$)	8	17	9	12
Mol. weight (kDa)	15	45	2	17
Component sugars (mol/100 mol [Gal + methylated Gals][a]				
Xyl	35	42	34	51
2-*O*-Me-L-Gal	9	9	10	11
3-*O*-Me-D-Gal	2	2	1	2
3-*O*-Me-L-Gal	5	4	6	4
6-*O*-Me-D-Gal	5	2	4	2
D-Gal	42	48	41	47
L-Gal	30	27	31	25
3,6-An-D-Gal	5	—	4	—
3,6-An-L-Gal	2	8	3	8
D-Man	2	1	4	1
D-Glc	170	8	444	7
Ratios				
D/L	54:46	52:48	50:50	50:50
Gals/Xyl	2.7	2.4	2.9	2.0

[a] Calculated as an average of determinations made as alditol acetates, aldononitrile acetates, and acetylated 1-deoxyaminoalditols.

Fraction **PA1d** was separated preparatively into subfractions by means of ion-exchange chromatography on DEAE-Sephadex A-50[3,4] using increasing concentrations of NaCl as eluant. The separation profile is shown in Figure 1: ten fractions were isolated. Seven of them appeared when changes in the NaCl concentration were made (from 0.1 to 0.7 M), whereas the other three were 'tails' of the main peak at concentrations of NaCl between 0.3 and 0.5 M. No products were obtained at NaCl concentrations higher than 0.7 M (up to 2.5 M). The yields and analyses of the fractions can be seen in Table 2, and their component sugars in Table 3. The total yield is ca. 65%. About 70% of the recovered material corresponds to a group of five fractions (**4a**–**6**, centered in **5a**, the most abundant one) which share most of their analytical characteristics. The late-eluting fraction **7** is also similar, whereas the early-eluting fractions show different characteristics, becoming stepwise closer to those of the main group as the concentration of NaCl to elute



Scheme 1. Residues to the left, supernatants to the right.

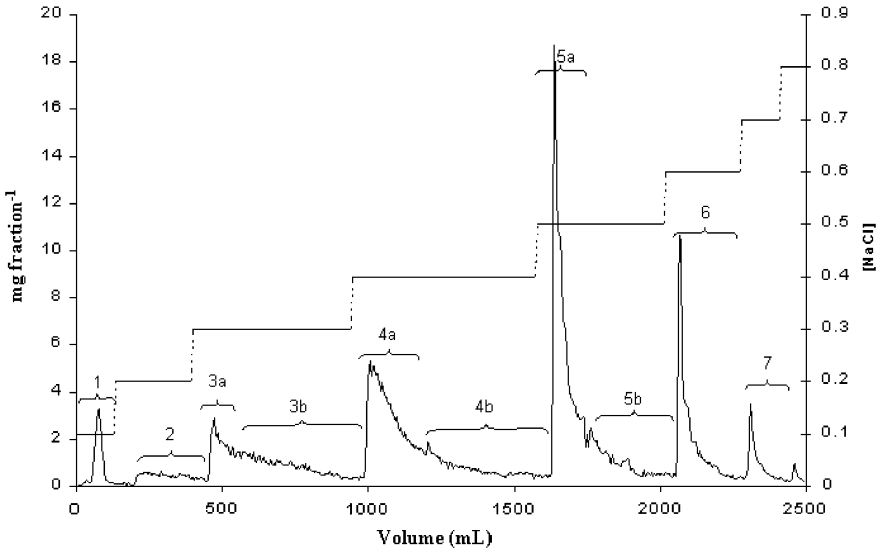


Figure 1. Elution profile of ion-exchange chromatography on DEAE-Sephadex A-50 of polysaccharide **PA1d**, with the acronyms of the isolated fractions.

Table 2
Yields and analyses of the fractions obtained by ion-exchange chromatography of the polysaccharides extracted from *Jania rubens*

	1	2	3a	3b	4a	4b	5a	5b	6	7
Yield (%)	1.2	3.7	4.5	8.2	13.0	5.1	13.6	3.3	9.2	2.7
Carbohydrates (%)	51	40	57	67	72	84	63	55	65	55
Protein (%)	9	28	10	6	2	1	2	1	2	2
Sulfate (% $NaSO_3$)	ND	12	10	10	11	14	11	15	11	14
Mol. weight (kDa)	ND	3.1	6.0	14	29	19	49	20	43	33
$[\alpha]_D$	+36	−49	−72	−61	−74	−65	−83	−70	−71	−71

Table 3
Proportions of component sugars[a] of the fractions obtained by ion-exchange chromatography of the polysaccharides extracted from *Jania rubens* (expressed as mol/100 mol [Gal + methylated Gals])

	2	3a	3b	4a	4b	5a	5b	6	7
Xyl	35	37	42	42	40	43	42	43	37
D-Gal	32	35	45	50	51	50	51	50	51
3-*O*-Me-D-Gal	9	9	6	4	2	1	–	–	–
6-*O*-Me-D-Gal	5	4	1	1	1	1	1	1	2
2,3-di-*O*-Me-D-Gal	3	2	1	–	–	–	–	–	–
L-Gal	26	25	23	25	28	26	29	28	30
2-*O*-Me-L-Gal	10	10	10	10	9	9	10	11	10
3-*O*-Me-L-Gal	9	9	6	4	3	2	2	2	3
3,6-An-L-Gal	5	5	7	7	6	10	6	7	3
3,6-An-2-*O*-Me-L-Gal	1	1	1	–	–	2	1	1	–
Man	4	tr.	tr.	–	–	–	–	–	1
Glc	5	2	1	–	–	–	–	–	–
Ratios									
D/L	1.0	1.0	1.1	1.2	1.2	1.0	1.1	1.1	1.1
Gals/Xyl	2.9	2.7	2.4	2.4	2.5	2.3	2.4	2.4	2.7

[a] Calculated as an average of determinations made as alditol acetates, aldononitrile acetates, and acetylated 1-deoxyaminoalditols.

them was increased. Fraction **1** was obtained in low yield. Although it also contained a xylogalactan, it concentrated almost all the glucose present in **PA1d** (8 mol/mol Gals). Highly contaminated with starch (as also shown by the optical rotation), this fraction was not studied any further. For the remaining fractions, the proportion of sulfate is very evenly distributed (10–12%). Only in the 'tail' fractions **4b** and **5b**, its proportion increased slightly (14–15%). The early-eluting fractions contain larger amounts of protein and lower molecular weights, two facts which can be related.[4] For the fractions not corresponding to a 'tail', an increase of the molecular weight with the order of elution can be observed (Table 2). The 'tail' fractions (**b**), however, have lower molecular weights, as does the latest eluting fraction **7**. Xylose and 2-*O*-methylgalactose are distributed evenly among all the fractions (37–43% and 9–11%, respectively). 3,6-AnGal and its 2-*O*-methyl ether are also distributed with no specific trend among the fractions, but with larger ranges of values (3–10% and 0–2%, respectively). The presence of 3,6-AnGal in all the fractions can be considered as evidence of its incidence within the corallinan chain, diminishing the possibility of a contaminating polysaccharide. On the other hand,

some sugars appear preferentially or exclusively in the early-eluting fractions. They are the contaminating ones Man and Glc, but also 2,3-diMe-D-Gal, 3-MeGal (especially the D-enantiomer), and 6-Me-D-Gal. In order to compensate for these appearances, the proportion of both D- and L-galactoses increases in the main fractions. The D/L-galactose ratio always keeps very close to 1 (Table 3), suggesting no major deviation from a classical agaran structure. The behavior found for 2,3-di- and 3-Me-D-Gal (abundance in early-eluting fractions, scarcity in the remaining ones) is similar to that encountered by Cases et al.[4,5] for its 4-*O*-methyl analog in the system of polysaccharides from *C. officinalis*. The latter sugar was found to occur as side chains of the main structure in the early-eluting fractions of those polysaccharides. **PA1d** was devoid of cyclizable 6-sulfate units, as determined chemically.

2.2. Structural analysis by chemical methods

The nine fractions were submitted to methylation analysis. After a single addition of NaOH and methylating agent in DMSO, the results shown in Table 4 were obtained. For fraction **5b**, undermethylation was put in evidence by the large proportions of non-methylated and monomethylated galactoses found (ca. 20%). Although the remaining pattern was similar to those of the other fractions, results for this fraction are not shown in Table 4. For the current fractions, the proportions of non-methylated and monomethylated are small, suggesting that undermethylation, if exists, is not a problem. In most of the fractions, 2,3,4-tri-*O*-methylxylose, 2,3,6-tri-*O*-methylgalactose, and 2,4-di-*O*-methylgalactose are the main sugars, together with lesser amounts of 2,4,6-tri-, 2,3-di-, 3,6-di-*O*-methylgalactose, and 2-*O*-methyl-3,6-anhydrogalactose. The early-eluting fractions also give substantial amounts of 2,3,4,6-tetra-*O*-methylgalactose by hydrolysis (Table 4), suggesting the presence of higher branching. 2,6-Di-*O*-methylgalactose is also important in these fractions. The proportion of methylated xylose units appears to be lower than that of xylose in the original fractions. This might be due to the volatility of the derivatives of the permethylated xylose monosaccharide which precludes its safe quantization. As 2,6-di-*O*-methylgalactose in agarans can originate in either 4-linked L-galactose units substituted at O-3, or in 3-linked D-galactose units also substituted at O-4, the configuration of the methylated sugar was determined[13] for fraction **3a**, where this sugar is in a high proportion. It was shown that it is entirely of the L-configuration. Thus, it should correspond to a 4-linked unit, sulfated or substituted at O-3. With the same reaction of reductive amination, it was possible to see that the high amounts of 2,3,4,6-tetra-*O*-methylgalactose correspond to the two enantiomers with a D:L ratio of 1.8, indicating that units with both configurations are acting as side chains.

In order to determine the location of naturally methylated sugars in the fractions of *J. rubens*, ethylation[5,14] was carried out on six fractions. Results are shown in Table 5. The ethylation data agree with those obtained by methylation. Also, the proportions of naturally methylated sugars determined by direct analysis (Table 3) are similar to those encountered after ethylation. The results show that the original 2,3-di-MeGal (in **3a**) gives rise only to the tetra-O-alkylated product, indicating that it acts as a side chain; 2-MeGal yields 2,3,6-tri- and 2,6-di-O-alkylated products; 3-MeGal yields 2,3,4,6-tetra- and 2,3,6-tri-O-alkylated products, whereas 6-MeGal yields exclusively a 2,4,6-tri-O-alkylated galactose. These results show that in most cases methyl groups do not appear in the same monomeric unit with another substituent, the presence of 3-*O*-ethyl-2-*O*-methylgalactose being the only exception.

In order to complete the chemical determination of the structure, a desulfation analysis followed by methylation is necessary. Such analysis was carried out first by microwave-assisted solvolytic desulfation and then by the in situ methylation procedure developed in our lab[15] with four fractions. The proportion of sulfate went down from 10.2–11.3% to 1.7–4.6%, that is, a decrease of 55–85% of the sulfate originally present. The solvolytic method produced a yield of desulfated product of 56–84%, being larger for the higher molecular weight products. As a control, the monosaccharide composition was determined for the desulfated products, yielding very similar values to those of the original fractions, even for 3,6-AnGal, indicating that the desulfation process did not produce any specific degradation. The proportion of alditol acetates after methylation and hydrolysis of the four desulfated fractions is shown in Table 6. A rough analysis of the results indicate that the amount of 2,3,6-tri-*O*-methylgalactose increased at the expense of 3,6-di-*O*-methylgalactose (ca. 6–10%), indicating that the L-galactose unit is partially sulfated at O-2. An increase of 2,4,6-tri-*O*-methylgalactose at the expense of 2,4-di-*O*-methylgalactose is also observed, suggesting the presence of some 6-sulfate in the 3-linked D-galactose units. No major change in the proportion of 2,6- and 2,3-di-*O*-methylgalactose is observed, suggesting that the units leading to those methylated sugars are either not sulfated or carry a sulfate group that is not susceptible to solvolytic desulfation. A small increase in the proportion of tetramethylated galactose can be attributed to a decrease in the

Table 4
Methylation analysis of the fractions obtained by ion-exchange chromatography of the polysaccharides of *J. rubens* (mol/100 mol Gals)[a]

Position of *O*-Me[b]	Structural unit	2	3a	3b	4a	4b	5a	6	7
2,3,4-Xyl	Xyl-t	20	25	37	32	25	29	32	36
2,3-Xyl	→4)-Xyl	4	–	–	1	1	–	–	–
2,3,4,6-Gal	Gal-t	15	18	10	3	2	1	1	1
2,4,6-Gal	→3)-Gal	10	9	6	6	4	5	5	6
2,3,6-Gal	→4)-Gal	22	24	21	20	20	21	24	24
2,3,4-Gal	→6)-Gal	3	–	–	2	1	1	1	1
2,6-Gal	→4,3)-Gal or →3,4)-Gal	9	10	5	3	2	1	1	1
4,6-Gal	→3,2)-Gal	–	–	–	1	–	–	–	–
3,6-Gal	→4,2)-Gal	4	6	8	9	10	10	9	9
2,3-Gal	→4,6)-Gal	3	1	1	2	4	2	5	5
2,4-Gal	→3,6)-Gal	30	27	42	41	44	45	42	43
2-Gal	→4,3,6)-Gal	–	1	–	1	4	3	2	2
3-Gal	→4,2,6)-Gal	–	–	–	1	2	1	1	2
4-Gal	→3,2,6)-Gal	–	–	–	1	1	1	2	2
3,6-An-2-Gal	→4)-3,6-AnGal	4	3	4	7	4	6	5	3
3,6-AnGal	→4,2)-3,6-AnGal	–	1	3	3	2	3	2	1

[a] Proportions lower than 1% are not indicated. Small proportions (<2%) of non-methylated Gal and Xyl were found in some fractions. Calculated as an average of the alditol and aldononitrile acetates results.
[b] 2,3,4-Xyl = 2,3,4-tri-*O*-methylxylose, etc.

Table 5
Ethylation analysis of some fractions obtained by ion-exchange chromatography of the polysaccharides of *J. rubens* (mol/100 mol Gals)[a]

Position of *O*-alkyl	Position of *O*-methyl	**3a**	**3b**	**4a**	**4b**	**5a**	**6**
2,3,4-Xyl[b]	—	29	30	29	31	31	32
2,4-Xyl	—	3	—	—	—	3	3
2,3-Xyl	—	3	1	3	2	—	3
2,3,4,6-Gal	—	2	—	—	—	—	1
2,3,4,6-Gal	3	11	6	9	3	2	1
2,3,4,6-Gal	2,3	4	—	—	—	—	—
2,4,6-Gal	—	5	5	4	5	5	3
2,4,6-Gal	6	3	—	1	—	1	2
2,3,4-Gal	—	3	6	1	4	1	—
2,3,6-Gal	—	9	20	11	17	10	9
2,3,6-Gal	2	5	6	6	8	4	5
2,3,6-Gal	3	4	1	2	1	1	1
2,6-Gal	—	6	5	5	2	3	5
3,6-Gal	—	5	5	9	9	11	10
2,4-Gal	—	29	31	34	41	40	34
2,3-Gal	—	1	6	2	4	2	3
2,3-Gal	2	1	3	—	3	1	2
2-Gal	—	1	—	3	—	5	3
3-Gal	—	—	—	2	—	2	2
4-Gal	—	1	—	2	—	3	5
Gal	—	1	—	—	—	3	—

[a] Determined as aldononitriles (3,6-AnGal derivatives not shown). Proportions lower than 1% are not indicated.
[b] 2,3,4-Xyl = 2,3,4-tri-*O*-alkylxylose, etc.

Table 6
Methylation analysis of four fractions obtained by ion-exchange chromatography of the polysaccharides of *J. rubens* after solvolytic desulfation (mol/100 mol Gals)[a]

Sugar	**3b-D**	**4a-D**	**5a-D**	**6-D**
2,3,4-Xyl[b]	28 (+11)	54 (+32)	31 (+14)	41 (+17)
2,3-Xyl	1 (−1)	6 (+4)	1 (0)	2 (0)
2,3,4,6-Gal	11 (+1)	3 (+2)	— (0)	3 (+3)
2,4,6-Gal	9 (+3)	11 (+4)	8 (+1)	10 (+3)
2,3,6-Gal	32 (+6)	34 (+5)	34 (+10)	36 (+2)
2,3,4-Gal	2 (+1)	— (−1)	1 (+1)	— (0)
2,6-Gal	6 (−1)	5 (+1)	3 (+1)	3 (+1)
3,6-Gal	2 (−6)	2 (−8)	4 (−9)	3 (−8)
2,3-Gal	3 (0)	3 (0)	4 (+3)	5 (0)
2,4-Gal	34 (−5)	42 (−2)	46 (−7)	40 (−1)

[a] Determined as alditol acetates, with normal hydrolysis (3,6-AnGal derivatives not detected and thus excluded from the table). Differences from original values with the same method of hydrolysis are given in parentheses.
[b] 2,3,4-Xyl = 2,3,4-tri-*O*-methylxylose, etc.

molecular weight of the products. The proportion of 2,3,4-tri-*O*-methylxylose increased significantly, reaching values closer to those of the original analysis. This increase in the proportion of permethylated xylose derivative after desulfation has been previously observed.[5] However, no explanation can be provided for this fact.

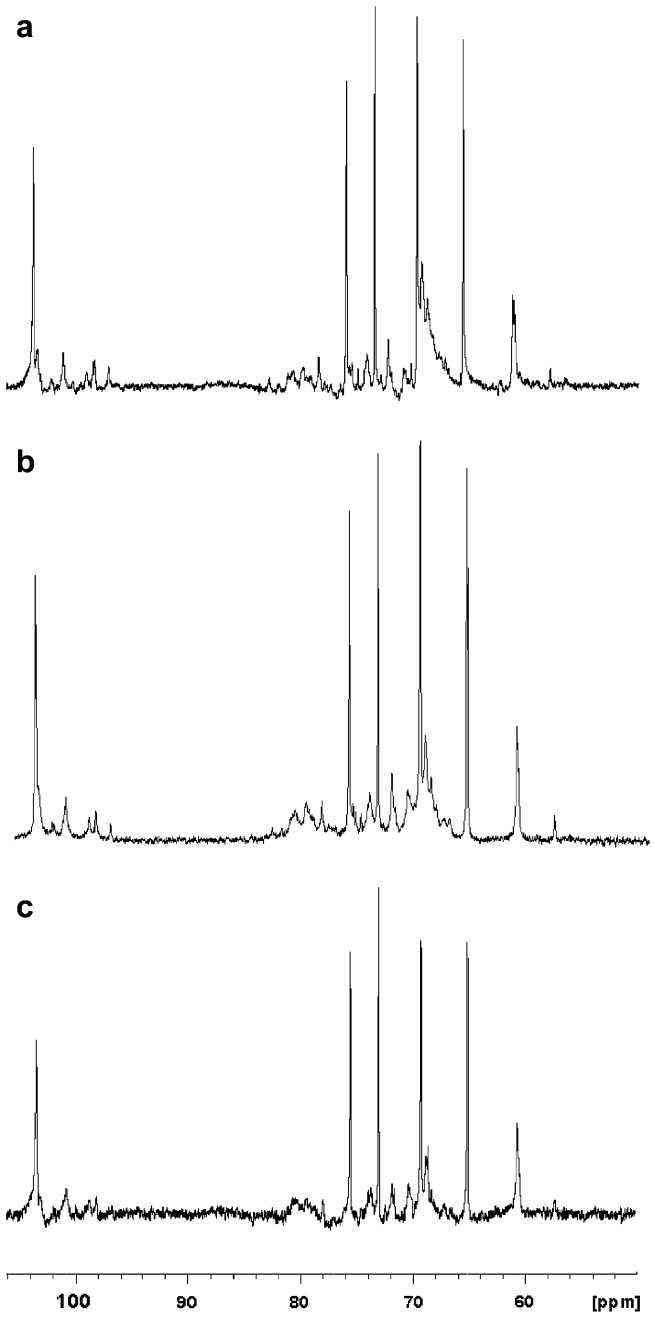


Figure 2. 125 MHz proton-decoupled ^{13}C NMR spectra of fractions (a) **4a**, (b) **5a**, and (c) **6**.

2.3. Structural analysis by NMR spectroscopy

The ^{13}C NMR spectra of fractions **4a**, **5a**, and **6** are shown in Figure 2. The spectra of the three fractions are very similar, and are also very similar to those already published for other corallinans.[7–10] Five sharp peaks dominate the spectra: they correspond to the signals assigned to the β-D-xylosyl side chains at 104.3 (C-1), 76.4 (C-3), 73.9 (C-2), 70.1 (C-4), and 65.9 ppm (C-5). This is consistent with a constant upfield shift of 0.6 ppm in respect to the work of Usov et al.[7] For the remaining signals, the spectra were too complex. However, the similarity with previously published spectra allowed the confirmation of many of the previous assignments. Seven anomeric signals were found: some of them were assigned with the aid of previous work, whereas the others were assigned by using two-dimensional techniques and some degradative experiments (see later). The ^{1}H NMR spectra (see Supplementary data) also showed severe overlapping. Although the region of the anomeric α-protons showed a better resolution, two-dimensional experiments (see later) showed that they also corresponded to the overlapping of several different units.

The HSQC spectra of fractions **3a**, **3b**, and **4a** were obtained. Figure 3 shows the two-dimensional spectrum of **4a**, whereas the remaining spectra are shown as Supplementary data. With these spectra, it was possible to assign the ^{1}H NMR chemical shifts corresponding to the xylosyl units as 4.49 (H-1), 3.28 (H-2), 3.45 (H-3), 3.61 (H-4), 3.32 (H-5), and 3.94 ppm (H-5′). These values are similar (but not identical) to those found for β-D-xylose units acting as side stubs of a galactan at the O-3 position,[16] and of a

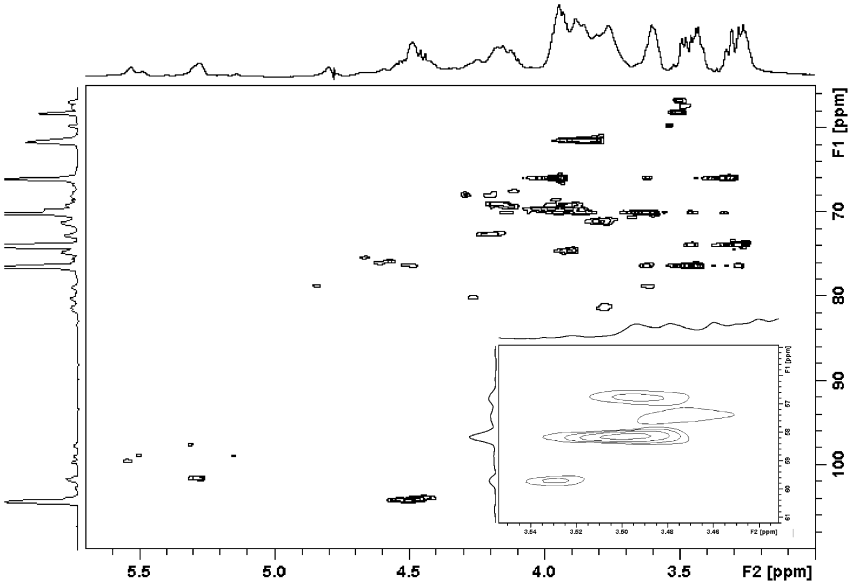


Figure 3. HSQC spectrum of fraction **4a**. The inset represents an enlargement of the methoxyl region.

mannan at the O-6 position.[17] Given the complexity of the spectra, we have focused on identifying and assigning the signals at the methoxyl and anomeric regions. The methoxyl region of the HSQC spectra shows five signals: their assignments are shown in Table 7, whereas an enhancement of the spectrum in this region is shown as an inset of Figure 3 for fraction **4a** (the signal for 6-MeGal, which is very scarce in this fraction, can be observed only in the spectrum of **3a**). The assignments for the methoxyl groups in 6-Me-D-Gal, 2- and 3-Me-L-Gal present in the main chain were made by comparison of the work of Usov et al. for the corallinan of *C. pilulifera*.[7] The signal for the methyl ether of 3,6-AnGal (many times considered to overlap that of 6-MeGal) was assigned considering its presence in another agaran.[18] At last, the remaining signal was assigned to the methoxyl group in side chains of 3-Me-D-Gal. Besides the match found with previous references,[19] a semiquantitative determination of the integrals of the HSQC spectra reveals that the volume ratios of the peaks at 56.7 and 58.2 ppm (expected to have similar $^{1}J_{C-H}$ values) are 0.3, 0.6 and 1.0 for fractions **4a**, **3b**, and **3a**, respectively. This compares very favorably with the 3-Me-D-Gal/2-Me-L-Gal ratios found by GC (0.4, 0.6, and 0.9, respectively).

The anomeric region of the corallinans also shows a straightforward HSQC spectrum. This region for fraction **4a** is shown in Figure 4a, whereas those of other three fractions are shown as Supplementary data. Table 8 shows the assignments of the eight peaks observed for all the fractions and the additional two observed only for fraction **3a**. As shown, some peaks are only distinguishable in the two-dimensional spectrum, as their ^{13}C NMR chemical shifts are identical or very similar (Table 8). Five of the signals corresponding to β-Xyl, β-Gal (and 6-substituted derivatives), α-Gal, α-Gal2S, and 2-Me-α-Gal were assigned by direct comparison with the data already given for the corallinans of *C. pilulifera*.[7] Two different assignments were given to the C-1 of 3-Me-α-L-Gal: in one paper[7] it was assumed that it overlapped the signal of 2-Me-L-Gal, whereas in another paper it was matched with the signal of non-methylated L-Gal.[10] The second assignment appears to be more consistent, as the 2-*O*-methyl group is expected to introduce an upfield shift to C-1. Besides, the calculated volumes of the spectra agree better with this assignment. The signal at 101.3/5.07 ppm, present only in the spectrum of **3a**, was assigned to C-1 of an α-L-Gal substituted at O-3 by a substituent different from a methyl group. This assignment was already made to a 3-sulfated unit,[20] but in this case no sulfate appears to be present at this position. O-3 bears a side chain of a branching sugar (see above). The signal at 99.4/5.41 ppm, which is only present in the spectrum of **3a**, corresponds to a small contamination with floridean starch (the fraction contains 2% of Glc), as demonstrated in a separate spectrum made for the starch-rich fraction **PW2**. Three signals remained to be assigned, that is, those appearing at 102.7/4.63, 98.9/5.14, and 97.6/5.30 ppm. The first one can be clearly assigned to a β-Gal unit linked to a 3,6-An-L-Gal. Such an upfield shift for changing the neighboring unit from a regular L-Gal unit to its 3,6-anhydro derivative was already observed, giving rise to precisely this value for the chemical shift in the ^{13}C NMR spectroscopy.[21] The other two assignments were made with the help of chemical degradation reactions: the HSQC spectrum of fraction **4a** submitted to desulfation (Fig. 4b) showed the disappearance of the signals at 99.5/5.54 and 97.6/5.30 ppm, indicating that they correspond to sulfated units. The volumes of the signals at 101.6/5.28 and 98.9/5.14 ppm increased accordingly, indicating that they correspond to their respective non-sulfated units. For the first-mentioned signal, this is compatible with the presence of L-Gal 2S units; the second one can only be explained in terms of a 3,6-An-L-Gal 2S signal (97.6/5.30 ppm) which shifts to the 3,6-AnGal one (98.9/5.14 ppm) upon desulfation. This was confirmed by a partial depolymerization[22] of fraction **4a** which breaks specifically at the 3,6-AnGal linkages, followed by reduction, in a way to eliminate the 3,6-anhydro anomeric signal. The corresponding spectrum is shown in Figure 4c. The disappearances of the signals at 97.6/5.30 ppm and 98.9/5.14 ppm (as well as that at 102.7/4.63 ppm) confirm the previous assignments. These results suggest that in all the frac-

Table 7
Chemical shifts assignments of the NMR spectra of the corallinans of *Jania rubens* in the methoxyl region (56–60 ppm in ^{13}C)

Type of unit	Chemical shift of CH_3 (ppm)	Chemical shift of CH_3 (ppm)
2-*O*-Me-L-Gal	58.2	3.50
3-*O*-Me-D-Gal	56.7	3.49
3-*O*-Me-L-Gal	57.5	3.47
6-*O*-Me-D-Gal	59.2	3.37
3,6-An-2-*O*-Me-L-Gal	59.7	3.53

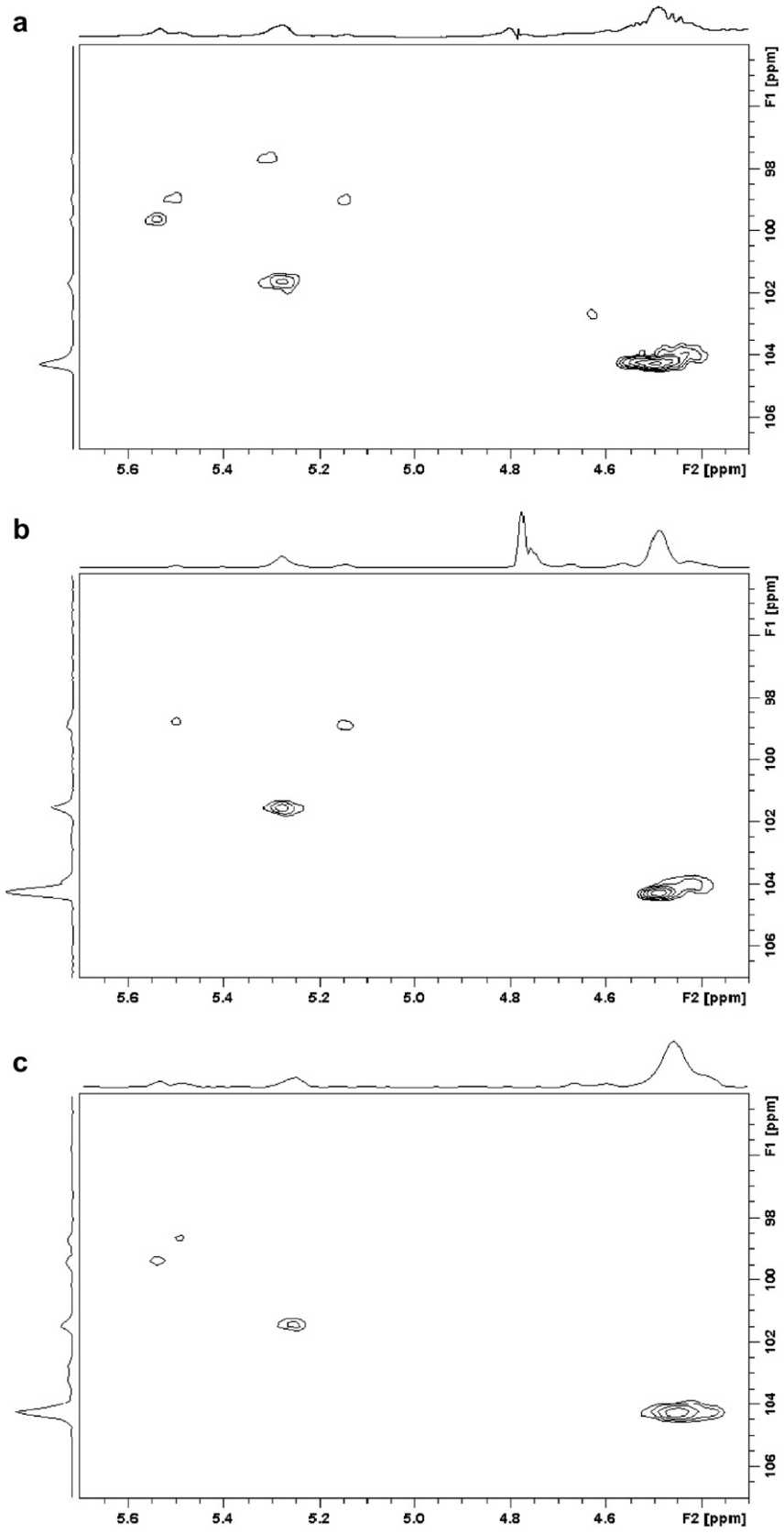


Figure 4. HSQC spectrum of fraction **4a** in the anomeric region: (a) original polysaccharide, (b) desulfated sample, and (c) partly depolymerized-reduced sample.

tions under study, the amount of 3,6-AnGal 2S is larger than that of 3,6-AnGal. Methylation analysis (Table 4) gave the opposite results. A possible explanation for this fact is a partial desulfation of the 3,6-AnGal units during the methylation procedure. Previous assignments for C-1 of 3,6-An-L-Gal 2S gave values shifted upfield from the current value.[23] However, they appeared in environments

Table 8
Chemical shifts assignments of the NMR spectra of the corallinans of *Jania rubens* in the anomeric region (95–110 ppm in ^{13}C)

δ ^{13}C (ppm)	δ ^{1}H (ppm)	Assignment of anomeric signal
104.3	4.49	β-D-xylosyl side stubs
104.0	4.43	β-D-Gal (with any substitution on O-6) linked to α-L-Gal
102.7	4.43	β-D-Gal (with any substitution on O-6) linked to 3,6-An-α-L-Gal and derivatives
101.6	5.28	α-L-Gal and 3-Me-α-L-Gal
101.3[a]	5.07[a]	α-L-Gal with sugar substitution on O-3
99.5	5.54	α-L-Gal 2-sulfate
99.4[a]	5.41[a]	α-Glc in floridean starch
98.9	5.50	2-Me-α-L-Gal
98.9	5.14	3,6-An-α-L-Gal and its 2-*O*-methyl ether
97.6	5.30	3,6-An-α-L-Gal 2-sulfate

[a] Only found for fraction **3a**.

quite different from that of the corallinans, with heavy substitution on O-6 of the β-D-Gal residue.

3. Discussion

The polysaccharides from only four species from the order Corallinales have been studied in detail up to the moment.[3–10] The structural resemblance between them is so patent that their description through a common name, as corallinans,[1,5] appears to be valid. The feature common to all of them is to carry an agaran backbone in which C-6 of the β-D-Gal unit is almost completely substituted, mostly with β-xylosyl side stubs, but also by sulfate, while C-2 of α-L-Gal residues is partly substituted by methoxyl and sulfate groups. Other features were considered to be also common to all corallinans, as the presence of some sulfate on C-3 of the α-L-Gal units, and the absence of 3,6-AnGal. This report tears down these last assertions. Furthermore, each species gives rise to different peculiarities. The simplest corallinans were found in *Joculator maximus*:[8] about 80% of the C-6 of the β-D-Gal units appear to be substituted by xylosyl side stubs (50%) or sulfate ester groups (30%). On the other hand, about half of the α-L-Gal units were substituted, in similar amounts at C-2 by a methoxyl group, at C-2 by a sulfate group, and at C-3 by a sulfate group (i.e., 2-MeGal is the only monomethylated galactose present). The xylogalactan from *Corallina pilulifera* is similar but less simple.[6,7] For the D-Gal unit, there are few residues unsubstituted: the xylose substitution increased, and some methoxyl substitution appears. For the other unit, a small proportion of 3-MeGal also appears. Thus, three different methoxylated Gal units are occurring. In *Bossiella cretacea*,[9,10] the α-L-Gal units have similar substitution patterns to those of *J. maximus*. However, a unique characteristic appears at the β-D-Gal units, where C-4 appears partly (ca. 15%) sulfated, a fact usual in carrageenans but not in agarans.[2] The total proportion of sulfate is, however, similar to that of *C. pilulifera*: 4-sulfated units have taken the place of the 6-sulfated ones. The products from *C. officinalis* have been studied in detail because they were fractionated.[1,4,5] Although they appear to be more complex, the general patterns are similar to those previously described. For the main fractions, which represent two-thirds of the extract, the substitution of the β-D-Gal unit is similar to that of *C. pilulifera*. However, a larger proportion of the C-6 substitution originates in the xylose side stubs, and less in the sulfate groups. Besides, substitution by 4-Me-D-Gal side stubs also appears in the same position. For the α-L-Gal unit, the qualitative pattern is similar to that of *C. pilulifera*, but it is much more substituted (ca. 80% against 55% in *C. pilulifera*): the main difference appears by larger sulfation of C-3 and larger methoxylation on C-2. In *C. officinalis*, other fractions were also isolated. Those eluting later than the main group appear to be very similar, with only small quantitative differences (less xylose, more 6-sulfate, 2-Me and 6-Me). On the other hand, the early-eluting fractions show much more complicated patterns, which have not been completely established:[1,4,5] for the β-D-Gal units, the proportion of branching with 4-MeGal increases, whereas for the α-L-Gal units, additional branching at C-3 with Xyl and 4-MeGal side stubs appears, and the proportion of sulfate on this unit appears markedly diminished. A preliminary study of *Bossiella orbigniana* showed that its polysaccharides are uniquely rich in 6-Me-D-Gal, and that it also contains 2-Me-D-Gal.[12]

Extraction of the polysaccharides from *J. rubens* was effected by destroying the calcium carbonate cover with acid. However, a previous hot-water extraction was effected in order to reduce the proportion of floridean starch in the main product.[12] Fractionation by ion-exchange chromatography allowed for the separation of 10 fractions (Fig. 1) with variations in their analytical data (Tables 2 and 3). The general elution pattern can be compared with that of *C. officinalis*:[4] (a) Both give rise to the most abundant fractions at 0.4–0.5 M NaCl; (b) in *C. officinalis* the two more abundant fractions appear after another peak appearing at the same concentration; in *J. rubens* the most abundant fractions appear right after changing the concentration; (c) the proportion of xylose is higher in the central, most abundant fractions and falls down at the early-eluting and late-eluting fractions. However, this fall was very pronounced for *C. officinalis* and slight for *J. rubens*; (d) the proportion of 2-MeGal is evenly distributed for *J. rubens*, but shows a trend to increase with NaCl concentration for *C. officinalis*; (e) the proportion of 4-MeGal (assumed to be a side chain) in *C. officinalis* follows the same pattern as that of 3-Me-D-Gal in *J. rubens*, that is, substantial amounts in the early-eluting fractions, negligible ones in the main and late-eluting group, and (f) the proportion of 6-MeGal follows the same pattern for both in the beginning, being moderate in early-eluting fractions and negligible in the middle ones. However, in *J. rubens* they keep low values for the late-eluting ones, increasing to substantial amounts in the late-eluting fractions of *C. officinalis*.[4]

In order to determine the structure of the system of xylogalactans from *J. rubens*, several different techniques were utilized: constituent monosaccharides (including their configuration), methylation, ethylation, and desulfation–methylation analysis, complemented in some cases with a determination of configuration to assess the actual structural meaning, and NMR analyses by different procedures. These methods allow one to determine a general structural pattern which can be characterized by a basic agaran structure (alternating 3-linked and 4-linked Gal units, with the former being β-D, and the latter α-L), modulated by (a) the replacement of some L-Gal units by its 3,6-anhydro derivative, which is a characteristic never found previously in other members of the Corallinales; (b) C-6 of the β-D- Gal unit substituted mainly by β-D- xylosyl stubs, with minor proportions of sulfate, methoxyl, and perhaps 3-MeGal stubs; (c) no substitution at all on C-2 or C-4 of the β-D-Gal units; (d) C-2 of the α-L-Gal and 3,6-An- α-L-Gal partly substituted by methoxyl or sulfate; (e) C-3 of the α-L-Gal unit partly substituted by methoxyl, and (f) C-3 and C-6 of the α-L-Gal unit partly substituted by glycosidic stubs (mainly 3-MeD-Gal, but possibly others as Xyl, 2,3-di-Me-D-Gal, or 3-Me-L-Gal). The presence of 3-Me-Gal and 2,3-di-Me-Gal as side stubs is (up to this moment) also unique to this species. It should be considered possible that the presence of double stubs of xylose [β-D-Xyl-(1→4)-β-D-Xyl→] in some fractions, as methylation or ethylation analysis showed, both derivatizing to aldononitriles or alditols, the presence of small amounts of 2,3-di-*O*-alkylxylose. The structure depicted in Table 9 can be applied to corallinans, taking into account an average of all the data, and making certain assumptions, as disregarding the small amounts of monomethylated units or 2,3,4-tri-*O*-methylgalactose present after methylation analysis,

Table 9
Structure of the corallinan fractions from *Jania rubens*, expressed per 100 monosaccharide units in the main backbone

→ 3-β-D-Gal-(1 → 4)-α-L-Gal-(1 →

	2	3a	3b	4a	4b	5a	6	7
3-Linked unit								
β-D-Gal	7	7	5	5	3	4	4	4
6-*O*-Me-β-D-Gal	6	5	1	1	1	1	1	2
β-D-Gal 6-sulfate	3	3	3	3	4	5	6	6
6-glycosyl-β-D-Gal	34	35	41	41	42	40	39	38
4-Linked unit								
α-L-Gal	8	14	17	15	15	15	16	17
2-*O*-Me-α-L-Gal	8	8	5	8	6	7	7	6
α-L-Gal 2-sulfate	6	7	8	9	11	10	11	11
3-*O*-Me-α-L-Gal	6	6	2	3	2	1	2	3
3-glycosyl-α-L-Gal	11	6	3	2	2	1	1	1
6-glycosyl-α-L-Gal	4	1	1	2	4	4	5	6
6-glycosyl-2-*O*-Me-α-L-Gal	1	1	3	1	2	1	2	2
3,6-An- α-L-Gal	3	3	5	4	3	4	2	2
3,6-An-2-*O*-Me-α-L-Gal	1	1	1	—	—	2	1	—
3,6-An-α-L-Gal 2-sulfate	2	3	5	6	5	5	3	2
Glycosyl								
β-D-Xyl (1→	32	28	40	40	47	45	46	47
3-*O*-Me-D-Gal (1→	10	8	7	5	3	1	—	—
3-*O*-Me-L-Gal (1→	4	4	1	1	—	—	—	—
2,3-di-*O*-Me-D-Gal (1→	4	3	tr.	—	—	—	—	—

extrapolating some data to the fractions for which ethylation was not performed, etc. Some analysis demonstrated clearly specific substitution patterns, for example, the absence of sulfate at C-3 and C-6 of the 4-linked units. For the main fractions (**4a**–**6**), the structure can be depicted as having the β-D-Gal unit substituted in about 80% of the C-6 by xylosyl units, 2% by a methoxyl group, and 10% by a sulfate group, that is, with more xylose and less sulfate than those from *C. officinalis*, *J. maximus*, or *C. pilulifera*.[5–8] The pattern for the 4-linked unit is more complex: about 15–20% corresponds to 3,6-anhydrogalactose units, most of them sulfated or methoxylated at C-2 (Table 9). Almost half of the remaining units are substituted at C-2 by either sulfate or methoxyl groups (about the same proportion of each). Little (ca. 6% of the α-Gal units) substitution at C-3 appears, either with methoxyl or glycosyl groups, as well as with some glycosyl substitution at C-6 (ca. 10% of the α-Gal units). The remaining α-L-Gal units (ca. 30%) are not substituted. The pattern for this unit is the most complicated one ever studied: besides the presence of 3,6-anhydro derivatives, the glycosylation at C-3 and C-6, the latest one coexisting with some methoxylation at C-2, generate ten different structures (Table 9), well above the simple structures found for the simplest one, *J. maximus*,[8] or for more complex products in *C. officinalis*.[5] Although it is assumed here that the substitution at C-3 arises from glycosylation and not from sulfation, based on the desulfation analysis, it can also be possible that the sulfate in this position resists the solvolytic desulfation effected in this work (a second desulfation did not change at all the observed patterns). In *J. rubens* the late-eluting fractions (**7** and **6** in part) are very similar to those of the mainstream, in contrast to those of *C. officinalis*,[5] which were clearly different by being more heavily methoxylated and less xylosylated. On the other hand, the early-eluting fractions (**3b**, **3a**, and especially **2**) show clearly different substitution patterns (besides the lower molecular weight), some of which were not completely established. The β-D-Gal unit appears to have a very similar substitution pattern, though some methoxyl and other glycosyl (3-Me-Gal, 2,3-di-Me-Gal) groups at C-6 replace the xylosyl substitution. On the α-L-Gal unit the differences are larger; less unsubstituted and 2-sulfated units and more substitution at C-3 (by either methoxyl groups or glycosyl substituents) appear. The variety of glycosyl substituents (although not the total substitution) also increases markedly.

One of the novelties present in these polysaccharides is the presence of 3-MeGal acting as the side chain. In the past, this sugar has been found only rarely in red seaweed polysaccharides,[24] but its presence has been reported in the highly methoxylated carrageenans from *Rhabdonia verticulata* as terminal units.[25] This sugar was considered to be a taxonomical marker for the Lycophyta.[26] However, it was also found in glycoproteins, fungi, pectins, and polysaccharides from the Chlorophyceae.[19c,27]

This study shows that as the structure of polysaccharides from new species from the Corallinales is determined, more and more variations to the main structure appear. For red seaweed galactans, a triangular prism analogy has been presented.[2b] In that model, corallinans are located on one of the six vertices, by being devoid of 3,6-AnGal and of its precursor 4-linked Gal 6-sulfate. The polysaccharides from *J. rubens* show a different pattern; they are devoid of the precursor, but have significant amounts of the anhydro sugar. Thus, within the prism model, corallinans should be located on the only edge of the front triangular side for which there are no examples, and not on the upper vertex.

4. Experimental

4.1. Materials

Samples of *J. rubens* (L.) Lamoroux were collected near Miramar (Buenos Aires Province, Argentina). The seaweeds were sorted, air dried, cleaned manually very carefully (with microscopic observation), and milled to a fine powder before extraction. All chemicals were of analytical grade.

4.2. General methods

Total carbohydrates were determined by the phenol–H_2SO_4 method[28] that was standardized as described by Cases et al.[4] Uronic acids were determined using the method of Filisetti-Cozzi and Carpita[29] using glucuronolactone as the standard. The percentages of sulfate were measured by turbidimetry[30] after hydrolysis with 1 M HCl, and also by ion chromatography,[31] while the soluble proteins were determined by the procedure of Lowry et al.[32] Average molecular weights were estimated as described by Park and Johnson.[33] Optical rotations of aqueous solutions of the samples (0.4–0.6%) were measured at the sodium D line using a Perkin–Elmer 343 polarimeter. Cyclizable 6-sulfate was determined by measuring the 3,6-AnGal produced after alkaline treatment of the products.[34]

The proportions of monosaccharides constituting the polysaccharides were determined by gas chromatography (GLC on an HP 5890A apparatus equipped with a flame-ionization detector) of the hydrolyzates, using different derivatives. The regular hydrolysis procedure was performed by treating the polysaccharides with 2 M trifluoroacetic acid (TFA, 90 min at 120 °C). The TFA was evaporated. Aliquots of the hydrolyzates were converted to their aldononitrile acetates[35] and analyzed by GLC as described elsewhere.[36] Other aliquots of the hydrolyzates were converted to the acetylated aminodeoxyalditols using (*S*)-1-amino-2-propanol and (*S*)-α-methylbenzylamine[37] and analyzed by GLC as stated therein. In order to avoid destruction of the 3,6-anhydrogalactose, alditol acetates were obtained from the products of a reductive hydrolysis procedure[22] slightly modified as shown elsewhere.[38] The configuration of the 3,6-anhydrogalactose was determined after mild hydrolysis and derivatization with (*S*)-α-methylbenzylamine, and analyzed as described.[34] The GLC–MS analyses were carried out on a Shimadzu QP 5050 A GC/MS apparatus working at 70 eV using

conditions similar to those described above, but using He as gas carrier at a flow rate of 7 mL/min and a split ratio of 11:1.

4.3. Extraction and fractionation

The extraction procedures are summarized in Scheme 1. In short, the milled seaweed (300 g) was extracted successively with water (2.5 L) at 100 °C,[8] water with the addition of 1 M HCl (pH 6)[4] first with a total of 4.4 L, and then with 1.6 L, and water (2.5 L) at 100 °C again. All the extractions were carried out under mechanical stirring for 24 h. The residues from each step were recovered by centrifugation, and the supernatants were concentrated and precipitated with 3.5 vol of 2-PrOH. The precipitates were redissolved in water (small amounts of insoluble material were discarded), and lyophilized to yield fractions **PW1**, **PA1**, and **PW2**. **PA1** was redissolved again in water, dialyzed with mwco 6000–8000 membrane, and lyophilized to give **PA1d**.

The product **PA1d** was used in this work. A column of DEAE-Sephadex A-50 (50 × 3.5 cm) was used, equilibrated with 0.1 M NaCl. The sample (820 mg) in 5 mL of the same solvent was adsorbed onto the top of the column, and increasing concentrations of NaCl solutions were used as eluants. Fractions (4.6 mL) were collected and analyzed for carbohydrates. When no elution was found, the concentration of NaCl was increased in 0.1-M steps. The fractions corresponding to peaks (Fig. 1) were pooled, dialyzed, and lyophilized, yielding the 10 fractions shown in Table 2.

4.4. Desulfation, methylation, and ethylation

The triethylammonium salts of the fractions (5 mg) were methylated as described by Ciucanu and Kerek,[39] using NaOH and CH_3I. The ethylation was carried out by a similar procedure, but using ethyl iodide as the alkylating agent.[14] The alkylated products (isolated by dialysis and lyophilization) were hydrolyzed (2 M TFA, 90 min, 120 °C), and the partially alkylated monosaccharides were derivatized as their aldononitrile acetates or hydrolyzed by reductive hydrolysis and derivatized to the alditol acetates, which were analyzed by GC under the conditions described elsewhere,[36] and characterized by GLC–MS as described above. The determination of the configuration of 2,6-di-*O*-methylgalactose was carried out by reductive amination, as reported elsewhere.[13] With the same procedure, the configuration of the 2,3,4,6-tetra-*O*-methylgalactose was determined, using as standard a methyl glycoside with the D-configuration.

Solvolytic desulfation was carried out by the microwave-assisted procedure of Navarro et al.[15] An aliquot was isolated by dialysis and lyophilization (mwco 3500), and another one was subjected to an in situ methylation procedure.[15] The desulfated–methylated product, after hydrolysis, was derivatized as the corresponding alditol acetates.

4.5. Nuclear magnetic resonance spectroscopy

The spectra were obtained on a Bruker Avance II 500 spectrometer at 500.13 (^{1}H) and 125.77 (^{13}C) MHz provided with a 5-mm probe, at room temperature, using ca. 20 mg polysaccharide in 0.4 mL of D_2O. Acetone was added as the internal standard (referred to Me_4Si by calibrating the acetone methyl group to 31.1 ppm in ^{13}C, 2.22 ppm in ^{1}H). Multiplicity determinations and 2D spectra were obtained using standard Bruker software. The sample **4a** was depolymerized by reaction with 0.1 M TFA (80 °C, 3 h), further reduction with $NaBH_4$, and purification of the products by dialysis.[22,34]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2008.06.015.

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