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Sulfated xylomannans isolated from red seaweeds *Chondrophycus papillosus* and *C. flagelliferus* (Ceramiales) from Brazil

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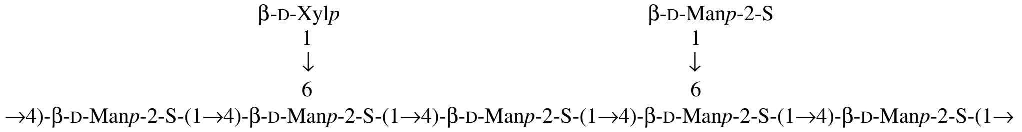
Abstract—Sulfated xylomannans were isolated from two species of genus *Chondrophycus* by aqueous extraction followed by KCl fractionation. Structural determination of the native, desulfated and Smith-degraded KCl-precipitated polysaccharides carried out by composition and methylation analysis and NMR spectroscopy (1D and 2D experiments) showed the following general structure:



$$\begin{array}{ccccc}
 & \beta\text{-D-Xyl}p & & \beta\text{-D-Man}p\text{-2-S} & \\
 & 1 & & 1 & \\
 & \downarrow & & \downarrow & \\
 & 6 & & 6 & \\
\rightarrow 4)\text{-}\beta\text{-D-Man}p\text{-2-S-}(1\rightarrow & 4)\text{-}\beta\text{-D-Man}p\text{-2-S-}(1\rightarrow & 4)\text{-}\beta\text{-D-Man}p\text{-2-S-}(1\rightarrow & 4)\text{-}\beta\text{-D-Man}p\text{-2-S-}(1\rightarrow & 4)\text{-}\beta\text{-D-Man}p\text{-2-S-}(1\rightarrow
\end{array}$$

These xylomannans present different degrees of branching (15–25%) by β-D-Xyl*p* (70–80%) and β-D-Man*p*-2-S (20–30%) and molecular weights (33–222 kDa). This is the first report of the presence of a sulfated xylomannan in species of order Ceramiales.

Keywords: Algal polysaccharides; Sulfated xylomannan; NMR spectroscopy; Methylation analysis; Red seaweeds; *Chondrophycus*; Ceramiales

1. Introduction

Carrageenans and agarans are the principal sulfated polysaccharides synthesized by the great majority of red seaweeds (Rhodophyta). They are constituted by alternating 3-linked β-D-galactopyranosyl and 4-linked α-galactopyranosyl (or 3,6-anhydrogalactopyranosyl) units. The configuration of the 4-linked units D- or L- defines the group of carrageenans and agarans, respectively.[1] A third group of galactans, named DL-hybrids,[2] described for some species, presents the 4-linked units in both D- and L-configuration. All these galactans can present substitution by sulfate and/or methyl groups, acetals of pyruvic acid and single stubs of xylose.[1,2]

Nevertheless, other types of polysaccharides are synthesized by red algae. Sulfated xylomannans[3–11] have been described for species of Nemaliales as well as neutral xylans were isolated from Nemaliales[12–15] and Palmariales.[15–17] The former are constituted by a main chain of 3-linked α-D-mannopyranosyl[3–10] or β-D-mannopyranosyl residues[11] with different patterns of substitution by sulfate groups and branches.

Here, we describe the structure of a xylomannan sulfate isolated from two species of order Ceramiales collected in northeastern and southern Brazil. *Chondrophycus flagelliferus* and *Chondrophycus papillosus* are

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cosmopolitan algae and in Brazil both species are widely distributed along the coast.[18] As far as we know this is the first report of the presence of a xylomannan sulfate in red seaweeds of order Ceramiales.

2. Results and discussion

2.1. Extraction and fractionation of polysaccharides

The carefully cleaned and milled seaweeds *C. flagelliferus* and *C. papillosus* collected in the northeastern coast of Brazil were submitted to aqueous extraction at 25 °C giving rise to crude extracts F1 and P1 (3.0% and 3.1% w/w, respectively). The algal residues were further extracted with water at 80 °C yielding F2 and P2 (10.6% and 8.7% w/w, respectively). *C. flagelliferus* collected in the southern coast of Brazil was carefully examined to avoid contamination with epiphytes and endophytes, and its water-soluble polysaccharides were extracted at 80 °C, rendering FH fraction (15% w/w). The hot-water extracts F2, P2 and FH contain sulfate (17.5–22.2%) and are constituted by galactose (40.0–49.5 mol %), 3,6-anhydrogalactose (11.1–13.8), 3,6-anhydro-2-*O*-methylgalactose (1.0–1.3), 6-*O*-methylgalactose (5.9–9.0), xylose (7.4–11.0) and important amounts of mannose (20.3–26.0). Additionally, the fraction FH showed small amounts of 2-*O*-methylgalactose (Tables 1 and 2). Considering that red seaweeds from Ceramiales produce agarans[19–28] as the principal water-soluble polysaccharides, the monosaccharide composition of these crude extracts was suggestive of the presence of galactans besides other types of polysaccharides such as xylomannans, mannans and/or xylans.

In order to fractionate these extracts, F2 and P2 were treated with increasing concentrations of KCl (0–2 M) yielding the precipitated fractions F2a–F2d (24% w/w) and P2a–P2e (19% w/w) and the respective soluble fractions in 2 M KCl (F2-S and P2-S). FH was treated with 2 M KCl to afford the KCl-precipitated fraction FH-p (28% w/w) and the KCl-soluble fraction FH-S. Yields, analyses, optical rotations and monosaccharide composition of the crude extracts and fractions obtained by KCl treatment are given in Tables 1 and 2. Polysaccharidic fractions F2a and F2b showed an unusual monosaccharide composition, made up of mannose (90.7 and 85.0 mol %) and xylose (9.3 and 15.0 mol %), whereas F2c and F2d besides major amounts of the same sugars (Man plus Xyl ~64 mol %) contained the characteristic galactan units, such as galactose, 3,6-anhydrogalactose and/or its 6- and 2-methylated derivatives, respectively.

Likewise, P2 also gave rise to KCl-precipitated fractions (P2d and P2e) made up of only mannose and xylose, besides others containing the same set of monosaccharides (P2a–P2c) observed for F2c and F2d fractions. FH-p fraction contained principally mannose (68.2 mol %) and xylose (15.5 mol %) together with minor quantities of other monosaccharides. On the other hand, all the KCl-soluble fractions (F2-S, P2-S and FH-S) showed a different monosaccharide composition in relation to the precipitated ones, with galactose and

Table 1. Yield, analyses and optical rotation of the fractions obtained from *C. flagelliferus* and *C. papillosus*

Fractions[a]	Range of fractionation (M KCl)	Yield (%)	$[\alpha]_D$ (°)	Carbohydrate (%)	Sulfate (SO_3Na) (%)
F2	—	10.5[b]	−17.3	58.3	20.2
F2a	0.25	16.7[c]	−26.6	57.0	24.5
F2a-D	—	52.0	n.d.	n.d.	2.9
F2a-sm	—	70.0	n.d.	n.d.	n.d.
F2b	0.5	4.2[c]	−31.5	59.2	24.0
F2b-D	—	71.7	n.d.	n.d.	—
F2c	0.75	1.6[c]	−27.7	63.4	13.4
F2d	1.0	1.2[c]	−29.4	61.6	12.9
F2-S	Soluble 2.0	76.3[c]	−28.7	56.8	19.3
P2	—	8.7[b]	−12.0	61.2	17.5
P2a	0.25	9.1[c]	−28.8	51.5	19.1
P2b	0.5	2.6[c]	−36.2	44.0	20.9
P2c	0.75	0.6[c]	−40.3	45.2	17.4
P2d	1.0	3.2[c]	−31.9	67.0	28.1
P2d-D	—	75.3	n.d.	n.d.	—
P2e	1.5	3.7[c]	−32.4	55.7	25.1
P2e-D	—	58.8	n.d.	n.d.	3.4
P2-S	Soluble 2.0	80.8[c]	−27.4	57.2	16.6
FH	—	15.0[b]	−18.0	59.0	22.2
FH-p	2.0	28.0	−30.5	57.0	24.1
FH-S	Soluble 2.0	72.0[c]	−29.0	60.0	27.2

n.d. = not determined.

[a] Fractions are defined in the text.

[b] Percentage based on milled seaweed.

[c] Percentage based on material recovered from KCl fractionation. Yields of fractionation were 88.6, 88.4 and 93.0% for F2, P2 and FH, respectively.

Table 2. Monosaccharide composition of the fractions obtained from *C. flagelliferus* and *C. papillosus*

Fractions[a]	Monosaccharide (mol %)[b]						
	Xyl	Man	Gal	3,6-AnGal	6Me-Gal	2Me-AnGal	2Me-Gal
F2[c,d]	7.4	23.1	49.5	11.1	7.6	1.3	t.r.
F2a[e]	9.3	90.7	—	—	—	—	—
F2a-D[e]	10.0	90.0	—	—	—	—	—
F2a-sm[e]	—	100.0	—	—	—	—	—
F2a-sm-D[e]	—	100.0	—	—	—	—	—
F2b[e]	15.0	85.0	—	—	—	—	—
F2b-D[e]	15.0	85.0	—	—	—	—	—
F2c[c]	5.9	58.4	8.1	14.4	10.9	2.3	—
F2d[c]	10.0	54.6	26.2	5.3	3.9	—	—
F2-S[c,d]	5.7	4.1	66.8	10.8	11.3	1.3	t.r.
P2[c,d]	11.0	20.3	49.0	13.8	5.9	—	t.r.
P2a[c]	5.8	46.6	33.9	11.0	1.5	1.2	—
P2b[c]	5.3	47.7	29.6	13.9	1.8	1.7	—
P2c[c]	5.4	54.4	35.6	2.6	2.0	—	—
P2d[e]	6.1	93.9	—	—	—	—	—
P2d-D[e]	8.5	91.5	—	—	—	—	—
P2e[e]	13.2	86.8	—	—	—	—	—
P2e-D[e]	13.5	86.5	—	—	—	—	—
P2-S[c,d]	12.3	7.0	56.3	16.9	7.5	—	t.r.
FH[c,d]	10.0	26.0	40.0	12.0	9.0	1.0	2.0
FH-p[c]	15.5	68.2	10.5	3.5	2.5	—	—
FH-S[c,d]	5.0	5.6	64.0	8.6	14.8	t.r.	2.0

(—) Not detected.

t.r. Percentages lower than 1.0% are considered as trace amounts.

[a] Fractions are defined in the text.

[b] Gal corresponds to galactosyl, 3,6-AnGal to 3,6-anhydrogalactosyl, etc.

[c] Compositional analyses determined after reductive hydrolysis.[54,55]

[d] Normalized to exclude glucose, 3–5%.

[e] Compositional analyses determined after hydrolysis with M TFA for 4 h at 100 °C.

its derivatives as major components. The presence of these sugars, together with the negative optical rotations (Tables 1 and 2) and NMR data (see later) determined for KCl-soluble fractions indicative of agaran structures as major constituents.

Thus, the KCl-treatment of the polysaccharide extracts afforded three general types of fractions: one constituted exclusively by mannose and xylose, a second one indicative of agaran-like structures and a third one, with intermediate characteristics between the previous two. This last fraction type could result from the co- precipitation of agarans as observed in xylomannans and agarans fractionation carried out with the water-soluble polysaccharides obtained from *Nothogenia fastigiata.*[3–7]

2.2. Homogeneity analysis

HPSEC–MALLS analysis of F2a, F2b, P2d and P2e fractions gave a homogeneous profile with a MW of 180, 222, 33 and 41 kDa (*dn*/*dc* of 0.157, 0.131, 0.142 and 0.142), respectively. Figure 1 shows the HPSEC-RID elution profiles of fractions F2b and P2d. Considering the homogeneity, sugar composition and methylation analysis (see later), these fractions were named xylomannans. The KCl-soluble fractions (F2-S, P2-S and FH-S) showed an asymmetric peak when analyzed by the same technique, indicating that further purification of these fractions would be necessary.

2.3. Methylation analyses of native and desulfated polysaccharides

Xylomannans F2a, F2b, P2d and P2e were converted into the triethylammonium salt form[29] and methylated by the Ciucanu and Kerek method.[30] The results of linkage analyses are shown in Table 3. The principal derivatives were 3,6-Man (64.7–79.9 mol %) and 3-Man (9.6–15.1 mol %), indicating the presence of 2,4-di- and 2,4,6-trisubstituted mannopyranosyl residues, respectively. Furthermore the occurrence of 2,3,4-Xyl (5.5–11.5 mol %) and 3,4,6-Man (3.4–5.8 mol %) are indicative of xylopyranosyl single stubs and 2-substituted mannopyranosyl residues, respectively.

In order to determine the glycosidic linkages, sulfation and glycosylation positions, the xylomannans were submitted to solvolytic desulfation,[31] followed by methylation of the desulfated polymers. Compositional sugar analyses of the desulfated polysaccharides (F2a-D, F2b-D, P2d-D and P2e-D) gave similar xylose-to-mannose ratios than those obtained for the native polysaccharides, indicating lack of degradation during the

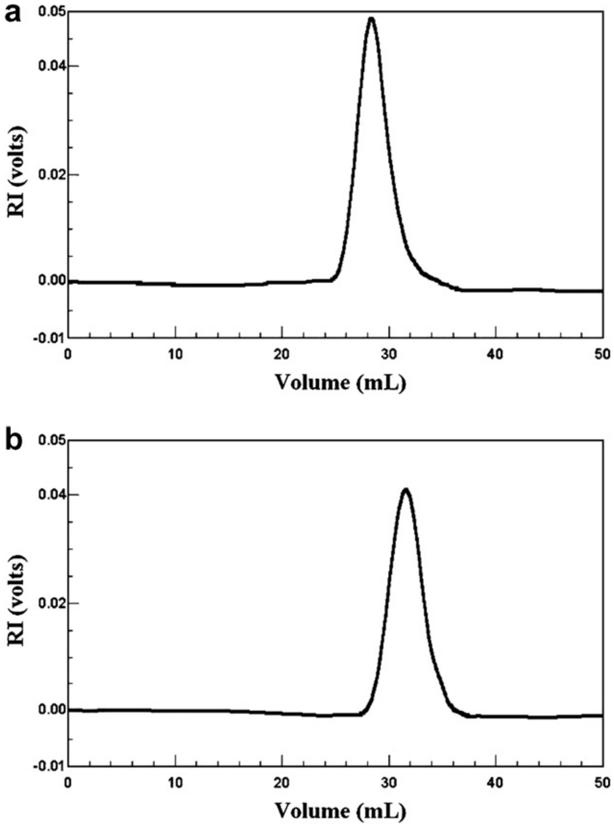

Figure 1. HPSEC elution profiles of F2b (a) and P2d (b), using a refractive index (RI) detector.

solvolytic treatment (Table 2). The xylomannans were completely (F2b and P2d) or highly (∼85%, F2a and P2e) desulfated (Table 1). Methylation analyses of these polysaccharides, when compared with those of the native polymers, showed the appearance of 2,3,6-Man and 2,3-Man as major derivatives with the concomitant 3,6-Man and 3-Man disappearance and/or decrease (Table 3). These data are in agreement with the removal of C-2 sulfate from 4-linked and 4,6-disubstituted mannopyranosyl residues. As well as in the linkage analyses of the native polysaccharides, xylose was detected only as branching units (2,3,4-Xyl, 5.7–11.2 mol %, Table 3). However, these amounts are lower than those obtained for xylose in the native (6.1–15.0 mol %) and desulfated polymers (8.5–15.0 mol %), as shown in Table 2. This decrease can be due to oxidative degradation[32] and/or preferential losses of the 2,3,4-tri-*O*-methylxylose during the methylation/derivatization process.[33] Additionally, 3,4,6-Man was not detected amongst the permethylated desulfated derivatives along with the appearance of equivalent amounts of 2,3,4,6-Man (2.6–5.9 mol %) indicating the presence of non-reducing mannopyranosyl 2-sulfated units. Moreover, the amounts of 2,3-Man derivative present in permethylated products of F2a-D, F2b-D, P2d-D and P2e-D are consistent with 15, 25, 15 and 20% of glycosylation, respectively, by xylopyranosyl (∼70–80% of branches) and mannopyranosyl 2-sulfated residues (∼20–30% of branches).

2.4. Smith degradation and methylation of the degraded product

In order to determine the position of attachment of the branches, xylomannan F2a was submitted to Smith degradation,[34] giving rise to the Smith-degraded product (F2a-sm) that was also sequentially submitted to desulfation (F2a-smD). Monosaccharide composition of both products showed only mannose (Table 2) and their methylation analysis 98 mol % of 3,6-Man plus 2 mol % of 2,3,6-Man for F2a-sm and 100 mol % of 2,3,6-Man for F2a-smD, as reported in Table 3. These results combined with the methylation analysis described above of the native and desulfated xylomannans are in agreement with a 4-linked mannan 2-sulfated

Table 3. Methylation analysis of the native xylomannans (F2a, F2b, P2d and P2e), Smith-degraded (F2a-sm) and their partially desulfated (F2a-D, F2b-D, P2d-D, P2e-D and F2a-sm-D) products obtained from *C. flagelliferus* and *C. papillosus*

Fractions[a]	Derivative[b]						
	3,6-Man[c]	2,3,6-Man	3-Man[d]	2,3-Man	3,4,6-Man	2,3,4,6-Man	2,3,4-Xyl
F2a	72.6	2.0	13.9	—	5.0	—	6.5
F2a-D	6.5	69.9	—	13.5	—	4.0	6.2
F2a-sm	98.0	2.0	—	—	—	—	—
F2a-sm-D	—	100.0	—	—	—	—	—
F2b	64.7	2.9	15.1	—	5.8	—	11.5
F2b-D	—	62.9	—	20.0	—	5.9	11.2
P2d	79.5	t.r.	10.6	—	4.4	—	5.5
P2d-D	—	79.0	—	11.6	—	3.7	5.7
P2e	71.5	2.6	14.3	—	3.4	—	8.2
P2e-D	8.2	62.8	—	16.6	—	3.0	10.0

t.r. Percentages lower than 1.0% are considered as trace amounts.

[a] Fractions are defined in the text.

[b] Mol % of monosaccharide bearing methyl groups at the positions indicated.

[c] 3,6-Man analyzed as 1,2,4,5-tri-*O*-acetyl-3,6-di-*O*-methylmannitol, etc.

[d] Determined after acid hydrolysis, NaBD$_4$ reduction, acetylation and GC–MS analyses (*m*/*z* 139, 159, 190 and 219).

backbone, partially C-6 substituted by single stubs of mannopyranosyl 2-sulfate and xylopyranosyl units.

2.5. NMR analyses

^{13}C NMR spectra of native (F2a and P2d), desulfated (F2a-D and P2d-D) and Smith-degraded (F2a-sm) xylomannans are shown in Figures 2 and 3, and their chemical shift assignments are reported in Table 4.

^{1}H NMR spectra of the native xylomannans showed a singlet at 4.86 ppm attributed to H-1 of the mannopyranosyl residues and a doublet at 4.45 ppm ($J_{\text{H-1,H-2}}$ 7.2 Hz) assigned to H-1 of the xylopyranosyl residues in the β-configuration.[22,35] The anomeric configuration of the former residue was determined by ^{13}C NMR proton-coupled experiment. The C-1,H-1 coupling constant of 161 Hz is consistent with a β-linkage.[36]

^{13}C NMR spectra of the native xylomannans (F2a, F2b, P2d and P2e) were qualitatively similar, but differed slightly in the intensity of their signals. These spectra showed in the anomeric region a major signal at 99.1 ppm (F2a and F2b) or at 99.0 ppm (P2d and P2e) corresponding to 4-linked β-D-mannopyranosyl 2-sulfate residues (C-2 at 78.1 ppm). These signals are shifted upfield (~1.0 ppm) and downfield (8.1 ppm), respectively, when compared to the spectrum of its corresponding desulfated polymers (F2a-D, F2b-D; P2d-D and P2e-D), in accordance with the β- and α-effect of sulfation. Moreover, the spectra of F2a-D and P2e-D showed a very low intensity signal at 99.1 and 99.0 ppm, respectively, consistent with the presence of remaining sulfate groups after solvolytic desulfation (Table 1) and the corresponding derivative (3,6-Man, 6.5 and 8.2 mol %, respectively, Table 3) amongst the products of methylation of the partially desulfated polymers.

The signal at 103.4 ppm present in both native and desulfated xylomannans corresponds to C-1 of xylopyranosyl[22] single stub units attached to C-6 of the 4-linked mannan backbone. DEPT NMR spectrum of the desulfated xylomannan showed inverted signals at 68.3, 65.2 and 60.6 ppm, attributed to substituted C-6,[37] C-5 of xylose and non-substituted C-6, respectively (insert in Fig. 3b). C-6 and C-5 (73.6 ppm) signals of 4,6-disubstituted mannopyranosyl residues are shifted downfield (7.7 ppm) and upfield (1.4 ppm), respectively, in comparison with those of unbranched residues[37] (Table 4).

The signal of very low intensity at 66.8 ppm that was present only in the spectra of xylomannans P2d (Fig. 3a) and P2e was assigned to C-4 of the β-D-mannopyranosyl side chain units[38] in agreement with results of methylation. Considering that these xylomannans have low MW (33 and 41 kDa, respectively), 6.7–4-fold lower than those of F2a and F2b, part of this resonance would arise from C-4 of the β-D-mannopyranosyl non-reducing end of the main chain.[38]

The ^{13}C NMR spectrum of the Smith-degraded polysaccharide (F2a-sm, Fig. 2c) contains only six sharp

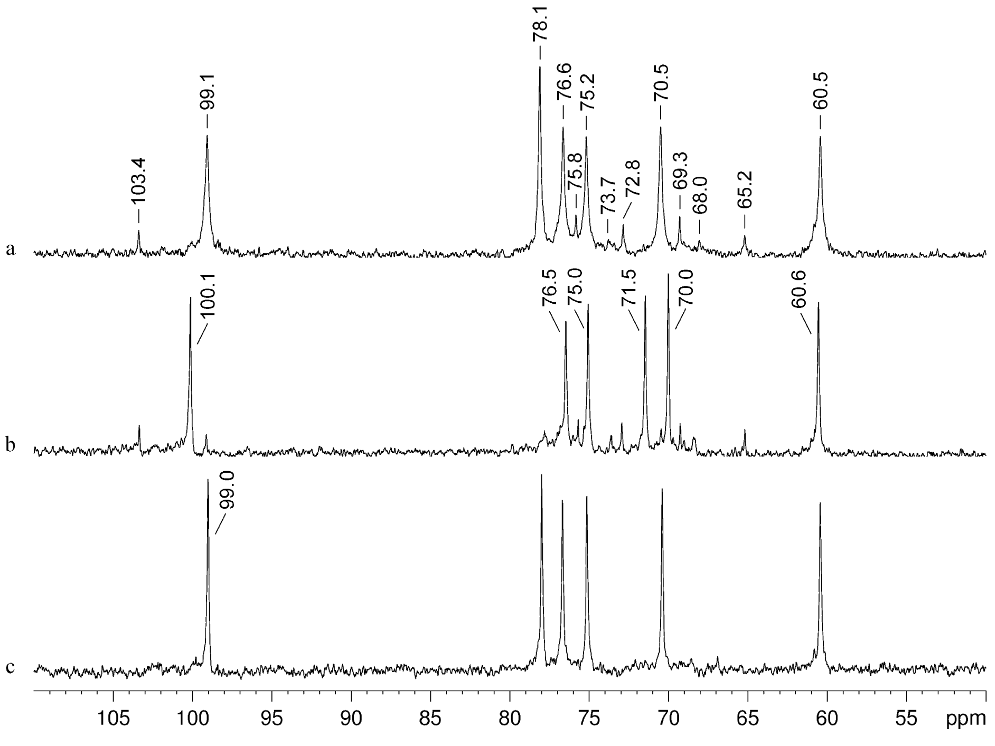

Figure 2. ^{13}C NMR spectra of the native xylomannan F2a (a), desulfated derivative F2a-D (b), Smith-degraded product F2a-sm (c) isolated from *C. flagelliferus*.

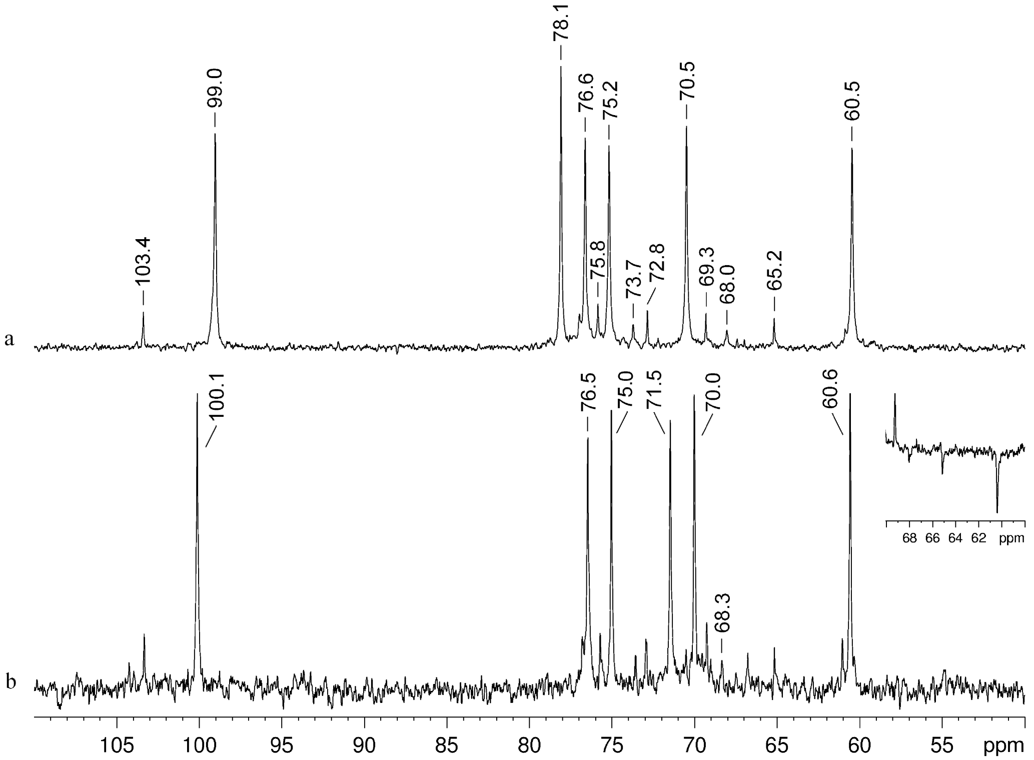

Figure 3. ^{13}C NMR spectra of the native xylomannan P2d (a), desulfated derivative P2d-D (b) isolated from *C. papillosus.*

Table 4. Chemical shift assignments of the NMR spectra of the native[a] xylomannans (F2a, F2b, P2d and P2e) and Smith-degraded[b] polysaccharide (F2a-sm) and desulfated[c] xylomannans (F2a-D, F2b-D, P2d-D and P2e-D)

Type of unit	Chemical shift (ppm)													
	C-1	C-2	C-3	C-4	C-5	C-6	H-1	H-2	H-3	H-4	H-5a	H-5b	H-6a	H-6b
→4)-β-D-Man*p*-2-S-(1→														
Unbranched at O-6[a,b]	99.0	78.1	70.5	76.6	75.2	60.5	4.86	4.81	3.96	3.87	3.59		3.83	3.94
Branched at O-6[a,c]	99.0	78.1	70.5	76.6	73.7	68.0								
→4)-β-D-Man*p*-(1→														
Unbranched at O-6[c]	100.1	70.0	71.5	76.5	75.0	60.6								
Branched at O-6[c]	100.1	70.0	71.5	76.5	73.6	68.3								
β-D-Xyl*p*-(1→[a,c]	103.4	72.8	75.8	69.3	65.2		4.45	3.37	3.48	3.66	3.37	4.00		

[a] NMR signals in the spectrum of the native sulfated xylomannan. In F2a and F2b the C-1 signal appears at 99.1 ppm.
[b] NMR signals in the spectrum of the Smith-degraded polysaccharide.
[c] NMR signals in the spectrum of the desulfated xylomannan.

signals corresponding to C-1–C-6 of 4-linked mannopyranosyl 2-sulfate residues (Table 4) in agreement with the removal of the side-chain units and consistent with its methylation analysis.

The 2 M KCl-precipitated fraction, FH-p, obtained from a southern Brazilian sample of *C. flagelliferus* that was carefully examined to avoid contamination with epiphytes and endophytes, showed the same set of resonances present in the spectra of all the other native xylomannans (Fig. 4c). This result clearly shows that this type of polysaccharide is synthesized by *Chondrophycus* species and eliminates the possibility of exogenous contamination.

^{1}H chemical shifts of 4-linked β-D-mannopyranosyl 2-sulfate and β-D-xylopyranosyl residues were assigned using 1D and 2D COSY, TOCSY and HMQC NMR techniques. The HMQC spectrum of xylomannan P2d with its complete correlation assignment is shown in Figure 5. ^{1}H Assignments were in agreement with NMR data previously reported for methyl glycosides of mannose[39] and oligo- and polysaccharides containing 4-linked mannopyranosyl residues,[40–43] considering the deshielding effect of the sulfate group on geminal and vicinal protons.[44] Chemical shift assignments of β-D-xylopyranosyl side-chain residues are in accordance with the literature for terminal β-D-xylopyranosyl residues.[22]

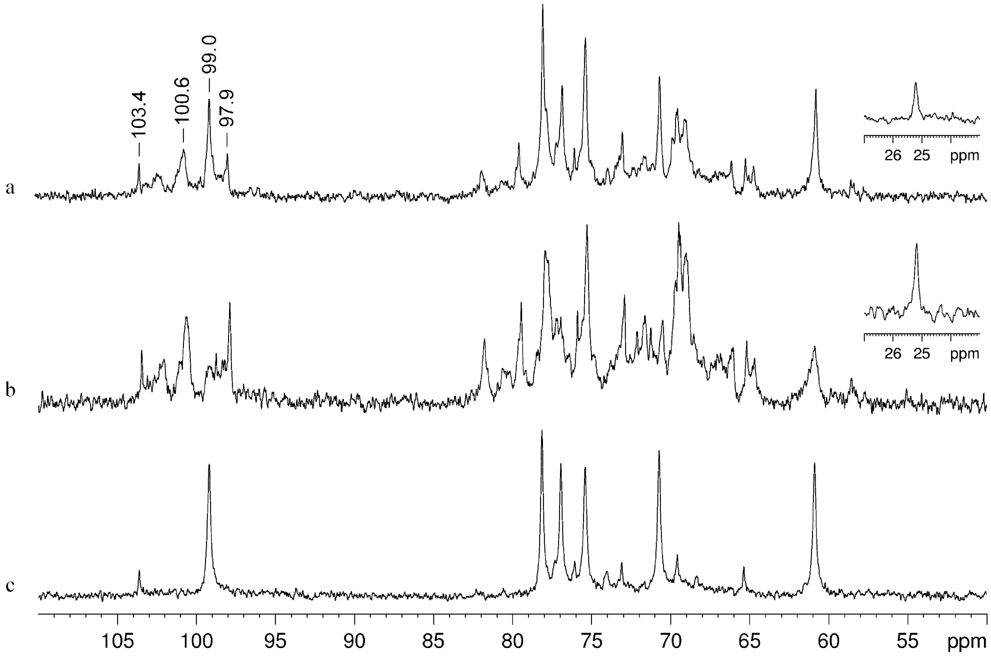

Figure 4. ^{13}C NMR spectra of the crude extract FH (a), 2 M KCl-soluble fraction FH-S (b), 2 M KCl-precipitated fraction FH-p (c) isolated from *C. flagelliferus*.

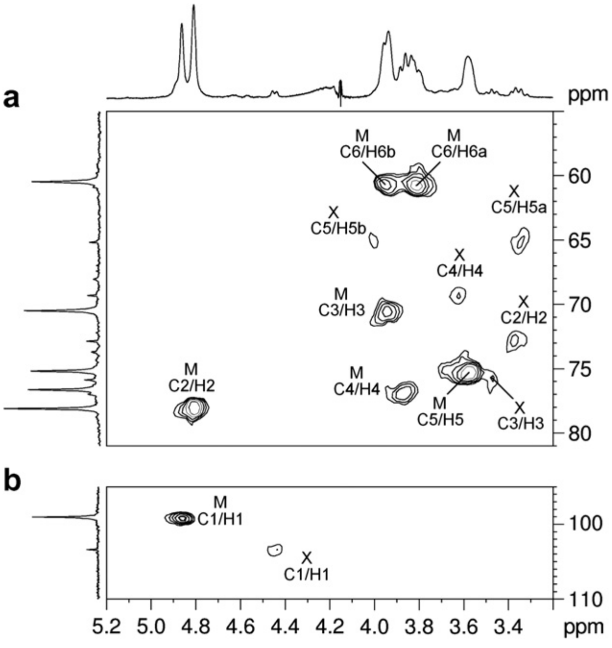

Figure 5. HMQC spectrum of P2d, C-2, C-6/H-2, H-6 region (a), anomeric region (b). M and X correspond to [→4)-β-D-Man*p*-2-S-(1→] and [β-D-Xyl*p*-(1→] residues, respectively.

Therefore, NMR data confirm methylation analyses in terms of glycosidic linkage, sulfate and side-chain positioning of the xylomannans isolated from *C. flagelliferus* and *C. papillosus* and additionally indicated that the sugar constituents were β-glycosidically linked.

Xylomannans have also been isolated from several red seaweeds of the order Nemaliales, but in this case, they are constituted by a α-D-mannopyranosyl 3-linked backbone.[3–10] Included in this group are the xylomannans isolated from *N. fastigiata*, sulfated on C-2 and/or C-6 and partially glycosylated by single stubs of β-D-xylopyranosyl units attached to C-2.[3–7] The α-mannan backbone of the water-soluble polysaccharide isolated from *Liagora valida* is sulfated on C-4 and C-6 and substituted on C-2 by single stubs of β-D-xylopyranosyl residues or less often on C-6 by short chains of 4-linked β-D-xylopyranosyl units or single stubs of β-D-xylopyranosyl or its 3-*O*-methylated derivative.[9] A structurally similar sulfated xylomannan has been described for *Nemalion vermiculare*.[8] In order Nemaliales there is only one description of a xylomannan backbone made up of β-D-mannopyranosyl residues.[11] This is the case of the xylomannan isolated from *Galaxaura squalida* in which the 3-linked β-D-mannopyranosyl backbone is substituted on C-4 by a sulfate group or xylopyranosyl or L-galactopyranosyl units.[11] Therefore, the structural characteristics described in the present work for the sulfated xylomannan produced by *Chondrophycus* species are different from those mentioned above and could represent a chemotaxonomic marker for this genus.

The KCl-soluble fractions (F2-S, P2-S and FH-S) gave similar ^{13}C NMR spectra between them with a complex anomeric region containing at least nine signals. The spectrum of one of these fractions (FH-S) is shown in Figure 4b. The major signal at 100.6 ppm was attributed, at least in part, to C-1 of β-D-galactose 2-sulfate linked to 3,6-anhydro-α-L-galactose (C-1 at 97.9 ppm).[45] The signal of medium intensity at 25.1 ppm, besides that at ~175.8 ppm arose from methyl and carboxyl groups of the acetal of pyruvic acid in the *R* form.[46] These spectroscopic data, together with sugar composition and the negative optical rotations, are indicative of the presence of pyruvylated agaran structures as major water-extractable polysaccharides. Our results are in agreement with the agarophyte character of *Laurencia* complex species (genera *Laurencia*, *Chondrophycus* and *Osmundea*)[47,48] as reported by several authors.[19–24]

It is noteworthy that the ^{13}C NMR spectra of the crude extracts FH (Fig. 4a), F2 and P2 contain, besides the above-mentioned signals, one of high intensity at 99.0, 99.1 and 99.0 ppm, respectively (C-1 of 4-linked β-D-mannopyranosyl 2-sulfate). Combined with mannose and xylose detection, this signal could be used as a diagnostic resonance to indicate the presence of xylomannan 2-sulfate in crude extracts of other *Chondrophycus* species.

Agarans with different patterns of substitution have been described for representatives of Ceramiales.[19–28] Together with these polysaccharides, another type of galactan, DL-hybrid, has been isolated from some species of this order.[49,50] The isolation of a xylomannan sulfate from two species of *Chondrophycus* adds a new type of polysaccharide to this group of red seaweeds. Studies are underway to determine if this class of sulfated polysaccharide is a general feature of the red seaweeds from genus *Chondrophycus* or even of the *Laurencia* complex.

3. Experimental

3.1. Collection of specimens

The specimens of *C. flagelliferus* (J. Agardh) K. W. Nam and *C. papillosus* (C. Agardh) Garbary and Harper were collected from the northeastern coast of Brazil (Cabo, Pernambuco State, and in Praia do Forte, Bahia State, respectively). The voucher specimens were deposited in the herbarium of the Department of Botany, Federal University of Paraná (Curitiba, Brazil) with the herbarium numbers UPCB-55549 and UPCB-55343, respectively. The algal material was carefully cleaned by hand and washed with tap water and sun-dried. Whole thalli were used for polysaccharide extractions. A second sample of *C. flagelliferus* was collected on the southern coast of Brazil (Bombinhas, Santa Catarina State). For inspection of the existence of epiphytes and endophytes, the specimens were exhaustively examined using a stereomicroscope, and when necessary, a microscope. About 1 g of the apical regions of these thalli, free of any kind of contaminants, was selected to be used as standard material.

3.2. Extraction

The milled seaweeds *C. flagelliferus* and *C. papillosus* (both collected on the northeastern coast of Brazil) were separately extracted with water (3% w/v) at 25 °C with mechanical stirring for 16 h. After centrifugation the supernatants were concentrated and treated with EtOH (3 vols). The precipitated materials were resuspended in distilled water, dialyzed, concentrated and freeze-dried yielding the crude extracts F1 and P1, respectively (Table 1). The algal residues were sequentially extracted with water (3% w/v) at 80 °C with mechanical stirring for 3 h and treated as described above, giving the crude extracts F2 and P2, respectively. The polysaccharides from *C. flagelliferus* (collected on the southern coast of Brazil) were obtained by aqueous extraction with hot water (80 °C) as described above, to give the crude extract FH (Table 1).

3.3. Fractionation of the crude extracts with KCl

The crude extracts were submitted to KCl treatment.[51] Briefly, F2 and P2 were dissolved in water (0.25% w/v), and KCl was added under mechanical stirring so that the concentration was increased by 0.25 M each time, up to 2 M. After 2 h stirring at 25 °C, the materials were maintained at 4 °C for 15 h, and the precipitated polysaccharides were centrifuged, dialyzed, concentrated and freeze-dried.

Four and five KCl-insoluble fractions were obtained from F2 (F2a–F2d) and P2 (P2a–P2e), respectively. The ranges of precipitation of each fraction are shown in Table 1. The soluble materials (in 2.0 M KCl) were dialyzed, concentrated and freeze-dried yielding the KCl-soluble fractions F2-S and P2-S, respectively (Table 1). FH extract (0.25% w/v) was treated with 2 M KCl giving rise to the precipitated (FH-p) and soluble (FH-S) fractions.

3.4. Analytical methods

Optical rotations of aqueous solutions of the polysaccharide samples (0.2% w/v) were measured at 20 °C, using a 10-cm cell and the sodium D line (589.3 nm) with a Rudolph Autopol III automatic polarimeter. Total carbohydrate and sulfate contents were determined by the phenol–sulfuric acid method[52] and the turbidimetric method of Dodgson and Price,[53] respectively. For monosaccharide composition, fractions F2a, F2b, P2d, P2e and FH-p were hydrolyzed using M TFA at 100 °C for 4 h. Hydrolysis products were reduced with $NaBH_4$ and acetylated (Ac_2O, 1 h, 120 °C), and the resulting alditol acetates were analyzed by

GC–MS. Monosaccharide composition of the other fractions was carried out by reductive hydrolysis[54] using the modifications introduced by Jol et al.[55] GC–MS analyses were performed with a Varian 3800 chromatograph, equipped with a fused-silica capillary column (30 m × 0.25 mm) coated with DB-225MS (Durabond), and a Varian Saturn 2000 R ITD spectrometer. The chromatograph was programmed to run at 50 °C for 1 min, then 50–215 °C at 40 °C min^{-1}, using helium as carrier gas at 1 mL min^{-1}.

3.5. Smith degradation

F2a (50 mg) was oxidized[34] with $NaIO_4$ (0.05 M, 20 mL) in the dark, at 25 °C for 48 h. Following the addition of 1,2-ethanediol (2 mL), the solution was dialyzed for 48 h against distilled water, and concentrated, and the resulting polyaldehydes were reduced with $NaBH_4$ for 15 h. The solution was acidified with HOAc, and dialyzed, and the material was partially hydrolyzed with M TFA at 25 °C for 20 h. After neutralization with M NaOH, the oxidized and reduced material was dialyzed and freeze-dried to give the Smith-degraded fraction F2a-sm (70% yield).

3.6. Desulfation

Desulfation of the polysaccharides was carried out as previously described.[31] The polysaccharidic fractions, in the pyridinium salt form, were treated with a mixture of 89:10:1 Me_2SO–MeOH–pyridine, for 4 h at 100 °C. The native xylomannans F2a, F2b, P2d, P2e and the Smith-degraded fraction (F2a-sm) were submitted to this solvolytic treatment, giving rise to F2a-D, F2b-D, P2d-D, P2e-D and F2a-sm-D, respectively. Yields and chemical analyses of these fractions are shown in Tables 1 and 2.

3.7. Methylation

The native xylomannans (F2a, F2b, P2d and P2e), their desulfated fractions (F2a-D, F2b-D, P2d-D and P2e-D) and the Smith-degraded product before (F2a-sm) and after desulfation (F2a-sm-D) were methylated by the method of Ciucanu and Kerek[30] with the polysaccharides in the triethylammonium salt form.[29] The samples (10 mg) were dissolved in Me_2SO (1 mL), then powdered NaOH (200 mg) was added, and the mixture was stirred for 30 min at 25 °C, followed by CH_3I (1 mL) addition. After 15 h at 25 °C the reaction was interrupted by addition of water, neutralized with HOAc, dialyzed against distilled water and freeze-dried. The permethylated polysaccharides were obtained after two further steps of methylation carried out in the same way as described above. These polysaccharides were hydrolyzed with aq formic acid (45% v/v) for 16 h at 100 °C, followed by $NaBD_4$ reduction, acetylation and GC–MS analysis. Partially methylated alditol acetates were identified by their typical electron-impact breakdown profiles and retention times[56,57] using the same column and GC–MS conditions described above.

3.8. HPSEC–MALLS analysis

The determination of the homogeneity and molar mass (MW) was performed on a Waters High-Performance Size-Exclusion Chromatography (HPSEC) apparatus coupled to a refractive index detector (RID) and a Wyatt Technology Dawn-F Multi-Angle Laser Light Scattering detector (MALLS). Waters Ultrahydrogel Columns (2000, 500, 250 and 120) were connected in series and coupled with multi-detection equipment, using a $NaNO_2$ solution (0.1 M) as eluent, containing NaN_3 (200 ppm) as preservative. The samples (2 mL^{-1}) were dissolved in the same solvent under magnetic stirring for 2 h and filtered through 0.45- and 0.22-μm nitrocellulose membranes (GSWP, Millipore). HPSEC data were collected and analyzed by the Wyatt Technology ASTRA program. The light-scattering signal was detected simultaneously at 11 scattering angles, θ, ranging from 35° to 132°. All experiments were carried out at 25 °C.

3.9. Spectroscopic analysis

NMR spectra were recorded at 70 °C using a Bruker Avance DRX400 spectrometer, equipped with a 5-mm multi-nuclear inverse detection probe, at a base frequency of 100.63 MHz for ^{13}C and 400 MHz for ^1H nuclei. Chemical shifts are reported in ppm using acetone as internal standard at 30.2 and 2.224 ppm for ^{13}C and ^1H, respectively. For coupled and decoupled-^{13}C and ^{13}C–DEPT (DEPT135) analyses, the lyophilized samples were dissolved in D_2O (30–40 mg mL^{-1}). For ^1H and 2D NMR experiments, the samples were deuterium exchanged by successive freeze-drying steps in D_2O (99.9%) and then dissolved in D_2O (20–30 mg mL^{-1}). ^1H, ^{13}C and ^{13}C–DEPT acquisition parameters were previously reported.[58] 2 D ^1H, ^1H COSY, TOCSY and ^1H, ^{13}C HMQC experiments were carried out using the pulse programs supplied with the apparatus.

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