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Carbohydrate Research 339 (2004) 97–103
Carbohydrate RESEARCH

The extracellular polysaccharide of *Porphyridium* sp.: an NMR study of lithium-resistant oligosaccharidic fragments

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Received 8 July 2003; accepted 12 September 2003

Abstract—This study deals with the chemical characterization of an extracellular polysaccharide produced by the unicellular red alga *Porphyridium* sp. The sugar moiety of this polymer is composed of three neutral monosaccharides (Xyl, Glc, and Gal) and one uronic acid (GlcA). Proteins represent 5.5% of the dry weight of the polymer. Uronic degradation of this exopolysaccharide with lithium in ethylenediamine yielded two different oligosaccharides. The absolute configuration of the constitutive monosaccharides was chemically determined and revealed the presence of D-Xyl, D-Glc, D-, and L-Gal. The following oligosaccharide structures were established by NMR spectroscopy:

β-D-Xyl*p*
1
↓
2
β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-L-Gal*p*

and

β-D-Xyl*p*
1
↓
2
β-D-Gal*p*-(1→3)-β-D-Glc*p*-(1→3)-β-D-Xyl*p*-(1→4)-β-D-Xyl*p*-(1→4)-L-Gal*p*

Keywords: *Porphyridium* sp.; Extracellular polysaccharide; Anionic polysaccharide; Lithium degradation; NMR; Structure

1. Introduction

As reported by Ramus,[1] cells of red algae are always generously coated with 'gel state' polysaccharides, that is, '*a fairly permanent network structure formed from polymer solutions*'. Typically, the interconnected network

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doi:10.1016/j.carres.2003.09.020

gives rise to the characteristic texture and properties of the polysaccharides. Their solubility in water depends on both the chemical nature and the number of bonds between the polymer chains, but most of these polysaccharides tend to be water soluble. Their biological function(s) in a marine environment are, up to now, not well defined. These cell wall polysaccharides could be involved in mechanical, hydric, and/or ionic regulations.[2] Most authors tracing the first attempt at determining the possible taxonomic significance of cell-wall polysaccharides in red algae refer to Stoloff and Silva.[3] They first demonstrated the potential of the properties of water extractable polysaccharides as chemotaxonomic tools in red algae. The main groupings of algae were based on the colloids they produce, in a scheme that has suffered few modifications over the past decades: the agarophytes producing agar, the carrageens producing carrageenin, and a group of 'other' producing mostly heteropolysaccharides.[4]

Porphyridium sp., a unicellular red alga, belongs to the third family. It produces an exocellular matrix mainly composed of a sulfated polyanionic polysaccharide containing a number of neutral sugars (Xyl, Glc, and Gal) and GlcA,[5–7] with a molar mass of $2.3\times10^6\,g\,mol^{-1}$.[8] Small amounts of methylated sugars (3-*O*-methyl-Xyl, 3-*O*- and 4-*O*-methyl-Gal, 2-*O*-methyl-GlcA) have also been found.[9] Arad and co-workers attempted to elucidate the fine structure of the extracellular sulfated polysaccharides produced by different red unicells such as *Porphyridium* sp., *P. cruentum*, *P. aerugineum*, *Rhodella reticulata*.[10–19]

Their work were based on two complementary approaches: nonspecific chemical hydrolysis or enzymatic degradation of the polysaccharide, combined with spectroscopic analyses of oligosaccharides by NMR spectroscopy, and GC–MS and physicochemical studies with special emphasis on rheological properties. The results obtained indicate that the presence of aldobiuronic acid 3-*O*-(α-D-glucopyranosyluronic acid)-L-galactopyranose seems to be a characteristic common to all of the polysaccharides of the studied red unicells. Unfortunately, acidic hydrolysis of the polymer led quickly to its complete depolymerization without liberation of oligosaccharide and, to date, no efficient enzymatic hydrolysis has been described. Lastly, the presence of uronic acid units often complicates the investigations on the primary structure of the polysaccharides since the uronic acid glycosides are resistant to acid hydrolysis.[20,21]

This lack of structural information impairs the interpretation of the physiological role(s) of this polymer and consequently the utilization of its interesting rheological and potential biotechnological properties, for example, strong resistance to physical and chemical attacks, tertiary oil recovery, waste water treatment, thickener, stabilizer, and emulsifier in food industry.[22–24] Another interesting aspect of the polysaccharides of the red alga unicells is that they present biological properties with potential applications in medicine. Red microalgae are considered as a source of pharmacologically valuable polysaccharides.[25] These polysaccharides could be used as hypocholesterolemic or antiviral agents, potential pharmacological effects are expected in immune modulatory and antithrombotic activities, another application was described as oral drug delivery matrix.[26–29]

Elucidating the structure of a complex carbohydrate requires first its fragmentation into characteristic oligosaccharidic units. In the present paper, the polysaccharide from *Porphyridium* sp. was successfully subjected to uronic acidic degradation with lithium in ethylenediamine[30,31] to generate specific fragments of this polymer for structural analysis purposes, making use of NMR spectroscopy.

2. Experimental

2.1. Biological material

Porphyridium sp. biomass was obtained from the Centre d'Etude Nucléaire at Cadarache (France). This biomass was harvested and cleaned by washing with deionized water and freeze-dried for storage.

2.2. Isolation and purification of the exocellular polysaccharide

Ground algal powder (1 g) was extracted overnight in 50 mL acetone and washed several times with acetone and EtOH, until the filtrate became clear. The dried powder was refluxed with 50 mL of ethanol for 1 h and then with distilled water (100 mL) at 100 °C for 1 h. The resulting supernatant was recovered after centrifugation (5000g, 15 min) by filtration through cellulose nitrate membranes (2.7 μm). The extracellular polysaccharides were isolated, after centrifugation (5000g, 15 min), by precipitation with 3% hexadecyl trimethyl ammonium bromide. The precipitated acidic polysaccharide was purified by four successive cycles of re-solubilization in KCl of decreasing concentration (1.5, 0.75, 0.3 M, water), and re-precipitation by 50% EtOH–water, before centrifugation at 5000g (30 min). Finally, this purified material was dialyzed against water and lyophilized *prior* to lithium degradation.

2.3. Fragmentation of the extracellular polysaccharide with lithium in ethylenediamine

Fragmentation of the anionic exocellular polysaccharide with lithium in ethylenediamine was adapted from the procedure described by Mort and Bauer[30] and Lau et al.[31] The carbohydrate (10 mg) was added to a screw

capped tube (20×120 mm) containing a small stirring bar. The sample was dried under diminished pressure overnight. Ethylenediamine (1 mL/5 mg carbohydrate) was added and the mixture was stirred in a sonication bath until dissolution of the carbohydrate. Small pieces of lithium (2–5 mm) were then added to the soln. After the soln turned deep blue, additional small pieces of lithium were added. The amount of lithium added was determined by the amount required to maintain the deep blue color. After 2 h, the sample was cooled in an ice bath to freeze the mixture and water (5 mL) was slowly added. The mixture was vortexed, with intermittent cooling in the ice bath, until dissolution was complete. The resulting clear soln was transferred to a 50 mL flask, and toluene (25 mL) was added. The flask was shaken and the mixture rotoevaporated to dryness. This step was repeated twice. The resulting powdery, white residue (LiOH and carbohydrate) was cooled in an ice bath and dissolved in water (5 mL). The soln was brought to pH 4–5 with glacial AcOH, and passed through a column of Dowex 50×8 (H^+) ion-exchange resin to remove the lithium ions. Lithium-resistant oligosaccharides were then purified on a TSK-HW40 column (E. Merck; 70×2 cm) with AcOH (0.5%) as eluent, with monitoring by refractometric detection; fractions (1.5 mL) were collected. Oligosaccharide fractions, which were homogeneous were combined, desalted using Biogel P2 (Bio-Rad; 60×2 cm), and freeze-dried for chemical analysis.

2.4. Composition of the polysaccharide

Total carbohydrate was measured by the phenolsulfuric acid method.[32] Hexuronic acids were determined according to Blumenkrantz and Asboe-Hansen[33] and soluble protein by the Lowry assay.[34] Monosaccharide determination was carried out after methanolysis (MeOH/HCl 0.5 N, 24 h, 80 °C) by GLC of pertrimethylsilylated methylglycosides according to Kamerling et al.,[35] modified by Montreuil et al.[36] GLC was performed on a Perichrom gas chromatograph fitted with a flame-ionization detector. A capillary column, CPSIL-5CB (Chrompack; 0.32 mm×50 m), was used with the following temperature programs: 120–240 °C at 2 °C min^{-1}. Nitrogen was the carrier gas at 0.5 atm. The absolute configuration of the monosaccharides was determined according to Gerwig et al.[37]

2.5. NMR spectroscopy experiments

Prior to spectroscopic analysis, oligosaccharides were exchanged in D_2O (99.97% ^2H, Euriso-top, Saclay, France) with intermediate freeze-drying and then dissolved in 250 μL D_2O. Chemical shifts were expressed in ppm downfield from the signal of methyl group of internal acetone (δ $^1H = 2.225$ ppm and $^{13}C = 31.55$ ppm at 300 K). The samples were analyzed in 5 mm susceptibility matched tubes (Shigemi, Japan) on Bruker ASX-400-WB spectrometer (Centre Commun de RMN, Villeneuve d'Ascq; ^1H 400.33 MHz, ^{13}C 100.66 MHz) equipped with a double resonance ($^1H/X$) Broad Band Inverse *z*-gradient probe head. All NMR data were recorded without sample spinning using NOESY, COSY, HMQC sequences.

3. Results and discussion

3.1. Isolation and characterization of the extracellular polysaccharide

The purified extracellular polysaccharide represented a significant proportion of the biomass, reaching 4% (w/w) of the dry weight. Analysis of the polysaccharide by GLC of the trimethylsilylated methyl glycoside derivatives showed the presence of three major neutral sugars (xylose, glucose, and galactose) and one glucuronic acid. Finally, the purified polymer contained 5.5% (w/w) of protein.

3.2. Fragmentation of the extracellular polysaccharide with lithium in ethylenediamine

Fragmentation of the extracellular polysaccharide produced by *Porphyridium* sp. with lithium in ethylenediamine resulted in a complex mixture of products that were isolated in <30–40% yield, and which were distributed between the void vol and the partially included vol of the TSK-HW40 column. This mixture was mainly composed of two oligosaccharides (oligosaccharides **1** and **2**), which proved homogeneous by TLC and by HPAEC-PAD (data not shown).

3.3. NMR characterization of the purified oligosaccharides

3.3.1. Oligosaccharide 1. The ^1H NMR spectrum of oligosaccharide **1** was assigned using 2D COSY and relayed COSY experiments (Fig. 1 and Table 1). Based on the vicinal coupling constant values, seven sugar spin systems were assigned to α-Gal*p*, β-Gal*p*, and β-Xyl*p*. Actually, the two anomeric signals labeled as Iα and Iβ originate from the terminal reducing Gal unit. The influence of anomerization on the chemical shift of Xyl^{II} H-1 (δ 4.497 and 4.714) and Xyl^{III} H-1 (δ 4.374 and 4.356) is apparent. The ROESY experiment (Fig. 1) showed the following interresidue correlations between the *trans*-glycosidic protons: β-Xyl^{IIIB} H-1, β-Gal^{IB} H-4 at δ 4.356/4.130; β-Xyl^{IIIA} H-1, α-Gal^{IA} H-4 at δ 4.374/4.206; β-Xyl^{IIB} H-1, β-Gal^{IB} H-2 at 4.714/3.634; β-Xyl^{IIA}

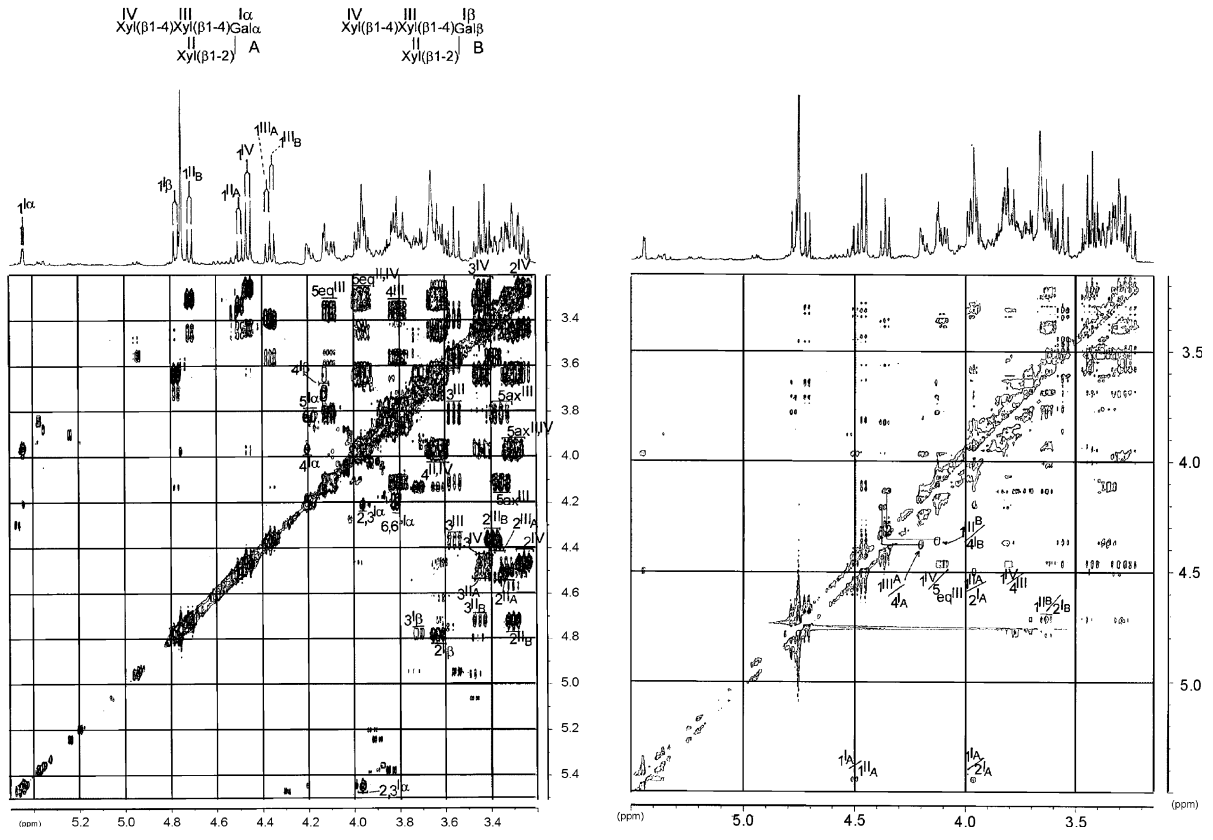


Figure 1. Homonuclear ^{1}H–^{1}H NMR spectra of oligosaccharide **1**. Left, expanded region (δ $^{1}H = 5.5$–3.2 ppm, in both dimensions) of one relayed COSY spectrum; right, expanded region (δ $^{1}H = 5.5$–3.15 ppm, in both dimensions) of 400 ms ROESY spectrum.

Table 1. ^{1}H and ^{13}C chemical shifts (ppm) of monosaccharide residues for oligosaccharides **1** and **2**

Unit	H-1	H-2	H-3	H-4	H-5_{ax}	H-5_{eq}	H-6,6′	C-1	C-2	C-3	C-4	C-5	C-6
Oligosaccharide **1**													
Iα	5.443	3.966	3.963	4.206	4.193	—	3.818	91.56	**77.26**[a]	67.89	**79.61**	71.89	61.45
Iβ	4.775	3.634	3.715	4.130	3.772	—	3.68	97.44	**80.33**	71.66	**78.45**	76.13	61.83
IIA	4.497	3.331	3.447	3.632	3.301	3.970	—	103.08	74.04	76.91	70.44	66.47	—
IIB	4.714	3.303	3.445	3.632	3.301	3.970	—	103.99	74.04	76.91	70.44	66.47	—
IIIA	4.374	3.384	3.562	3.811	3.355	4.107	—	104.70	74.50	74.97	**77.64**	64.29	—
IIIB	4.356	3.396	3.562	3.811	3.355	4.107	—	104.70	74.50	74.97	**77.64**	64.29	—
IV	4.461	3.256	3.429	3.632	3.301	3.970	—	103.08	74.04	76.92	70.44	66.47	—
Oligosaccharide **2**													
Iα	5.43	3.97	3.97	4.21	4.18	—	3.83	91.54	**77.18**	67.92	**79.61**	71.83	61.56
Iβ	4.77	3.66	3.71	4.15	3.76	—	N.D.	97.22	**80.47**	71.66	**78.53**	75.95	N.D.
IIA	4.51	3.33	3.50	3.65	3.29	3.97	—	102.64	74.26	76.83	70.46	66.41	—
IIB	4.72	3.33	3.50	3.65	3.29	3.97	—	103.72	74.26	76.83	70.46	66.41	—
IIIA	4.39	3.41	3.58	3.81	3.83	4.14	—	104.47	74.46	74.96	**77.64**	64.27	—
IIIB	4.38	3.41	3.58	3.81	3.83	4.14	—	104.47	74.46	74.96	**77.64**	64.27	—
IV	4.51	3.48	3.70	3.73	3.34	4.04	—	102.62	73.52	**85.26**	69.07	66.03	—
V	4.77	3.48	3.67	3.67	3.49	—	3.93 3.19	103.72	73.52	**83.80**	71.08	76.83	61.92
VI	5.36	3.88	3.89	4.03	4.25	—	3.75	100.23	69.83	70.70	70.46	72.04	61.15

N.D. = not determined.
[a] Linkage carbons are indicated in bold.

H-1, α-Gal^{IA} H-2 at δ 4.497/3.966; β-Xyl^{IV} H-1, β-Xyl^{III} H-4 at δ 4.461/3.811. The ^{13}C NMR spectrum of oligosaccharide **1** was assigned using H-detected ^{1}H, ^{13}C HMQC experiments (Fig. 2 and Table 1). Significant downfield displacements of the signals for C-2, C-4 of Gal^{I}, and C-4 of $Xyl^{IIIA,B}$, as compared with their posi-

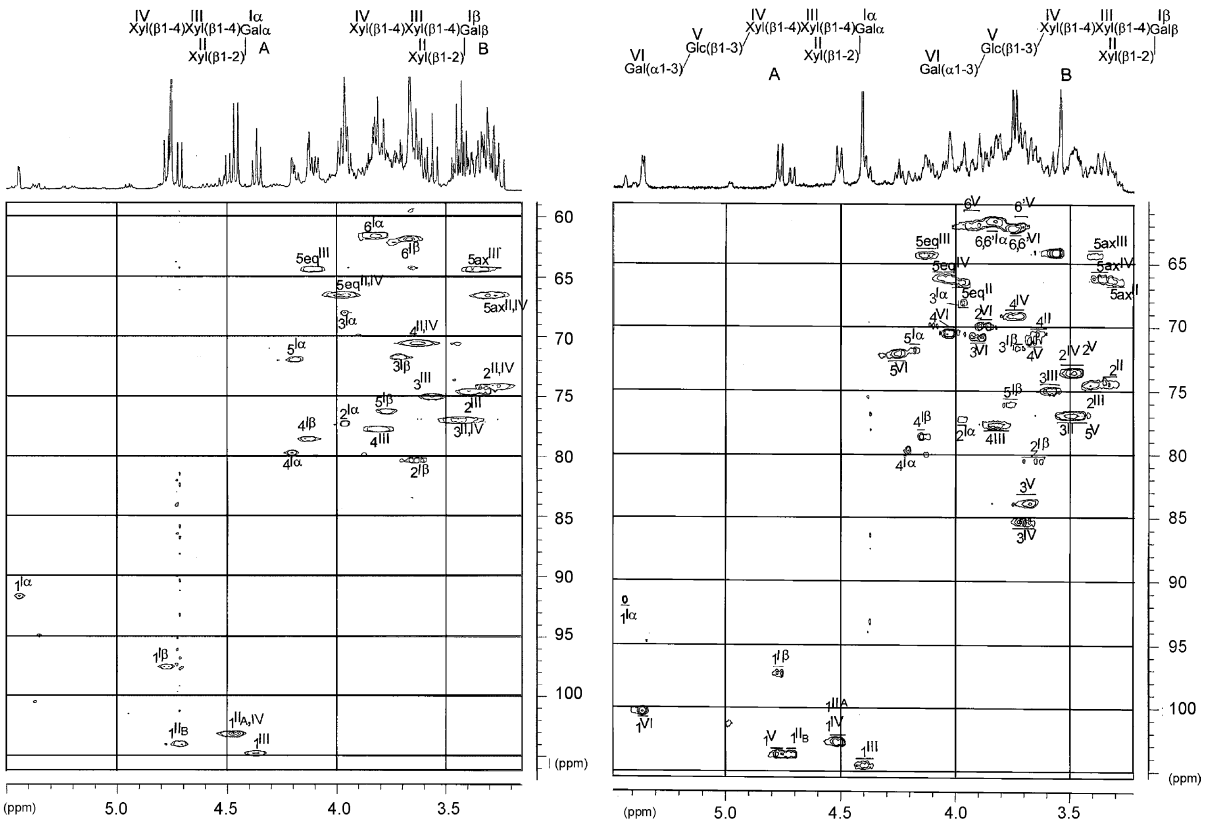


Figure 2. Parts of heteronuclear ^{1}H–^{13}C HMQC spectra of oligosaccharides **1** (left) and **2** (right). Expanded regions (δ $^{1}H = 5.5$–3.15 ppm, δ $^{13}C = 106$–59 ppm) for compound **1** and (δ $^{1}H = 5.5$–3.2 ppm, δ $^{13}C = 105$–60 ppm) for oligosaccharide **2**.

tion in the spectra of corresponding nonsubstituted monomers,[38] confirmed the linkage positions. These data confirmed the substitution pattern and revealed the sequence of residues in the oligosaccharide:

$$\begin{array}{c} \text{II} \\ \beta\text{-Xyl}p \\ 1 \\ \downarrow \\ 2 \\ \beta\text{-Xyl}p\text{-}(1\rightarrow4)\text{-}\beta\text{-Xyl}p\text{-}(1\rightarrow4)\text{-Gal}p \\ \text{IV} \qquad\quad \text{III} \qquad\quad \text{I} \end{array}$$

3.3.2. Oligosaccharide 2. Based on the measure of vicinal coupling constants on the 2D relayed COSY spectrum of oligosaccharide **2**, sugar units were assigned to β-Xyl$p^{II,III,IV}$, α-Gal$p^{I,VI}$, β-Galp^{I}, and β-Glcp^{V}. Combining the COSY and HMQC data demonstrated ^{13}C chemical shifts of sugar units I(α,β), II, and III to be unchanged, in comparison with oligosaccharide **2** (Fig. 2). On the other hand, two significant downfield displacements to δ 85.26 and 83.80 defined the new linkage positions. The assignment of these two signals was achieved by a HMQC-TOCSY experiment (not shown), which revealed intraresidue correlations between ^{1}H and ^{13}C resonances: β-Xyl^{IV} H-1, β-Xyl^{IV} C-3 at δ 4.51/85.26 and β-Glc^{V} H-1, β-Glc^{V} C-3 at δ 4.77/83.80. These data confirmed the substitution pattern and revealed the sequence of residues in the oligosaccharide, which, thus has the following structure:

$$\begin{array}{c} \text{II} \\ \beta\text{-Xyl}p \\ 1 \\ \downarrow \\ 2 \\ \beta\text{-Gal}p\text{-}(1\rightarrow3)\text{-}\beta\text{-Glc}p\text{-}(1\rightarrow3)\text{-}\beta\text{-Xyl}p\text{-}(1\rightarrow4)\text{-}\beta\text{-Xyl}p\text{-}(1\rightarrow4)\text{-Gal}p \\ \text{VI} \qquad\quad \text{V} \qquad\quad \text{IV} \qquad\quad \text{III} \qquad\quad \text{I} \end{array}$$

3.4. Determination of the absolute configuration

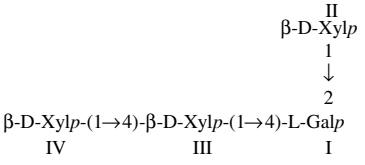
The D and L configurations of monosaccharides, established according to Gerwig et al.[37] showed the presence of both L-Gal and D-Gal isomers (Table 2). Since the commercial (−)-2 butanol contains ~6% of the (+)-enantiomer, a correction factor was calculated in function of data obtained with pure L- and D-Gal samples. In spite of this correction, an excess of 17–20% D-Gal was observed in compounds **1** and **2**. Nevertheless, the analysis of NMR data showed that Xyl^{II,III} (Fig. 1), present in oligosaccharide **1**, are linked to only one L (or D)-Gal unit, since we do not observe any doubling of the anomeric signals of Xyl. From these observations, the sugar compositions of oligosaccharides **1** and **2** were finally considered to be 3-D-Xyl, 1-L-Gal, and 3-D-Xyl, 1-L-Gal, 1-D-Gal, 1-D-Glc, respectively. Moreover, the

Table 2. GLC analysis of trimethylsilylated (−)-2-butylglycosides of the monosaccharides occurring in compounds **1** and **2**

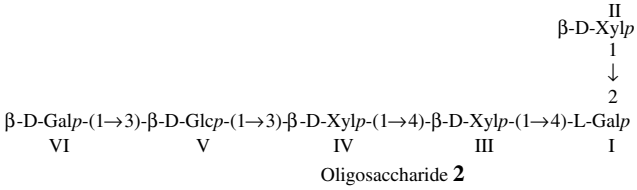
Monosaccharide	Molar ratios					
	Oligosaccharide **1**			Oligosaccharide **2**		
	a	b	c	a	b	c
D-Xyl	3.0	3.0	3	3.0	3.0	3
L-Gal	0.65	0.80	1	0.76	0.83	1
D-Gal	0.35	0.20	0	1.24	1.17	1
D-Glc	—	—	—	1.0	1.0	1

a: Before correction; b: after correction due to the contamination of (−)-2-butanol with (+)-2-butanol; c: after examination of NMR data.

fact that compound **1** is part and parcel of compound **2** strongly suggests that the terminal nonreducing α-Gal unit present in **2** occurs in the D-configuration. Therefore, the structures of oligosaccharides **1** and **2** were established as follows:



Oligosaccharide **1**



Oligosaccharide **2**

The presence of both L- and D-forms of galactose seems to be a characteristic common to the polysaccharide of red algae. This property has been described by Medcalf et al.[39] and Percival and Foyle[9] in the case of various red microalgae. It is also well known in the case of agar and/or carrageenan producing red macroalgae.[4,40] The fragmentation of the extracellular polysaccharide of *Porphyridium* sp. through uronic acid degradation with lithium in ethylenediamine, proved a very powerful methodological tool. The results obtained allowed us to propose the structures of various oligosaccharides, which are representative of the architecture of the polysaccharide. The chain structure found in the extracellular polysaccharide produced by the red alga *Porphyridium* sp. may not be comparable with other biopolymers secreted by the red alga of the agarophyte or carrageen families. The question of the periodicity of chemical structures in term of distribution of oligosaccharides **1** and **2** is still open. The complexity of this extracellular polysaccharide is apparent regarding the variety of monosaccharides and linkages, but also in the number of oligosaccharidic units, which constitute its architecture; it certainly implies a complex biosynthetic pathway with coordinated action of enzymatic activities.

Acknowledgements

The authors gratefully acknowledge Y. Leroy for help in GCMS analysis, Prof. M. Guilloton for his help in ellaboration of the manuscript and the Conseil Regional du Limousin for financial support.

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