Carbohydrate Research 344 (2009) 343–349

Contents lists available at ScienceDirect

Carbohydrate Research

journal homepage: www.elsevier.com/locate/carres

Isolation and characterization of poly- and oligosaccharides from the red microalga *Porphyridium* sp.

Shimona Geresh [a,b,✠], Shoshana (Malis) Arad [a], Oshrat Levy-Ontman [a], Wang Zhang [a], Yoram Tekoah [a], Robert Glaser [b,*]

[a] Department of Biotechnology Engineering, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel
[b] Department of Chemistry, Ben-Gurion University of the Negev, Beer-Sheva 84105, Israel

ARTICLE INFO

Article history:
Received 14 July 2008
Received in revised form 19 November 2008
Accepted 20 November 2008
Available online 6 December 2008

Keywords:
Porphyridium sp.
Extracellular polysaccharide
Oligosaccharide
Hydrolysis
NMR spectroscopy

ABSTRACT

The current study forms part of an ongoing research effort focusing on the elucidation of the chemical structure of the sulfated extracellular polysaccharide of the red microalga *Porphyridium* sp. (UTEX 637). We report here on the chemical structure of a fraction separated from an acidic crude extract of the polysaccharide, as investigated by methylation analysis, carboxyl reduction–methylation analysis, desulfation–methylation analysis, partial acid hydrolysis, Smith degradation, together with 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. This fraction with a molar mass of 2.39×10^{5} g mol^{-1} is comprised of D- and L-Gal, D-Glc, D-Xyl, D-GlcA, and sulfate groups in a molar ratio of 1.0:1.1:2.1:0.2:0.7. The almost linear backbone of the fraction is composed of (1→2)- or (1→4)-linked D-xylopyranosyl, (1→3)-linked L-galactopyranosyl, (1→3)-linked D-glucopyranosyl and (1→3)-linked D-glucopyranosyluronic acid and comprises a possible acidic building unit:

[(2 or 4)-β-D-Xyl*p*-(1→3)]$_m$-α-D-Glc*p*-(1→3)-α-D-Glc*p*A-(1→3)-L-Gal*p*(1→

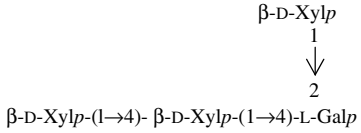
Attached to the backbone are sulfate groups and nonreducing terminal D-xylopyranosyl and galactopyranosyl residues, which occur at the O-6 positions of Glc-derived moieties in the main chain.

1. Introduction

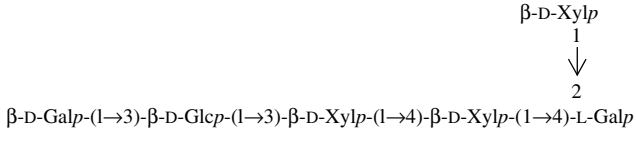
Porphyridium sp., the most abundant species of red microalga of the division Rhodophyta, has been the subject of intensive study by our group for a number of years.[1,2] The cells of this red microalga are encapsulated within a cell-wall polysaccharide complex. The external part of the complex dissolves continuously into the medium,[3,4] and is thus designated 'soluble polysaccharide'. However, most of the polysaccharide remains bound to the cell-wall and is designated 'bound polysaccharide'.[1,2]

Due to the complexity of the polysaccharide, only limited information is available on its structure. Our group has, however, shown that the soluble polysaccharide, with a molar mass of 2.3×10^{6} g mol^{-1},[5] is composed mainly of a sulfated complex heteropolymer containing a number of neutral sugars (mainly Xyl, Glc and Gal) and GlcA.[2,6,7] Minor amounts of methylated sugars are also present.[6,8] The polysaccharide is anionic (negatively charged) due to the presence of GlcA and half-ester sulfate groups[6,9] and is made up of 67% carbohydrate, ~10% ash, ~9% uronic acid, and ~10% half-ester bound sulfate.[6] Two polysaccharide fractions differing in charge and sugar compositions have been obtained by fractionation.[6] These two fractions accounted for ~89% of the total carbohydrates.[6] A primary disaccharide building block, α-D-glucopyranosyluronic acid-(1→3)-L-galactopyranose has been isolated and characterized from acid hydrolysates of *Porphyridium* sp.[10,11] The monosugar sulfates have also been characterized.[9]

Gloaguen et al.[12] have characterized the chemical structure of an anionic polymer separated from the bound polysaccharide produced by *Porphyridium* sp. Analysis of this polymer showed the presence of three major neutral monosaccharides, Xyl, Glc and Gal and of GlcA. Uronic degradation of this polymer with lithium in ethylenediamine yielded the following two oligosaccharides:



and



* Corresponding author. Tel.: +972 8 646 9812; fax: +972 8 647 2943.
E-mail address: glaser@bgu.ac.il (R. Glaser).
✠ Deceased 15 October 2004.

0008-6215/$ - see front matter © 2008 Elsevier Ltd. All rights reserved.
doi:10.1016/j.carres.2008.11.012

The studies of our group on the rheology and chemistry[6,7,13–15] of the soluble polysaccharide of *Porphyridium* sp. have revealed that the polysaccharide exhibits special rheological properties, which makes it suitable for a wide range of potential industrial applications.[2,16,17] Biotechnological investigations have also indicated its potential applications in medicine.[18–20]

For advanced biotechnological development, it was necessary to have access to detailed structural information about the polymer complex of *Porphyridium* sp. To supplement the limited information available to date, we isolated a sulfated glucuronoxylan from the soluble polysaccharide of *Porphyridium* sp. and undertook a structural study utilizing classical chemical methods and spectroscopic analysis.

2. Results

The soluble polysaccharide isolated from cultures of *Porphyridium* sp. was subjected to preliminary fractionation by precipitation with cetyltrimethylammonium bromide (CTAB) to produce an acidic crude polysaccharide and a neutral crude polysaccharide. The former (major) fraction comprised about 96% of the total soluble polysaccharide.

Since the high viscosity of the acidic crude polysaccharide complicated further purification and analysis, the crude preparation was first depolymerized by ultrasound to facilitate isolation and purification of smaller fractions. After 40 min of sonication, the viscosity of the acidic crude polysaccharide solution was markedly reduced (by about 98%).

After ultrasonic depolymerization, the crude preparation was separated into four fractions by anion-exchange chromatography on DE-52 by elution with the following media: water (fraction I), 0.5 M NaCl (fraction II), 1.0 M NaCl (fraction III), and hot urea (fraction IV). Fraction III comprised the major fraction of the acidic polysaccharide from *Porphyridium* sp. and accounted for 30.4% of the total soluble polysaccharide. The homogeneity of this fraction was evident from the single symmetrical high-performance size-exclusion chromatography (HPSEC) peak and the absence of proteins, as indicated by negative responses in both the assay by Lowry et al.[21] and the one by Bradford.[22] The molar mass of this fraction was 2.39×10^5 g mol^{-1}, as determined by the gel-permeation chromatography multiple angle laser light scattering (GPC–MALLS) method.

Sugar composition analysis, by thin-layer chromatography (TLC) and gas chromatography (GC) of the alditol acetate derivatives of fraction III, showed this fraction to be composed of Xyl, Glc and Gal in a molar ratio of 2.1:1.0:1.1 (Table 1). Polysaccharide fraction III also contained uronic acid and sulfate groups, as shown by the *m*-hydroxybiphenyl assay[23] and the sodium rhodizonate assay,[24] respectively. IR spectroscopy of fraction III gave absorption bands at 1250 cm^{-1} and 820 cm^{-1}, indicating the presence of sulfate groups and thus confirming the analytical results. The presence of only one absorption band at 820 cm^{-1}, the region characteristic of sulfated primary hydroxyl groups, suggested that the majority of sulfate groups occurred at C-6 of the sugar residues.[25]

It is known that both D- and L-Gal are present in extracellular polysaccharides extracted from other species of red algae.[12,26–31] In our previous studies[10,11], we obtained an α-D-glucopyranosyluronic acid-(1→3)-L-galactopyranose disaccharide from the acid hydrolysate of *Porphyridium* sp., indicating that the absolute configuration of GlcA and Gal was D- and L-, respectively. This disaccharide was shown to be part of a basic building block found in polysaccharides from various red microalgae.[10,11] To reveal whether this disaccharide is related to fraction III, we determined the absolute configuration of the Gal component of fraction III by means of an enzymatic assay with D-Gal oxidase (Fig. 1).[29] D-Gal, determined in this way, accounted for about 50% of the total Gal in the hydrolysate from fraction III (Fig. 1). This finding thus indicated that fraction III contained both D- and L-Gal units, thereby suggesting that other Gal units exist in this polysaccharide with a D-configuration.

The nature of the glycosidic linkage, the number of uronic acid residues, and the position of the sulfate groups in fraction III were elucidated by methylation analysis of the native (designated III), carboxyl-reduced (III-Du) and desulfated (III-Ds) fractions, respectively. For this purpose, fraction III was methylated by the method of Needs and Selvendran,[32] and the fully methylated polysaccharide was then subjected to acid hydrolysis, reduction, and acetylation. The neutral sugar composition of the permethylated fraction III was in good agreement with the GC analytical data for the unmethylated polymer. As shown in Table 2, GC–MS analysis revealed the presence of 2,3,4-tri-*O*- and 3,4- or 2,3-di-*O*-methylxylitol acetates, 2,3,4,6-tetra-*O*- and 2,4,6-tri-*O*-methylgalactitol acetates, and 2,4,6-tri-*O*- and 2,4-di-*O*-methylglucitol acetates. These results indicated that the neutral sugar residues in this polysaccharide existed as (1→2)- or (1→4)-linked xylopyranosyl, (1→3)-linked galactopyranosyl, (1→3)-linked glucopyranosyl,

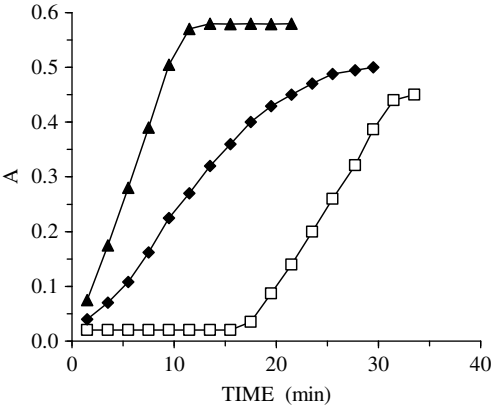


Figure 1. Determination of the absolute configuration of Gal in the polysaccharide III by the D-Gal oxidase assay. Right-most curve (white square symbol): standard L-Gal plus addition of standard D-Gal after 15 min; middle curve (black diamond symbol): standard D-Gal; Left-most curve (black triangle symbol): complete hydrolysate of the polysaccharide III.

Table 1
Molecular weight and sugar composition of fraction III and subfraction III-P

Molar ratio					MW	Samples
SO_2ONa%[a]	Uronic acid[b]	Glu	Gal	Xyl		
0.7	0.2	1.1	1.0	2.1	2.39×10^5	III
0.8	0.5	1.0	1.0	3.0	1.13×10^4	III-P

[a] Determined by the sodium rhodizonate assay.
[b] Determined by the *meta*-hydroxybiphenyl assay.

Table 2
Methylation analysis of the polysaccharide III and its derivatives

Mass fragments (*m*/*z*)	Molar ratio			Sugar (alditol acetates)
	III-Du	III-Ds	III	
43, 73, 101, 117, 129, 161, 205	9.31	9.36	9.35	2,3,4-Me_3Xyl*p*
43, 73, 101, 117, 129, 161, 189, 233	31.86	32.44	32.97	3,4-Me_2Xyl*p* or 2,3-Me_2Xyl*p*
43, 45, 101, 117, 129, 145, 161, 205	3.88	3.97	3.98	2,3,4,6-Me_4Gal*p*
43, 87, 101, 117, 129, 161, 173, 233	17.25	18.53	18.98	2,4,6-Me_3Gal*p*
43, 87, 101, 117, 129, 161, 173, 233	8.28	19.58	3.37	2,4,6-Me_3Glc*p*
43, 87, 117, 129, 159, 189, 233	25.42	10.70	25.74	2,4-Me_2Glc*p*

(1→3,6)-linked glucopyranosyl and terminal xylopyranosyl and galactopyranosyl residues, which were attached at the branching point of (1→3,6)-linked glucopyranosyl residues.

Comparison of the methylation analysis of the native fraction (III) with that of the carboxyl-reduced fraction (III-Du)[33] showed that, while no new partially methylated alditol acetate appeared in the reduced sample, the proportion of 2,4,6-tri-*O*-methylglucitol acetate in the reduced fraction increased as a result of glucuronic acid conversion into Glc (Table 2). This finding revealed that the uronic acid in this polysaccharide most likely existed as a (1→3)-linked glucopyranosyluronic acid, but further analysis needs to be done to confirm this.

Desulfation of fraction III by a pyromellitic acid assay[29] resulted in elimination of 82.6% of the sulfate content of the III-Ds desulfated derivative. Methylation analysis showed an increase of 2,4,6-tri-*O*-methylglucitol acetate together with a concomitant decrease of 2,4-di-*O*-methyl glucitolacetate (Table 2). This finding suggested that the sulfate groups were linked at the O-6 position of (1→3,6)-linked glucopyranosyl residues, as was consistent with the IR spectrum. Thus, Glc was present as (1→3)-linked units, with 11.6% of the units being 3-substituted (in 2,4,6-di-*O*-methyl glucitol acetate); 64.2% of the units also being substituted at O-6 with a sulfate group (in 2,4-di-*O*-methylglucitol acetate); and 24.2% of the units also carrying a substitution at the O-6 position (also in 2,4-di-*O*-methylglucitol acetate) as the branching point of this polymer with terminal galactopyranosyl and xylopyranosyl residues. The low proportion of branching points suggested that the backbone of this polysaccharide was almost linear. The polymer was most probably composed of (1→2)- or (1→4)-linked xylopyranosyl, (1→3)-linked galactopyranosyl, (1→3)-linked glucopyranosyl, and (1→3)-linked glucopyranosyluronic acid. In several other pooled samples, it was shown that the ratio of terminal to branched residues deviated from unity. This finding indicated undermethylation (which is generally observed for uronic-acid-containing polysaccharides[34]) or elimination of partially sulfated groups during methylation and preparation of alditol acetates.

Partial acid hydrolysis of fraction III followed by dialysis against distilled water gave a dialysate containing only the monosaccharides Gal, Glc and Xyl in a molar ratio of 1:1:1, as detected by TLC and GC analyses. Fractionation of the mixture of monosaccharides by Sephadex G-10 column chromatography gave a pure Xyl fraction. The optical rotation of the Xyl fraction of +16.5 (*c* l, H_2O) suggested that the absolute configuration of Xyl in fraction III was D-, since the observed value was similar to the rotation of D-Xyl [+18.9 (*c* 10, H_2O) from Aldrich.

The nondialysate fraction was also a homogeneous polysaccharide (III-P) with a molar mass of 11,300 as measured by GPC–MALLS. The degraded fraction III-P was composed of Xyl, Gal, Glc, GlcA and sulfate in a molar ratio of 3.0:1.0:1.0:0.5:0.8 (Table 1). The relatively high-molecular weight of III-P and a sugar composition similar to that of the native fraction (III) suggested that the main chain of III-P consisted of Xyl, Glc, Gal and GlcA with minimal branching. This finding was in agreement with the previous methylation analysis. On the other hand, III-P contained more Xyl and uronic acid than fraction III, suggesting that mainly neutral sugars were removed by partial acid hydrolysis.

The ^{13}C NMR spectrum of homogeneous polysaccharide III-P (Fig. 2) deserves some comment. The broad peak at δ 174.89 was assigned to the carboxyl group of GlcA. The β-configuration of the D-xylopyranosyl residues was indicated by the presence of the anomeric signal at δ 104.56 and by the C-5 peak of this residue at δ 65.39 [together with a negatively phased δ 65.39 CH_2 signal in the DEPT-135 spectrum (not shown)]. The δ 103.22 resonance signal was assigned to the anomeric carbon of β-D,L-galactopyranosyl residues, and the α-configuration of D-Glc and GlcA was confirmed by a broad anomeric peak in the region of δ *101.7–99.1*. The intensities of these three anomeric carbon peaks were consistent with the results of our sugar composition analysis. Two additional slightly broadened signals with relatively lower intensity were observed in this anomeric carbon region, and their combined intensities roughly correspond to those expected for anomeric carbons of the β- and α-configuration terminal reducing sugar residues in the degraded polysaccharide. These signals at δ 96.5 ± 0.3 and δ 93.5 ± 0.4 were assigned to the respective α-Gal*p* and δ 93.17 β-Gal*p* anomeric carbons, since they are both similar in intensity and chemical shifts to those expected for the respective δ 97.18 α-Gal*p* and δ 93.17 β-Gal*p* terminal anomeric carbons reported earlier for purified α-D-Glc*p*A(1→3)-L-Gal*p* aldobiouronic acid disaccharide isolated by us from various marine algae (including the present example under investigation, see III-O_1 below).[10,11] Evidence for the presence of a sulfate group ligated at *O*-6-Glc was provided by an O-substituted CH_2 signal at δ 68.1 in the DEPT-135 spectrum and by an unsubstituted C-6 peak at δ 60.8. The multiplicity of signals in the region at δ 81.9–78.1 was ascribed to the presence of the O-substituted positions in these sugar residues: (1→2)- or (1→4)-linked xylopyranosyl, (1→3)-linked galactopyranosyl, (1→3)-linked glucopyranosyl or glucopyranosyluronic acid residues. All the above resonance signals were in agreement with the methylation analysis of fraction III.

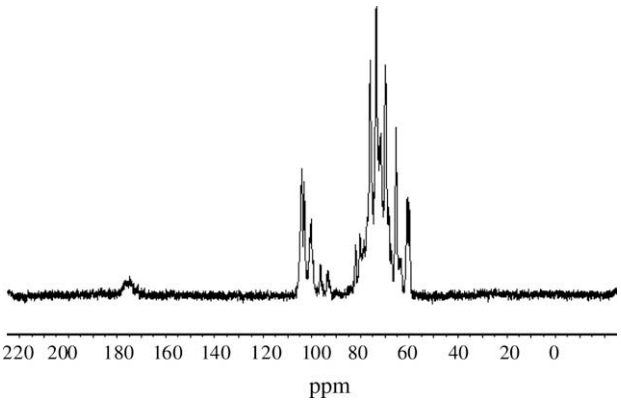


Figure 2. $^{13}C\{^1H\}$ NMR spectrum of the degradated polysaccharide III-P.

Exposure of the degraded fraction III-P to more extreme conditions [0.4 M trifluoroacetic acid (TFA) at 100 °C for l h], followed by dialysis, resulted in almost complete destruction of the polysaccharide, as shown by the very small amount (only about 2% of the original quantity of III-P) of nondialysed material. Fractionation of the dialysate on a Sephadex G-10 column, followed by a Bio-Gel P-2 column, gave two main acidic oligosaccharides, which were designated III-O_1 and III-O_2.

The acidic oligosaccharide III-O_1 was shown to be the same disaccharide as that mentioned above, that is, α-D-glucopyranosyluronic acid-(1→3)-L-galactopyranose, as confirmed by its NMR spectral parameters (Fig. 3 (top) and Table 3). All the ^{13}C NMR spectral data (Table 3) for the oligosaccharide were in good agreement with the literature values.[11] The ^{13}C NMR spectrum showed three anomeric carbon signals at δ 101.47 (C-1 of α-D-glucopyranosyluronic acid), δ 97.10, and δ 93.13 (anomeric C-1 of the reducing terminal β- and α-L-galactopyranose, respectively). The signal of the carboxyl group was also observed at δ 177.10. Therefore, disaccharide fragment III-O_1 had the following structure

α-D-Glc*p*A-(1 → 3)-L-Gal*p*

The ^{13}C NMR (Fig. 3 (middle)) and ^1H NMR spectra of the other acidic oligosaccharide III-O_2 also contained the signals of the above-described aldobiouronic acid (δ 101.21 for C-1 and δ 5.15 for H-1 of the α-D-glucopyranosyluronic acid; δ 97.18 and δ 93.16 for anomeric C-1 and δ 4.59 and δ 5.19 for the anomeric

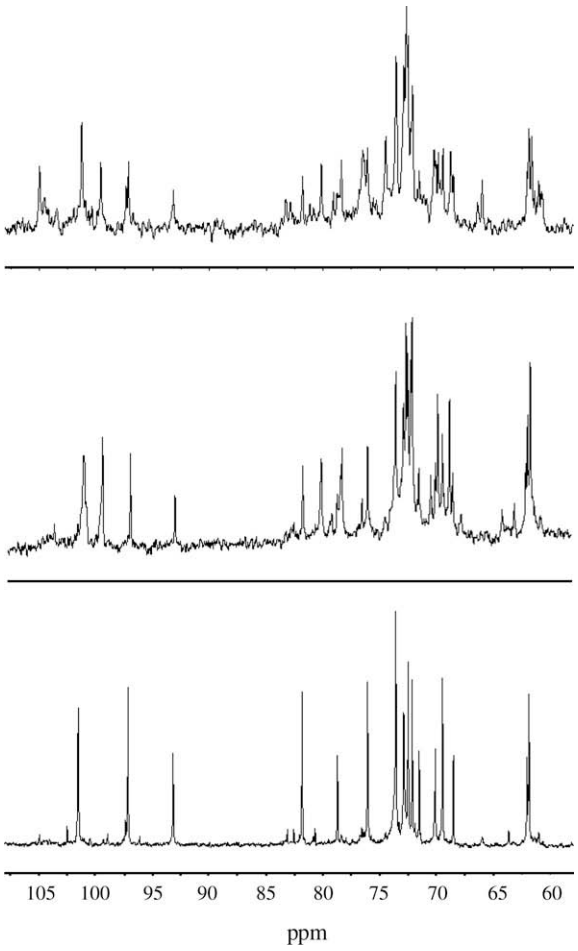


Figure 3. Partial $^{13}C\{^1H\}$ NMR spectra of the oligosaccharides: tetrasaccharide III-O_2 [top]; trisaccharide III-O_3 [middle]; and disaccharide III-O_1 [bottom].

Table 3
^{13}C NMR data for the oligosaccharide III-O_1

Literature values[11] (ppm)	III-O_1 (ppm)	Sugar composition
α, β-L-Gal*p*		
97.18, 93.17	97.10, 93.13	C-1
72.49 (72.23), 68.59	72.46, 68.46	C-2
81.73, 78.63	81.79, 78.63	C-3
69.46, 70.09	69.45, 70.09	C-4
76.01, 71.48	76.03, 71.49	C-5
61.86, 62.01	61.84, 62.01	C-6
α-D-Glc*p*A		
101.29	101.47	C-1
72.49 (72.23)	72.11	C-2
73.65	73.56	C-3
72.81	72.85	C-4
73.40	73.56	C-5
177.11	177.1	C-6

H-1 peaks of the reducing terminal β, α-L,D-galactopyranose, respectively). The ^{13}C NMR spectrum showed two more anomeric carbon peaks at δ 99.54 and δ 104.96 in addition to those for the aldobiouronic acid, which suggested that III-O_2 was a tetrasaccharide. The ^1H NMR spectrum also revealed the corresponding anomeric proton signals at δ 4.99 and δ 4.30, respectively. The δ 104.96 characteristic anomeric carbon signal was assigned to C-1 of β-D-xylopyranose, whose presence was also consistent with the observation of an O-substituted methylene carbon (C-5) peak for β-D-xylopyranosyl residues at δ 65.99 in the DEPT-135 spectrum. Among the neutral sugars in this polymer only Glc existed in an α-configuration, and therefore the δ 99.54 and δ 4.99 peaks were ascribed to the respective C-1 and H-1 nuclei of the α-D-glucopyranosyl residues. An O-substituted methylene carbon signal at δ 61.62 for C-6 was also observed. Augmentation of the ^1H and ^{13}C NMR spectra utilizing the 2D techniques of ^1H–^1H COSY, TOCSY, and XHCORR (long-range) enabled the structure elucidation of the III-O_2 oligosaccharide to be completed (Table 4). Evidence for O-substitution at C-3 of the α-D-glucopyranosyluronic acid and at C-3 of α-D-glucopyranose was provided by movement of their chemical shifts to a relatively lower field at δ 80.14 and δ 83.30, respectively. Thus, III-O_2 was a tetrasaccharide with the following sequence

β-D-Xyl*p*-(1 → 3)-α-D-Glc*p*-(1 → 3)-α-D-Glc*p*A-(1 → 3)-L-Gal*p*

Additional structural information was obtained from periodate oxidation, reduction and mild hydrolysis of fraction III: Since Gal, Glc and glucuronic acid in this polysaccharide are linked mainly at the O-3 position, only Xyl and terminal Gal would be destroyed during a periodate oxidation.[35] The degraded mixture was fractionated chromatographically on a Bio-Gel P-2 column to afford three fragments: glycerol (originating principally from Xyl and terminal Gal), a mixture of monosaccharides (containing mainly Gal and Glc, by TLC), and a main oligosaccharide, designated III-O_3. No high-molecular-weight fragments were obtained, indicating that Xyl was part of the main chain of the native polysaccharide, a conclusion that was consistent with the above results.

NMR spectroscopic data for the oligosaccharide III-O_3 were similar to those of the above acidic oligosaccharides obtained by partial acidic hydrolysis (Fig. 3 (bottom)), which suggested that fraction III-O_3 also contained the aldobiouronic acid unit, that is, δ 101.28 for C-1 of α-D-glucopyranosyluronic acid and δ 97.13 and δ 93.16 (anomeric C-1 of the reducing terminal β- and α-L-galactopyranose, respectively). Comparison of the ^{13}C NMR spectra of III-O_2 and III-O_1 showed that the III-O_3 spectrum exhibited an additional anomeric carbon signal at δ 99.59, which indicated that III-O_3 contained Glc in addition to GlcA in III-O_1. In addition, in contrast to the ^{13}C NMR spectrum of the tetrasaccharide III-O_2, all the signals assigned to β-D-Xyl carbons disappeared in the spectrum of III-O_3. All these observations suggest that III-O_3 was a trisaccharide, with the following structure

α-D-Glc*p*-(1 → 3)-α-D-Glc*p*A-(1 → 3)-L-Gal*p*

The results of the study thus indicated that fraction III, which was separated from the extracellular sulfated polysaccharide, was an acidic galactoglucoxylan having an almost linear main chain composed of β-D-xylopyranosyl, α-D-glucopyranosyl, and β-D,L-galactopyranosyl residues. Models for the possible acidic repeating unit in this polysaccharide are depicted as structures 1 and 2 in Figure 4.

3. Discussion

Sulfated polysaccharides are the major components of the cell-wall of marine red algae.[1,2,9,36–41] Among the various sources of

Table 4
^1H and ^{13}C NMR data for the oligosaccharide III-O_2

Xyl*p* (ppm)		GlcpA (ppm)		Glc*p* (ppm)		Gal*p* (ppm)				Sugars
						α		β		
^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	^1H	^{13}C	Nucleus
4.30	104.96	5.15	101.21	4.99	99.54	4.57	97.18	5.19	93.16	1
3.22	74.47	3.55	72.50	3.85	72.87	3.57	72.44	3.90	68.77	2
3.36	76.43	3.56	80.14	4.07	83.30	3.81	81.81	3.87	78.37	3
3.52	70.18	4.12	72.87	3.80	72.61	4.02	69.66	4.03	70.14	4
3.62	65.99	3.57	73.56	3.93	73.57	3.69	76.45	4.01	71.53	5
			177.44	3.58	61.62	3.65	61.85	3.67	62.03	6

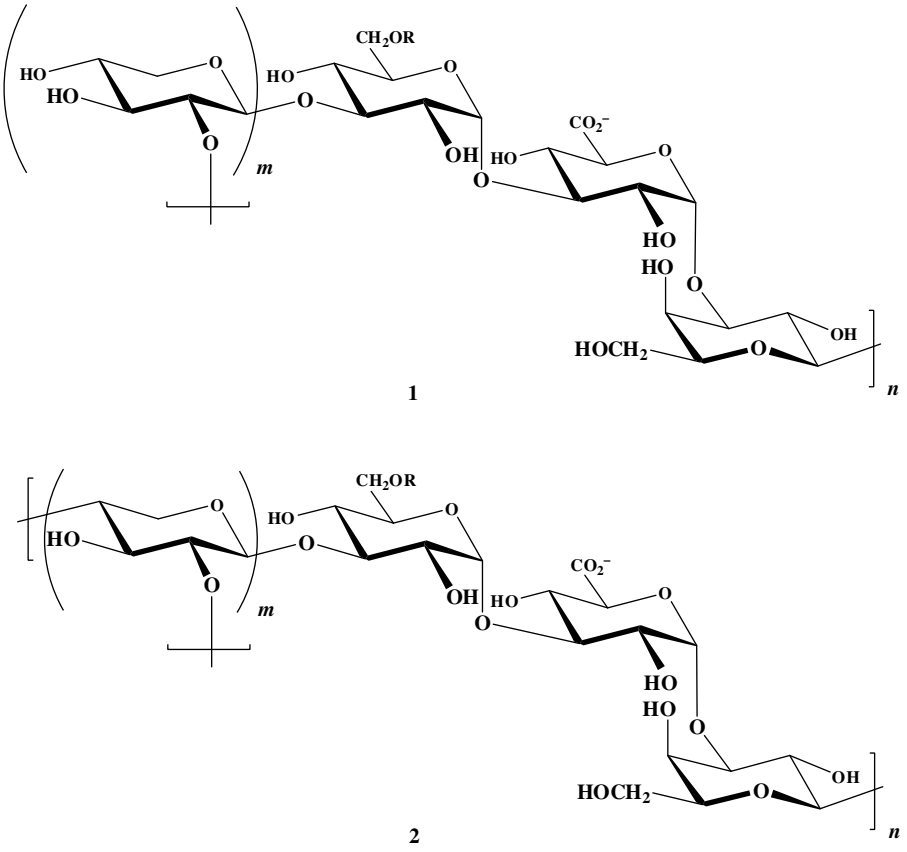


Figure 4. Models 1 or 2 for the possible acidic repeating unit in polysaccharide III. R = H, SO_2O, terminal Gal or terminal Xyl, *m* = 2 or 3.

sulfated polysaccharides, those from red seaweed are the best known; their backbone, which consists of alternating 3-linked β-D-galactopyranosyl and 4-linked α-galactopyranosyl units, is classified either as a carrageenan,[37] if the 4-linked residue is in the D-configuration, or as an agaran,[39] if the 4-linked residue is in the L-configuration. These regular backbones are usually substituted as sulfate esters.[37–40,42] To date, the structures of the sulfated polysaccharides of red microalgae have been less well studied than those of red seaweeds. The present study suggests that the backbone of the extracellular sulfated polysaccharide of the red microalga *Porphyridium* sp. is composed of (1→2)- or (1→4)-linked xylopyranosyl, (1→3)-linked galactopyranosyl, and (1→3)-linked glucopyranosyl or glucopyranosyluronic acid residues. The content of α-D-glucuronic acid in the backbone repeat unit is higher than that in fraction III or that in the degraded polysaccharide III-P. This finding suggests that there are additional neutral units within the polymer that could not be accounted for by the analytical methodology employed in this present study. These neutral units are sensitive to acid hydrolysis, and thus all the oligosaccharide units found in this study contained only glucuronic acid structures. The degraded fraction III-P obtained by 0.2 M TFA hydrolysis contained more glucuronic acid than the native polysaccharide, showing that some neutral units were hydrolyzed in this step, which further confirmed the above premise.

Since the partially methylated alditol acetates of (1→2)-linked and (1→4)-linked β-*O*-D-xylopyranosyl residues afforded the same mass spectra in GC–MS analyses, the linkage of β-D-xylopyranose in this polymer could not be determined by methylation analysis. Despite reports that the C-5 chemical shifts of β-D-xylopyranose linked at the O-4 position should be lower than corresponding values for those of β-D-xylopyranosyl residues linked at other positions,[43,44] lower C-5 chemical shifts for β-D-xylopyranose were not observed in the *Porphyridium* sp. polysaccharide. Thus, it is more likely that β-D-xylopyranose exists as a (1→2) linkage in this polymer, i.e., the structure of the acidic repeating unit is most probably that of model 1.

^{13}C NMR substituent shifts (calculated from the chemical shifts) for the carbons on both sides of the glycosidic linkage [i.e., C-1′ in α-D-Glc*p*A and C-3 in β,α-L-Gal*p*] can be correlated with the relative stereochemistry of sugars in diastereomeric disaccharide units.[10] We have previously shown that the substituent shifts calculated both for the α-D-Glc*p*A-(1→3)-β-L-Gal*p* disaccharide isolated from the red alga *Dixionella grisea* and for its α-D-Glc*p*A-(1→3)-α-L-Gal*p* epimer are similar in magnitude to those expected for a heterochiral (1→3)-linked aldobiouronic acid, while they differ markedly from those values for a homochiral diastereomer.[10] The tetrasaccharide III-O_2, the trisaccharide III-O_3, and the disaccharide III-O_1 isolated from the polysaccharide of *Porphyridium* sp. all contain this identical aldobiouronic acid unit. Thus, it comes as no surprise that the analogous ^{13}C NMR substituent shifts calculated for all the aldobiouronic acid residues in the oligosaccharides of this study are also consistent with a heterochiral (1→3) configuration. On this basis, it is suggested that the acidic repeating unit in the polysaccharide from *Porphyridium* sp. should contain only L-Gal, while D-Gal should exist in neutral parts of this polysaccharide.

Our group has previously shown that the aldobiouronic acid α-D-glucopyranosyluronic acid-(1→3)-L-galactopyranose disaccharide is the basic building block of the polysaccharides of various unicellular red algae.[10,11] In this work, we obtained a larger building block, which incorporates the same aldobiouronic acid unit. It remains to be elucidated whether other species of red microalga (such as *P. aeurgineum* and *D. grisea*) have the same larger building block. It is interesting to note that the oligosaccharide segments isolated from the soluble polysaccharide of *Porphyridium* sp. which

we report in this work, are different than those isolated by Gloaguen et al.[12] from the bound polysaccharide. Various reasons could explain these different structures. The use of different methods in the two studies could lead to structure variability, or a difference of origin of alga strains. In addition, the soluble polysaccharide was analyzed in this study, while the Gloaguen and co-workers studied the structure of the bound polysaccharide.

4. Experimental

4.1. General methods

Uronic acid content was determined by the *m*-hydroxybiphenyl method.[23] Sulfate content was determined by the sodium rhodizonate assay.[24] ^{1}H and ^{13}C NMR spectra were obtained in D_2O on a Bruker DMX-500 spectrometer at 500 and 125 MHz, respectively. The spectral reference was based on acetone added to the sample after the first acquisition of data. IR spectra were recorded on a Bruker Equinox 55; a KBr pellet or a paraffin oil suspension was used. Optical rotation was measured with a Perkin–Elmer MC-141 polarimeter. HPSEC was performed with a Waters instrument, using a TSK gel column, G5000 PW_{XL}. GC was performed with Hewlet–Packard 5890 series GC system equipped with an FID detector and a DB-1 capillary column (30 m, 0.25 mm ID). GC–MS was determined with a MD800 instrument equipped with a DB-1 column. Visible absorption spectra were recorded on a V-530 UV–vis spectrophotometer.

4.2. Preparation of the sulfated extracellular polysaccharide

Porphyridium sp. (UTEX 637) was obtained from the culture collection of the University of Texas, Austin. The extracellular polysaccharide of *Porphyridium* sp. was isolated as we previously described.[6,45] The medium was collected, and the polysaccharide was precipitated with NaOH (2.5%). The precipitate was mixed with distilled water and redissolved by addition of HC1 (2 M) to bring pH to 7.0. EtOH was then added to precipitate the polysaccharide from solution.

The precipitated polysaccharide was dissolved in a 0.5 M NaCl solution, and then an 8% aqueous solution of CTAB was slowly added, followed by stirring overnight. The CTAB-complex was removed by centrifugation and suspended in distilled water. NaCl was added, with constant stirring, up to a concentration of 2.5 M, until the complex was once again dissolved, and the resulting solution was then stirred overnight. Two volumes of ethanol were added, and the resulting precipitate (the acidic crude polysaccharide) was centrifuged off, washed three times with acetone, and dried at 40 °C under vacuum. The supernatant from the CTAB precipitation step was dialyzed and poured into a threefold volume of EtOH. The neutral crude polysaccharide precipitate was washed and dried as above.

4.3. Ultrasonic depolymerization

The crude acidic polysaccharide (3.0 g) was dispersed in distilled water (180 mL). Sonication was performed at 0 °C with a Sonicor ultrasonic probe for 40 min (pulse: on 5.0 off 9.9 ampl: 85%). The sonicated solution was centrifuged and filtered to remove metal particles.

4.4. Ion-exchange chromatography

The sonicated polysaccharide was fractionated on a 6 × 50 cm column packed with DE52 cellulose and equilibrated with distilled water. After the polysaccharide solution had been loaded on the column, fractions I, II, and III were eluted with distilled water, 0.5 M NaCl, and 1.0 M NaCl, respectively. The residual polysaccharide on the column was released by heating the gel at 80 °C for 2 h with a 6 M urea solution (hot urea fraction IV). Each fraction was filtered, dialyzed and then lyophilized.

4.5. Homogeneity and molecular weight

Homogeneity was detected by HPSEC. The absolute molar mass was determined by the GPC–MALLS technique on an instrument equipped with a differential index detector (DRI, Optilab DSP) using a GPC-Guard-Column PSS SUPREMA, as the protection column and two serial analytical GPC columns (Polymer Standards Service GmbH, 10 μm/3000 Å and PSS SUPREMA l0 μm/10000 Å) connected in series. Elution was performed at 25 °C with 0.1 M $NaNO_3$ containing 0.02% NaN_3 as a bactericide and 1.0 mM imidazole, pH 7.0.

4.6. Sugar constituent analysis

Sugar constituent analysis was performed by TLC and GC according to previously described methods.[6,9,11]

4.7. Reduction of uronic acid

Uronic acid was reduced by the method of Taylor and Conrad.[33] As the reaction proceeded, the pH of the reaction mixture was maintained at 4.75 by automatic titration with 0.1 M HCl using a 719 Set Titrino Metrohm instrument, which plotted the total milliliters of added acid versus time. After hydrogen ion uptake had ceased, a 4 M aqueous $NaBH_4$ solution was slowly added. The pH of the mixture was maintained at 7.0 by automatic titration with 4 M HCl. A total of 25 mL of the $NaBH_4$ solution was usually required for reduction. All reactions were allowed to proceed for at least 2 h.

4.8. Desulfation

The polysaccharide was desulfated[46] as follows: 100 mg was dissolved in dried dimethyl sulfoxide (18 mL); pyridine (1 mL) was then added, followed by pyromellitic acid (0.13 g), NaF (0.12 g), and additional pyridine (2 mL). The mixture was stirred at 120 °C for 3 h, and thereafter the solution was cooled and poured into 3% $NaHCO_3$ solution (10 mL). The desulfated polysaccharide in the reaction mixture solution was purified by dialysis and then recovered by lyophilization. The desulfated polysaccharide was analyzed by methylation.

4.9. Methylation analysis

The polysaccharide was methylated twice by the method of Needs and Selvendran.[32] The completeness of methylation was confirmed by IR spectroscopy. The permethylation product was depolymerized with 90% HCO_2H at 100 °C for 6 h and then hydrolyzed with 2 M TFA at 110 °C for 4 h. The partially methylated aldoses were converted into the corresponding acetylated alditols. The mixture of alditol acetates was analyzed by GC–MS with a DB-1 column (30 m). The temperature program was isothermal at 70 °C for 4 min, followed by a 10 °C/min gradient up to 280 °C.

4.10. Partial acid hydrolysis

The polysaccharide was cleaved by the two steps of mild acidic hydrolysis: first, 0.2 M TFA at 100 °C for l h; second, 0.4 M TFA at 100 °C for l h. The initial polysaccharide concentration in each step was 2 mg/mL. In the first hydrolysis step, the hydrolysate was

evaporated to dryness; then methanol was added and evaporated to dryness again until no acidic vapor was present. The residue was dissolved in a small volume of distilled water and dialyzed against 800 mL of distilled water. The non-dialysate was purified by Sephacryl S-300 column chromatography and then subjected to the second hydrolysis step. The dialysate was collected and analyzed by TLC with a solvent system of methanol/butanol/water (3:5:2, v/v/v). Sugar spots were identified by spraying the plates with 0.1% orcinol in 20% H_2SO_4, followed by heating at 100–110 °C for 10 min. The oligosaccharide complexes were further purified via column chromatography on a Sephadex G-10 column followed by a Bio-Gel P-2 column. Elution was performed with distilled water.

4.11. Periodate oxidation and Smith degradation

The polysaccharide (200 mg) was oxidized with 0.02 M $NaIO_4$ (200 mL) at 4 °C in the dark for 14 days. When consumption of the oxidant had ceased (monitored by the decrease in the optical density of the solution at 223 nm), ethylene glycol (8 mL) was added to stop the reaction. The reaction mixture was then dialyzed and concentrated to 100 mL. $NaBH_4$ (400 mg) was added, and the mixture was stirred at room temperature for 24 h. The solution was acidified with 0.1 M HOAc, dialyzed, and lyophilized to give a polysaccharide (103 mg) resulting from the consecutive oxidation, reduction, and partial degradation sequences. This preparation was hydrolyzed with 0.1 M TFA at 100 °C for 2 h and then concentrated to dryness. A solution of the residue in distilled water was analyzed by TLC, and then chromatographically purified on a 100 × 3 cm Bio-Gel P-2 column (elution with distilled water).

4.12. Absolute configuration determination of Gal

D-Gal, L-Gal, or the hydrolysate of the polysaccharide was incubated at room temperature with D-Gal oxidase (26 U/mL), peroxidase (60 U/mL), and *o*-toluidine in phosphate buffer (0.1 M, pH 6.0). The absorbance was measured periodically at 425 nm on a Spectronic spectrophotometer.

Acknowledgments

The late Professor Shimona Geresh was the Director of the Center for Glycobiology at BGU and the first Ph.D. degree research advisor of O. L.-O. This paper represents the culmination of her many years of careful structure elucidation of the polysaccharide from *Porphyridium* sp. Her knowledge and wisdom in the field of organic chemistry and polysaccharide research were invaluable assets to all who came in contact with her. It is with great sorrow that we note her passing on 15 October 2004.

References

1. Arad (Malis), S. In *Chemicals from Microalgae*; Cohen, Z., Ed.; Taylor and Francis: New York, 1999; pp 282–287.
2. Arad (Malis), S.; Richmond, A. In *Handbook of Microalgal Culture: Biotechnology and Applied Phycology*; Richmond, A., Ed.; Blackwell Publishing Ltd: Oxford, 2004; pp 289–297.
3. Ramus, J. *J. Phycol.* **1972**, *8*, 97–111.
4. Arad (Malis), S.; Freidman (Dahan), O.; Rotem, A. *Appl. Environ. Microbiol.* **1988**, *54*, 2411–2414.
5. Geresh, S.; Adin, I.; Yarmolinsky, E.; Karpasas, M. *Carbohydr. Polym.* **2002**, *50*, 183–189.
6. Geresh, S.; Lupescu, N.; Arad (Malis), S. *Phytochemistry* **1992**, *31*, 4181–4186.
7. Geresh, S.; Arad (Malis), S. *Bioresour. Technol.* **1991**, *38*, 195–201.
8. Arad (Malis), S.; Keristovesky, G.; Simon, B.; Barak, Z.; Geresh, S. *Phytochemistry* **1993**, *32*, 287–290.
9. Lupescu, N.; Arad (Malis), S.; Geresh, S.; Bernstein, M. A.; Glaser, R. *Carbohydr. Res.* **1991**, *210*, 349–352.
10. Geresh, S.; Dubinsky, O.; Arad (Malis), S.; Christiaen, D.; Glaser, R. *Carbohydr. Res.* **1990**, *208*, 301–305.
11. Jaseja, M.; Perlin, A. S.; Dubinsky, O.; Christiaen, D.; Arad (Malis), S.; Glaser, R. *Carbohydr. Res.* **1989**, *186*, 313–319.
12. Gloaguen, V.; Ruiz, G.; Morvan, H.; Mouradi-Givernaud, A.; Maes, E.; Krausz, P.; Strecker, G. *Carbohydr. Res.* **2004**, *339*, 97–103.
13. Arad (Malis), S.; Rapoport, L.; Moshkovich, A.; van-Moppes, D.; Karpasas, M.; Golan, R.; Golan, Y. *Langmuir* **2006**, *22*, 7313–7317.
14. Eteshola, . E.; Karpas, M.; Arad (Malis), S.; Gottlieb, M. *Acta Polym.* **1998**, *49*, 549–556.
15. Gourdon, D.; Lin, Q.; Oroudjev, E.; Hansma, H.; Golan, Y.; Arad, S.; Israelachvili, J. *Langmuir* **2008**, *24*, 1534–1540.
16. Tannin-Spitz, T.; Bergman, M.; van-Moppes, D.; Grossman, S.; Arad (Malis), S. *J. Appl. Phycol.* **2005**, *17*, 215–222.
17. Matsui, M. S.; Muizzuddin, N.; Arad, S.; Marenus, K. *Appl. Biochem. Biotechnol.* **2003**, *104*, 13–22.
18. Arad (Malis), S.; Weinstein, Y. *Biomedicine (Israel)* **2003**, *1*, 32–37.
19. Arad (Malis), S.; Ginzberg, A.; Huleihel, M. In *Recent Advances in Marine Biotechnology: Biomaterials from Aquatic and Terrestrial Organisms*; Fingerman, M., Ed.; Science: Enfield, New Hampshire, 2006; pp 37–62.
20. Huleihel, M.; Ishanu, V.; Tal, J.; Arad (Malis), S. *J. Appl. Phycol.* **2001**, *13*, 127–134.
21. Lowry, O. H.; Rosebrough, N. J.; Farr, A. L.; Randall, R. J. *J. Biol. Chem.* **1951**, *193*, 265–275.
22. Bradford, M. *Anal. Biochem.* **1976**, *72*, 248–254.
23. Kintner, P. K.; VanBuren, J. P. *J. Food Sci.* **1982**, *47*, 756–764.
24. Dodson, K. S. *Biochem. J.* **1962**, *84*, 106–112.
25. Gretz, M. R.; McCandless, E. L.; Aronson, J. M.; Sommerfeld, M. R. *J. Exp. Bot.* **1983**, *34*, 705–711.
26. Heaney-Kieras, J.; Chapman, D. J. *Carbohydr. Res.* **1976**, *52*, 169–177.
27. Percival, E.; Foyle, R. A. J. *Carbohydr. Res.* **1979**, *72*, 165–176.
28. Zhang, Q.; Qi, H.; Zhao, T.; Deslandes, E.; Ismaeli, N. M.; Molloy, F.; Critchley, A. T. *Carbohydr. Res.* **2005**, *340*, 2447–2450.
29. Painter, T. J.. In *The Polysaccharides*; Academic Press: New York, 1983; Vol. 2. pp 195–285.
30. Usov, A. L.; Bilan, M. L.; Shashkov, A. S. *Carbohydr. Res.* **1997**, *303*, 93–102.
31. Knutsen, S. H.; Myslabodski, D.; Larsen, B.; Usov, A. I. *Bot. Mar.* **1994**, *37*, 163–169.
32. Needs, P. W.; Selvendran, R. R. *Carbohydr. Res.* **1993**, *245*, 1–10.
33. Taylor, R. L.; Conrad, H. E. *Biochemistry* **1972**, *11*, 1383–1388.
34. Duarte, M. E. R.; Cardoso, M. A.; Noseda, M. D.; Cerezo, A. S. *Carbohydr. Res.* **2001**, *333*, 281–293.
35. Dixon, J. S.; Lipkin, D. *Anal. Chem.* **1954**, *26*, 1092–1093.
36. Ramus, J. In *Biogenesis of Plant Cell Wall Polysaccharides*; Loewus, F., Ed.; Academic Press: New York, 1973; pp 333–359.
37. Kolender, A. A.; Matulewicz, M. C. *Carbohydr. Res.* **2002**, *337*, 57–68.
38. Amimi, A.; Mouradi, A.; Givernaud, T.; Chiadmi, N.; Lahaye, M. *Carbohydr. Res.* **2001**, *333*, 271–279.
39. Falshaw, R.; Furneaux, R. H.; Stevenson, D. E. *Carbohydr. Res.* **1998**, *308*, 107–115.
40. Craigie, J. S. *Biology of the Red Algae*; Cambridge Univ. Press: Cambridge, 1990. pp 221–257.
41. Laos, K.; Ring, G. S. *J. Appl. Phycol.* **2005**, *17*, 461–464.
42. Chiovitti, A.; Bacic, A.; Craik, D. J.; Kraft, G. T.; Liao, M. L.; Falshaw, R.; Furneanx, R. H. *Carbohydr. Res.* **1998**, *310*, 77–83.
43. Nishimura, T.; Ishihara, M.; Ishii, T.; Kato, A. *Carbohydr. Res.* **1998**, *308*, 117–122.
44. Igartuburu, J. M.; Pando, E.; Luis, F. R.; Serrano, A. G. *J. Nat. Prod.* **2001**, *64*, 1174–1178.
45. Dubinsky, O.; Simon, B.; Karamanos, Y.; Geresh, S.; Barak, Z.; Arad (Malis), S. *Plant Physiol. Biochem.* **1992**, *30*, 409–414.
46. Miller, I. J.; Blunt, J. W. *Carbohydr. Res.* **1998**, *309*, 39–43.