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Structure of a highly pyruvylated galactan sulfate from the Pacific green alga *Codium yezoense* (Bryopsidales, Chlorophyta)☆

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Dedicated to the memory of Professor Nikolay K. Kochetkov

Abstract—A polysaccharide fraction consisting of D-galactose, sulfate, and pyruvate in a molar proportion of 4:2:1 was isolated from the green seaweed *Codium yezoense* by water extraction followed by ion-exchange chromatography. To elucidate its structure, modified polysaccharides were prepared by desulfation, depyruvylation, and by total removal of non-carbohydrate substituents. Structures of the native polysaccharide and of the products of its chemical modifications were investigated by methylation analysis as well as by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy. The polysaccharide devoid of sulfate and pyruvate was subjected to two subsequent Smith degradations to afford a rather low-molecular and essentially linear (1→3)-β-D-galactan. A highly ramified structure was suggested for the native polysaccharide, which contains linear backbone segments of 3-linked β-D-galactopyranose residues connected by (1→6) linkages, about 40% of 3-linked residues being additionally substituted at C-6, probably by short oligosaccharide residues also containing (1→3) and (1→6) linkages. Sulfate groups were found mainly at C-4 and in minor amounts at C-6. Pyruvate was found to form mainly five-membered cyclic ketals with O-3 and O-4 of the non-reducing terminal galactose residues. The minor part of pyruvate forms six-membered cyclic ketals with O-4 and O-6. The absolute configurations of ketals (*R* for six-membered ketals and *S* for five-membered ones) were established using NMR spectral data.

Keywords: Pyruvylated galactan sulfate; NMR; Seaweed; Green algae; *Codium yezoense*

1. Introduction

Marine macrophytes belonging to Chlorophyta (green seaweeds) are widely distributed in the coastal waters. Some of them, as, for example, representatives of the genera *Caulerpa*, *Codium*, and *Ulva*, are extremely invasive and proliferate in great amounts in eutrophicated lagoons.[2–4] The utilization of green algal biomass could be based on specific properties of their sulfated polysaccharides, which are known to act as anticoagulants,[5] antiviral agents,[6] or immunomodulators.[7] To understand better the biological properties of green algal polysaccharides, a detailed description of their chemical structures is necessary, but up to now most of these structures were investigated only tentatively. Although there are several excellent papers describing the structure of ulvans,[8,9] very complex sulfated heteroglycans isolated from representatives of the genus *Ulva*, structures of sulfated polysaccharides of many other species are far from detailed characterization. In the genus *Codium*, the presence of sulfated galactan and sulfated arabinan (or arabinogalactan) in *C. fragile*,[10] sulfated (1→5)-α-L-arabinan in *C. latum*[11] and sulfated glucan in *C. pugniforme*[12] was described with some chemical evidence about the structures of these polysaccharides. Other authors paid more attention to biological properties of polysaccharides describing sulfated arabinans from *C. divaricatum*, *C. adhaerens*, *C. latum*, *C. fragile*,[5] *C. dwarkense*,[13] and a sulfated galactan

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from *C. cylindricum*[14] as biopolymers possessing high level of anticoagulation activity. Antiangiogenic properties were also mentioned for the latter polysaccharide,[15] whereas a fraction of sulfated galactans and/or arabinogalactans isolated from *C. fragile* was shown to interact specifically with several immune cytokines.[7]

The present work is devoted to the structural analysis of a sulfated galactan isolated from *Codium yezoense* (Tokida) K. L. Vinogradova, a species that is abundant on the Russian coast of the Sea of Japan. According to our preliminary evidence,[16] the polysaccharide contains, besides galactose and sulfate, a rather high amount of pyruvic acid residues. Now we describe the elucidation of the polysaccharide structure carried out by combination of chemical methods of structural analysis and NMR spectroscopy.

Table 1. Composition (in M %) of pyruvylated galactan sulfate (G-II) and its chemically modified preparations

Sample	Ara	Xyl	D-Gal	SO_3Na	Pyruvate
G-II	3	2	52	28	15
deS	2	2	78	—	18
deP	2	3	65	29	1
deSdeP	2	2	96	—	—

2. Results and discussion

2.1. Isolation of pyruvylated galactan sulfate

Water-soluble polysaccharides were extracted from dried and milled biomass with water at room temperature and fractionated by anion-exchange chromatography on DEAE-Sephacel using water and then aqueous sodium chloride solutions of increasing concentration as eluants.[16] One of the fractions obtained (G-II, eluted with 0.8 M NaCl) was essentially a pyruvylated galactan sulfate containing D-galactose, sulfate, and pyruvate in a molar ratio of about 4:2:1 (Table 1).

2.2. Preliminary characterization of G-II

The IR-spectrum of G-II contained a broad intense absorption band at 1240 cm^{-1} (S=O) common to all the sulfate esters. An additional sulfate absorption band at 848 cm^{-1} (C–O–S, secondary axial sulfate) indicated that the majority of sulfate groups occupy position 4 of galactose residues.

A very complex ^{13}C NMR spectrum of the intact polysaccharide G-II was typical of non-regular polymers (Fig. 1a). The spectrum contained two groups of broadened signals in the resonance region of carbons bearing

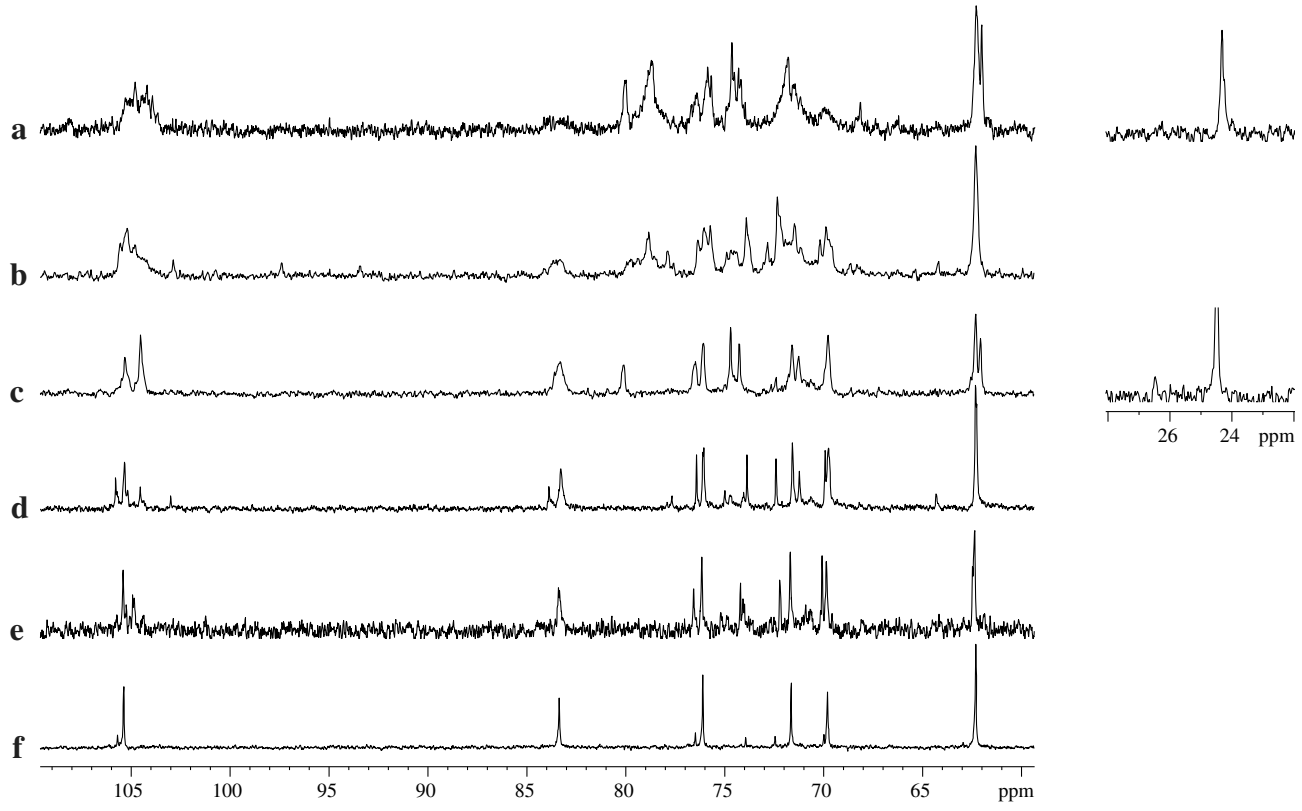


Figure 1. ^{13}C NMR spectra of polysaccharide preparations: native galactan G-II (a), depyruvylated derivative deP (b), desulfated derivative deS (c), desulfated and depyruvylated derivative deSdeP (d), product of the first Smith degradation deSdePSm-1 (e), product of the second Smith degradation deSdePSm-2 (f).

two oxygen atoms (δ_C 103.8–105.3 and 108.3), many signals of carbons bearing one oxygen (δ_C 62–84), two peaks of C–CH_3 groups (δ_C 24.4, major, and 26.4, minor) and a signal of carbonyl group at δ_C 178.5 (not shown in Fig. 1a). According to the APT spectrum (Fig. 2), signal at δ_C 108.3 belongs to a quaternary carbon, signals at δ_C 62.2 and 62.5 belong to $HOCH_2$-groups, whereas signals at δ_C 68.5, 68.7, and 70.8–71.4 belong to $–CH_2O$-groups bearing a substituent on the oxygen atom.

The ^1H NMR spectrum of G-II contained broadened signals in the region of δ_H 4.9–3.5 and two singlets of C–CH_3 at δ_H 1.62 (major) and 1.48 (minor). Absence of lower-field signals (δ >5) was the evidence that the polysaccharide contained β-pyranoses only.

The ^1H and ^{13}C NMR spectra of the native polysaccharide were partially assigned using 2D COSY, TOCSY, ROESY, gHSQC, and gHMBC experiments. In particular, $^1H/^1H$ 2D experiments revealed that G-II is built up of β-galactopyranose residues, a part of which being substituted with electronegative (sulfate) groups at C-4 (correlation peak in the HSQC spectrum δ_H/δ_C 4.67–4.91/78.2–79.5). The HSQC experiment (Fig. 2) displayed also glycosylation of the residues at C-3 (correlation peaks δ_H/δ_C 3.75–3.90/83.1–83.4) and/or at C-6 (δ_H/δ_C 4.20/70.8–71.4 and 3.88/70.8–71.4). Correlation peaks δ_H/δ_C 1.62/24.4 and 1.48/26.4 were found for the major and minor methyl groups, respectively.

The HMBC spectrum (Fig. 3) showed correlation peaks δ_H/δ_C 1.48/102.0 and 1.48/177.2 indicating the presence of galactose residues with 4,6-*O*-(1′-carboxy)-ethylidene cyclic ketals as a minor component of the polysaccharide. Signal positions of protons (δ_H 1.48) and carbon (δ_C 26.4) of the methyl group evidenced unambiguously that the six-membered ketals are in the *R* configuration.[17,18] Correlation peaks δ_H/δ_C 1.62/108.3 and 1.62/178.5 proved that the major methyl group belongs also to a pyruvic acid residue. The low-field chemical shift of its quaternary carbon atom (108.3 ppm) was typical of pyruvate involved in a five-membered ketal cycle.[19,20] The proportion of about 1:8 between six- and five-membered ketals was calculated from the ^1H NMR spectrum of G-II.

There was no possibility to give more detailed spectral assignments due to the very complex character of the spectra obtained. This fact may be explained either by molecular heterogeneity of G-II or by its highly ramified structure, where signals belonging to outer parts of the molecule were resolved much better, as compared with the signals of the inner core. Therefore, several chemical modifications of G-II were carried out to simplify its structure.

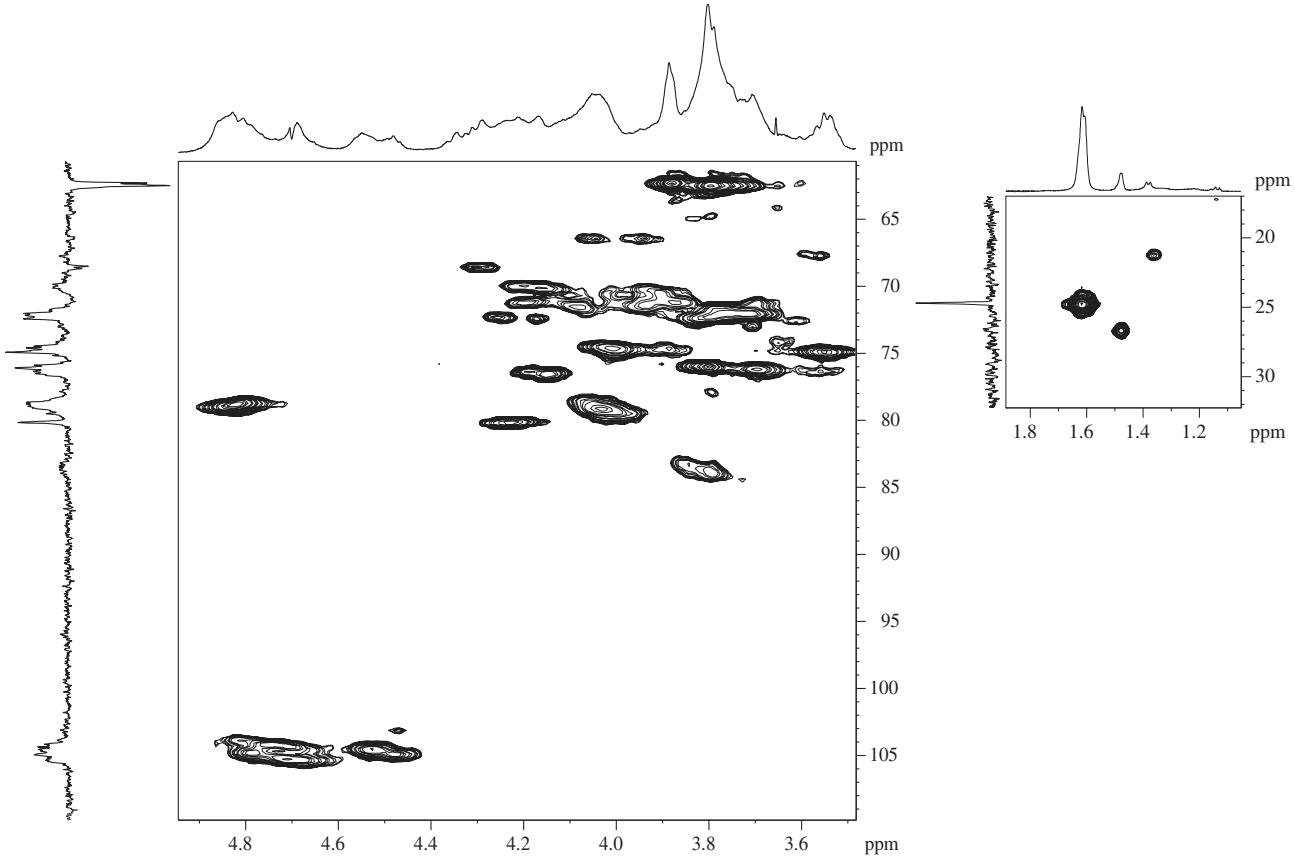


Figure 2. 2D HSQC spectrum of native galactan G-II.

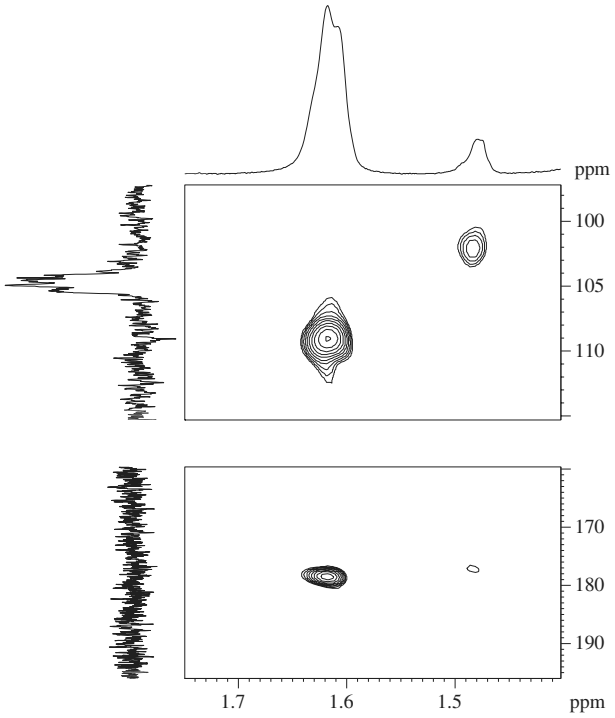


Figure 3. A part of the 2D HMBC spectrum of native galactan G-II.

2.3. Chemical modifications of G-II

Three modified polysaccharide preparations were obtained as the result of desulfation (deS), depyruvylation (deP), and both desulfation and depyruvylation (deSdeP) of G-II. Molar proportions of constituents of the native polysaccharide (G-II) and modified preparations are given in Table 1. A solvolytic desulfation procedure was used to remove sulfate groups. The yield of desulfated polysaccharide (deS) was 76% of theoretical value. The preparation contained practically no sulfate, whereas the ratio of D-Gal and pyruvic acid differed only slightly from that of the native polysaccharide G-II. Mild acid hydrolysis of G-II resulted in removal of all pyruvic acid residues without marked changes in the proportion of galactose and sulfate. The depyruvylated sulfated galactan (deP) was obtained in 66% yield. Finally, the polysaccharide devoid of any non-carbohydrate constituents (deSdeP) was obtained as a result of mild acid hydrolysis of desulfated galactan (deS) in 58% yield. It is interesting to mention that removal of all pyruvic acid residues from desulfated galactan required 2.5-fold longer time than the corresponding reaction of the native galactan G-II. Hence, acid hydrolysis of pyruvate ketals proceeds much easier in the presence of sulfate groups.

The desulfated and depyruvylated galactan (deSdeP) was subjected to methylation analysis. The polysaccharide was methylated with methyl iodide in the presence of sodium hydroxide in methyl sulfoxide two times to achieve complete methylation. The methylated galactan was hydrolyzed, and the resulting mixture of partially methylated monosaccharides was analyzed as alditol acetates by GC–MS. It is evident from the results of methylation (Table 2) that deSdeP is a highly branched polysaccharide built up of 3- and 3,6-linked galactopyranose residues. The presence of two branching points and two non-reducing terminal residues per every seven galactose residues was detected.

Smith degradation of deSdeP was used to simplify further the structure of the polysaccharide. During the oxidation step the consumption of periodate was nearly 2 mol per every terminal galactose residue. The oxidized polymer was reduced with sodium borohydride and subjected to mild acid hydrolysis according to the usual Smith degradation conditions. After separation from low-molecular-mass fragments, a Smith-degraded galactan (deSdePSm-1) was obtained in 47% yield.

The Smith-degraded galactan (deSdePSm-1) was subjected to methylation analysis as above. The results showed (Table 2) that deSdePSm-1 remained to be a branched polysaccharide similar to the starting deSdeP built up mainly of 3-linked and also of 3,6- and 6-linked galactopyranose residues. Therefore, it was decided to oxidize this Smith-degraded galactan once more. The consumption of periodate in the second oxidation was also about 2 mol per every terminal and 6-linked galactose residue. A double Smith-degraded galactan (deSdePSm-2) was obtained in 43% yield. It was subjected to methylation analysis. Since it was readily soluble in

Table 2. Methylation analysis of pyruvylated galactan sulfate (G-II) and its chemically modified preparations (M % of partially methylated galactitol acetates)

Position of *O*-methyl groups	deSdePSm-2	deSdePSm-1	deSdeP	deS	deP	G-II
2,3,4,6	9	20	27	3	15	—
2,3,6	1	1	2	2	9	4
2,4,6	76	51	38	35	20	5
2,3,4	—	11	2	—	6	—
2,6	5	2	3	22	20	11
2,3	—	—	2	1	6	—
2,4	6	15	26	22	10	2
2	2	—	—	1	14	8
Gal	1	—	—	14	—	70

Table 3. NMR data for pyruvylated galactan sulfate (G-II) and its chemically modified preparations

Sample and residue	Chemical shifts, δ					
	C-1 H-1	C-2 H-2	C-3 H-3	C-4 H-4	C-5 H-5	C-6 H-6,6′
deSdePSm-2						
β-D-Gal*p*-(1→3	105.7 4.61	72.5 3.61	73.9 3.68	70.0 3.93	76.5 3.69	62.3 3.79
→3)-β-D-Gal*p*-(1→3	105.4 4.70	71.5 3.79	83.4 3.86	69.8 4.21	76.1 3.73	62.3 3.79
deSdePSm-1						
β-D-Gal*p*-(1→3	105.7 4.62	72.2 3.54	73.9 3.65	69.9 3.92	76.4 3.70	62.2 3.78
β-D-Gal*p*-(1→6	104.9 4.43	72.2 3.53	74.0 3.63	69.9 3.92	76.4 3.70	62.2 3.78
→6)-β-D-Gal*p*-(1→6	104.8 4.47	72.2 3.57	73.9 3.69	69.9 3.97	74.8(a) 3.92	70.5(b) (4.04, 3.91)(c)
→3)-β-D-Gal*p*-(1→3	105.4 4.69	71.5 3.79	83.2 3.86	69.7 4.21	76.0 3.73	62.3 3.79
→3,6)-β-D-Gal*p*-(1→3	105.4 4.69	71.5 3.79	83.2 3.86	69.7 4.23	75.2(a) 3.92	70.9(b) (4.06, 3.93)(c)
deSdeP						
β-D-Gal*p*-(1→3	105.9 4.61	72.4 3.62	74.0 3.67	70.0 3.93	76.5 3.69	62.2 3.78
→3)-β-D-Gal*p*-(1→6	104.6 4.48	71.3 3.68	84.0 3.81	69.8 4.19	76.2 3.71	62.2 3.78
→3,6)-β-D-Gal*p*-(1→6	104.5 4.53	71.3 3.72	83.7(d) 3.85	69.8 4.23	74.9 3.92	70.8 4.02; 3.92
→3)-β-D-Gal*p*-(1→3	105.5 4.68	71.7 3.78	83.5(d) 3.85	69.8 4.19	76.2 3.71	62.3 3.79
→3,6)-β-D-Gal*p*-(1→3	105.5 4.68	71.7 3.78	83.5(d) 3.85	69.8 4.23	74.5 3.99	70.5 4.04; 3.92
deS						
β-D-Gal*p*(3,4-*S*-Pyr)-(1→3(e)	104.4 4.67	74.7 3.55	80.2 4.27	76.5 4.19	74.3 4.04	62.1 3.86
→3)-β-D-Gal*p*-(1→6	104.5 4.47	71.2 3.69	83.5 3.80	69.7 4.16	76.1 3.71	62.3 3.76
→3,6)-β-D-Gal*p*-(1→6	104.5 4.52	71.6 3.75	83.3 3.85	69.7 4.22	74.4 3.91	70.7 4.03; 3.91
→3)-β-D-Gal*p*-(1→3	105.2 4.67	71.6 3.81	83.6 3.85	69.7 4.16	76.1 3.71	62.3 3.76
→3,6)-β-D-Gal*p*-(1→3	105.2 4.69	71.6 3.78	83.6 3.85	69.7 4.20	74.8 3.91	70.7 4.03; 3.94
deP						
β-D-Gal*p*-(1→3	105.6 4.62	72.4 3.62	74.0 3.67	70.4 3.89	76.5 3.70	62.3 3.80
→6)-β-D-Gal*p*-(1→3	105.6 4.62	72.4 3.62	74.0 3.67	70.1 3.89	74.3 3.91	71.0 n.d.(f)
→3)-β-D-Gal*p*-(1→3	105.5 4.68	71.7 3.78	83.8 3.84	70.0 4.20	76.2 3.71	62.3 3.77
→3)-β-D-Gal*p*-(1→6	104.8 4.49	71.3 3.69	84.1 3.79	70.2 4.18	76.3 3.72	62.3 3.77
β-D-Gal*p*($4SO_3^-$)-(1→3	105.6 4.65	72.4 3.62	71.9 3.85	78.2 4.67	n.d. n.d.	62.3 3.77(g)
β-D-Gal*p*($4SO_3^-$)-(1→6	104.4 4.54	72.4 3.62	71.9 3.87	78.2 4.69	n.d. n.d.	62.3 3.77(g)
→3)-β-D-Gal*p*($4SO_3^-$)-(1→3	105.4 4.70	72.2 3.82	79.0 4.08	78.8 4.82	76.0 3.81	62.3 3.74(g)
→3)-β-D-Gal*p*($4SO_3^-$)-(1→6	104.4 4.55	72.2 3.70	80.0 4.00	78.8 4.85	76.0 3.81	62.3 3.74(g)
→3,6)-β-D-Gal*p*($4SO_3^-$)-(1→3	105.4 4.71	72.2 3.82	80.0 4.05	79.2 4.85	74.9 4.05	n.d. n.d.

Table 3 (*continued*)

Sample and residue	Chemical shifts, δ					
	C-1 H-1	C-2 H-2	C-3 H-3	C-4 H-4	C-5 H-5	C-6 H-6,6′
→3,6)-β-D-Gal*p*(4SO_3^-)-(1→6	104.4 4.58	72.2 3.73	80.0 4.05	79.5 4.91	74.9 4.05	n.d. n.d.

For (a)–(d), (g), assignments could be interchanged.
(e) 3,4-Pyr: CH_3 24.4 (^{13}C) and 1.64 (^{1}H), O–*C*–O 108.3, *C*OOH 178.5.
(f) Not determined.

methyl sulfoxide, complete methylation was achieved after a single treatment with methylating agents, and repeated methylations did not change significantly the proportion of methylated monosaccharides in the hydrolyzate. It may be concluded from the results of methylation (Table 2) that deSdePSm-2 remains to be slightly branched, but the main structural feature of the doubly degraded polysaccharide is a linear galactan chain built up mainly of 3-linked galactopyranose residues.

2.4. Methylation analysis of native (G-II), desulfated (deS), and depyruvylated (deP) galactans

The native (G-II), desulfated (deS), and depyruvylated (deP) polysaccharide preparations were methylated to localize the positions of sulfate groups and pyruvic acid residues (Table 2). Three consecutive treatments with methylating reagents were applied, while the fourth methylation did not change significantly the results. It is known that highly branched polysaccharides carrying electronegative substituents do not always yield reliable proportions of methylated alditols, and so the presence of non-methylated galactose in deS and especially in G-II was attributed to incomplete methylation of these preparations. Nevertheless, comparing the results of methylation of deSdeP and deS (Table 2), it may be concluded that pyruvic acid residues are located mainly at C-3 and C-4 of terminal galactose residues (decrease of 2,3,4,6-tetra-*O*-methylgalactose with the corresponding increase of 2,6-di-*O*-methylgalactose in the methylated deS). The presence of 2,3,4,6-tetra-*O*-methylgalactose

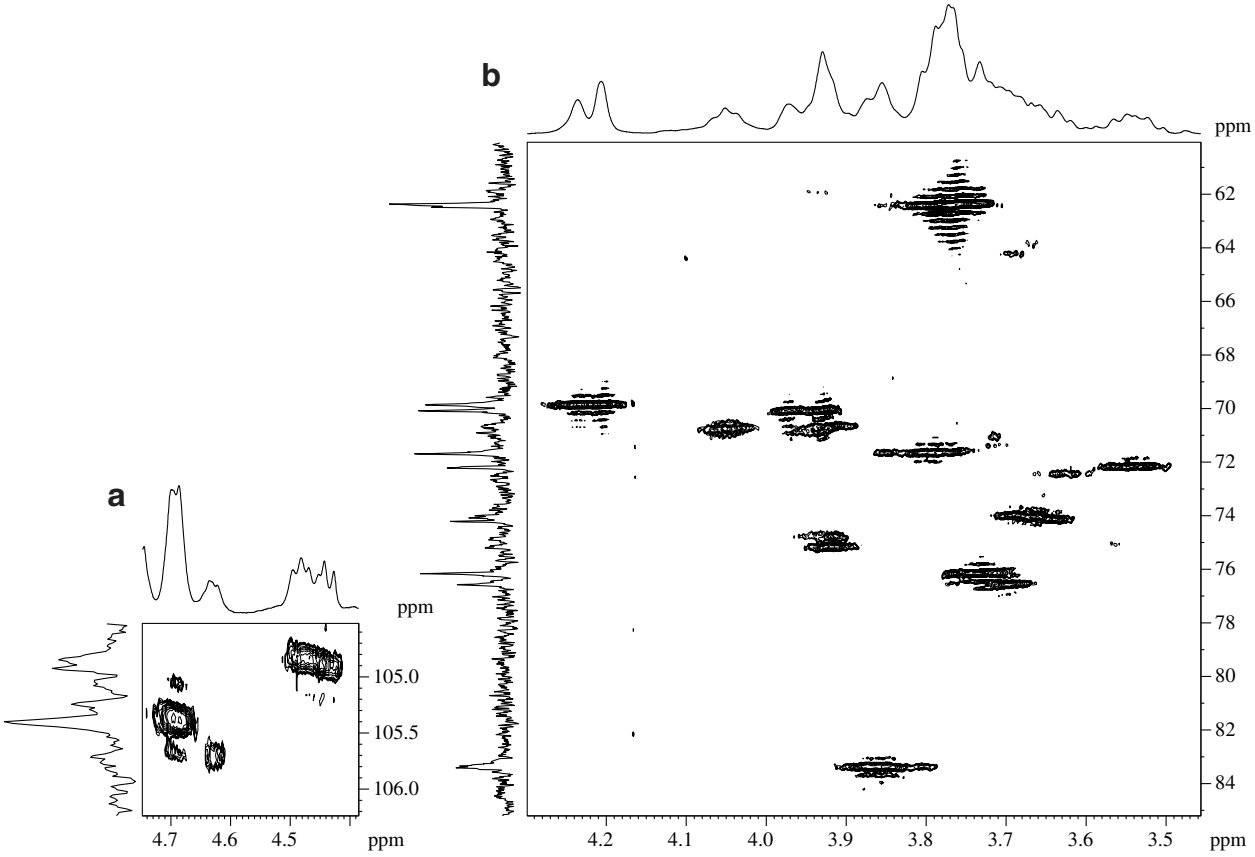


Figure 4. 2D HSQC spectrum of deSdePSm-1 (the product of the first Smith degradation of desulfated and depyruvylated polysaccharide deSdeP): (a) anomeric region and (b) C-2-C-6/H-2-H-6 region.

in methylated deP and its absence in methylated G-II (as well as in deS) were consistent with the location of pyruvate both at C-3,C-4 and at C-4,C-6 of terminal galactose residues. Comparison of methylation products of deSdeP and deP indicated that majority of sulfate groups occupy positions 4 of some 3- and 3,6-linked galactose residues (increase of 2,4,6-tri-*O*-methylgalactose and 2,4-di-*O*-methylgalactose and decrease of 2,6-di-*O*-methylgalactose and 2-*O*-methylgalactose upon desulfation).

2.5. NMR analysis of chemically modified polysaccharide preparations

The ^{1}H and ^{13}C NMR spectra of compounds were assigned using 2D homonuclear $^{1}H/^{1}H$ COSY, TOCSY, NOESY, ROESY and heteronuclear $^{1}H/^{13}C$ gHSQC, gHMBC, and HMQC-TOCSY experiments.

The ^{13}C NMR spectrum of deSdePSm-2 (Fig. 1f) was typical of a more or less regular polysaccharide containing only one type of glycosidic linkage. Its ^{1}H NMR spectrum was well resolved and revealed the presence of monosaccharide residues with β-galactopyranose configuration only. The signal assignments in the spectra were completed using 2D NMR experiments (Table 3). The low-field position of C-3 resonance of all residues except the terminal ones confirmed the presence of (1→3) linkages between galactose residues. Integration of the signals belonging to terminal and 3-linked residues in the ^{1}H NMR spectrum allowed to estimate the average chain length as about 10–12 galactose residues.

The ^{13}C NMR spectrum of deSdePSm-1 (Fig. 1e) was typical of a non-regular polysaccharide containing at least two types of glycosidic linkages. Its ^{1}H NMR spectrum (Fig. 4a) contained two separated groups of signals at δ_H 4.61–4.72 and 4.41–4.50 in the anomeric resonance region. The 1D spectra were assigned (Table 3) using the full set of 2D experiments mentioned above. Analysis of 2D spectra revealed two types of substitution, namely, (1→3) and (1→6), in galactopyranose residues. The presence of (1→6) linkages was confirmed by correlation peaks δ_H/δ_H 4.43/4.04, 4.43/3.91, 4.47/4.06, and 4.47/3.93 in the ROESY spectrum as well as by correlation peaks δ_H/δ_C 4.43/70.5 and 4.47/70.9 in the HMBC spectrum. The HSQC spectrum (Fig. 4a and b) showed that all the signals of anomeric protons belonging to the 3-linked residues were located in the region of δ_H 4.61–4.72, while the corresponding signals of 6-linked residues appeared in the region of δ_H 4.41–4.50. The signals of H-4 of 3-linked residues were found in the more low-field region (δ_H 4.21 and 4.23) than those of non-substituted at position 3 residues (δ_H 3.92 and 3.97). Among them, the signals at 4.43 and 3.92 ppm belonging to the 3-linked residues additionally substituted at position

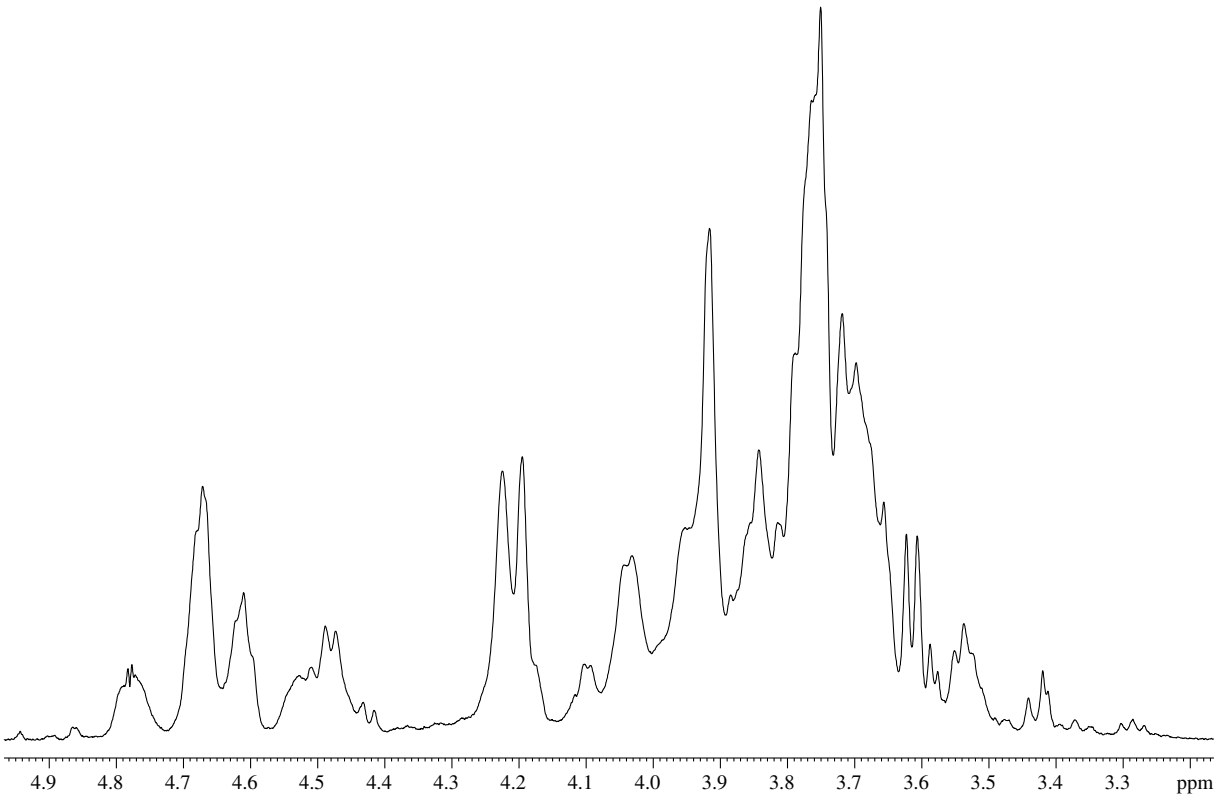


Figure 5. ^{1}H NMR spectrum of desulfated and depyruvylated polysaccharide deSdeP.

6. These observations were used for interpretation of the spectra of the more complicated compounds deSdeP, deS, deP, and G-II.

The ^{13}C NMR spectrum of deSdeP (Fig. 1d) was markedly more complicated as compared with the spectrum of deSdePSm-1. It contained an intense anomeric signal of terminal (1→3)-linked residues at δ_C 105.9, whereas many other signals were significantly broadened. The ^{1}H NMR spectrum (Fig. 5) contained only broadened signals even in the anomeric proton resonance region. Analysis of 2D spectra showed the same set of structural units as in deSdePSm-1 (Table 3), but the content of non-reducing terminal residues in deSdeP was much higher than in deSdePSm-1. It was concluded that deSdeP contained rather short branches built up of (1→3)-linked galactose residues.

Signals of both five- and six-membered cyclic pyruvate ketal groups were detected in the ^{13}C NMR spectrum of desulfated polysaccharide deS (Fig. 1c, Table 3). Signals of methyl protons were found also in its ^{1}H NMR spectrum. Signals of galactose residues bearing major pyruvate can be identified by analysis of 2D spectra (see, e.g., the HSQC spectrum, Fig. 6). Both proton and carbon resonances practically coincided with the corresponding signals of terminal 3,4-*O*-pyruvylated β-galactose residues present in the repeating oligosaccharide unit of O-specific polysaccharide from the bacterium *Proteus mirabilis* O24.[20] Position 3,4 of pyruvate ketal was additionally confirmed by low-field shifts of both C-3 and C-4 signals of the substituted residues and by high-field shift of C-5 (Table 3). The absolute configuration of the five-membered ketal was determined as *S* by the presence of an intense correlation peak δ_H/δ_H 1.64/3.55 in the ROESY spectrum (Fig. 7) indicating the spatial proximity of H-2(Gal) and methyl group of pyruvate. The H-1 chemical shift (δ_H 4.67) of pyruvylated galactose residues corresponded to their location at C-3 of the neighboring monosaccharides. Since C-2 and C-6 of 3,4-*O*-pyruvylated galactose residues carry no substituents, all these residues are terminal ones. The corresponding location of minor 4,6-*O*-pyruvylated residues could not be determined.

The ^{13}C NMR spectrum of depyruvylated polysaccharide deP (Fig. 1b) differed from the spectrum of native galactan G-II (Fig. 1a) by the absence of pyruvate signals, but it was too complex for analysis due to considerable broadening of peaks. Proton signals in ^{1}H NMR spectrum of deP were also broadened. Nevertheless, the main structural features of the polysaccharide were characterized by analysis of 2D spectra (Table 3). The location of the sulfate at C-4 (low-field shifts of corresponding resonances due to sulfation effects) and its

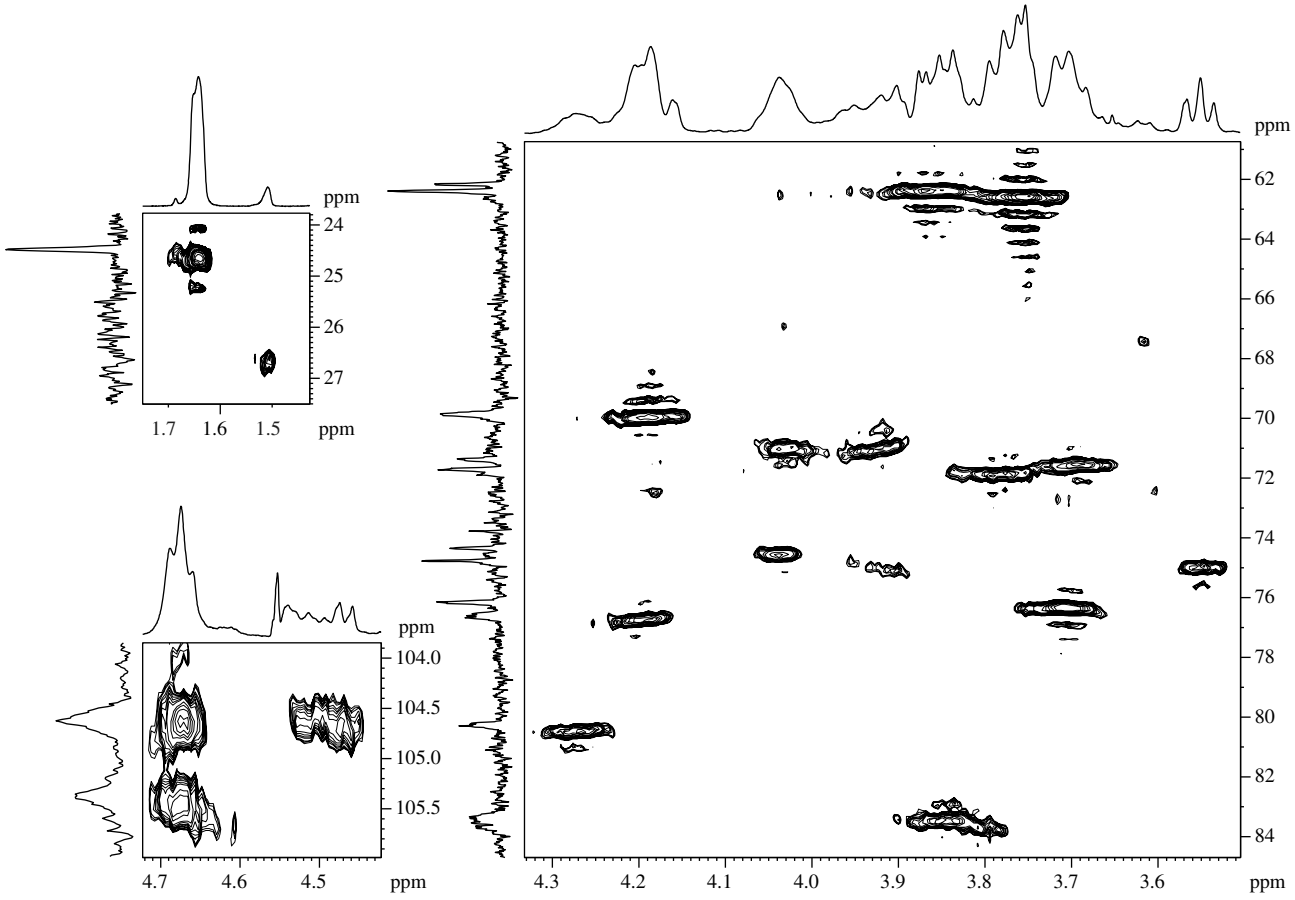


Figure 6. 2D HSQC spectrum of desulfated polysaccharide deS.

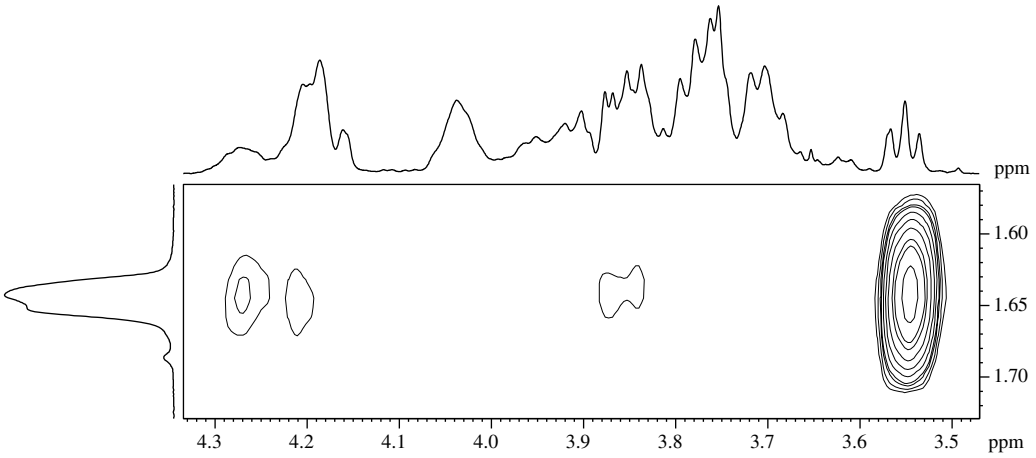


Figure 7. A part of the 2D ROESY spectrum of desulfated polysaccharide deS.

absence at C-2 was confirmed. At the same time no distinct correlation C-6/H-6 was found due to considerable broadening of C-6 resonances (which was observed also in more effectively resolved spectra of deSdePSm-1, deSdeP, and deS), and hence, the spectra did not confirm the sulfation at C-6.

Surprisingly the ^{13}C NMR spectrum of native polysaccharide G-II (Fig. 1a) contained two distinct resonances of sulfated C-6 (δ_C 68.5 and 68.7), and HSQC spectrum contained distinct correlation peaks δ_H/δ_C 4.30/68.5,68.7 (Fig. 2). Moreover, in the ROESY spectrum of G-II, in addition to an intense correlation peak 1.62/3.55 (CH_3Pyr/H-2Gal*p*3,4Pyr), a minor signal 1.62/3.88 was detected. The broadened singlet δ_H 3.88 belongs to protons of a non-substituted CH_2OH group according to the data of HSQC experiment (correlation peak δ_H/δ_C 3.88/62.2). Thus, the weak correlation peak 1.62/3.88 in the ROESY spectrum might be interpreted as CH_3Pyr/H-6Gal*p*3,4Pyr. Such long range interactions are characteristic of high-molecular compounds due to intense spin diffusion within their locked spin systems. It may be concluded that pyruvylated residues in G-II are not sulfated, and hence, sulfate groups occupy C-6 of some other galactose residues.

3. Conclusion

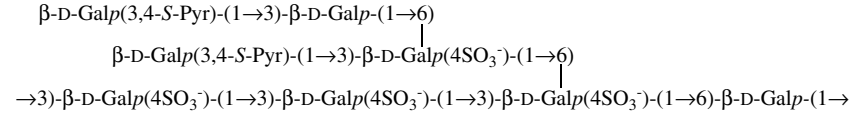
As it was mentioned in our preliminary communication,[16] the most unusual feature of water-soluble polysaccharides of *C. yezoense* was the high pyruvate content. While the total aqueous extract gave several monosaccharides upon acid hydrolysis, a fraction of pyruvylated galactan sulfate containing one pyruvate and two sulfate per every four galactose residues was isolated by anion-exchange chromatography.

Elucidation of the galactan structure required several modifications of the polysaccharide, for example, desulfation and depyruvylation, followed by methylation analysis. Chemical data were supported by NMR spectroscopy. The results corresponded to highly branched molecules with (1→3) and (1→6) linkages between β-D-Gal. The branched structure was retained after the first Smith degradation of the polysaccharide devoid of non-carbohydrate substituents. Essentially linear, but rather short (1→3)-linked fragments were obtained only after the second Smith degradation. It is evident that the polysaccharide contains a backbone of mainly 3-linked β-D-Gal with some (1→6) linkages. Branchings are attached mainly to C-6 of the backbone and may be either monosaccharide or short oligosaccharide residues. Hence, the carbohydrate moiety of the native polysaccharide is similar in many respects to (1→3,1→6)-galactans of terrestrial plants present usually as type II arabinogalactans.[21]

A common and specific feature of the polysaccharides of marine macrophytes is the presence of sulfate. The galactan of *C. yezoense* is sulfated mainly at C-4 and to a lesser extent at C-6. Practically the same sulfation pattern was found in the earlier work on the structure of sulfated (arabino)galactan from the closely related species *C. fragile*.[10]

In addition to sulfate, the galactan contains another ionizable group, the pyruvate residue. This feature is common to red algal galactans often containing 3-linked 4,6-*O*-(1′-carboxy)ethylidene-D-galactopyranose units.[22] Both six- and five-membered cyclic pyruvate ketals of different monosaccharide residues were detected in bacterial polysaccharides.[23] At the same time pyruvate ketals have been seldom found in green algal polysaccharides. There is one example of an extracellular galactan containing 4,6-*O*-(1′-carboxy)ethylidene-D-galactose residues, produced by unicellular fresh-water alga *Palmella texensis*,[24] but this alga is taxonomically very far from the genus *Codium*. Recently indication on the presence of pyruvate was found in NMR spectra of a sul-

fated arabinogalactan isolated from *C. fragile*, but the position of the pyruvate in the polysaccharide molecules was not established.[7] The galactan from *C. yezoense* seems to be the first algal polysaccharide containing five-membered cyclic pyruvate ketals. Evidently the polysaccharide contains no real repeating units. Nevertheless, its putative fragment containing all the main structural features in accordance with the data observed may be depicted as follows:



Thus, the pyruvylated galactan sulfate isolated from *C. yezoense* resembles red algal galactans in composition, but is quite different from these galactans in structure, having ramified carbohydrate moiety similar to that of the (1→3,1→6)-β-D-galactans of terrestrial plants, and bearing cyclic pyruvate ketals linked mainly to C-3 and C-4 of non-reducing terminal galactose residues, as in bacterial polysaccharides.

4. Experimental

4.1. General methods

The pyruvylated galactan sulfate G-II, $[\alpha]_D^{20}$ +24 (*c* 1.0, water), was isolated from algal biomass as described earlier.[16] Neutral monosaccharides in hydrolyzates of polysaccharide samples were determined using GC of acetylated alditols.[25] Pyruvic acid was estimated by colorimetry as 2,4-dinitrophenylhydrazone[26] or enzymatically with lactate dehydrogenase.[27] The D-configuration of galactose was confirmed by quantitative determination with D-galactose dehydrogenase.[16] Sulfate was estimated turbidimetrically[28] after hydrolysis of polysaccharides in 2 M CF_3COOH. IR spectra of polysaccharides were recorded with Perkin–Elmer 577 spectrometer in KBr pellets. Optical rotations were measured using a digital polarimeter PU-07 (Russia).

4.2. NMR spectroscopy

The spectra were recorded using a Bruker DRX-500 spectrometer at 303 K (at 333 K for native polysaccharide G-II). Samples were deuterium-exchanged by lyophilization two times with D_2O and then examined as 2–3% soln in 99.97% D_2O, TSP (δ_H 0), and methanol (δ_C 50.15) were taken as the internal standards. 2D NMR experiments were performed using standard Bruker software. The TOCSY spectra were acquired with 200 ms duration of MLEV17 spin-lock; the ROESY spectra were acquired with 200 ms duration of spin-lock, the NOESY spectra were acquired with 300 ms duration of mixing time, the HMBC spectra were recorded with 60 ms delay for evolution of long-range couplings. The parameters used for other 2D experiments were described previously.[29]

4.3. Desulfation procedure

Solvolytic desulfation of G-II (as pyridinium salt) was carried out as described earlier.[30] Briefly, the polysaccharide was dissolved in Me_2SO (18 mL) and absolute MeOH (2 mL) was added. The soln was heated at 80 °C for 6 h, dialyzed, and lyophilized to give desulfated galactan (deS), yield was 90 mg from 130 mg of the starting material, $[\alpha]_D^{20}$ +26 (*c* 1.0, water).

4.4. Removal of pyruvic acid residues from polysaccharides

Sample of G-II (50 mg) was heated in 1% CH_3COOH (10 mL) for 4 h at 100 °C, the soln was neutralized with $NaHCO_3$, dialyzed, and lyophilized to give depyruvylated galactan (deP) (33 mg), $[\alpha]_D^{20}$ +24 (*c* 1.0, water). Sample of deS (38 mg) was similarly treated with 1% CH_3COOH (10 mL) for 10 h at 100 °C, neutralized with $NaHCO_3$, dialyzed, and lyophilized to give desulfated and depyruvylated galactan (deSdeP) (22 mg), $[\alpha]_D^{20}$ +32 (*c* 0.4, water).

4.5. Smith degradation of polysaccharides

A soln of $NaIO_4$ (0.02 M, 25 mL) was added to a soln of deSdeP (60 mg) in water (50 mL), and the mixture was left in the dark for 120 h at room temperature, when the consumption of the oxidant ceased (monitored by decrease in optical density of the soln at 305 nm). The consumption of periodate was 0.50 mol per every galactose residue. Ethylene glycol (0.5 mL) was added to the reaction mixture, which was then dialyzed, concentrated to about 30 mL, $NaBH_4$ (200 mg) was added, and the mixture was left overnight. The soln was neutralized with HOAc, dialyzed, and lyophilized to give the oxidized and reduced polysaccharide (50 mg), containing Gal (62.2%), Ara (2.9%), and Xyl (2.2%). This preparation was dissolved in 1% HOAc (10 mL), the soln was heated for 2 h at 100 °C, and concentrated to dryness. A soln of the residue in water (2 mL) was placed on a column (76 × 2.6 cm) containing TSK HW-40(S) Toyopearl gel (Toyo Soda Manufacturing, Japan) and eluted with water at the rate of 2 mL/min, a differential refractometer (Knauer, Germany) was used as detector. A

polymeric fraction was lyophilized to afford a Smith-degraded galactan deSdePSm-1 (28 mg), $[\alpha]_D^{20}$ +17 (*c* 1.0, water).

Similarly a soln of $NaIO_4$ (0.02 M, 35 mL) was added to a soln of deSdePSm-1 (70 mg) in water (50 mL), and the mixture was left in the dark for 27 h at room temperature, when the consumption of the oxidant ceased. The consumption of periodate was 0.53 mol per every galactose residue. The oxidized polysaccharide was reduced and partially hydrolyzed with 1% HOAc as above. A polymeric fraction was obtained by gel-permeation chromatography as above and lyophilized to afford a double Smith-degraded galactan deSdePSm-2 (30 mg), $[\alpha]_D^{20}$ +21 (*c* 1.0, water).

4.6. Methylation analysis of polysaccharides

According to the procedure of Ciucanu and Kerek,[31] powdered NaOH (20–30 mg) and CH_3I (0.4 mL) were added to a soln (or suspension) of a polysaccharide (5–8 mg) in Me_2SO (0.5 mL). The mixture was stirred for 1 h at room temperature, water (4 mL) and $CHCl_3$ (4 mL) were added, and the resulting soln was dialyzed, concentrated, and lyophilized. HCOOH (85%, 1 mL) was added to the residue, the soln was heated for 1.5 h at 100 °C, and then co-evaporated with water. CF_3COOH (2 M, 1 mL) was added to the residue, the soln was heated for 8 h at 100 °C, then co-evaporated with EtOH, and the resulting partially methylated monosaccharides were converted into alditol acetates and analyzed by GC/GC–MS according to conventional procedures.[32]

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References

1. Bilan, M. I.; Zakharova, A. N.; Grachev, A. A.; Shashkov, A. S.; Nifantiev, N. E.; Usov, A. I. *Bioorg. Khim.* **2007**, *33*.
2. Verlaque, M.; Afonso-Carillo, J.; Gil-Rodrigues, C.; Durand, C.; Boudouresque, C. F.; Le Parco, Y. *Biol. Invasions* **2004**, *6*, 269–281.
3. Kusakina, J.; Snyder, M.; Kristie, D. N.; Dadswell, M. J. *Bot. Mar.* **2006**, *49*, 1–9.
4. Fletcher, R. L. The Occurrence of 'Green Tides'—A Review. In *Marine Benthic Vegetation. Recent Changes and the Effects of Eutrophication*; Schramm, W., Nienhuis, P. H., Eds.; Springer: Berlin, 1996; pp 7–43.
5. Hayakawa, Y.; Hayashi, T.; Lee, J.-B.; Srisomporn, P.; Maeda, M.; Ozawa, T.; Sakuragawa, N. *Biochim. Biophys. Acta* **2000**, *1543*, 86–94.
6. Lee, J. B.; Hayashi, K.; Maeda, M.; Hayashi, T. *Planta Med.* **2004**, *70*, 813–817.
7. Nika, K.; Mulloy, B.; Carpenter, B.; Gibbs, R. *Eur. J. Phycol.* **2003**, *38*, 257–264.
8. Lahaye, M.; Brunel, M.; Bonnin, E. *Carbohydr. Res.* **1997**, *304*, 325–333.
9. Lahaye, M.; Cimadevilla, E. A.-C.; Kuhlenkamp, R.; Quemener, B.; Lognone, V.; Dion, P. *J. Appl. Phycol.* **1999**, *11*, 1–7.
10. Love, J.; Percival, E. *J. Chem. Soc.* **1964**, 3338–3345.
11. Uehara, T.; Takeshita, M.; Maeda, M. *Carbohydr. Res.* **1992**, *235*, 309–311.
12. Matsubara, K.; Matsuura, Y.; Hori, K.; Miyazawa, K. *J. Appl. Phycol.* **2000**, *12*, 9–14.
13. Siddhanta, A. K.; Shanmugam, M.; Mody, K. H.; Goswami, A. M.; Ramavat, B. K. *Int. J. Biol. Macromol.* **1999**, *26, 151–154.*
14. Matsubara, K.; Matsuura, Y.; Bacic, A.; Liao, M.-L.; Hori, K.; Miyazawa, K. *Int. J. Biol. Macromol.* **2001**, *28*, 395–399.
15. Matsubara, K.; Mori, M.; Matsumoto, H.; Hori, K.; Miyazawa, K. *J. Appl. Phycol.* **2003**, *15*, 87–90.
16. Bilan, M. I.; Vinogradova, E. V.; Shashkov, A. S.; Usov, A. I. *Bot. Mar.* **2006**, *49*, 259–262.
17. Garegg, P. J.; Lindberg, B.; Kvarnström, I. *Carbohydr. Res.* **1979**, *77*, 71–78.
18. Gonçalves, A. G.; Ducatti, D. R. B.; Duarte, E. R.; Noseda, M. D. *Carbohydr. Res.* **2002**, *337*, 2443–2453.
19. Garegg, P. J.; Jansson, P.-E.; Lindberg, B.; Lindh, F.; Lönngren, J.; Kvarnström, I.; Nimmich, W. *Carbohydr. Res.* **1980**, *78*, 127–132.
20. Shashkov, A. S.; Senchenkova, S. N.; Vinogradov, E. V.; Zatonsky, G. V.; Knirel, Y. A.; Literacka, E.; Kaca, W. *Carbohydr. Res.* **2000**, *329*, 453–457.
21. Stephen, A. M. Other Plant Polysaccharides. In *The Polysaccharides*; Aspinall, G. O., Ed.; Academic Press: New York, 1983; Vol. 2, pp 97–193.
22. Painter, T. J. Algal Polysaccharides. In *The Polysaccharides*; Aspinall, G. O., Ed.; Academic Press: New York, 1983; Vol. 2, pp 195–285.
23. Lindberg, B. Bacterial Polysaccharides: Components. In *Polysaccharides: Structural Diversity and Functional Versatility*; Dumitriu, S., Ed.; Marcel Dekker: New York, 1998; pp 237–273.
24. Ha, Y. W.; Thomas, R. L. *FEMS Microbiol. Lett.* **1991**, *78*, 49–52.
25. Sloneker, J. H. Gas–Liquid Chromatography of Alditol Acetates. In *Methods in Carbohydrate Chemistry*; Whistler, R. L., BeMiller, J. N., Eds.; Academic Press: New York, 1972; Vol. 6, pp 20–24.
26. Sloneker, J. H.; Orentas, D. G. *Nature* **1962**, *194*, 478–479.
27. Duckworth, M.; Yaphe, W. *Chem. Ind.* **1970**, 747–748.
28. Dodgson, K. S.; Price, R. G. *Biochem. J.* **1962**, *84*, 106–110.
29. Bilan, M. I.; Grachev, A. A.; Ustuzhanina, N. E.; Shashkov, A. S.; Nifantiev, N. E.; Usov, A. I. *Carbohydr. Res.* **2002**, *337*, 719–730.
30. Usov, A. I.; Smirnova, G. P.; Bilan, M. I.; Shashkov, A. S. *Bioorg. Khim.* **1998**, *24*, 437–445 (*Russ. J. Bioorg. Chem.* **1998**, *24*, 382–389 (English translation)).
31. Ciucanu, I.; Kerek, F. *Carbohydr. Res.* **1984**, *131*, 209–217.
32. Bjorndal, H.; Hellerquist, C. G.; Lindberg, B.; Svensson, S. *Angew. Chem., Int. Ed. Engl.* **1970**, *9*, 610–619.