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A sulfated glucuronofucan containing both fucofuranose and fucopyranose residues from the brown alga *Chordaria flagelliformis* ☆

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ABSTRACT

A fucoidan fraction composed of L-fucose, sulfate, and D-glucuronic acid in a molar proportion of about 1:1:0.25 and small amount of acetyl groups was isolated from the brown alga *Chordaria flagelliformis*. Several modified polysaccharides were prepared from the native fucoidan using solvolytic desulfation, carboxyl reduction, and partial acid hydrolysis. Polysaccharide structures were elucidated by methylation analysis and 1D and 2D NMR spectroscopy. The fucoidan was shown to contain a backbone of 3-linked α-L-fucopyranose residues, about one-third of which are glycosylated at C-2 by α-D-glucopyranosyluronic acid residues. About half of the latter residues are glycosylated at C-4 by single α-L-fucofuranose residues or by disaccharides α-L-Fuc*f*-(1→2)-α-L-Fuc*f*-(1→. Fucofuranose residues are mono- and disulfated at different positions, whereas some additional sulfate groups occupy C-2 and C-4 of the backbone, the latter position being also partially acetylated.

1. Introduction

Sulfated polysaccharides of brown algae, usually named fucoidans, are known as readily accessible biopolymers possessing a wide spectrum of biological activities.[2,3] From the chemical point of view, fucoidans represent a heterogeneous group of polysaccharides, which structural diversity is not fully characterized.[4] The simplest fucoidans contain L-fucose, sulfate, and acetate, but more complex polysaccharides may additionally contain several neutral monosaccharides and uronic acids. In particular, glucuronic acid residues were found in different fucoidans both as components of the backbone[5–8] or as single branches linked to the backbone built up of fucose residues. An example of the latter structure[9–11] namely a polysaccharide having a linear backbone of 3-linked α-L-fucopyranose residues with sulfate groups at C-4 and α-D-glucopyranosyluronic acid residues at C-2 of the main chain was isolated from *Cladosiphon okamuranus*, the alga belonging to the order Ectocarpales. The present paper is devoted to the structural analysis of the next fucoidan containing fucose and glucuronic acid, which was isolated from the Pacific brown alga *Chordaria flagelliformis* (O.F. Müller) C. Agardh, also belonging to Ectocarpales. In our previous papers[12,13] the algal biomass was shown to be rich in fucose-containing polysaccharides, and hence the alga was regarded as a good practical source of fucoidan.

2. Results and discussion

2.1. Isolation of fucoidans

Water-soluble polysaccharides were isolated from the defatted algal biomass by extraction with dilute aqueous calcium chloride at 85 °C. The resulting crude polysaccharide preparation (F, for composition see Section 4) was purified and fractionated by ion-exchange chromatography using water and then aqueous sodium chloride solutions of increasing concentration as eluants. The yields and composition of five fractions obtained are given in Table 1. Fraction F0 gave glucose and some mannitol upon acid hydrolysis, and hence was a practically pure laminaran. Agarose gel electrophoresis of three sulfated fractions F2, F3, and F4 showed the presence of at least two distinct polysaccharides in F2, whereas F3 and F4 produced single bands (Fig. 1), indicating that F3 and F4 were electrophoretically homogeneous polysaccharides. Fucoidans F3 and F4 differed very slightly in their composition. They contain fucose, sulfate, and uronic acid in a molar ratio of about 1:1:0.25 together with traces of other monosaccharides and small amount of acetate groups. Due to the higher yield, F3 was the main fraction selected for structural analysis. L-Fucose was identified in the acid hydrolyzate of F3 by GLC. Glucuronic acid was identified by ion-exchange chromatography. It has the D-configuration, since

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Table 1
Yields and composition of polysaccharide fractions (F0–F4) obtained by ion-exchange chromatography of crude fucoidan preparation F

Fraction	Yield (% of F)	Neutral monosaccharides (%)					SO_3Na (%)	Uronic acids (%)	Acetate (%)
		Fuc	Xyl	Gal	Man	Glc			
F0	9.5	—	—	—	3.3	100.0	—	n.d.	n.d.
F1	1.0	23.0	8.8	3.6	4.4	4.2	3.0	19.3	n.d.
F2	40.0	40.9	2.7	6.2	tr.	1.1	18.3	16.1	1.0
F3	21.9	40.1	tr.	1.7	—	tr.	26.6	13.5	1.1
F4	14.8	41.6	tr.	tr.	—	tr.	26.6	10.3	1.9

tr.—amounts lesser than 1%.
n.d.—not determined.

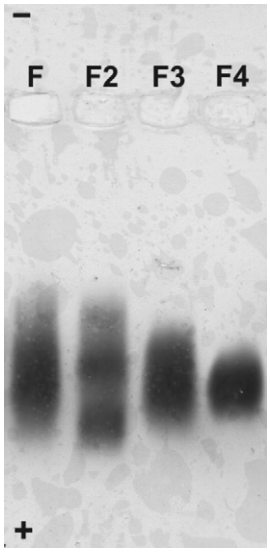

Figure 1. Agarose gel electrophoresis of fucoidans F, F2, F3 and F4.

D-glucose was identified as a constituent of a carboxyl-reduced polysaccharide (see below).

2.2. Preliminary characterization and chemical modifications of F3

As expected, fucoidan F3 had a complex ^{13}C NMR spectrum (Fig. 2), which was difficult to interpret completely. It contained two groups of signals in the anomeric region (93–96 and 99–102 ppm). The signals at 21–22 ppm confirmed the presence of *O*-acetyl groups in the polysaccharide. Another high-field group of resonances at 16.6–17.3 ppm was typical of CH_3 carbons of fucopyranosides. In addition, the very interesting feature of the ^{13}C NMR spectrum of F3 was the presence of two rather intense signals at 19.5–19.9 ppm, which could be attributed to CH_3 groups of fucofuranose residues. The ^{1}H NMR spectrum of F3 (Fig. 3) was also poorly resolved, so it was impossible to apply 2D procedures for assignment of other resonances in the ^{13}C NMR spectrum of native polysaccharide. Therefore, several chemical modifications of F3 were made for further structural analysis. Four modified polysaccharide preparations were obtained as the result of carboxyl group reduction (F3-red), desulfation (F3-deS), both carboxyl group reduction and subsequent desulfation (F3-red-deS) of the native fucoidan, and by mild partial acid hydrolysis of desulfated polysaccharide (F3-deS-H).

Activation of carboxyl groups of uronic acid residues by treatment with water-soluble carbodiimide according to Taylor and Conrad[14] was used for carboxyl reduction. It should be noted that the procedure proposed for capsular polysaccharide from *Aerobacter aerogenes* as well as for homopolyuronides was not effective in our case, whereas F3 was reduced according to the modified procedure recommended for heparin.[15] Several experiments were made to test the influence of different pH values during the carbodiimide addition. It was noted that pH maintenance exactly at 4.75 was not necessary, since different pH within a wide range of 4.5–6.0 gave the same extent of carboxyl reduction in F3. The carboxyl-reduced fucoidan (F3-red) contained L-fucose, sulfate, and D-glucose practically at the same ratio as fucose, sulfate, and glucuronic acid in native F3 (Table 2). According to colorimetric estimation, the preparation F3-red still contained about 2–3% of residual uronic acid, but the same result was obtained with an equivalent artificial mixture of methyl α-L-fucopyranoside and methyl α-D-glucopyranoside, evidently due to some non-specific coloration of neutral monosaccharides in the uronic acid determination procedure. It was concluded that complete reduction of glucuronic acid residues in F3 was achieved giving rise to D-glucose residues in F3-red.

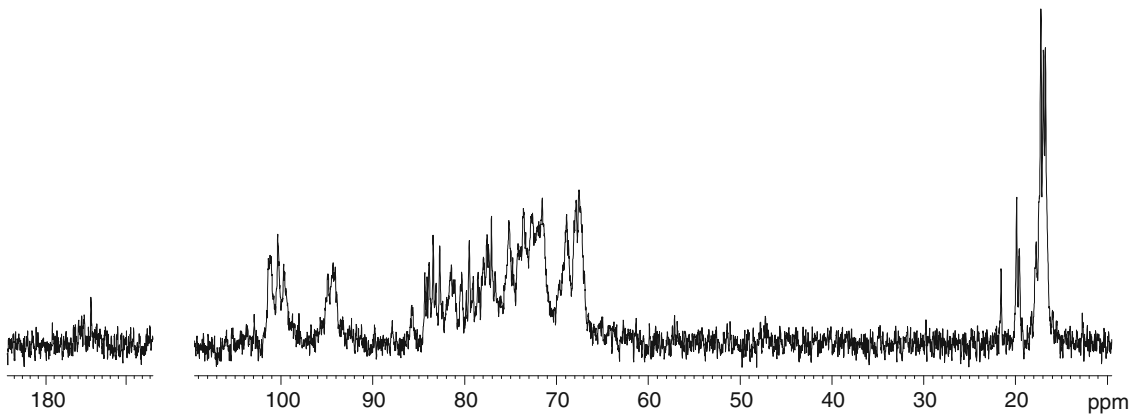

Figure 2. ^{13}C NMR spectrum of native fucoidan F3.

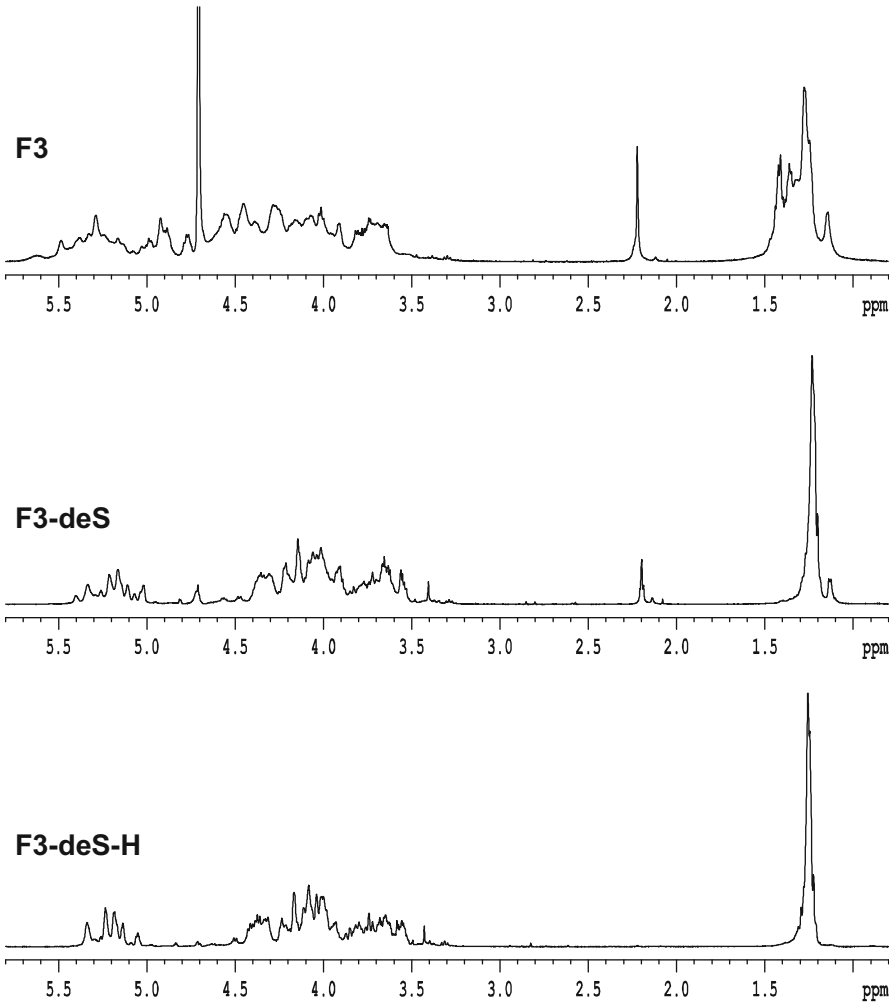

Figure 3. ^{1}H NMR spectra of F3, F3-deS and F3-deS-H.

Table 2
Composition (in M %) and optical rotation of polysaccharide preparations

Sample	Fuc	SO_3Na	GlcA	Glc	$[\alpha]_D^{16}$
F3	46	43	11	—	−101.6
F3-deS	78	—	22	—	−153.0
F3-deS-H	68	—	32	—	−143.6
F3-red	43	46	tr.	11	−104.2
F3-red-deS	78	—	tr.	22	−140.1
F4	48	43	9	—	−115.7
F4deS	82	—	18	—	−176.8

A solvolytic desulfation procedure was used to remove sulfate groups from F3. Desulfated preparation F3-deS contained no sulfate, whereas the ratio of fucose and glucuronic acid differed only slightly from that of the native polysaccharide F3 (Table 2). Surprisingly, attempts to reduce desulfated polymer F3-deS according to the procedure described above was unsuccessful. At the same time desulfation of reduced fucoidan F3-red gave rise to the carboxyl-reduced and desulfated polysaccharide F3-red-deS without any problem. The molar proportions of fucose and glucose in F3-red-deS were exactly the same as the corresponding values of fucose and glucuronic acid in F3-deS.

Partial acid hydrolysis of F3-deS was performed in an attempt to split off all the fucofuranose residues leaving fucopyranose and glucuronic acid residues in the polymeric molecule. Unfortunately, appropriate conditions for complete removal of furanosides were not found. Treatment of F3-deS with 0.1 M CF_3COOH at 100 °C for 2 h (conditions successfully used for removal of terminal α-D-Fuc*f* residues from a bacterial fucorhamnan[16]) gave rise to a complex mixture of oligosaccharides and fucose, the polymeric product

Table 3
Methylation analysis of fucoidan F3, its chemically modified preparations and F4-deS (M % of partially methylated fucitol and glucitol acetates)

Position of *O*-methyl groups in	Deduced positions of substitution:	F3-deS	F3	F3-red-deS	F3-red	F4-deS
Fucitol						
2,3,5	Fuc*f*-(1→	10	—	10	1	5
2,3,4	Fuc*p*-(1→	5	—	3	—	6
2,5	→3)-Fuc*f*-(1→	tr.	2	tr.	4	tr.
2,3		tr.	3	—	5	—
3,5	→2)-Fuc*f*-(1→	8	4	5	6	5
2,4	→3)-Fuc*p*-(1→	45	23	37	13	59
5	→2,3)-Fuc*f*-(1→	—	4	—	4	—
2		6	18	4	12	4
4	→2,3)-Fuc*p*-(1→	24	14	21	17	19
Fuc		2	32	tr.	23	2
Glucitol						
2,3,4,6	GlcA*p*-(1→			11	3	
2,3,6	→4)-GlcA*p*-(1→			9	12	

being obtained with the yield of about 13%. Milder hydrolysis of F3-deS (0.01 M CF_3COOH, 100 °C, 1.5 h) resulted in partial loss of fucofuranose residues together with complete deacetylation, giving rise to preparation F3-deS-H with the yield of 56.4% (Table 2). The L-configuration of fucose liberated by partial hydrolysis was proved in a separate experiment, thus confirming the same absolute configuration of fucofuranose and fucopyranose residues in the polysaccharide. The NMR spectra of F3-deS-H contained the same signals as the spectra of F3-deS but were better resolved and were used to confirm the assignment of resonances in the spectra of F3-deS (see below).

2.3. Methylation analysis

The native fucoidan F3 and its chemically modified preparations F3-deS, F3-red-deS, and F3-red were subjected to methylation analysis. The polysaccharides were treated with methyl iodide in the presence of sodium hydroxide in methyl sulfoxide, the negatively charged polymers F3, F3-deS, and F3-red being first converted into the PyH^+-salts to enhance its solubility in DMSO. The methylated fucoidans were hydrolyzed, and the resulting mixtures of partially methylated monosaccharides were analyzed as alditol acetates by GLC–MS. It is evident from the results of methylation

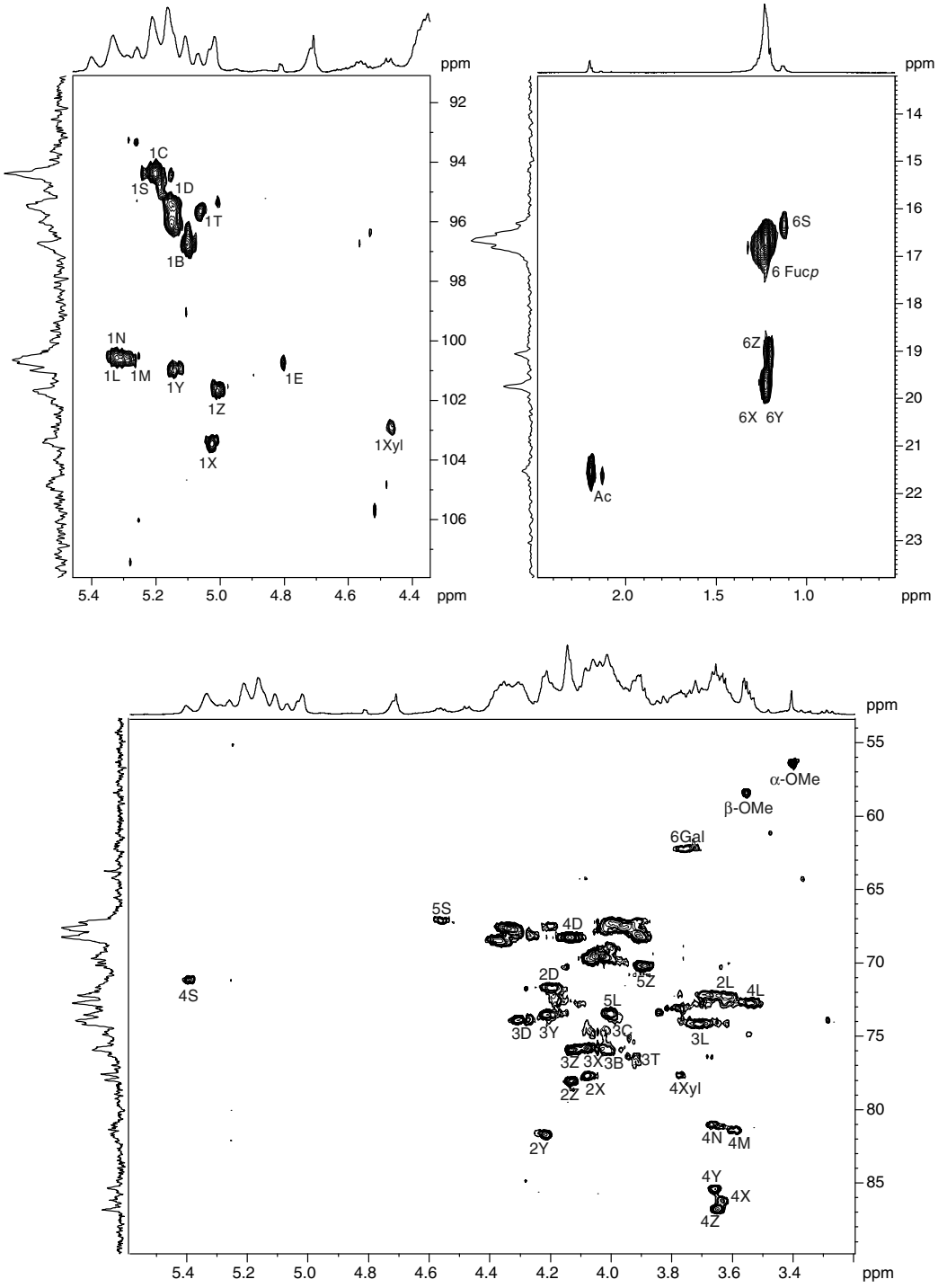

Figure 4. Fragments of 2D HSQC spectrum of F3-deS. Some important correlation peaks are marked on the spectra (abbreviations as in Table 4 and Scheme 1).

of F3-deS and F3-red-deS (Table 3) that these preparations are branched polysaccharides built up mainly of 3-linked and 2,3-linked fucopyranose residues in a molar ratio of about 2:1. Besides that the presence of terminal and 2-linked fucofuranose residues (about 20% of all fucose) was detected. Similarly both terminal and 4-linked glucopyranose residues practically in equimolar amounts were detected in F3-red-deS. It was suggested that fucofuranose and glucuronic acid residues are present in F3 not only as single branches, but also as side chains of more complex structure. This suggestion was then confirmed by NMR spectroscopy.

2.4. NMR spectroscopy

Both ^{1}H (Fig. 3) and ^{13}C NMR spectra of desulfated polysaccharide F3-deS were resolved enough to apply 2D spectroscopy for the assignment of resonances in the 1D spectra. Analysis of COSY, TOCSY, HSQC (Fig. 4), and HMQC–TOCSY spectra revealed the presence of α-fucose, α-glucuronic acid, and traces of β-xylose and β-galactose residues in the sample, α-fucose being in both pyranose and furanose forms at a ratio of about 3.5:1. Resonance assignments for the main monosaccharide residues in ^{1}H and ^{13}C NMR spectra of F3-deS are given in Table 4. It was shown that the main fucoidan chain was built up of (1→3)-linked α-L-fucopyranose residues (residue **B**, Scheme 1, Table 4). The mode of substitution in this chain was proved by the downfield location of C-3_B at 76.0 ppm and was confirmed by the presence of correlation peak 5.10/4.05 in ROESY spectrum corresponding to inter-residue H-1_B/H-3_B and H-1_B/H-4_B interactions typical of (1→3)-linked α-L-fucopyranoside chains.[17]

A part of residues (~35%) in the main chain is substituted at positions 2 (residue **D**, Scheme 1, Table 4) by α-D-glucopyranosyluronic acid (residue **L**). The presence of 2,3-disubstitution in fucopyranose residues **D** was confirmed by position of correlation peaks H-2_D/C-2_D (4.19/71.8), H-3_D/C-3_D (4.29/73.9), and H-4_D/C-4_D (4.14/68.2) in HSQC spectrum (Fig. 4), by downfield location of C-2_D and C-3_D resonances at 71.8 and 73.9 ppm, respectively, and by upfield location of C-4_D resonance at 68.2 ppm. Furthermore, ROESY spectrum (Fig. 5) contains correlation peaks 5.31/4.19 and 5.31/4.29 corresponding to H-1_L/H-2_D and H-1_L/H-3_D interactions, respectively, as well as a correlation peak 5.20/4.14 corresponding to H-1_C/H-4_D interaction. The presence of inter-residue correlation H-1_C/H-4_D seems to be the specific feature of (1→3)-linkage in 2,3-disubstituted fucopyranose residue **D** in this polymer. The absence of (1→4)-linkage between residues **C** and **D** was confirmed by the values of C-1_C and C-4_D chemical shifts (94.5 and 68.2 ppm, respectively), which could not be characteristic of (1→4)-linkage in any case. The presence of fragment **C**→**D** in the main fucoidan chain was confirmed by correlation peak 5.15/4.05 in ROESY spectrum corresponding to H-1_D/H-3_B and H-1_D/H-4_B and by correlation peaks 5.10/4.07 and 5.10/4.05 corresponding to H-1_B/H-3_C and H-1_B/H-4_C.

The ^{1}H NMR spectrum of F3-deS contained an intense singlet at 2.19 ppm and a small one at 2.12 ppm corresponding to acetyl groups. Analysis of 2D NMR spectra revealed that some of fucopyranose residues **S** of the main fucoidan chain (about 7%) were 4-O-acetylated (characteristic correlation peak 5.39/71.1 in HSQC spectrum corresponding to H-4_S/C-4_S, downfield location of H-4_S resonance at 5.39 ppm). It was evident that an intense singlet at 2.19 ppm corresponded to acetyl group linked to C-4 of residue **S**. However, the location of the second acetyl group (2.12 ppm) was not determined due to the absence of the corresponding correlations in 2D spectra.

Three different fucofuranose residues (**X**, **Y**, **Z**) were found by analysis of NMR spectra. These residues have characteristic correlation peaks H-4/C-4 in HSQC spectrum with downfield shift at ^{13}C axis (Fig. 4, Table 4). Analysis of ^{1}H and ^{13}C NMR chemical shifts (Table 4) of fucofuranoses showed that two of them (**X** and **Z**) were terminal, whereas residue **Y** was substituted at C-2 or C-3. Substitution at C-3 should be excluded, since only C-2-substituted fucofuranose residues were found in methylated F3-deS (Table 3). In addition, ROESY spectrum confirmed substitution of residue **Y** at C-2 by terminal fucofuranose residue **Z** (Fig. 5, correlation peak 5.01/4.21 corresponding to H-1_Z/H-2_Y). Furthermore, the ROESY spectrum contained correlation peaks 5.02/3.59 and 5.14/3.67 of anomeric protons of fucofuranoses **X** and **Y** (H-1_X and H-1_Y), respectively (Fig. 5), obviously not corresponding to intra-residue correlations. This conclusion was confirmed by the presence of correlation peaks 3.59/81.4 and 3.67/81.2 with downfield ^{13}C chemical shifts corresponding to substituted carbons in HSQC spectrum. The real positions of both residues **X** and **Y** were determined from the NMR spectra of F3-deS-H (see below).

The ^{1}H (Fig. 3) and ^{13}C NMR spectra of F3-deS-H were similar to the spectra of F3-deS, but were better resolved and contained no acetate signals. At the same time, the spectra contained signals of all three fucofuranose residues (**X**, **Y**, and **Z**), and their relative intensities were close to those in the spectra of F3-deS. Analysis of 2D NMR spectra of F3-deS-H confirmed again that the main fucoidan chain was built up of (1→3)-linked α-L-fucopyranose residues **B** (Scheme 1) and was branched at position 2 of residues **D** by the residues **L** of α-D-glucuronic acid. Furthermore, the spectra gave evidence that fucofuranose residues **X** and **Y** are linked to C-4 of some α-D-glucuronic acid residues (units **M** and **N**, respectively; Table 4). At the same time, no evidence was obtained on the direct substitution of the main chain by fucofuranose residues or on the substitution of glucuronic acid residues by other glucuronic acid residues. The non-reducing terminal residues in the spectra of F3-deS and F3-deS-H were represented by α-L-fucopyranose residues **A** of the main chain. The reducing terminal residues

Table 4
^{13}C and ^{1}H NMR data for desulfated fucoidan F3-deS

Residue	Chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
	H-1	H-2	H-3	H-4	H-5	H-6
A α-L-Fuc*p*-(1→3	96.7	69.3	71.1	73.3	n.d.	n.d.
	5.01	3.75	n.d.	3.80	n.d.	n.d
B →3)-α-L-Fuc*p*-(1→3	96.9	67.7	76.0	69.8	n.d.	n.d.
	5.10	3.95	4.05	4.05	n.d.	1.23
C →3)-α-L-Fuc*p*-(1→3	94.5	67.6	75.0	69.5	68.4[a]	16.7
	5.20	4.00	4.07	4.05	4.36[b]	1.22
D→2,3)-α-L-Fuc*p*-(1→3	95.9	71.8	73.9	68.2	67.8[a]	16.7
	5.15	4.19	4.29	4.14	4.34[b]	1.20
E →3)-α-L-Fuc*p*-OMe	100.8	67.3	75.5	69.3	n.d.	n.d.
	4.81	3.93	4.04	4.06	n.d.	n.d.
F →3)-β-L-Fuc*p*-OMe	97.7	71.3	78.9	69.2	n.d.	n.d.
	4.60	3.59	n.d.	4.00	n.d.	n.d.
S →3)-α-L-Fuc*p*4OAc(1→3	94.5	67.9	72.7	71.1	67.2	16.3
	5.23	4.04	4.17	5.39	4.55	1.12
T →3)-α-L-Fuc*p*-(1→3	95.7	67.3	76.5	69.7	n.d.	n.d.
	5.06	3.89	3.92	3.81	n.d.	n.d.
L α-D-GlcA*p*-(1→2	100.8	72.2	74.1	72.7	73.5	176.0
	5.31	3.62	3.71	3.54	4.00	
M →4)-α-D-GlcA*p*-(1→2	100.8	72.7	73.1	81.4	72.9	176.0
	5.29	3.70	3.80	3.60	4.10	
N →4)-α-D-GlcA*p*-(1→2	100.8	72.7	72.7	81.2	n.d.	176.0
	5.33	3.68	3.75	3.68	3.98	
X α-L-Fuc*f*-(1→4	103.5	77.7	75.9	86.2	68.2	19.8
	5.02	4.08	4.08	3.63	3.91	1.22
Y →2)-α-L-Fuc*f*-(1→4	101.0	81.7	73.5	85.4	68.0	19.8
	5.14	4.21	4.21	3.65	3.91	1.23
Z α-L-Fuc*f*-(1→2	101.7	78.0	75.9	86.8	70.2	19.1
	5.01	4.13	4.13	3.64	3.90	1.20

[a,b] The assignment may be reversed.
n.d.—signal not detected.

A **B** **B**
α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→

B **C** **D** **B**
→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→
2
L ↑
α-D-GlcA*p*

D
→3)-α-L-Fuc*p*-(1→
2
M ↑
α-D-GlcA*p*
4
X ↑
α-L-Fuc*f*

D
→3)-α-L-Fuc*p*-(1→
2
N ↑
α-D-GlcA*p*
4
Y ↑
α-L-Fuc*f*
2
Z ↑
α-L-Fuc*f*

E
→3)-α-L-Fuc*p*-OMe

F
→3)-β-L-Fuc*p*-OMe

B **S** **T** **B**
→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→3)-α-L-Fuc*p*-(1→
4
↑
Ac

Scheme 1. Structural fragments of desulfated fucoidan F3-deS.

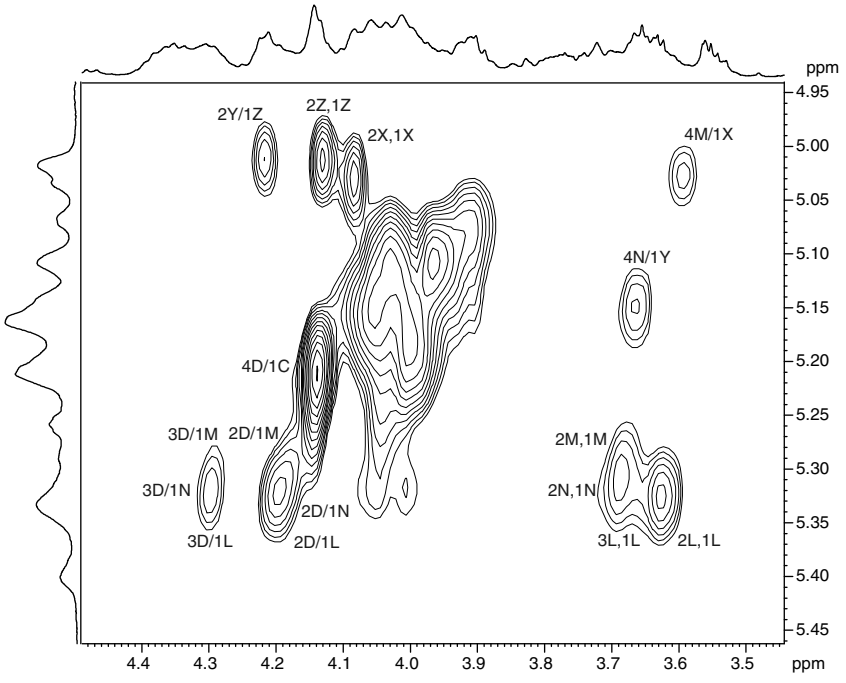

Figure 5. Fragment of 2D ROESY spectrum of F3-deS. Some important correlation peaks are marked on the spectrum (abbreviations as in Table 4 and Scheme 1).

were observed in the spectra as 3-linked methyl α- and β-fucopyranosides **E** and **F**, which evidently were formed by reaction with methanol under the solvolytic desulfation conditions. Structural fragments identified in F3-deS by spectral and chemical methods are shown in Scheme 1, whereas Table 4 represents the chemical shifts of signals in the ^{1}H and ^{13}C NMR spectra of these fragments.

NMR spectra of the native polysaccharide F3 were analyzed to localize the positions of sulfate groups. As mentioned above, the full resonance assignments for ^{1}H and ^{13}C NMR spectra of F3 were

not made due to insufficient signal resolution. Nevertheless, comparison of NMR spectra of F3 and F3-deS made it possible to conclude that most of the fucofuranose residues (about 60%) were mono- and disulfated (5-sulfated, 2,3-disulfated, and 3,5-disulfated residues at a ratio of about 1:1:1). Furthermore, the spectra contained several weak signals corresponding to 2-sulfated and 2,4-disulfated fucopyranose residues. The positions of sulfate groups were evidenced by the low-field shifts of the corresponding resonances in the 2D TOCSY spectrum of F3 and confirmed by methylation data (Table 3). The methylation analysis also indicates the presence of minor amounts of 3-sulfated terminal fucofuranose residues. It was formerly suggested from comparison of methylated glucitols derived from F3-red and F3-red-deS (Table 3) that some glucuronic acid residues are also sulfated, but no data on the presence of sulfated GlcA were obtained from the NMR spectra. Hence, the increase of terminal glucose residues in F3-red-deS was explained by substantial loss of fucofuranose residues under solvolytic desulfation conditions.

NMR spectra of native F3 contained practically no signals belonging to xylose and galactose residues, but the content of these monosaccharides slightly increased after chemical modifications of the polysaccharide. Analysis of β-xylose signals present in 2D spectra of F3-deS and F3-deS-H revealed (1→4)-linked β-xylopyranose residues, although the subspectrum of terminal xylose residues was not observed. There were no correlation peaks for anomeric protons of the xylose residues with any protons of fucose residues in the ROESY spectra. It was concluded that F3 contained a small amount of separate (1→4)-β-xylan. Attempts to obtain from the spectra some information about the position of galactose, another minor component of fucoidan, were unsuccessful.

Fraction F4 (Table 1) was analyzed by NMR spectroscopy as described above for F3 (data not shown). No marked structural differences between F3 and F4 were obtained, but (according to methylation data, Table 3) F4 was slightly less branched than F3.

3. Conclusion

A sulfated glucuronofucan isolated from the brown seaweed *C. flagelliformis* contains a backbone of 3-linked α-L-fucopyranose residues similar to several known fucoidans.[4] It has single branches in the form of α-D-glucopyranosyluronic acid residues at C-2 of the backbone, like the fucoidan from *Cladosiphon okamuranus*.[9–11] In addition, about half of these latter residues are substituted at C-4 by single α-L-fucofuranose residues or by disaccharides α-L-Fuc*f*-(1→2)-α-L-Fuc*f*-(1→.

The high level of fucofuranose residues, which are mono- and disulfated, is the most interesting structural feature of the fucoidan isolated from *C. flagelliformis*. Although the presence of terminal non-sulfated fucofuranosyl units was observed previously in several algal fucoidans, their position in the macromolecule was not determined. Such units in amounts of about 3–10% of the total fucose were detected during the methylation analysis of polysaccharides from *Ecklonia kurome*,[18] *Fucus vesiculosus*,[19] and *Adenocystis utricularis*.[20] Alternatively, the characteristic signal of CH_3 of fucofuranose residues at about 19 ppm was observed in ^{13}C NMR spectra of fucoidans from *Fucus distichus*[21] and *Analipus japonicus*.[22] Therefore, fucofuranose residues may be regarded as rather common constituents of the brown algal fucoidans. At the same time the presence of fucose in both pyranose and furanose forms in one molecule was rarely detected in the polysaccharides of other origin. Thus, both residues were found in an extracellular sulfated polysaccharide produced by the diatom *Chaetoceros curvisetus*.[23] According to methylation analysis data of the native polysaccharide, the authors proposed that fucofuranoses are not only present as end units, but also as a part of the chain and as branch points (however, the positions of sulfate groups in the polymer were not determined). Besides the algal polysaccharides, fucofuranose was also found in some lipopolysaccharides of bacteria belonging to the phyla Proteobacteria and Firmicutes.[24] In contrast to fucopyranose, which may occur in both D and L forms in many bacterial polymers, only D-fucofuranose units were identified in bacteria. There is an example of the antigenic bacterial polysaccharide[25] containing disaccharide side chains α-D-Fuc*f*-(1→2)-α-D-Fuc*f*-(1→, which coincide with the disaccharide side chains found in desulfated fucoidan F3-deS from *C. flagelliformis* in all aspects except the absolute configuration of fucose.

4. Experimental

4.1. General methods

Quantitative determination of monosaccharides by gas-liquid chromatography of alditol acetates, colorimetric determination of acetate, and turbidimetric determination of sulfate were carried out as described previously.[22,26] Uronic acids were estimated colorimetrically with 3,5-dimethylphenol using D-glucuronic acid as the standard.[27] Uronic acid obtained by hydrolysis of fucoidan with 2 M CF_3COOH (100 °C, 8 h) followed by removal of sulfate as $BaSO_4$ was identified by high-performance ion-exchange chromatography as described previously.[27] The absolute configurations of fucose and glucose were established by GLC analysis of the corresponding acetylated (S)-(+)-*sec*-butyl glycosides.[28] Optical rotations were measured using a digital polarimeter PU-07 (Russia) for 1% solutions in water. Electrophoresis of fucoidans was performed on 0.6% agarose gel plates (120 × 110 × 2 mm) using 0.05 M 1,3-diaminopropane/acetate buffer (pH 9.0), for 1 h at 100 V. The gel was fixed, dried, and stained as described in Ref. 29.

4.2. NMR spectroscopy

NMR spectra were recorded using a Bruker DRX-500 spectrometer at 303 K. Samples were deuterium-exchanged by lyophilization with D_2O and then examined as 2–3% solutions in 99.97% D_2O, TSP (δ_H 0.0) and methanol (δ_C 50.15) were taken as the internal standards. The parameters used for 2D experiments were described previously.[12] The TOCSY and HMQC–TOCSY spectra were acquired with 200 ms and 150 ms duration of MLEV17 spin-lock, respectively, the ROESY spectra were acquired with 200 ms duration of spin-lock, the HMBC spectra were recorded with 60 ms delay for evolution of long-range couplings.

4.3. Isolation of fucoidans

A sample of the alga *C. flagelliformis* was collected from the littoral of the Possjet Bay of the Sea of Japan on September 1977, dried in air and milled (particles about 0.25 mm). The algal biomass was treated subsequently with methanol and acetone in Soxhlet apparatus to remove lipids and colored matter and vacuum dried. Thirty grams of defatted material and 2% aqueous $CaCl_2$ solution (4 × 150–200 mL) were mechanically stirred at 85 °C for 5 h. The extracts were collected by centrifugation, dialyzed, and lyophilized to give crude polysaccharide fraction (F), yield 13.6 g (45.3% of dry defatted biomass), composition: fucose, 31.8%; SO_3Na, 20.3%; uronic acids, 13.4%; glucose, 11.6%; galactose, 2.2%; xylose, 1.3%; mannose, 0.8%. An aqueous solution of F (2.1 g in 80 mL) was placed on a column (24 × 4 cm), containing DEAE-Sephacel (Pharmacia) in Cl^--form, and eluted with water followed by NaCl solutions of increasing concentration (0.5, 1.0, 1.25, and 1.5 M), each time up to the absence of a positive reaction of eluate for carbohydrates with phenol and concd H_2SO_4.[30] All the solutions obtained

were dialyzed and lyophilized, yields of fractions F0–F4 being 0.20, 0.02, 0.84, 0.46, and 0.31 g, respectively. Composition of these fractions is given in Table 1.

4.4. Chemical modifications of fucoidans

Solvolytic desulfation of F3, F4, and F3-red (as pyridinium salts) was carried out as described earlier.[22,31] Yields of desulfated fucoidans (F3-deS, F4-deS, and F3-red-deS) containing no residual sulfate groups were 48.5%, 30.0%, and 20.6%, respectively. Carboxyl groups of glucuronic acid residues in F3 were reduced according to the method of Taylor and Conrad proposed for heparin[15] with minor modifications. Briefly, an aqueous solution of F3 (58 mg in 6 mL) was adjusted to pH 5.0 and solid 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma, 90 mg) was added gradually. The pH of the mixture was maintained at 5.0 with 0.1 M HCl in the course of reaction. After stabilization of the pH value sodium borohydride (530 mg) and few drops of *n*-butanol (to minimize the foam) were added to the reaction mixture with stirring. The resulting solution was kept at 50 °C for 2 h, cooled in an ice bath, and 4 M HCl was added dropwise until the excess of sodium borohydride was destroyed. The mixture was then dialyzed and lyophilized to yield 45 mg of reduced fucoidan (F3-red).

Partial acid hydrolysis: a solution of 39 mg of F3-deS in 4.0 mL of 0.01 M CF_3COOH was heated at 100 °C for 1.5 h, acid was evaporated with EtOH, the solution was dialyzed and lyophilized to yield 22 mg of partially hydrolyzed desulfated fucoidan (F3-deS-H).

Methylation of fucoidans (F3, F3-deS, and F3-red as pyridinium salts) followed by hydrolysis and GLC–MS of partially methylated alditol acetates was performed as previously described.[22,32]

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