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A highly regular fraction of a fucoidan from the brown seaweed *Fucus distichus* L.☆

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Abstract—A fucoidan fraction consisting of L-fucose, sulfate, and acetate in a molar proportion of 1:1.21:0.08 was isolated from the brown seaweed *Fucus distichus* collected from the Barents Sea. The ^{13}C NMR spectrum of the fraction was typical of regular polysaccharides containing disaccharide repeating units. According to 1D and 2D ^{1}H and ^{13}C NMR spectra, the fucoidan molecules are built up of alternating 3-linked α-L-fucopyranose 2,4-disulfate and 4-linked α-L-fucopyranose 2-sulfate residues: →3)-α-L-Fuc*p*-(2,4-di-SO_3^-)-(1→4)-α-L-Fuc*p*-(2SO_3^-)-(1→. The regular structure may be only slightly masked by random acetylation and undersulfation of several disaccharide repeating units.

Keywords: Fucoidan; NMR; Disaccharide repeating unit; Seaweed; Brown algae; *Fucus distichus*

1. Introduction

Natural polysaccharides built up essentially of sulfated α-L-fucose residues are known as fucoidans.[2] They are present in brown algae and some echinoderms. Sulfated fucans isolated from echinoderms have usually linear backbones and regular sulfation patterns resulting in the formation of oligosaccharide repeating units.[3] The structures of these repeating units can be determined by NMR spectroscopy, and hence, correlation between structures and biological activity of polysaccharides may be made.[4] The structures of algal fucoidans are usually much more complicated. Algal polysaccharides are heterogeneous and branched, they may contain additional monosaccharide constituents and acetyl groups, the sulfation pattern is not regular, and, as a result, chemical methods of structural analysis as well as NMR spectra of native algal fucoidans give only partial information on their structures. Recently it was shown that several representatives of the orders Chordariales and Laminariales (Phaeosporophyceae) contain polysaccharides with a linear backbone built up of (1→3)-linked α-L-fucopyranose residues,[5–7] whereas fucoidans from representatives of the order Fucales (Cyclosporophyceae), namely, *Fucus evanescens, Ascophyllum nodosum*, and *F. vesiculosus*, have a backbone built up of alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues.[1,8,9] To confirm this fundamental structural difference of fucoidans obtained from the two different classes of brown algae, Phaeosporophyceae and Cyclosporophyceae, we have studied fucoidans from several other species of the genus *Fucus*. The present work is devoted to the structural analysis of a fucoidan isolated from the brown alga *F. distichus* L.

2. Results and discussion

2.1. Isolation of fucoidan

Extraction of water-soluble polysaccharides from defatted algal biomass, precipitation of acid polysaccharides by hexadecyltrimethylammonium bromide (Cetavlon)

☆ Polysaccharides of algae, Part 57. For Part 56, see Ref. 1.

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Table 1. Yields and composition of fucoidan fractions obtained by ion-exchange chromatography of crude fucoidan (F)

Fraction	Yield (%) of F	Neutral monosaccharides (%)					SO_3Na (%)
		Fuc	Xyl	Gal	Man	Glc	
F		51.6	2.7	1.5	0.7	0.2	38.3
F_1	14.9	53.9	6.3	2.0	1.1	0.6	23.8
F_2	3.5	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
F_3	14.0	54.0	6.1	2.8	1.3	0.5	23.5
F_4	46.5	40.8	0.8	0.8	—	—	34.8
F_5	1.7	19.7	3.9	0.5	0.4	0.4	22.9

and transformation of the precipitate into water-soluble sodium salt were carried out as described in our previous paper[1]. The resulting crude fucoidan (F) was fractionated by stepwise elution from DEAE-Sephacel using aqueous sodium chloride of increasing concentration. The yields and composition of five fucoidan fractions obtained are given in Table 1. Fraction F_4, which was essentially a homofucan sulfate containing fucose and sulfate in a molar ratio of about 1:1.21 and only traces of other monosaccharide constituents and acetyl groups, was subjected to structural analysis.

2.2. IR spectral characterization of F_4

The IR spectrum of F_4 contained an intense absorption band at 1240–1272 cm^{-1} (S=O) common to all sulfate esters. An additional sulfate absorption band at 828 cm^{-1} (C–O–S, secondary equatorial sulfate) and a shoulder at 848 cm^{-1} (C–O–S, secondary axial sulfate) indicated that the majority of sulfate groups occupy positions C-2 and/or C-3, and only the lesser part of sulfate is located at C-4 of fucopyranose residues. An absorption band at 1732 cm^{-1} revealed the presence of some *O*-acetyl groups in the polysaccharide.

2.3. NMR analysis of F_4

Unlike many native algal fucoidans, F_4 had a very simple ^{13}C NMR spectrum (Fig. 1). It contained two intense signals (100.2 and 99.1 ppm) in the anomeric region and one broad intense signal in the high-field (16.8 ppm) region, which are typical of α-fucopyranosides. This evidence together with the high negative optical rotation of the polysaccharide, $[\alpha]_D^{20}$ −138.9° (*c* 0.92, water), revealed that fucose belongs to the L-series, like in other natural fucoidans. Two small signals at 22.2 and 175.2 ppm confirmed the presence of *O*-acetyl groups.

The ^1H NMR spectrum (Fig. 2) was also resolved satisfactorily. It contained several intense signals in the α-anomeric (5.0–5.6 ppm) and high-field (1–1.5 ppm) regions. There were some minor signals in the same regions. The small signals at about 2.2 ppm confirmed the presence of small amount of *O*-acetyl groups. The molar proportion of fucose and acetate was calculated as 1:0.08 by comparison of intensities of anomeric protons and *O*-acetyl protons in the ^1H NMR spectrum. Exactly the same ratio was obtained by colorimetric determination of fucose and acetyl group content.

Several variants of 2D NMR spectroscopy were applied to assign all the resonances in the 1D spectra (Table 2). The COSY and TOCSY spectra taken at 303 K revealed the presence of two types of α-fucose residues in the molecule (A and B) differing in the mode of substitution. Some important correlation peaks were found in ROESY spectrum, namely, H-1 (A, 5.38 ppm)/H-4 (B, 4.03 ppm), H-1 (A, 5.38 ppm)/H-6 (B, 1.33 ppm), H-1 (B, 5.40 ppm)/H-3 (A, 4.33 ppm), and H-1 (B, 5.40 ppm)/H-2 (A, 4.57 ppm). These correlations may indicate that all the fucose residues A glycosylated position 4 of residues B, whereas all the residues B glycosylated position 3 of residues A. The HSQC spectrum (Fig. 3) was consistent with glycosylation of residues A at position 3 (downfield location of C-3 resonance at 74.4 ppm) and residues B at position 4 (signal C-4 at 84.0 ppm). If this linkage pattern is correct, the low-field position of H-2 and C-2 signals in both 3- and 4-linked α-fucopyranose residues gives the evidence that all C-2 in the polysaccharide bear sulfate groups. Low-field shifts of H-4 and C-4 of 3-linked residues show that additional sulfate occupies C-4 (structure **1**).

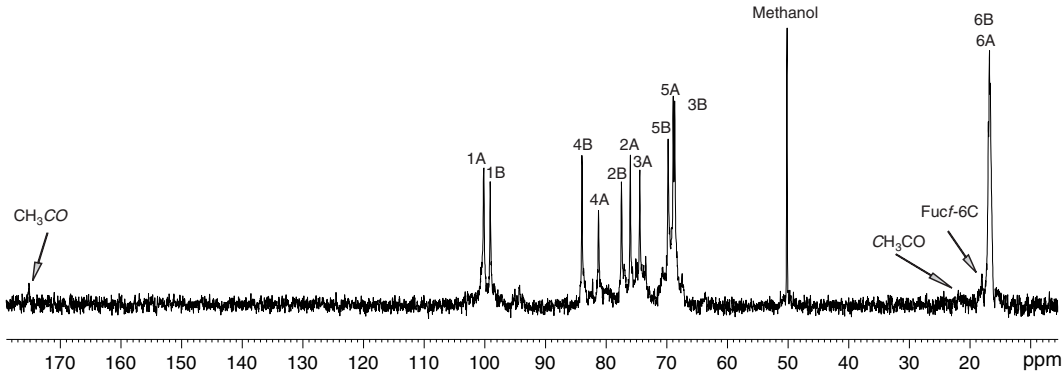


Figure 1. ^{13}C NMR spectrum of fucoidan F_4. 1A, 1B, etc. refer to the corresponding carbon atoms of residues A and B in the regular fucoidan structure **1**. Signals of small intensity belonging to acetyl groups and to C-6 of fucofuranose residues, marked by arrows, indicate a negligible deviation from the regular structure caused by the low content of these structural elements.

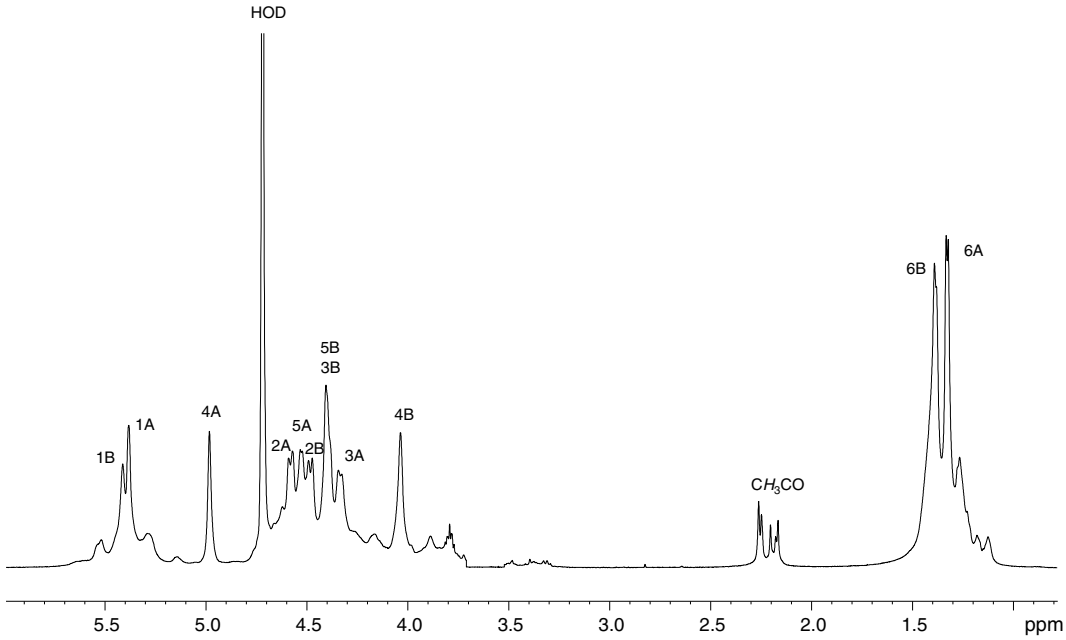
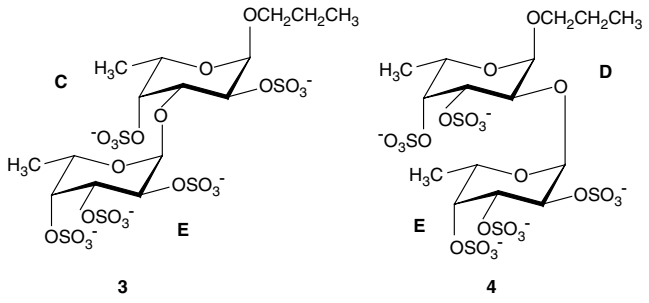
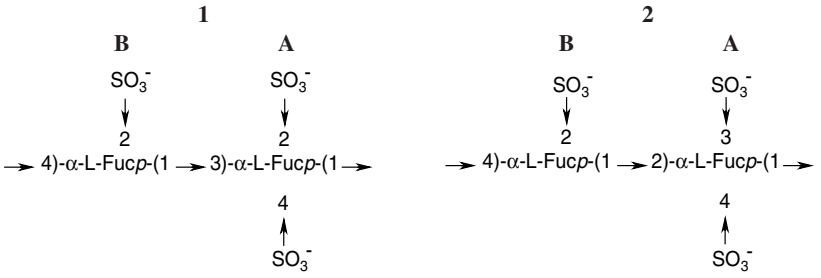


Figure 2. ^{1}H NMR spectrum of fucoidan F_4. 1A, 1B, etc. refer to the corresponding protons of residues A and B in the regular fucoidan structure **1**.

Since the H-1 signal of residue B (5.4 ppm) in ROESY spectrum correlated with two protons of residue A, H-2 and H-3, the real position of B → A inter-residue linkage was investigated more thoroughly. The low-field position of the signals of H-2, H-3, C-2, and C-3 of residue A may be equally explained by both glycosylation or sulfation of positions 2 and 3, and hence, two structural variants **1** or **2** for B → A fragment may be suggested according to these data:



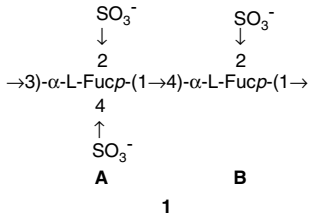


To determine the real position of glycosidic bond in B → A fragment, we compared the NMR chemical shifts of residue A in the polysaccharide molecule with the corresponding chemical shifts of synthetic totally sulfated (1 → 3)- and (1 → 2)-linked *n*-propyl *O*-α-L-fucopyranosyl-α-L-fucopyranosides **3** and **4**. These model compounds were prepared by sulfation of the corresponding disaccharide glycosides synthesized previously.[10] The NMR spectra of sulfated disaccharide derivatives **3** and **4** are presented in Table 3. The expected differences between the spectra of starting compounds[10] and their sulfation products confirmed the structures of **3** and **4**.

The model compounds had the same structural features near the inter-residue glycosidic bonds as the expected polysaccharide fragments **1** or **2**. It is important that they were sulfated at C-2 and C-4 of residue C (or C-3 and C-4 of residue D) and C-2 of residue E, whereas the presence of other sulfate groups in residue E should not alter significantly the properties of the inter-residue glycosidic bond due to their spatial remoteness.

Table 4 contains the ^{13}C and ^{1}H NMR chemical shifts for atoms, which are nearest to the linkage position, together with differences between the chemical shifts observed for the fucoidan fragment and the corresponding sulfated difucoside. As can be seen from these

Table 2. NMR data for fucoidan fraction F_4



1

Residue	^{1}H chemical shifts (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-6
A →3)-α-L-Fuc*p*-2,4-di-SO_3^--(1→	5.38	4.58	4.33	4.98	4.52	1.34
B →4)-α-L-Fuc*p*-2-SO_3^--(1→	5.40	4.48	4.38	4.03	4.40	1.40
	^{13}C chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	C-6
A →3)-α-L-Fuc*p*-2,4-di-SO_3^--(1→	100.2	76.0	74.4	81.2	68.9	16.8
B →4)-α-L-Fuc*p*-2-SO_3^--(1→	99.1	77.5	68.7	84.0	69.8	16.8

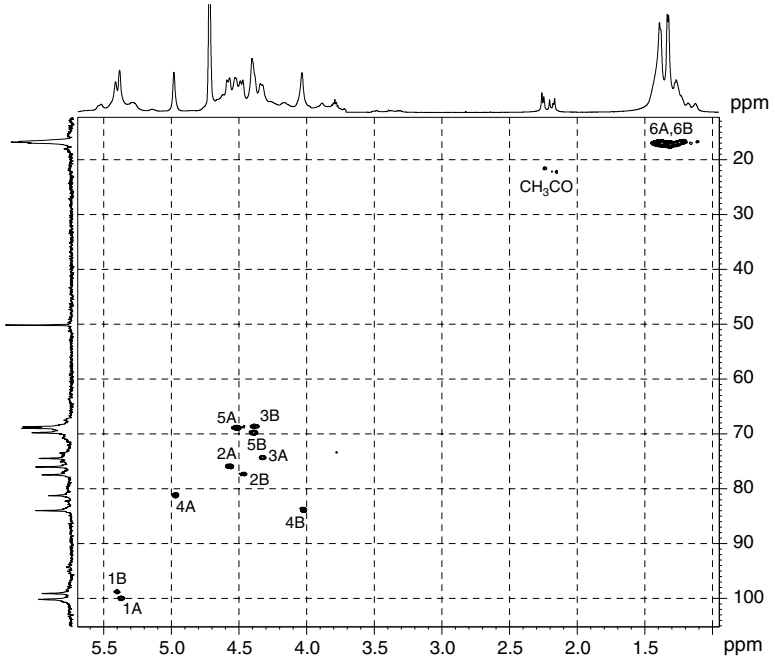


Figure 3. HSQC spectrum of fucoidan F_4. 1A, 1B, etc. refer to proton–carbon cross peaks in the regular fucoidan structure **1**.

Table 3. ^{1}H and ^{13}C NMR chemical shifts[a] for sulfated disaccharides **3** and **4**

Substance	Residue	H-1	H-2	H-3	H-4	H-5	H-6	C-1	C-2	C-3	C-4	C-5	C-6
3	α-L-Fuc-2,3,4-tri-SO_3^--(1→	5.43	4.56	4.98	4.96	4.50	1.31	99.0	74.8	73.7	80.8	68.5	17.2
	→3)-α-L-Fuc-2,4-di-SO_3^--OPr	5.26	4.59	4.35	4.96	4.24	1.33	97.4	76.0	75.2	81.3	67.9	17.0
4	α-L-Fuc-2,3,4-tri-SO_3^--(1→	5.36	4.56	4.75	4.96	4.66	1.32	98.3	73.1	73.5	80.6	67.9	17.0
	→2)-α-L-Fuc-3,4-di-SO_3^--OPr	5.14	4.04	4.72	4.98	4.30	1.34	97.7	74.7	74.3	80.5	67.1	17.0

[a] In ppm, recorded at 40 °C in D_2O with 0.05% acetone as an internal standard. Signals of *n*-propyl aglycon: $OCH_2CH_2CH_3$ δ 0.92; $OCH_2CH_2CH_3$ δ 1.62–1.64; $OCH_2CH_2CH_3$ δ 3.49–3.84; $OCH_2CH_2CH_3$ δ 11.1; $OCH_2CH_2CH_3$ δ 23.2–23.3; $OCH_2CH_2CH_3$ δ 71.3.

data, maximum deviations were obtained in the case of (1→2)-linked difucoside **4**. The mean square deviations in ^{1}H and ^{13}C chemical shift values for H-2–H-5 and C-2–C-5 atoms of 3-linked residue, calculated according to the known scheme,[11] were 0.13 and 0.61, respectively, whereas the corresponding deviations for 2-linked residue were 0.35 and 1.37. These data confirmed the presence of (1→3)-linkage in B→A fragment of the native fucoidan.

Final evidence on the positions of inter-residue linkages in polysaccharide molecule was obtained from the 2D HMBC and TOCSY spectra taken at 333 K. The following *trans*-glycosidic correlation peaks were observed in HMBC spectrum: H-1(A)/C-4(B), H-4(B)/C-1(A), and H-3(A)/C-1(B) (Fig. 4). The TOCSY spectrum recorded after the rather prolonged HMBC experiment showed that the polymer was stable enough during accumulation of signals at elevated temperature. Several signals of minor intensity found in these spectra could be attributed to residues A having no sulfate at position 4. It should be noted that structural elements deviating from the regular structure **1** (undersulfated disaccharide units and acetyl groups) were more distinctly represented in the spectra of fraction F_3 eluted before F_4 from DEAE-Sephacel (data will be published elsewhere).

Thus, according mainly to the spectral evidence, native fucoidan fraction F_4 has an essentially regular linear chain of alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues with sulfate groups at positions 2,4 of the former and at position 2 of the latter residues. This regularity may be slightly masked by small amount of *O*-acetyl groups, but their position was not determined due to low acetyl content. According to sulfate determination by chemical method and to several peaks of minor intensity in NMR spectra, additional deviation from the strictly regular structure may be caused by undersulfation of some disaccharide repeating units. A small peak at about 18.0 ppm in the ^{13}C NMR

Table 4. Comparison of several ^{1}H and ^{13}C NMR spectral signals of fucoidan F_4 and synthetic model compounds

Sample	Residue	^{1}H Chemical shifts (ppm)				^{13}C Chemical shifts (ppm)			
		H-2	H-3	H-4	H-5	C-2	C-3	C-4	C-5
Fucoidan (**1**)	A	4.57	4.33	4.97	4.52	76.0	74.5	81.2	68.9
Sulfated α-L-Fuc-(1→3)-α-L-Fuc-OPr (**3**)	→3)-α-L-Fuc-2,4-di-SO_3^--(1→	4.59	4.35	4.96	4.24	76.0	75.2	81.3	67.9
Differences between **1** and **3**		−0.02	−0.02	0.01	0.26	0.0	−0.7	−0.1	1.0
Sulfated α-L-Fuc-(1→2)-α-L-Fuc-OPr (**4**)	→2)-α-L-Fuc-3,4-di-SO_3^--(1→	4.04	4.73	4.97	4.31	74.5	74.0	80.0	66.6
Differences between **1** and **4**		0.53	−0.40	0.0	0.21	1.5	0.5	1.2	2.3

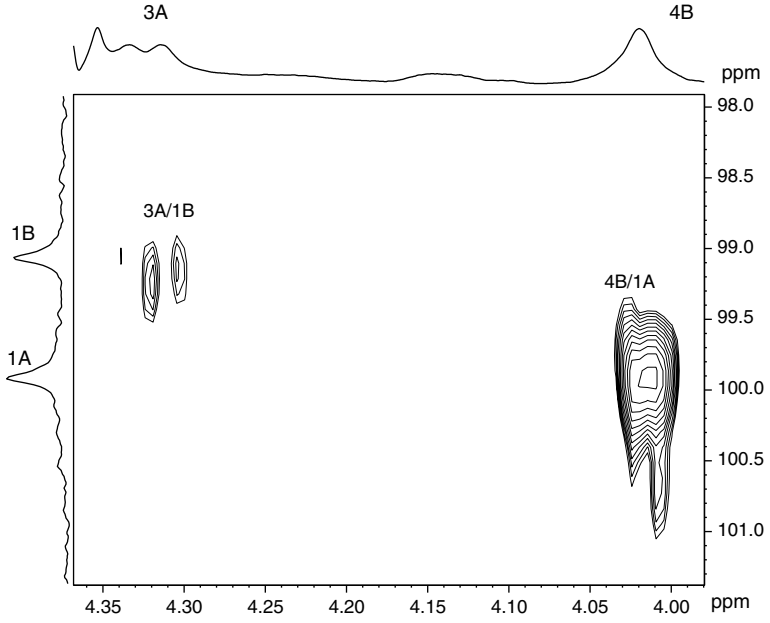


Figure 4. A part of HMBC spectrum of fucoidan F_4 demonstrating the most important inter-residue correlations of anomeric carbons 1A and 1B with the corresponding protons 4B and 3A of the neighboring residues, respectively, in the regular fucoidan structure **1**.

spectrum may indicate the presence of fucofuranose residues,[12] which were found previously in several other fucoidans.[13] Nevertheless, these deviations were too small to influence greatly the regular ^{13}C NMR spectrum of F_4 (Fig. 1). To our knowledge, it is a first example of such a highly regular fucoidan isolated from a brown alga.

It should be noted that structure **1** coincides with one of disaccharide repeating units of deacetylated polysaccharide from *F. evanescens*.[1] The ^1H NMR spectra of identical structural fragments found in both polysaccharides coincide completely, but the positions of several signals in ^{13}C NMR spectra are slightly different. Taking into account that the ^{13}C NMR spectrum of fucoidan from *F. distichus* was resolved much better than the corresponding spectrum of a sample from *F. evanescens*, we regard the present data as more reliable, and hence, the previous assignments[1] should be corrected according to the data of Table 2.

3. Conclusion

Fucoidans present in brown seaweeds are usually very complex nonregular polysaccharides, which may be even mixtures of molecules of different structural types.[14,15] As the result, their structural analysis is extremely difficult. In contrast, the highly sulfated fucoidan fraction isolated from *F. distichus* had essentially regular structure. Its backbone is built up of alternating 3- and 4-linked α-L-fucopyranose residues, as in several other fucoidans isolated from the representatives of the order Fucales. Each disaccharide fragment of the backbone is trisulfated giving rise to the repeating unit, the structure of which could be elucidated using NMR spectroscopy of the native polysaccharide without any chemical modifications. It is important to investigate, whether this regular structure may be regarded as a chemotaxonomic marker of the species *F. distichus*. In this connection we are planning to analyze further the polysaccharide content of other samples of this alga collected from different places, as well as to characterize the other fractions separated in the course of ion-exchange chromatography of the crude fucoidan.

4. Experimental

4.1. General methods

Quantitative determination of monosaccharides and sulfate, gas–liquid chromatography, recording of IR spectra, and optical rotation measurements were carried out as described previously.[1] Acetyl content was estimated by Hestrin procedure.[16]

4.2. NMR spectroscopy

The spectra were recorded using a Bruker DRX-500 spectrometer at 303 or 333 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 ppm) and methanol (δ_C 50.15 ppm) were taken as the internal standards. The parameters used for 2D experiments were described previously.[1] The TOCSY spectra were acquired with 200 ms duration of MLEV17 spin-lock; the HMBC spectra were recorded with 60 ms delay for evolution of long-range couplings.

4.3. Isolation of fucoidan

The alga *F. distichus* was collected from the littoral of the Barents Sea (Dalnie Zelentsy, the Murmansk region) in summer 2000 and dried in air. The milled algal biomass (64 g) was treated at room temperature with a 4:2:1 MeOH–$CHCl_3$–water mixture to remove colored matter, filtered and vacuum dried to yield 45 g (70.3%) of defatted algal biomass. This material and 2% aqueous $CaCl_2$ solution (5 × 250 mL) were mechanically stirred at 85 °C for 5 h. An aqueous hexadecyltrimethylammonium bromide solution (10%, 80 mL) was added to the combined extracts. The precipitate formed was centrifuged, washed with water, stirred with 20% ethanolic NaI solution (5 × 150 mL) for 2–3 days at room temperature, washed with ethanol, and dissolved in water. The solution was dialyzed and lyophilized to give the crude fucoidan fraction (F) as sodium salt, yield 9.7 g (21.5% of dry defatted biomass), composition is given in Table 1. An aqueous solution of F (1.14 g in 50 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, 1.5, and 2.0 M), each time up to the absence of a positive reaction of eluate for carbohydrates[17] with phenol and concd H_2SO_4. All the solutions obtained were dialyzed and lyophilized, yields and composition of fractions F_1–F_5 are given in Table 1.

4.4. Preparation of sulfated disaccharides 3 and 4

A solution of *n*-propyl 3-*O*-α-L-fucopyranosyl-α-L-fucopyranoside[10] or *n*-propyl 2-*O*-α-L-fucopyranosyl-α-L-fucopyranoside[10] (10.5 mg, 0.03 mmol) in DMF (0.5 mL) was treated with SO_3·Py (119 mg, 0.75 mmol) for 1 h at room temperature, then quenched with $NaHCO_3$ (80 mg) and stirred for 1 h. The solid was filtered off and washed with MeOH (10 mL). The filtrate was treated with KU-2 (Na^+) cation-exchange resin for 2 h, the resin was filtered off, and the filtrate was concentrated to a volume of 0.5 mL. Gel chromato-

graphy of the residue on a Sephadex G-10 column (2×20 cm) by elution with water gave amorphous totally sulfated disaccharides as Na-salts (22 mg, 85%), **3**, $[\alpha]_D$ −106° (*c* 1, H_2O), and **4** $[\alpha]_D$ −104° (*c* 0.5, H_2O). The ^1H NMR and ^{13}C NMR data for **3** and **4** are presented in Table 3.

Acknowledgements

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