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Structure of a fucoidan from the brown seaweed *Fucus evanescens* C.Ag.☆

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Abstract

A fucoidan consisting of L-fucose, sulfate and acetate in a molar proportion of 1:1.23:0.36 was isolated from the Pacific brown seaweed *Fucus evanescens*. The structures of its desulfated and de-*O*-acetylated derivatives were investigated by 1D and 2D ^{1}H and ^{13}C NMR spectroscopy, and the data obtained were confirmed by methylation analysis of the native and desulfated polysaccharides. The fucoidan was shown to contain a linear backbone of alternating 3- and 4-linked α-L-fucopyranose 2-sulfate residues: →3)-α-L-Fuc*p*(2SO_3^-)-(1→4)-α-L-Fuc*p*(2SO_3^-)-(1→. Additional sulfate occupies position 4 in a part of 3-linked fucose residues, whereas a part of the remaining hydroxyl groups is randomly acetylated.

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

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. Fucoidans have been extensively studied due to their diverse biological activities, since they are potent anticoagulant,[3,4] antitumor,[5] and antiviral[6,7] agents. In addition, they can act as ligands for selectins,[8,9] protect the gastric mucosa against the proteolytic activity of gastric juice,[10] block mammalian fertilization,[11] etc. The relationships between structure and biological activities in fucoidans are not clearly established due to many difficulties connected with determination of the fine structure of polysaccharides.

Sulfated fucans isolated from echinoderms have usually linear backbones and regular sulfation patterns resulting in the formation of oligosaccharide repeating units.[12] The structures of these repeating units can be determined unambiguously, especially by using high-field NMR spectroscopy, and hence, correlation between structures and biological action of polysaccharides may be outlined.[13] Unfortunately, the structures of algal fucoidans are much more complicated. The algal polysaccharides are usually 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, usually give only partial information on their structures. Controversial data may be found in the literature even about the structure of the most carefully studied fucoidan from *Fucus vesiculosus*, which is commercially available.[14–16] It is clear that structures of algal fucoidans vary with the algal species, but their possible structural diversity is also poorly understood. Only recently it was shown that representatives of the orders Chordariales and Laminariales (Phaeosporophyceae) may contain polysaccharides with a linear backbone built up of (1→3)-linked α-L-fucopyranose residues.[17–19] This backbone may have single branches at position 2 of several fucose residues (as α-D-glucopyranosyluronic acid residues in *Cladosiphon okamuranus*[18] or α-L-fucopyranosyl residues in *Chorda filum*[19]) resulting in

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the formation of quasi-regular carbohydrate chains with hexasaccharide repeating units; but in native fucoidans this regularity is masked by random sulfation and acetylation. In contrast, fucoidans from two representatives of the order Fucales (Cyclosporophyceae), namely, *Ascophyllum nodosum* and *F. vesiculosus*, were shown to have a backbone built up of alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues.[16,20] It is very probable that the difference in backbone structures reflects the fundamental difference in fucoidan biosynthesis in the two different classes of brown algae, Phaeosporophyceae and Cyclosporophyceae, respectively, but such a statement requires confirmation by additional examples of fucoidans with definitely elucidated structures.

The present work is devoted to the structural analysis of a fucoidan isolated from the next representative of the order Fucales (Cyclosporophyceae, Phaeophyta), the Pacific brown alga *Fucus evanescens* C.Ag.

2. Results and discussion

Isolation of fucoidan.—Before the extraction of polysaccharides, the algal biomass was pretreated with a MeOH–$CHCl_3$–water mixture to remove pigments and other low-molecular weight compounds.[21] Water-soluble polysaccharides were then extracted from defatted biomass with aqueous calcium chloride at 85 °C, acid polysaccharides were precipitated from the extract by the action of hexadecyltrimethylammonium bromide (Cetavlon) and transformed into water-soluble sodium salts. The resulting crude fucoidan (F) was purified and fractionated by ion-exchange chromatography on DEAE-Sephacel using aqueous sodium chloride of increasing concentration as eluant. 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 1:1.23 and only traces of other monosaccharide constituents, was subjected to structural analysis.

Preliminary characterization and chemical modifications of F_4.—The IR-spectrum of F_4 contained an intense absorption band at 1240 cm^{-1} (S=O) common to all the sulfate esters. An additional sulfate absorption band at 824 cm^{-1} (C–O–S, secondary equatorial sulfate) and a relatively small shoulder at 845 cm^{-1} (C–O–S, secondary axial sulfate) indicated that the majority of sulfate groups occupy positions 2 and/or 3, and only a minor part of sulfate is located at position 4 of fucopyranose residues. An absorption band at 1720 cm^{-1} revealed the possible presence of *O*-acetyl groups in this polysaccharide.

Like many other native algal fucoidans, fraction F_4 had a very complex ^{13}C NMR spectrum, which was difficult to interpret completely (Fig. 1). It contained several intense signals in the anomeric (97–102 ppm) and high-field (16.5–16.7 ppm) regions, which are typical of α-fucopyranosides. The signals at 19–20 ppm confirmed the presence of *O*-acetyl groups. Unfortunately, the ^1H NMR spectrum of F_4 was poorly resolved, so we could not apply 2D procedures to assign other resonances in the ^{13}C NMR spectrum of native polysaccharide.

Several chemical modifications were carried out to simplify the structure of F_4. Three modified polysaccharide preparations were obtained as the result of desulfation (deS), deacetylation (deAc), and both desulfation and deacetylation (deSdeAc). Molar proportions of constituents and specific optical rotation values of F_4 and modified preparations are given in Table 2. Deacetylation was carried out by treatment of polysaccharides with aqueous ammonia.[19] A solvolytic desulfation procedure[22] was used to remove sulfate groups, since acid methanolysis usually results in deep degradation of fucoidans.[17] The yield of desulfated polysaccharide (deS) was 62.3% from theoretical value. The preparation still contained about 7% of residual sulfate, but attempts to split it by additional solvolytic treatment resulted in considerable loss of the material. High negative values of optical rotation of all the four preparations were consistent with α configuration of L-fucopyranose residues in these polysaccharides.

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 (%)	Uronic acids (%)
		Fuc	Xyl	Gal	Man	Glc		
F_1	3.9	35.4	6.1		0.8	4.0	n.d.	n.d.
F_2	2.6	10.7	17.4	3.0	3.7	1.1	19.6	15.6
F_3	21.4	33.2	8.1	4.5	3.5		28.9	11.4
F_4	47.4	58.7	1.6	1.6			46.5	
F_5	4.5	34.0	3.8	5.4			32.5	

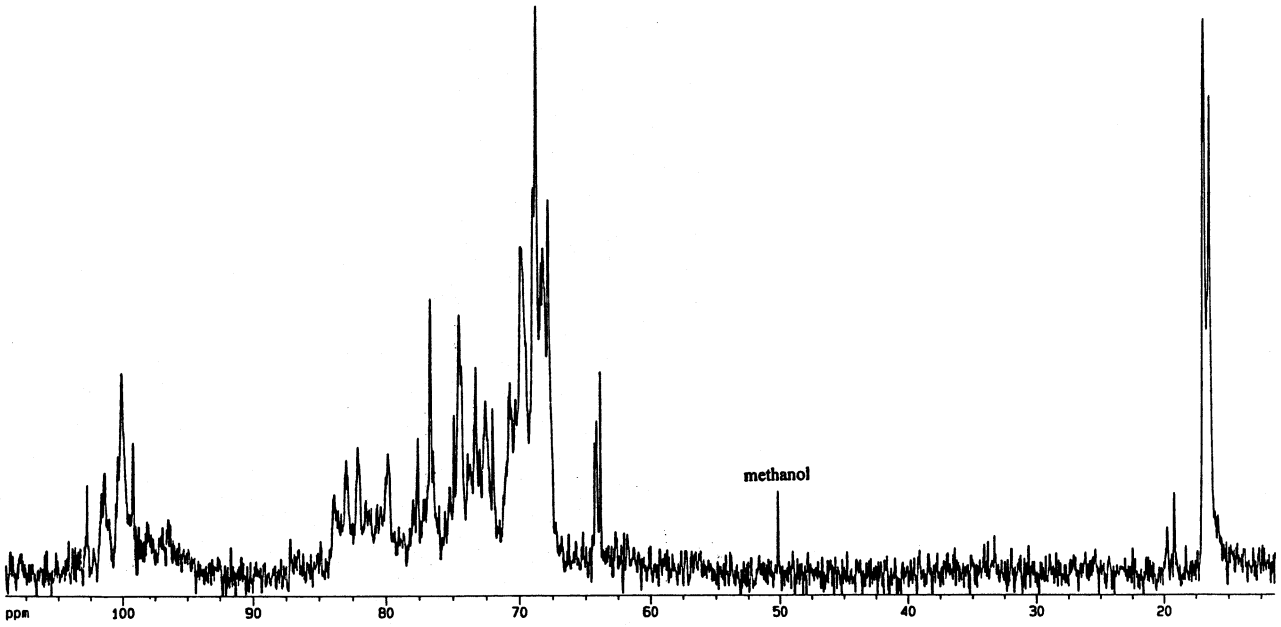


Fig. 1. ^{13}C NMR spectrum of native fucoidan F_4 (recorded at 333 K, the carbonyl region is not shown).

NMR analysis of polysaccharide preparations deSdeAc, deAc, and deS.—Both ^{1}H (Fig. 2(C)) and ^{13}C (Fig. 3) NMR spectra of desulfated and de-*O*-acetylated polysaccharide (deSdeAc) were resolved enough to apply 2D spectroscopy for the assignment of resonances in the 1D spectra. COSY and TOCSY (Fig. 4) spectra revealed the presence of α-fucose and β-xylose residues in the molecule, the former ones being of two types, **A** and **B**, differing in the mode of substitution. NOESY (Fig. 5) and ROESY spectra showed that all the fucose residues **A** (see Scheme 1 and Tables 3 and 4) were linked to C-4 of residues **B** (correlation peak 5.00/3.90 ppm), whereas all the residues **B** were linked to C-3 of residues **A** (correlation peaks 5.11/3.94 and 5.11/4.03 ppm). Analysis of the HSQC spectrum confirmed substitution of residues **A** at position 3 (downfield location of C-3 resonance at 77.2 ppm) and residues **B** at position 4 (signal C-4 at 81.2 ppm). Finally, type of substitution in these residues was confirmed by HMBC spectrum, where correlation peaks 5.00/81.2 and 5.11/77.2 ppm were observed.

Analysis of β-xylose signals present in 2D spectra revealed (1→4)-linked β-xylopyranose residues only (Table 5, cf.[28]); 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 NOESY or ROESY spectra. It was concluded that our fucoidan preparation contained a small amount of (1→4)-β-xylan, which was accidentally not separated during the purification steps. Signals corresponding to this xylan were observed only in the spectra of desulfated polysaccharides (deSdeAc and deS), where the relative xylose content was increased due to degradation of some fucoidan molecules under solvolytic desulfation conditions (Table 2). In contrast, spectra of sulfated preparations F_4 and deAc contained practically no signals belonging to xylose residues. Attempts to obtain from the spectra some information about the structural significance of galactose, another minor component of F_4, were unsuccessful.

Thus, according to spectral evidence, desulfated and de-*O*-acetylated fucoidan (deSdeAc) has a linear chain of alternating (1→3)- and (1→4)-linked α-L-fucopyranose residues (Scheme 1, structure **1**). To our knowledge, such a polysaccharide is obtained for the first time. It is interesting to compare its ^{13}C NMR spectrum with the spectra of some related polysaccharides and model compounds (Table 4). As expected, there are marked differences in the positions of anomeric and several other signals in our polysaccharide and linear α-L-fucopyranans of algal or invertebrate origin, containing (1→3) or (1→4) linkages only.[13] The chemical shifts in the spectrum of deSdeAc coincided more satisfactorily with values calculated according to additive

Table 2
Composition (molar proportions) and optical rotation of polysaccharide preparations

Sample	Fuc	Xyl	Gal	SO_3Na	$[\alpha]_D^{24}$ (° in H_2O)
F_4	44	1	1	54	−141.0
deAc	43	1	1	55	−136.0
deS	77	4	4	15	−198.8
deSdeAc	76	6	9	9	−177.0

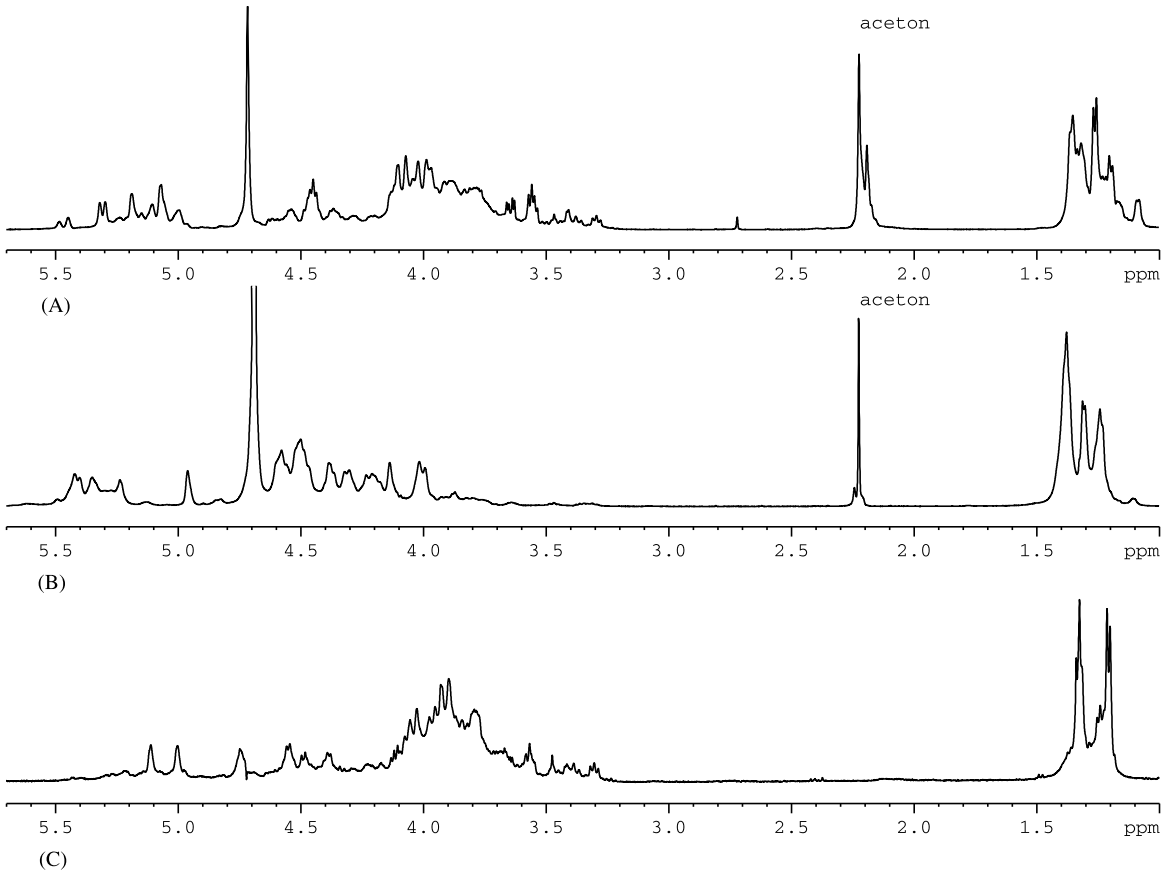


Fig. 2. ^{1}H NMR spectra of desulfated (deS, A), deacetylated (deAc, B), and desulfated and deacetylated polysaccharide (deSdeAc, C).

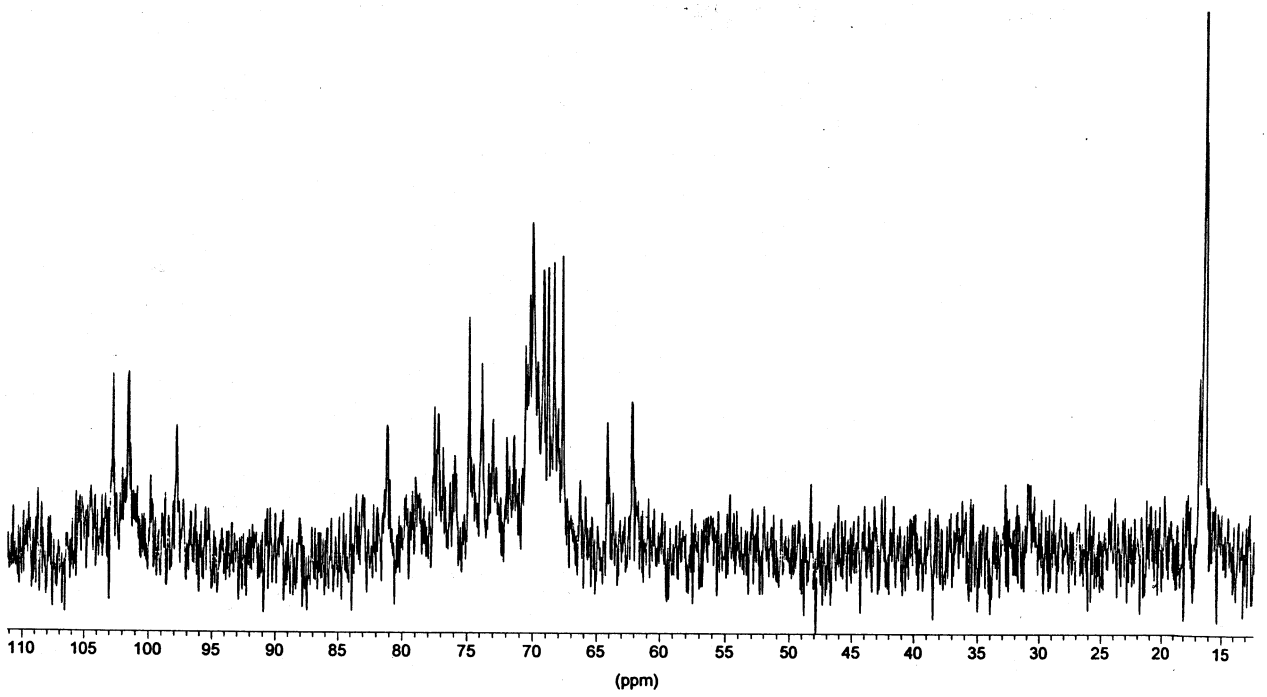


Fig. 3. ^{13}C NMR spectrum of desulfated and deacetylated polysaccharide (deSdeAc).

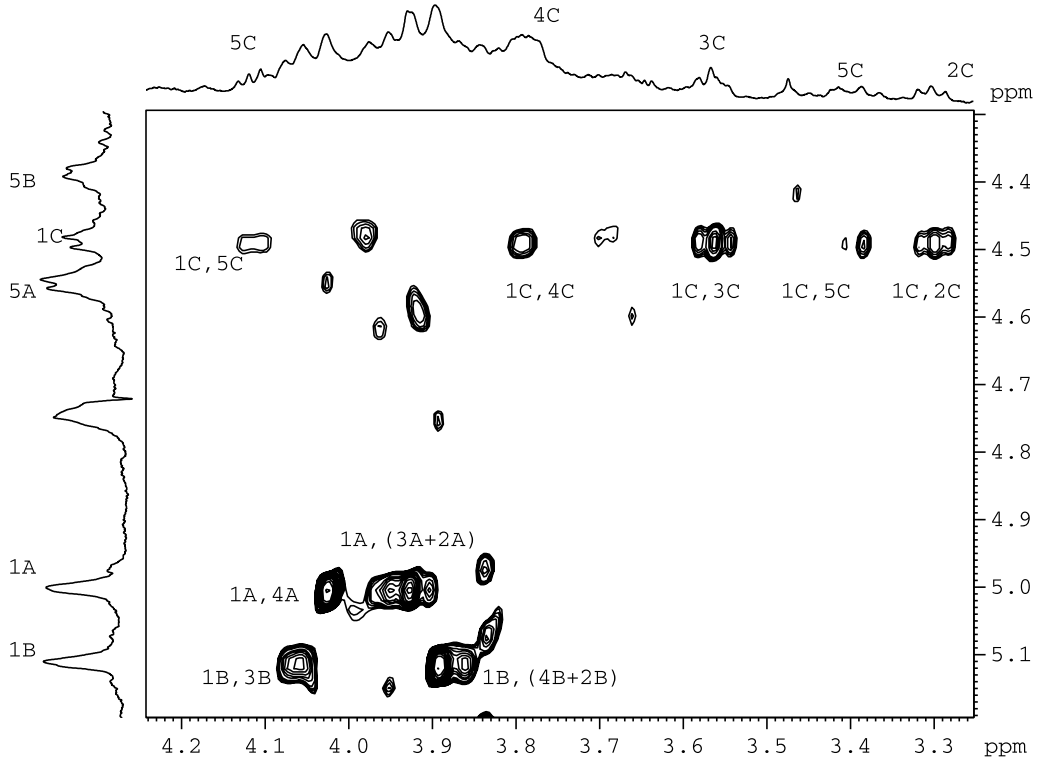


Fig. 4. A part of 2D TOCSY spectrum of desulfated and deacetylated polysaccharide (deSdeAc). Abbreviations: A, 3-linked α-L-fucopyranose; B, 4-linked α-L-fucopyranose; C, 4-linked β-D-xylopyranose.

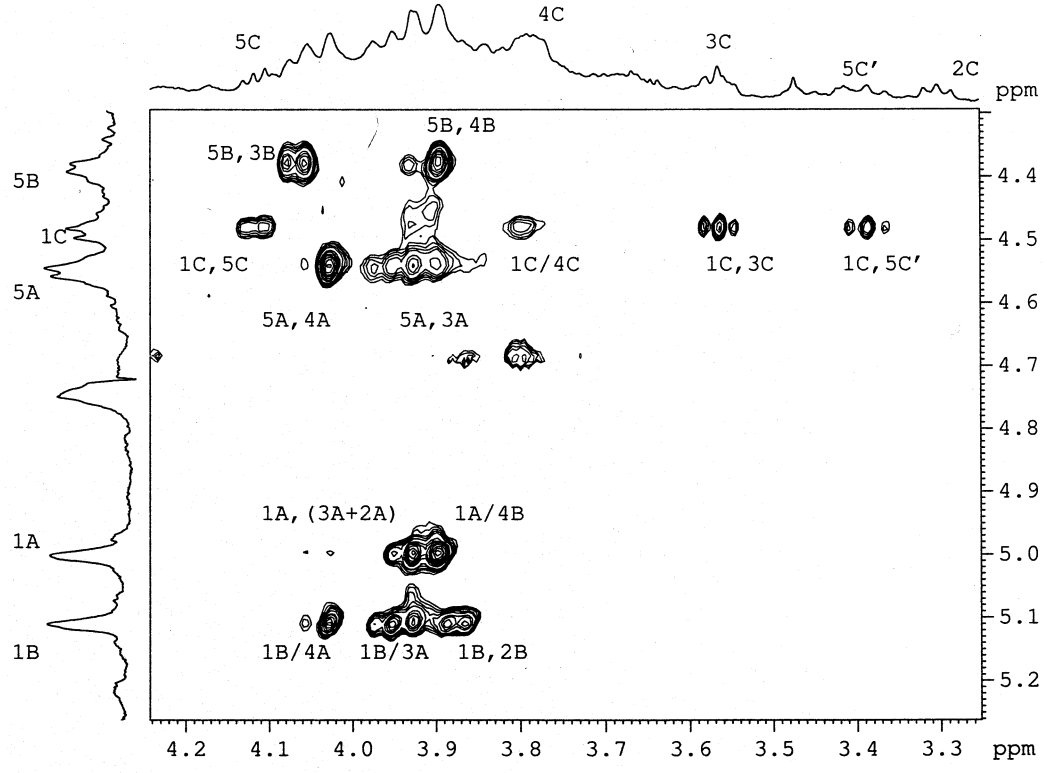
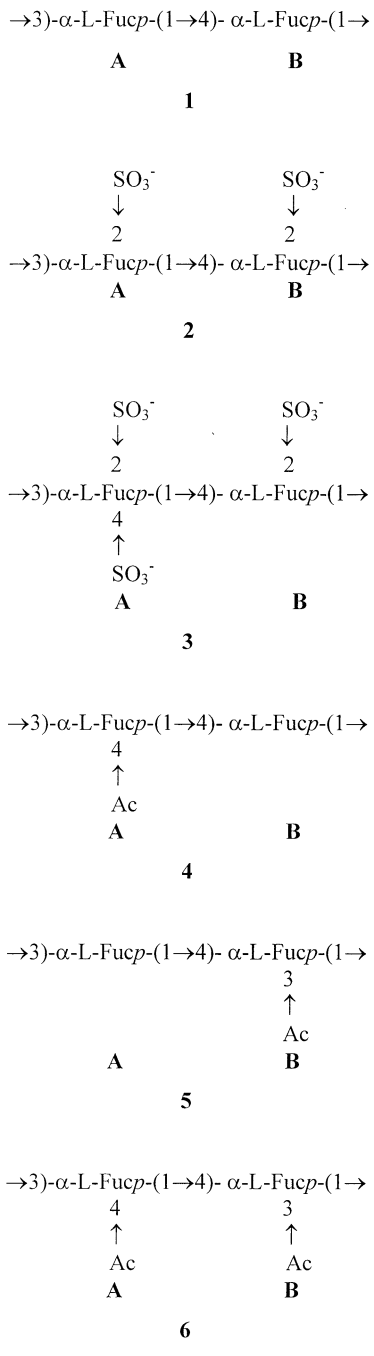


Fig. 5. A part of 2D NOESY spectrum of desulfated and deacetylated polysaccharide (deSdeAc). A, B, C, as in Fig. 4.



Scheme 1.

schemes[19,26] using ^{13}C NMR spectral data for synthetic model (1→3)- and (1→4)-α-L-fucobiosides and respective spectral glycosylation effects[27] (Table 4), but even in this case two remarkable deviations (each of 1.4 ppm) were observed for C-3 (unit **A**) and C-1 (unit **B**), which are involved in the (1→3)-bridge between units **A** and **B**. Most probably, this noticeable deviation of experimental and calculated chemical shift values in the spectrum of deSdeAc is connected with the difference of conformations of model disaccharides and respective fragments within the polysaccharide chain.

All the signals in ^{1}H (Fig. 2(B)) and ^{13}C (Fig. 6) NMR spectra of de-*O*-acetylated polysaccharide (deAc) were assigned using 2D techniques, as above (Table 6). As evidenced from the low-field shifts of H-2 and C-2 resonances in both 3- and 4-linked α-L-fucopyranose residues, all positions 2 in the polysaccharide were sulfated. Low-field shifts of H-4 and C-4 of some 3-linked residues showed that additional sulfate occupies position 4. According to the relative intensities of the corresponding signals in the assigned ^{1}H NMR spectrum, the proportion between structures **2** and **3** (see Scheme 1) was approximately 1:2.

NMR spectra of desulfated fucoidan (deS) (Figs. 2(A) and 7) were analyzed similarly to estimate the molar proportion of fucose and acetate (1:0.36) and to localize the positions of *O*-acetyl groups. It was found that O-4 of 3-linked residues and O-3 of 4-linked residues may be both free or acetylated, as followed from the low-field shifts of corresponding proton and carbon resonances (Table 7), to give structures **4**–**6** (see Scheme 1). The contents of each structure given in Table 7 were calculated from the ^{1}H NMR spectrum of deS.

Methylation analysis.—Methylation of polysaccharides was used to confirm the spectral data on their structure. Native fucoidan (F_4) and desulfated fucoidan (deS) were methylated with methyl iodide in the presence of sodium hydroxide in methyl sulfoxide.[29] F_4 was methylated in the form of both sodium and pyridinium (to enhance solubility) salts, but results of methylation were the same. Methylated polysaccharides were hydrolyzed, and the resulting mixtures of partially methy-

Table 3
^{1}H NMR data for desulfated, deacetylated fucoidan (deSdeAc)

Structure	Residue	^{1}H chemical shifts (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
1	→3)-α-L-$\overset{\mathbf{A}}{\text{F}}$ucp-(1→	5.00	3.91	3.94	4.03	4.55	1.20
	→4)-α-L-$\overset{\mathbf{B}}{\text{F}}$ucp-(1→	5.11	3.89	4.06	3.90	4.39	1.32

Table 4
^{13}C NMR data for desulfated, deacetylated fucoidan (deSdeAc, structure **1**) and some related polysaccharides

Sample	Residue	^{13}C chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
Desulfated fucoidan from *C. filum*[19]	→3)-α-L-Fuc*p*-(1→3)-	96.9	67.7	76.3	69.8	67.8	16.5
Desulfated fucoidan [a] from sea cucumber *Ludwigothurea grisea*[23,24]	→3)-α-L-Fuc*p*-(1→3)-	96.8	67.5	76.1	69.6	67.5	16.5
Desulfated fucoidan [a] from sea urchin *Arbacia lixula*[24,25]	→4)-α-L-Fuc*p*-(1→4)-	101.4			80.6		16.5
Experimental and calculated[19,26,27] (in parentheses) chemical shifts and their differences [in brackets] for structure **1**	→3)-α-L-$\overset{A}{\text{F}}$uc*p*-(1→4)	101.6	68.3	**77.2**	70.2	67.7	16.4
		(101.9)	(67.6)	(**75.8**)	(69.3)	(67.5)	(16.5)
		[−0.3]	[0.7]	**[1.4]**	[0.9]	[0.2]	[−0.1]
	→4)-α-L-$\overset{B}{\text{F}}$uc*p*-(1→3)	**97.8**	69.2	70.0	81.2	68.8	16.4
		(**96.4**)	(69.4)	(70.4)	(81.4)	(68.3)	(16.5)
		[1.4]	[−0.2]	[−0.4]	[−0.2]	[0.5]	[−0.1]

[a] Values corrected for the constant difference of −1.5 ppm.

Table 5
NMR data for xylose residues in desulfated polysaccharides (deSdeAc and deS)

Residue	^{1}H chemical shifts (ppm)					
	H-1	H-2	H-3	H-4	H-5	H-5′
→4)-β-D-Xyl*p*-(1→	4.48	3.30	3.56	3.80	4.12	3.38
	^{13}C chemical shifts (ppm)					
	C-1	C-2	C-3	C-4	C-5	
	102.8	73.8	74.8	77.5	64.1	

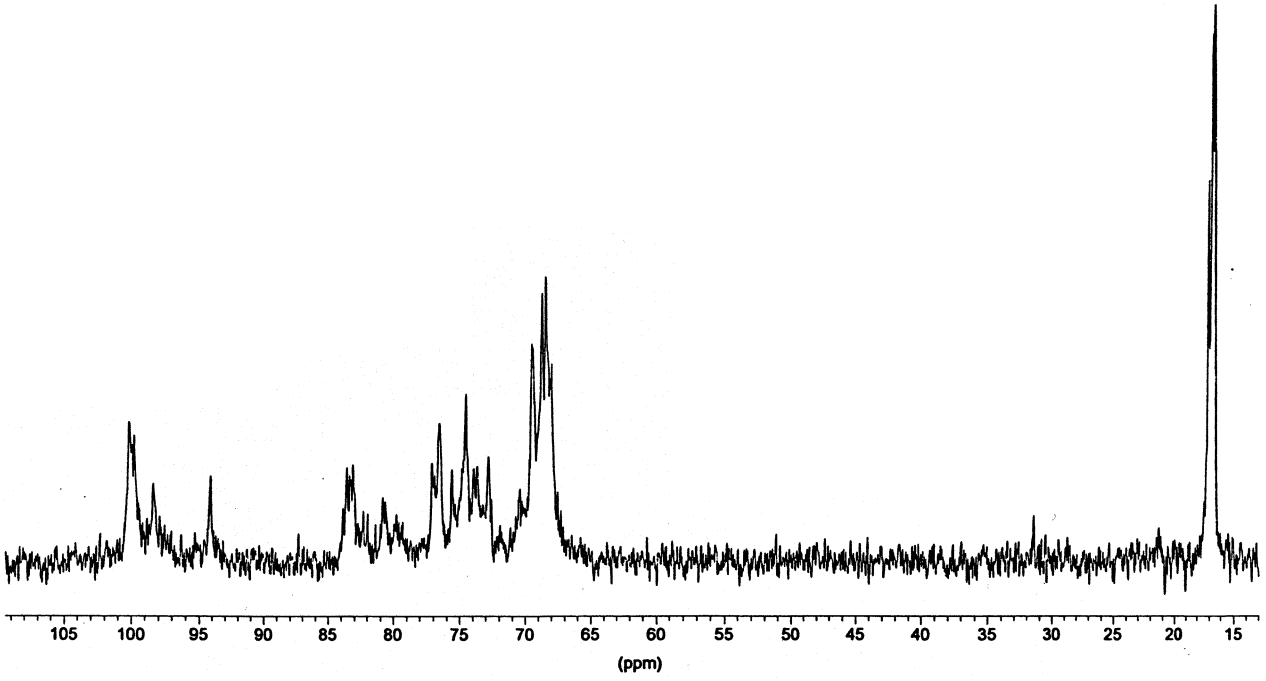


Fig. 6. ^{13}C NMR spectrum of deacetylated polysaccharide (deAc).

Table 6
NMR data for deacetylated fucoidan (deAc)

Structure	Residue	^{1}H chemical shifts (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
2 (38.5%)	**A** →3)-α-L-Fuc*p*2SO_3^--(1→	5.23	4.58	4.18	4.13	4.48	1.24
	B →4)-α-L-Fuc*p*2SO_3^--(1→	5.42	4.50	4.22	3.99	4.51	1.41
3 (61.5%)	**A** →3)-α-L-Fuc*p*2,4SO_3^--(1→	5.35	4.57	4.32	4.96	4.54	1.32
	B →4)-α-L-Fuc*p*2SO_3^--(1→	5.40	4.47	4.37	4.02	4.38	1.38
		^{13}C chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
2 (38.5%)	**A** →3)-α-L-Fuc*p*2SO_3^--(1→	100.2	74.5	72.8	69.4	68.3	16.7
	B →4)-α-L-Fuc*p*2SO_3^--(1→	94.2	76.5	68.4	83.1	68.7	16.8
3 (61.5%)	**A** →3)-α-L-Fuc*p*2,4SO_3^--(1→	100.2	75.6	73.7	80.8	68.7	17.1
	B →4)-α-L-Fuc*p*2SO_3^--(1→	98.4	76.5	68.4	83.1	69.4	16.8

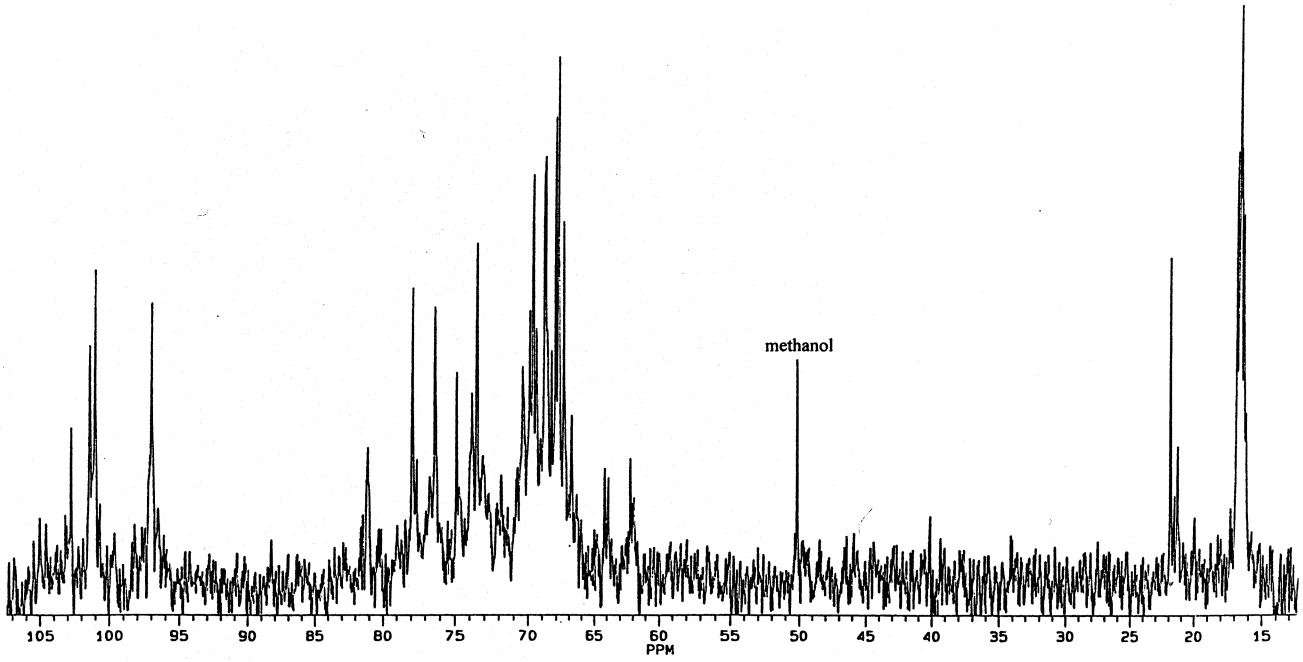


Fig. 7. ^{13}C NMR spectrum of desulfated polysaccharide (deS) (the carbonyl region is not shown).

Table 7
NMR data for desulfated fucoidan (deS)

Structure	Residue	^1H chemical shifts (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
1 (39%)	**A** →3)-α-L-Fuc*p*-(1→	5.00	3.93	3.95	4.02	4.54	1.20
	B →4)-α-L-Fuc*p*-(1→	5.16	4.07	4.05	3.88	4.37	1.31
4 (16%)	**A** →3)-α-L-Fuc*p*4OAc-(1→	5.05	3.99	4.14	5.45	4.71	1.08
	B →4)-α-L-Fuc*p*-(1→	5.16	4.07	4.05	3.88	4.37	1.31
5 (34%)	**A** →3)-α-L-Fuc*p*-(1→	5.07	3.98	4.01	4.07	4.44	1.26
	B →4)-α-L-Fuc*p*3OAc-(1→	5.18	4.12	5.30	4.11	4.45	1.36
6 (11%)	**A** →3)-α-L-Fuc*p*4OAc-(1→	5.11	4.03	4.21	5.48	4.61	1.16
	B →4)-α-L-Fuc*p*3OAc-(1→	5.18	4.12	5.30	4.11	4.45	1.36
		^{13}C chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
1 (39%)	**A** →3)-α-L-Fuc*p*-(1→	101.4	68.2	77.2	70.0	67.4	16.1
	B →4)-α-L-Fuc*p*-(1→	96.5	69.1	70.2	81.1	68.5	16.1
4 (16%)	**A** →3)-α-L-Fuc*p*4OAc-(1→	101.0	68.0	73.8	71.0	66.1	16.0
	B →4)-α-L-Fuc*p*-(1→	96.5	69.1	70.2	81.1	68.5	16.1
5 (34%)	**A** →3)-α-L-Fuc*p*-(1→	101.0	67.9	76.6	71.0	66.0	16.4
	B →4)-α-L-Fuc*p*3OAc-(1→	97.0	66.9	72.4	77.6	68.7	16.2
6 (11%)	**A** →3)-α-L-Fuc*p*4OAc-(1→	101.0	68.7	73.8	71.0	66.0	16.4
	B →4)-α-L-Fuc*p*3OAc-(1→	97.0	66.9	72.4	77.6	68.7	16.2

lated monosaccharides were analyzed as alditol acetates by GLC-MS.[30]

The molar ratios of partially methylated fucitol acetates obtained for desulfated fucoidan (deS) were as follows: acetates of 2,3,4-tri-*O*-methyl-:2,3-di-*O*-methyl-:2,4-di-*O*-methyl-:2-*O*-methyl-fucitol, 7:46:38:9. These results were consistent with the (1→3),(1→4)-backbone of fucoidan. A rather high content of terminal nonreducing fucose residues may be explained by a marked degradation of fucoidan backbone during the

desulfation procedure. Non-equal proportions of 3- and 4-linked fucose, as well as the presence of 2-*O*-methyl-fucose, are possibly due to the presence of residual sulfate at O-4 of some 3-linked fucose residues. Axial sulfate groups seem to be more resistant to solvolysis than equatorial ones: the presence of some 4-sulfated material after solvolytic desulfation of a sulfated fucan from sea cucumber was reported previously.[23]

There were three main components in the products of methylation of native fucoidan (F_4): acetates of 3-*O*-methyl-fucitol, 4-*O*-methyl-fucitol and fucitol in approximately equal amounts. Comparison of these data with the results of methylation of deS confirmed the conclusion that sulfate groups occupy position 2 in all the fucose residues. The presence of non-methylated fucitol acetate was attributed to 3-linked fucose-2,4-disulfate residues in the native polysaccharide.

3. Conclusion

Taking into account the results of spectral and chemical investigation, it may be concluded that the fucoidan isolated from the Pacific brown alga *F. evanescens* has a linear backbone of alternating 3- and 4-linked α-L-fucopyranose 2-sulfate residues. It means that the basic structure of the polysaccharide is regular and contains disaccharide-repeating units. In the native polysaccharide, this regularity is masked by partial sulfation of O-4 in 3-linked residues and by random acetylation of the remaining hydroxyl groups. A polysaccharide having similar backbone was found recently in *A. nodosum*.[16] It has a slightly different overall sulfation pattern, so direct comparison of its NMR spectra with those of our preparations is not possible. It should also be noted that its structural analysis was carried out, using an oligosaccharide fraction isolated from partial hydrolysis products of the starting polysaccharide with the yield of only 2%. Therefore, our data on the presence of alternating sequence of 3- and 4-linked α-L-fucopyranose residues as a backbone of fucoidans are more reliable. Complete assignments of resonances in the NMR spectra of this basic structure and of its sulfated and acetylated derivatives may be used for characterization of other fucoidans, which will be isolated from other species of brown seaweeds. Recently it was shown that some more complex sulfated heteropolysaccharides of brown seaweeds may also contain fucan chains consisting of 3- and 4-linked α-L-fucopyranose residues.[31] Further investigations should indicate whether the presence of alternating sequences found in *A. nodosum* and *F. evanescens* is a characteristic feature of fucoidans from all the algae belonging to the order Fucales.

4. Experimental

General methods.—Quantitative determination of neutral monosaccharides after hydrolysis of polysaccharide samples in 2 M CF_3COOH, 8 h at 100 °C, was performed using GLC of acetylated alditols and myo-inositol as an internal standard.[32] Quantitative determination of uronic acids by color reaction with concd H_2SO_4 and 3,5-dimethylphenol was carried out as described earlier.[33] Sulfate was estimated turbidimetrically[34] after hydrolysis of polysaccharides in 2 M CF_3COOH as above. Fucose was determined with concd H_2SO_4 and L-cysteine hydrochloride.[35]

GLC analyses were carried out with a Hewlett–Packard 5890A chromatograph.[17] IR spectra of polysaccharides were recorded with Perkin–Elmer 577 spectrophotometer in KBr pellets. Optical rotations were measured using a JASCO DIP-360 polarimeter for 0.9% solutions in water.

NMR spectroscopy.—The spectra were recorded using a Bruker DRX-500 spectrometer at 303 K (at 333 K for native fucoidan F_4). Samples were deuterium-exchanged by lyophilization three times with D_2O and then examined as 2–3% solutions in 99.97% D_2O, acetone (δ_H 2.225 ppm) and methanol (δ_C 50.15 ppm) were taken as the internal standards. The data were acquired and performed using XWINNMR 2.1 version. The parameters used for 2D experiments were as follows: COSY [512 × 1024 data matrix; zero-filled to 1024 data points in t_1; four scans per t_1 value; spectral width 2400 Hz; recycle delay 1 s; unshifted sine-square-bell filtering in t_1 and t_2]; ROESY [512 × 1024 data matrix; zero-filled to 1024 data points in t_1; 16 scans per t_1 value; spectral width 2400 Hz; mixing time 200 ms; shifted sine-squared filtering in t_1 and t_2]; NOESY [512 × 1024 data matrix; zero-filled to 1024 data points in t_1; eight scans per t_1 value; spectral width 2400 Hz; mixing time 600 ms; shifted sine-squared filtering in t_1 and t_2]; TOCSY [512 × 1024 data matrix; zero-filled to 1024 data points in t_1; eight scans per t_1 value; the duration of the MLEV17 spin-lock was 60 ms]; HSQC [256 × 1024 data matrix; zero-filled to 512 data points in t_1; 40 scans per t_1 value; spectral width in t_1 2400 Hz and in t_2 11970 Hz; recycle delay 1.0 s; shifted sine-squared filtering in t_1 and t_2]; HMBC [512 × 1024 data matrix; 56 scans per t_1 value; spectral width in t_2 2400 Hz and t_2 22680 Hz; recycle delay 1.0 s; optimization of the experiment for coupling constant 8 Hz].

Isolation of fucoidan.—The alga *F. evanescens* was collected from the littoral of Iturup island (Kuril Islands) in August of 1997, soaked in acetone and dried in air. The milled algal biomass was treated at rt with a 4:2:1 MeOH–$CHCl_3$–water mixture to remove colored matter, filtered and vacuum dried. Then the mixture of defatted algal biomass (15 g) and 2% aq $CaCl_2$ (4 × 150 mL) was mechanically stirred at 85 °C for 5 h. An aq

hexadecyltrimethylammonium bromide solution (10%, 50 mL) was added to the combined extracts. The precipitate formed was centrifuged, washed with water, stirred with 20% ethanolic NaI solution (3 × 60 mL) for 2–3 days at rt, washed with EtOH, and dissolved in water. The solution was dialyzed and lyophilized to give crude fucoidan fraction (F) as sodium salt, yield 1.94 g (12.9% of dry defatted biomass); composition: fucose, 42.4%; SO_3Na, 35.8%; xylose, 3.6%; galactose, 2.9%; mannose, 1.2%; glucose 0.7%. An aqueous solution of F (1.54 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[36] with phenol and concd H_2SO_4. All the solutions obtained were dialyzed and lyophilized, yields of fractions F_1–F_5 being 0.06, 0.04, 0.33, 0.73, and 0.07 g, respectively. Composition of these fractions is given in Table 1.

Desulfation of fucoidan.—To convert F_4 into pyridinium salt an aqueous solution of fucoidan was passed through a Dowex 50W × 4 (PyH^+-form) column, the eluate was concentrated and freeze-dried. Solvolytic desulfation of F_4 (as pyridinium salt) was carried out as described earlier.[17] Yield of desulfated fucoidan (deS) was 80 mg from 240 mg of the starting material, residual SO_3Na (6.7%).

De-O-acetylation of polysaccharides.—Samples of F_4 and deS were treated with aqueous ammonia at 37 °C to remove acetyl groups.[19]

Methylation analysis of polysaccharides.—Methylation of fucoidans followed by hydrolysis and GLC-MS of partially methylated fucitol acetates was performed as previously described.[17,19]

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