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A study of fucoidan from the brown seaweed *Chorda filum*☆

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Abstract

Fucoidan fractions from the brown seaweed *Chorda filum* were studied using solvolytic desulfation. Methylation analysis and NMR spectroscopy were applied for native and desulfated polysaccharides. Homofucan sulfate from *C. filum* was shown to contain poly-α-(1→3)-fucopyranoside backbone with a high degree of branching, mainly of α-(1→2)-linked single units. Some fucopyranose residues are sulfated at O-4 (mainly) and O-2 positions. Some α-(1→3)-linked fucose residues were shown by NMR to be 2-O-acetylated. The ^{1}H and ^{13}C NMR spectra of desulfated, deacetylated fucan were completely assigned. The spectral data obtained correspond to a quasiregular polysaccharide structure with a branched hexasaccharide repeating unit. Other fucoidan fractions from *C. filum* have more complex carbohydrate composition and give rather complex methylation patterns.

→3)-α-L-Fuc*p*$_{(0.7)}$-(1→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)
↑
α-L-Fuc*p*-(1

Keywords: Fucoidans; Sulfated polysaccharides; Methylation; Methylation analysis; NMR; *Chorda filum*

1. Introduction

Fucoidans, polysaccharides containing substantial percentages of L-fucose and sulfate ester groups, are constituents of brown algae (see recent review [2] and Refs. therein) and some marine invertebrates (see for example [3]). For the past decade algal fucoidans have been extensively studied due to their numerous biological activities: anticoagulant and an-

Abbreviations: HPAEC, high-performance anion exchange chromatography; SIM, selective ion mode; TIC, total ion current; tr., traces.

☆ Polysaccharides of algae, Part 54. For Part 53, see Ref. [1].

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tithrombotic [2], antiinflammatory [4,5], antitumour [6], contraceptive [7] and antiviral [8,9]. It has also been shown that fucoidans block cell–cell binding mediated by P- and L-selectin recognition of oligosaccharides terminated with either sialyl Lewis^x or sialyl Lewis^a sequence [5,10,11]. Biological activities of fucoidans occur mainly due to their high degree of sulfation, although these activities possibly depend on fine structural peculiarities and molecular weight [2].

Despite numerous structural studies of algal fucoidans, their fine structure remains unclear due to the absence of strict regularity and the presence of numerous minor components in some of them (pentoses, hexoses, uronic acids, and sometimes protein component; for review see [2]). Most biological studies have been done on commercial preparations of fucoidan from *Fucus vesiculosus*. From the early work of Conchie and Percival [12], the structure built up mainly of (1→2)-linked, 4-*O*-sulfated fucopyranose residues has been generally accepted for fucoidan from *F. vesiculosus*. Recently, the structure of this fucoidan was revised as a result of GLC–MS data obtained from methylation analysis. It was concluded that the main chain of the polysaccharide contains (1→3)-linked fucopyranose residues with some branches attached at O-2 or O-4 positions and some of the residues are 4-O-sulfated [13]. The (1→3)-linked fucopyranose residues were also established as the main component for fucoidans from *Ecklonia kurome* [14] and *Laminaria saccharina* [1].

The Pacific brown alga *Chorda filum* (L.) Lam. was shown previously to contain an unusual laminaran, which has been extensively studied by us [15–17], and a fucoidan, which was mainly a homofucan sulfate containing only traces of non-fucose constituents [15]. The present work is devoted to the structural analysis of fucoidan isolated from this alga.

2. Experimental

Materials.—Fucoidan fractions from *C. filum* were prepared by sequential extraction and ion-exchange chromatography on DEAE-Sephadex A-25. After extraction of low-molecular-weight components with a 2:4:1 $CHCl_3$–MeOH–water mixture followed by 80% aq EtOH, the algal biomass was extracted successively with 2% $CaCl_2$ at 20 and 70 °C (fucoidan A and laminaran), then with dilute (pH 2) aq HCl (fucoidan B) and 3% Na_2CO_3 (sodium alginate and fucoidan C); alginate was precipitated as the calcium salt. All of the polysaccharide extracts were dialysed and the resulting solutions were freeze-dried. Fraction A was applied on a DEAE-Sephadex A-25 column and eluted with water and aq NaCl (0.5, 1.0, 1.5, 2.0 M); saline eluates gave fucoidans A-0, A-1, A-2, and A-3, respectively [15]. Fractions C-1 and C-2 were obtained by ion-exchange chromatography on DEAE-Sephadex A-25 (1.0 and 1.5 M aq NaCl, respectively). Yields and carbohydrate composition for fucoidans from *C. filum* are given in Table 1.

The following reagents and solvents were used: Me_2SO (Romil), MeI (Lancaster Chemicals), $NaBH_4$ and sodium borodeuteride (Aldrich), NaOH (BDH), TFA, MeCN and $CHCl_3$ (HPLC grade, Rathburn), and sodium metaperiodate (Sigma).

General methods and analytical techniques.—Methylation of fucoidans was performed using a modification of the NaOH slurry method previously described [18]. Samples (1.1–3.5 mg) were solubilised in Me_2SO (1–2 mL) at 40 °C and powdered NaOH (100 mg) was added to the soln followed by MeI (0.2 mL). The mixture was vortexed for 20 min and then the addition of NaOH and MeI was repeated. The mixture was vortexed periodically for 1 h, then cooled on ice before the reaction was terminated by the addition of 1 mL of water. The excess MeI was removed by passing dry nitrogen for 10–15 min and the resulting soln was passed through a Sep-Pak® C18 cartridge (Waters Ltd.), which was washed with water (5 mL, discarded). Eluates with 15, 35, 50, and 75% MeCN (2 mL each) were collected and freeze-dried; the subsequent eluates with 100% MeCN and EtOH were dried under a flow of nitrogen. For GLC–MS analysis, methylated fucoidans were hydrolysed in 2 M TFA at 100 °C for 6 h then the resulting monosaccharides were reduced with $NaBH_4$ and acetylated with Ac_2O in Py. Eluates with aq MeCN gave

comparable chromatograms; the data for the eluate with 75% MeCN are presented in Section 3.

Desulfation of fucoidans was carried out using their pyridinium salts [19]. An aq soln of fucoidan A-2 (90 mg) was passed through a Dowex 50W × 4 (PyH^+) column, the eluate was concd and freeze-dried, the polysaccharide was dissolved in Me_2SO (10 mL) and Py (0.2 mL) was added. The soln was heated in a sealed tube at 100 °C for 10 h and then dialysed against pure water to remove Me_2SO. The soln was concd and freeze-dried, affording desulfated fucoidan D-2 (yield 13.3 mg (20%), residual SO_3Na 0.7%). Desulfation of the other fucoidan fractions A-1 and A-3 was carried out analogously, affording D-1 (yield 40%, residual SO_3Na tr.) and D-3 (yield 27%, residual SO_3Na ca. 2%), respectively.

For deacetylation of the desulfated fucoidan, the polysaccharide D-2 was dissolved in water (1.0 mL) and concd aq NH_3 (1.0 mL) was added. The soln was kept overnight at 37 °C and evaporated to dryness on a rotary evaporator. The residue was purified on a Sephadex G-15 column, and the high-molecular-weight fraction was pooled and evaporated to dryness; after isotopic exchange with D_2O (3 × 1 mL), the NMR sample of DD-2 was prepared.

For periodate oxidation/Smith degradation of fucoidans an aliquot of fucoidan D-2 (4.2 mg) was placed in a screw-capped tube, dissolved in water (1.0 mL), 0.01 M $NaIO_4$ (4.0 mL) was added and the soln was kept at 4 °C for 5 days; oxidation was monitored by decrease in absorption at λ 305 nm. Consumption of periodate was ca. 0.33 mol per mol of fucose residue. The reaction was terminated with ethylene glycol, the reaction mixture was dialysed (cutoff 3500 Da) and freeze-dried. The residue was dissolved in 0.1 mL of water and reduced with $NaBH_4$ (5 mg) overnight. Mild, acid hydrolysis was carried out with aq TFA (ca. 0.2 mL of TFA in 4 mL of water, adjusted to pH 1.3) at 37 °C for 16 h. TFA was neutralised with aq NH_3, the solvent was partially removed in a stream of nitrogen and then freeze-dried. The substance was reduced again as above and freeze-dried. To remove borate, the residue was dissolved in a 10:1 MeOH–AcOH mixture and evaporated in a stream of nitrogen; the procedure was repeated twice. Methylation, Sep-Pak procedure, and GLC–MS analysis for desulfated, Smith-degraded polysaccharide DSD-2 were carried out as above.

Partially methylated alditol acetates were analysed by GLC–MS with a Fisons MD-800 instrument (Rtx-5 (5% diphenyl methylpolysiloxane) Restek Thames capillary column, 30 m length, 0.25 mm internal diameter, 0.25 μm liquid phase thickness), temperature programme: 65 °C (1 min), 8 °C/min to 290 °C; carrier gas He, splitless mode. The EI mass spectra were interpreted using the data from Refs. [13,20] and the reference samples of partially methylated fucitol acetates. GLC analyses of alditol acetates were carried out with a Hewlett–Packard 5890A chromatograph as previously described [16].

Table 1
Yields and composition of fucoidan fractions from *C. filum*

Polysaccharide fraction	Yield [a]	Neutral carbohydrates, molar ratio [b] Fuc:Xyl:Man:Glc:Gal	SO_3Na (%)	Uronic acid (%)	Fucose (%)
A	21		n.d. [c]	n.d.	n.d.
A-0	(0.45)	1.00:0.14:0.15:0.40:0.10	n.d.	n.d.	n.d.
A-1	(5.6)	1.00:<0.01:0.075:0.02:<0.01	17	6	50
A-2	(28.6)	1.00:<0.01:<0.01:<0.01:0.02	26.5	0	64
A-3	(11.6)	1.00:<0.01:0.01:0.02:<0.01	26.5	0	65
B	10.5	1.00:<0.01:0.02:0.04:0.03	17	9 [d]	44
C	1.5	n.d.	9	8	20
C-1	(32)	1.00:0.04:0.10:0.05:<0.01	13	5.5	42
C-2	(24.3)	1.00:0.15:0.10:0.06:0.07	13	3.5	34

[a] In % from biomass for A, B, and C, and chromatographic recovery for sub-fractions is given in parentheses.
[b] GLC of alditol acetates.
[c] n.d., not determined.
[d] Mainly GlcA (HPAEC).

IR spectra of polysaccharides were recorded with Perkin–Elmer 577 (KBr tablets) and Specord M-80, Karl Zeiss (suspension in Nujol) spectrometers.

Sulfate content was determined turbidimetrically [22]. Uronic acids were determined with *m*-hydroxydiphenyl (Aldrich) using glucuronolactone (Fluka) for calibration [23]. Fucose was determined with cysteine hydrochloride [24].

For NMR spectroscopy, samples were deuterium-exchanged by evaporating three times with D_2O and then examined in solutions of 99.97% D_2O with acetone as the internal standard (δ 31.45). The spectra were recorded at 60 °C with a Bruker DRX-500 spectrometer. The data were acquired and performed using XWINNMR version 1.1. The parameters used for 2D experiments were as follows: COSY (512×1024 data matrix; zero-filled to 1024 data points in t_1; eight scans per t_1 value; spectral width 2250 Hz; recycle delay 1 s; unshifted sine-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 2250 Hz; mixing time 200 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; 16 scans per t_1 value; the duration of the MLEV17 spin-lock was 60 ms); HMQC (256×1024 data matrix; zero-filled to 512 data points in t_1; 40 scans per t_1 value; spectral width in t_1 2250 Hz and in t_2 11,300 Hz; recycle delay 1.0 s; shifted sine-squared filtering in t_1 and t_2); HMBC (512×2048 data matrix; 96 scans per t_1 value; spectral width in t_1 2250 Hz and t_2 11,300 Hz; recycle delay 1.0 s; optimisation of the experiment for coupling constant 8 Hz); HMQC-TOCSY [21] were done as for HMQC with mixing time 100 ms and recycle delay 0.5 s.

3. Results and discussion

Preparation and preliminary characterisation of polysaccharides.—The sequential extraction for preparation of fucoidan fractions from *C. filum* (L.) Lam. has been described in our previous paper [15] and briefly in Section 2 (see above). Yields and carbohydrate composition for fucoidans from *C. filum* are given in Table 1.

Size-exclusion chromatography on Sepharose CL-6B showed that fractions A-2 and A-3 have high molecular weight (higher than 100 kDa), whereas other fractions (A-1, B, and C-1) have a broad molecular-weight distribution (10–1000 kDa).

Methylation analysis of desulfated fucoidans from C. filum.—Solvolytic desulfation was shown to be milder than acid methanolysis; the latter results in deep depolymerisation of fucoidans [1,14]. Desulfated fucoidans D-1, D-2, and D-3 were prepared from A-1, A-2, and A-3, respectively. The TIC integral ratios of partially methylated fucitol acetates obtained for desulfated fucoidans are as follows (acetates of 2,3,4-*O*-methyl-:2,3-*O*-methyl:2,4-*O*-methyl-:2-*O*-methyl-:4-*O*-methyl-:fucitol): D-1, 23:3:41:16:16:1; D-2, 14:1:55:5:24:tr.; D-3, 9:2:75:3:9:tr. Thus, these fucans contain mainly the $(1 \rightarrow 3)$-linked backbone (1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylfucitol exhibits the most intense peak, for D-2; see also Fig. 1); D-2 is more branched than D-3. Branches are mainly attached by $(1 \rightarrow 2)$-linkages (for all three polysaccharides) and $(1 \rightarrow 4)$-glycosidic bonds (for D-1, to a much less extent for D-2 and D-3), but there might be only a negligible percentage of links bearing simultaneously two branches (very low percentage of fucitol acetate, the latter may arise for the presence of residual sulfate). Note that relative TIC values are not absolutely adequate for the molar percentage of the corresponding residues, and this feature may explain the fact that proportions of 2-*O*- and 4-*O*-methyl fucose derivatives (branching points in the central chain) are always above those of 2,3,4-tri-*O*-methyl fucose (non-reducing ends). GLC–MS makes it possible to distinguish isomeric fucitol acetates that have similar retention times (i.e., 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylfucitol and 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methylfucitol; 1,2,4,5-tetra-*O*-acetyl-3-*O*-methylfucitol and 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylfucitol for Rtx-5); SIM chromatograms are especially useful for these cases.

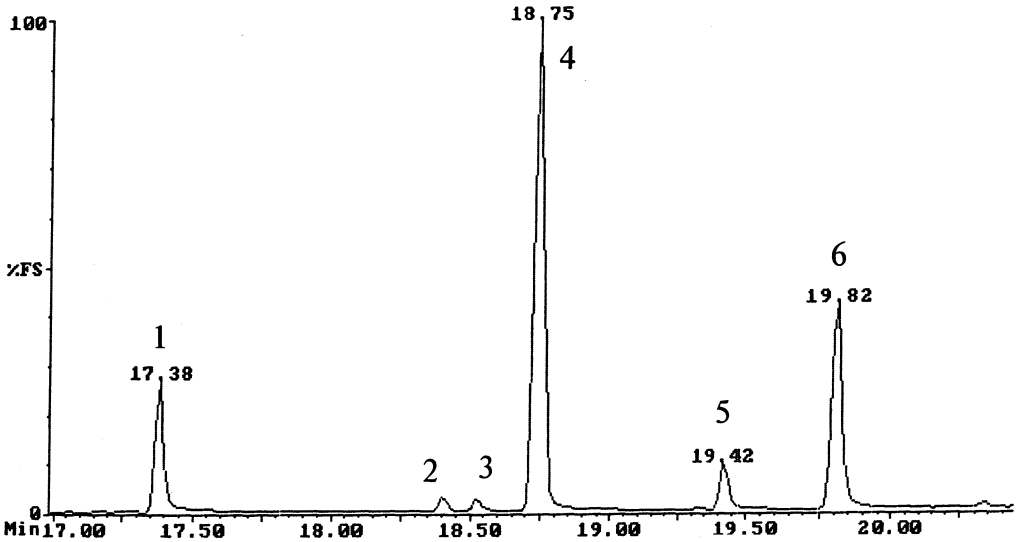


Fig. 1. GLC–MS of partially methylated alditol acetates derived from desulfated fucoidan D-2 (TIC chomatogram). **1**, 1,5-Di-*O*-acetyl-2,3,4-tri-*O*-methylfucitol; **2**, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylxylitol; **3**, 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methylfucitol; **4**, 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methylfucitol; **5**, 1,3,4,5-tetra-*O*-acetyl-2-*O*-methylfucitol; **6**, 1,2,3,5-tetra-*O*-acetyl-4-*O*-methylfucitol.

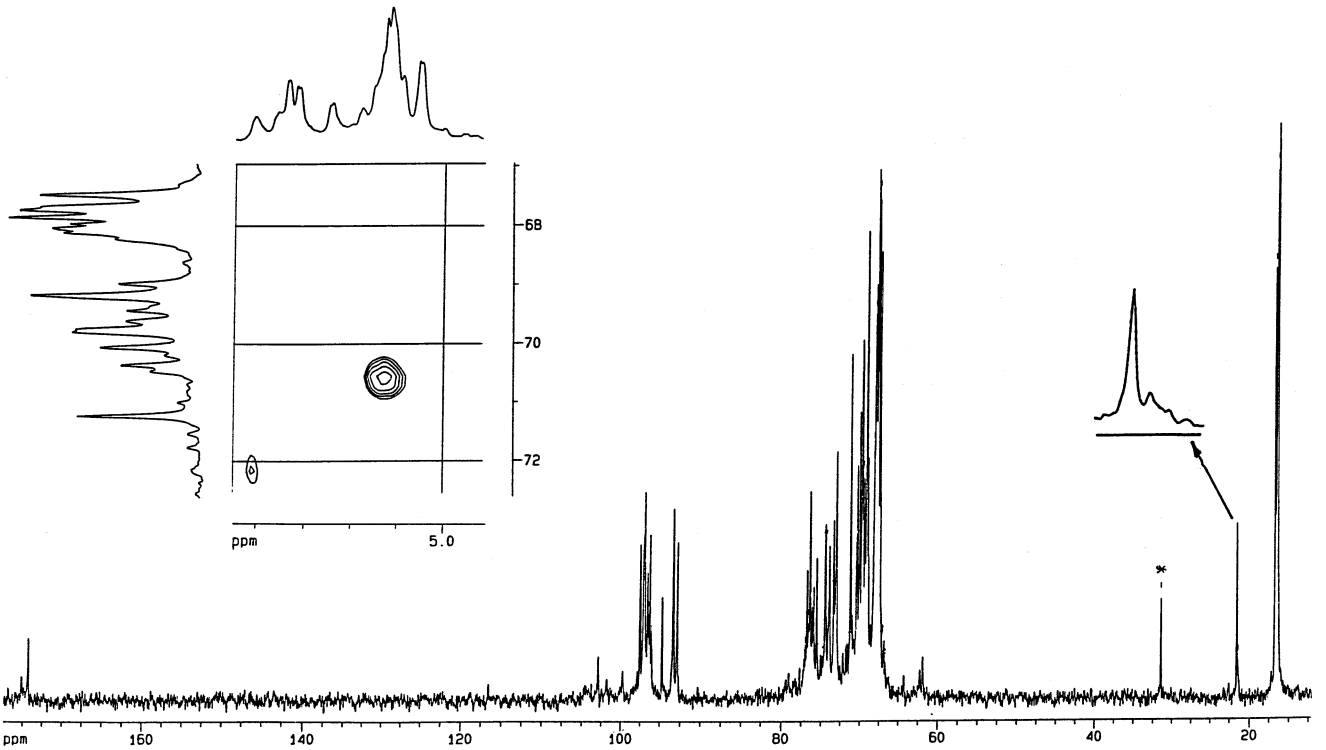
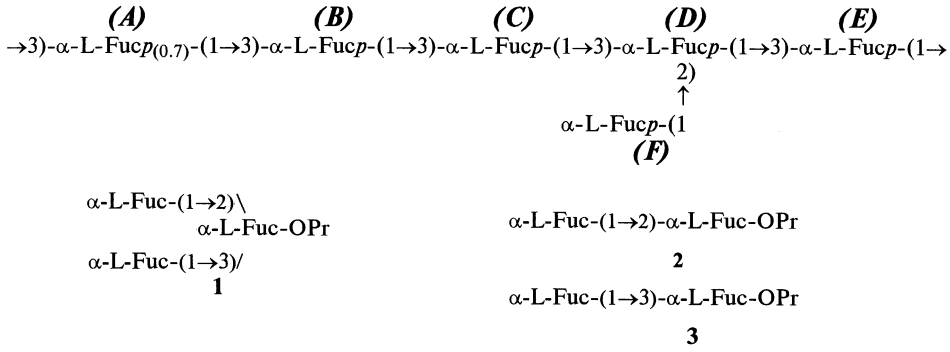


Fig. 2. ^{13}C NMR spectrum of desulfated fucoidan D-2. * Acetone signal. A fragment of HMQC spectrum is presented in an inset (left) to prove the presence of O-acetate. The acetate CH_3 area is expanded (right inset) (see text for details).

^{13}C NMR spectrum of desulfated fucoidan.—The ^{13}C NMR spectrum of D-2 is given in Fig. 2. In the anomeric region, at least ten signals were observed. All of them could be assigned to α-Fuc residues ([25,26] and reference samples, see below). Two signals were observed at δ 21.60 (larger) and δ 21.46 (smaller), which unequivocally indicate the presence of *O*-acetyl groups (ca. 20% mol. on average per fucosyl residue according to the ratio of the corresponding acetate signals at ca. δ 2.2 and that of deoxy protons at ca. δ



Scheme 1.

1.6 in the ^{1}H NMR spectrum). Previously, small acetate signals were reported for fucoidans from *F. vesiculosus* and *Laminaria brasiliensis*, but they were assigned to 'contaminating acetate, introduced during the purification' [3]. In fact, the presence of the corresponding cross-peak in the HMQC NMR spectrum (left inset) provides the evidence that acetate is a constituent of the polysaccharide, not an impurity. In fact, acetylation shifts significantly the resonance of the geminal proton, resulting in its overlap with peaks of anomeric protons, whereas the α-effect for corresponding carbon is relatively small (ca. +2–3 ppm); therefore, the aforementioned unusual HMQC cross-peak occurs, thus indicating the presence of acetate ester. Note that *O*-acetyl groups are completely removed in alkaline medium during methylation, thus not interfering with methylation analysis. From 2D homonuclear ^{1}H/^{1}H COSY, ROESY, and heteronuclear HMQC-TOCSY NMR spectra one can conclude that these acetyl groups are attached at O-2 in two different α-(1→3)-linked fucose residues (C-1: δ 94.7 and 93.4, H-1: δ 5.25 and 5.32, respectively). α-(1→3, 1→2)-Fuc residues give signals of anomeric carbons at δ 93.4 and 92.9; this splitting possibly arises due to the effect of the acetate attached to a neighbouring fucose residue.

^{13}C NMR spectrum of desulfated, deacetylated fucoidan.—The ^{13}C NMR spectrum of desulfated, deacetylated polysaccharide DD-2 is not as complex as that of D-2 and contains only five signals in the anomeric region (Fig. 3). Four of them are of approximately equal intensity and one signal at δ 96.9 is stronger (see also Tables 2 and 3 and Scheme 1).

The anomeric region of the ^{1}H NMR spectrum (Fig. 4) also displays five doublets (^3J ca. 4 Hz); four of them have similar integral intensities (δ 5.055, 5.15, 5.19, and 5.37) and the other signal at δ 5.12 is 1.7 times more intense.

Both ^{1}H and ^{13}C NMR spectra were assigned using 2D homonuclear ^{1}H/^{1}H COSY, TOCSY and ROESY spectra and 2D heteronuclear ^{1}H-detected HMQC, HMQC-TOCSY [21] and HMBC spectra. COSY and TOCSY spectra confirm that all of the anomeric signals belong to α-fucopyranoside residues. Since one cannot assign H-5 and H-6 signals of fucopyranoside residues from both COSY and TOCSY spectra due to the small value of $^3J_{H\text{-}4,H\text{-}5}$, a ROESY spectrum was recorded to determine the positions of all H-5 and H-6 using correlation of H-4 with H-5 and H-6 peaks of the same residue. The analysis of the ^{1}H chemical shifts revealed that the signal at δ 5.12 belongs to at least two residues having similar nearest environment, whereas the other four residues differ in environment and, possibly, the types of glycosylation from those two mentioned above. The ROESY spectrum was found to be insufficient for determination of linkage positions and sequence of the residues due to partial overlap of H-2 and H-3 signals for some residues. ROESY definitely revealed the presence of a (1→2)-linkage between residues ***F*** and ***D*** (correlation peaks H-1(***F***)/H-1(***D***) at δ 5.15/5.37).

The sequence and linkage positions were established using heteronuclear spectra. The analysis of HMQC (not shown) and HMQC-TOCSY spectra (Fig. 5) revealed the positions of ^{13}C signals for each residue (Table 2). The

Table 2
Experimental and calculated [a] (in parentheses) ^{13}C NMR chemical shifts for desulfated, deacetylated fucoidan from *C. filum* (DD-2)

	Residue	^{13}C chemical shifts (ppm)					
		C-1	C-2	C-3	C-4	C-5	C-6
(A,B)	→3)-α-L-Fuc*p*-(1→	96.9 (96.4)	67.7 (67.6)	76.3 (75.6)	69.75 (69.4)	67.85 (67.9)	16.5 (16.5)
(C)	→3)-α-L-Fuc*p*-(1→	96.0 (96.2)	67.4 (67.6)	76.3 (75.6)	69.6 (69.4)	68.2 (67.9)	16.7 [b] (16.5)
(D)	→2, 3)-α-L-Fuc*p*→	93.3 (92.3)	70.1 (70.6)	73.8 (72.9)	69.1 (68.6)	67.5 (67.6)	16.5 (16.5)
(F)	α-L-Fuc*p*-(1→	96.35 (96.6)	69.1 (68.95)	71.2 (71.1)	73.0 (72.9)	68.2 (68.3)	16.8 [b] (16.5)
(E)	→3)-α-L-Fuc*p*-(1→	97.0 (96.4)	67.7 (67.6)	76.1 (75.6)	69.6 (69.4)	67.7 (67.9)	16.5 (16.5)

[a] Chemical shifts were calculated as described [29] from the data [26] for 2,3-branched trifucoside **1** and constituting (1→2)- and (1→3)-linked difucosides **2** and **3**.
[b] The assignments may be interchanged.

Table 3
^{1}H NMR data for desulfated, deacetylated fucoidan from *C. filum* (DD-2)

	Residue	^{1}H chemical shifts (ppm)					
		H-1	H-2	H-3	H-4	H-5	H-6
(A,B)	→3)-α-L-Fuc*p*-(1→	5.12	3.99, 3.98	4.02	4.05	4.33	1.25
(C)	→3)-α-L-Fuc*p*-(1→	5.19	4.02	3.85	4.04	4.175	1.30 [a]
(D)	→2, 3)-α-L-Fuc*p*-(1→	5.37	4.145	4.23	4.11	4.33	1.23
(F)	α-L-Fuc*p*-(1→	5.15	3.86	3.755	3.805	4.175	1.28 [a]
(E)	→3)-α-L-Fuc*p*-(1→	5.055	3.99	4.03	4.05	4.33	1.25

[a] The assignments may be interchanged.

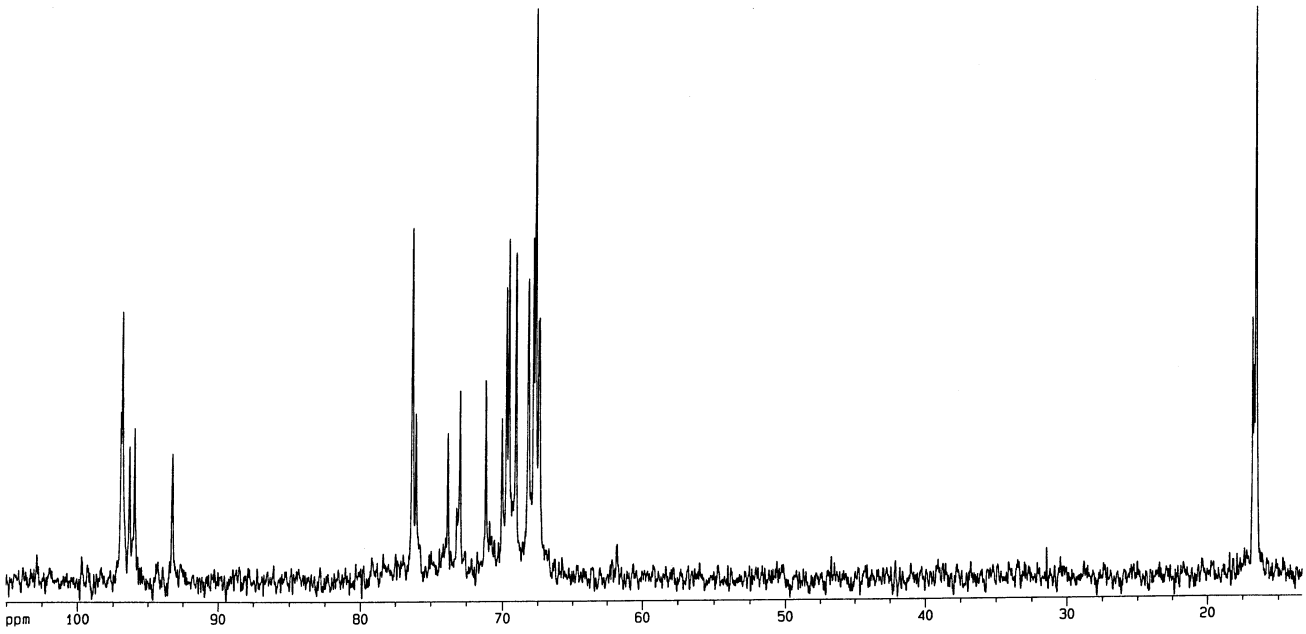


Fig. 3. ^{13}C NMR spectrum of desulfated, deacetylated fucoidan DD-2 (see text and Table 2 for assignments).

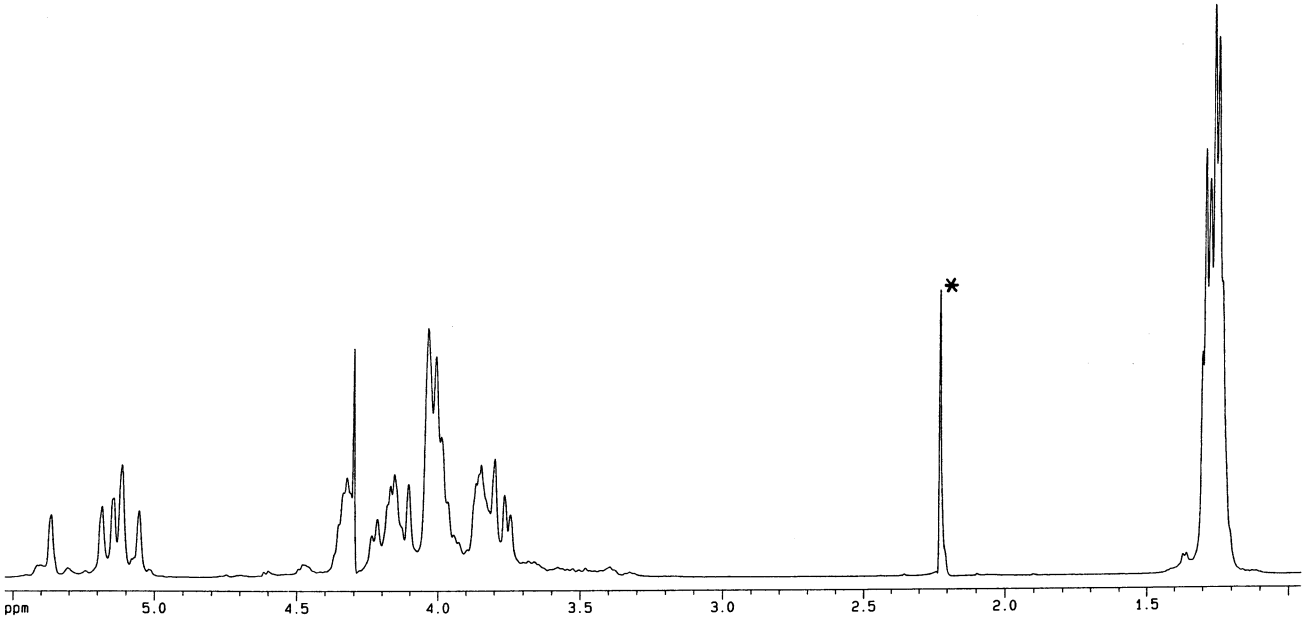


Fig. 4. ^{1}H NMR spectrum of desulfated, deacetylated fucoidan DD-2. * Acetone signal (see text and Table 3 for assignments).

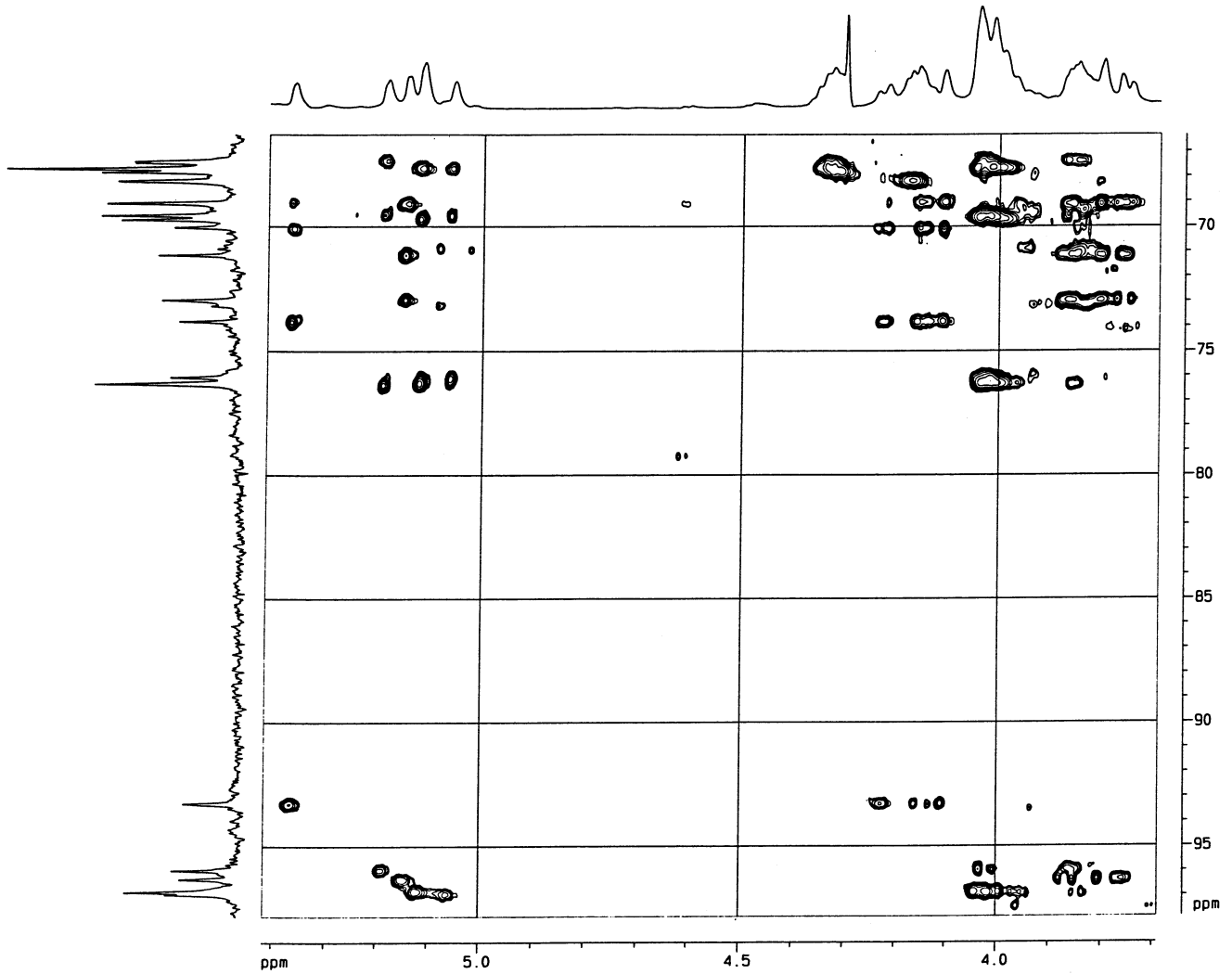


Fig. 5. HMQC-TOCSY NMR spectrum of desulfated, deacetylated fucoidan DD-2 (see text for details).

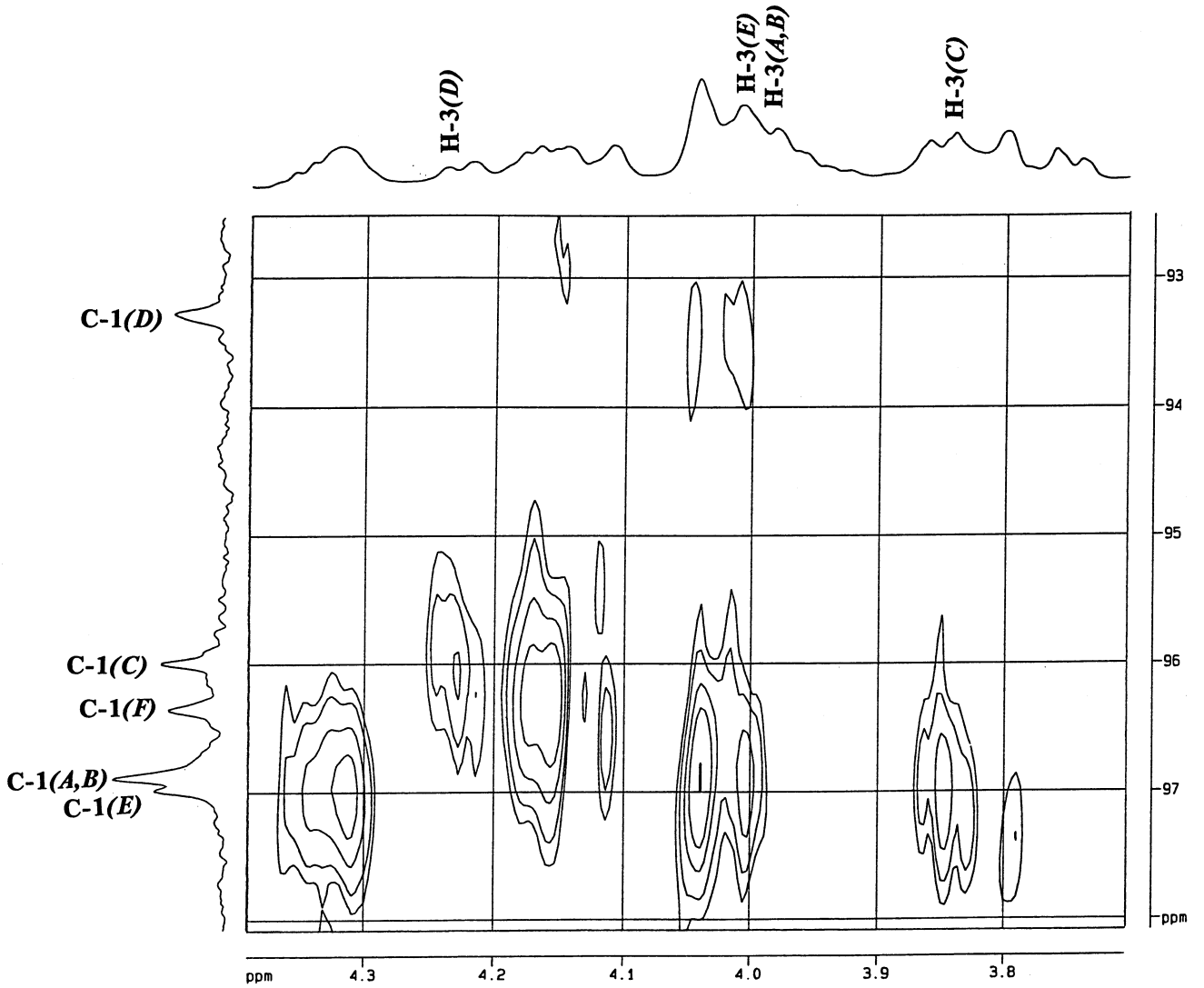


Fig. 6. 2D HMBC NMR spectrum (in part) of desulfated, deacetylated fucoidan DD-2 (see text for details).

chemical shifts of C-2–C-6 of the residue (***F***) practically coincide with those for the corresponding terminal unit in trisaccharide **1** [26], thus indicating that the residue (***F***) is terminal α-Fuc*p*. All other residues are substituted at C-3 since the signals of C-3 are shifted downfield and those of C-4 are shifted upfield from the corresponding signals of non-substituted α-Fuc*p* residue (***F***). Moreover, the absolute values of the β-glycosylation effects on C-4 (higher than 3 ppm) are typical for the same absolute configuration of all (1→3)-linked α-Fuc*p* residues under consideration [28,29]. The downfield position of the signal C-2 of (***D***) in comparison with other residues as well as the upfield shift of C-3 of (***D***) vs. other 3-substituted residues suggest O-2-glycosylation of (***D***) with residue (***F***) (see above, ROESY).

HMBC spectroscopy was used to reveal the definite sequence of the (1→3)-linked residues (Fig. 6). The spectrum contains intra-residue correlation peaks (all of H-1/C-5, H-1/C-3 and some of C-1/H-5, and C-1/H-3) for each of α-Fuc*p* and inter-residue peaks H-1(***D***)/C-3(***E***), H-1(***C***)/C-3(***D***), H-1(***E***)/C-3(***A***,***B***), C-1(***D***)/H-3(***E***), C-1(***C***)/H-3(***D***), and C-1(***A***,***B***)/H-3(***C***). Evidently, the inter-residue peaks for (***A***) and (***B***) are masked by the intra-residue peaks of these residues. ^{1}H NMR data are presented in Table 3.

Thus, the structure of desulfated, deacetylated fucoidan from *C. filum* is in accordance with that given in Scheme 1. We are currently

performing systematic synthesis [26,27] of fucoidan fragments in order to obtain a representative ^{13}C NMR database suitable for further computer-assisted [29] structural analysis. In particular, we prepared [26] the 2,3-branched trifucoside **1** and (1→2)- and (1→3)-linked difucosides **2** and **3** and used their ^{13}C NMR data [26] for calculating the chemical shifts for units (***A***)–(***F***) in fucoidan DD-2 (Table 2). Since (a) fucoidan DD-2 is built of approximately hexafucoside repeating units (as one can conclude from NMR data, see above) and practically does not contain other carbohydrate residues and (b) its NMR spectra are well resolved and were completely assigned by us, fucoidan DD-2 represents a very rare case of polysaccharides of this group for which it is possible to compare directly experimental chemical shifts and calculated ones using additive schemes [29].

^{13}C NMR data from Table 2 show a good coincidence between calculated and experimental chemical shift values. The small differences ($\Delta\delta \leq 1$ ppm) observed may be attributed to higher conformational rigidity of the polysaccharide in comparison to the corresponding oligosaccharides used as models. Some differences of the same value in experimental spectrum between C-1 chemical shifts for residues ***A***, ***B***, and ***E***, which are of similar type and formally are equal (within the additive scheme), are to be noted.

Periodate oxidation/Smith degradation of desulfated fucoidan.—An aliquot of desulfated fucoidan D-2 was subjected to Smith degradation (DSD-2). Methylation analysis of DSD-2 gave strong evidence for the presence of the main (1→3)-chain, which is resistant towards Smith degradation (for DSD-2, the TIC ratio of acetates of 2,3,4-*O*-methyl-:2,4-*O*-methyl-:2-*O*-methyl-:4-*O*-methyl-:fucitol is 5:86:1:7:tr.). Single-residue branches are cleaved during Smith degradation; however, one can assume that a small amount of branching retained in the DSD-2 fucoidan is due to the presence of several (1→3)-linked fucose residues in a minor amount of branches. No degradation of internal (1→2)- or (1→4)-fucose residues was observed in a separate experiment which include FAB control (data not shown).

Methylation analysis of native fucoidans A-1–A-3.—The TIC ratios of partially methylated fucitol acetates obtained from native fucoidans are as follows: (acetates of 2,3,4-*O*-methyl-:2,3-*O*-methyl-:2,4-*O*-methyl-:2-*O*-methyl-:4-*O*-methyl-:fucitol) for A-1, 18:13:26:tr.:15:20; for A-2, 17:tr.:28:2:20:27; and for A-3, 6:0:12:33:29:9. These data give the evidence that fucose residues in fucan sulfates A-2 and A-3 are mainly sulfated at O-4 and/or O-2 positions. As for the minor low-sulfated fraction A-1, it is more likely that the sulfate groups are located mainly at O-2 in the main chain. In all of the fractions, non-sulfated terminal fucopyranose residues are present in noticeable amounts. No peaks attributable to fucofuranose residues (cf. [13,14]) were observed.

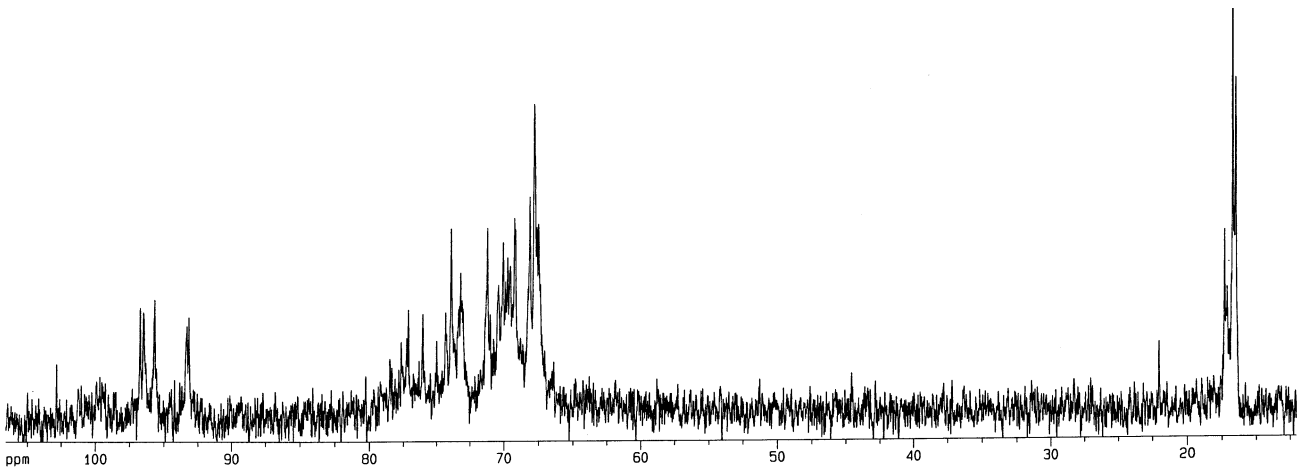


Fig. 7. ^{13}C NMR spectrum of native fucoidan from *C. filum* A-2.

^{13}C NMR spectrum of native fucoidan A-2.—The ^{13}C NMR spectrum of native fucoidan A-2 is given in Fig. 7. The unambiguous assignment of peaks is not possible due to peak overlapping; however, some assignments (e.g., deoxy carbons at δ 16.5–17.3 and minor peak of methyl in *O*-acetyl at 22.03 ppm) are clear. Deacetylation of the native fucoidan A-2 does not simplify the ^{13}C NMR spectrum significantly (data not shown).

Periodate oxidation/Smith degradation of native fucoidan A-2.—Methylation analysis data (TIC ratio of acetates of 2,3,4-*O*-methyl-: 2,4-*O*-methyl-:2-*O*-methyl-:4-*O*-methyl-: fucitol is 2:16:25:40:15) for Smith-degraded, sulfated fucoidan ASD-2 confirm moderate vulnerability of A-2 to periodate oxidation; mainly non-sulfated terminal fucose residues are cleaved by Smith degradation.

IR spectrum of native fucoidan A-2.—The IR spectrum of fucoidan fraction A-2 has mainly the axial sulfate band (857 cm^{-1}) and only a relatively small shoulder of equatorial sulfate, thus supporting the location of sulfate groups mainly at O-4 [13]. We also observed a small shoulder at 1720 cm^{-1} that may be assigned to ester carbonyl resonances, thus confirming the presence of acetate in this fucoidan. Note that the IR spectra of fucoidan from *F. vesiculosus* published in [13] and obtained by us (data not shown) exhibited bands of comparable intensity for axial (850 cm^{-1}) and equatorial (830 cm^{-1}) sulfate in KBr tablets (similar data were obtained for suspensions in Nujol).

Analysis of other fucoidan fractions from C. filum.—Methylation patterns of fractions B, C-1, and C-2 of *C. filum* fucoidan are more complex than those for homofucan sulfates (TIC ratios for acetates of 2,3,4-*O*-methyl-: 2,3-*O*-methyl-:2,4/3,4-*O*-methyl-:2-*O*-methyl-: 4/3-*O*-methyl-:fucitol are for B, 8:1:26: 15:28:12, C-1, 15:tr.:21:2:9:11, and C-2, 9:tr.:8:2:8:10, respectively). 1,2,5-Tri-*O*-acetyl-3,4-di-*O*-methylfucitol (practically absent for A-1, A-2, and A-3) is observed for B, C-1, and C-2 as well as small amounts of 1,2,4,5-tetra-*O*-acetyl-3-*O*-methylfucitol (SIM chromatograms at *m/z* 189); peaks of partially methylated hexitols and xylitol acetates are also present. The ^{13}C NMR spectrum of fucoidan B in D_2O (pH 1, adjusted with CF_3COOD to dissolve fucoidan B) has four signals in the deoxy C-6 region, three small acetate signals (21.7, 22.3, and 22.6 ppm), and poorly resolved peaks in the 68–78 ppm region along with a peak at 81.1 ppm (the latter may be putatively assigned to glycosylated or sulfated C-4); there are at least eight signals in the anomeric region (93.5–102.8 ppm). Glucuronic acid carboxyls give unresolved, weak peaks at 184 ppm. The ^{13}C NMR spectrum of fucoidan C-1 is similar; however, peak overlapping is even stronger than for fucoidan B (data not shown). Unfortunately, the linkage and position of uronic acid residues in polysaccharides B, C-1, and C-2 remain unclear. GLC–EIMS analysis conjugated with deuteroreduction of uronic acid residues in methylated polysaccharides [30] failed to reveal corresponding dideuterated *O*-methyl alditols (in contrast to [14]); possibly, uronide residues were decomposed during alkaline-medium methylation [31]. However, attempts to cleave selectively glucuronic acid residues in fucoidan B by the Li–ethylenediamine system [32] afforded a mixture of low-molecular oligofucosylfucitols and fucooligosaccharides (FABMS, GLC–EIMS methylation analysis, data not shown). Since this procedure causes substantial desulfation of sulfated oligo- and polysaccharides and, possibly, alkaline degradation of neutral links, its use for structural analysis seems unreliable. Thus, the study of these fucoidan fractions, which have more complex carbohydrate composition and give rather complex methylation patterns and NMR spectra needs different approaches and will be the subject of a future study.

4. Conclusions

A study of fucoidan fractions from *C. filum* using solvolytic desulfation and methylation analysis for both native and desulfated polysaccharides shows that homofucan sulfate A-2 contains mainly an (1→3)-linked poly-α-L-fucopyranoside backbone with a rather high degree of branching. Some fucopyranose residues are sulfated at O-4 and O-2 positions or 2-O-acetylated. Branching at O-4 and the

presence of multiple-unit branches cannot be excluded according to the data obtained. All of the signals presented in the ^{1}H and ^{13}C NMR spectra of desulfated, deacetylated fucoidan were assigned using 2D techniques and the reference spectra of synthetic fucooligosaccharides. The NMR data indicate the existence of a branched hexasaccharide repeating unit (five $(1\rightarrow3)$-linked α-L-fucopyranose residues with one $(1\rightarrow2)$-linked α-L-fucopyranose single-residue branch). Other fucoidan fractions from *C. filum* that have more complex carbohydrate composition and give rather complex methylation patterns and NMR spectra will be the subject of a future study.

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