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# Further data on the structure of brown seaweed fucans: relationships with anticoagulant activity

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**Abstract**

The composition, molecular weight (MW), anticoagulant activity and nuclear magnetic resonance spectra of various low-molecular-weight fucans (LMWFs) obtained by partial hydrolysis or radical depolymerization of a crude fucoidan extracted from the brown seaweed *Ascophyllum nodosum* are compared. Fucose units were found mainly sulfated at O-2, to a lesser extent at O-3, and only slightly at O-4, contrary to previously published results for fucoidans from other brown seaweeds, and fucose 2, 3-*O*-disulfate residues were observed for the first time. As the sulfation pattern excluded an α-(1→2)-linked fucose backbone and a high proportion of α-(1→4) linkages was found, it would appear that the concept of fucoidan structure needs to be revised. Anticoagulant activity is apparently related not only to MW and sulfation content, as previously determined, but also (and more precisely) to 2-*O*-sulfation and 2,3-*O*-disulfation levels. 

*Keywords:* Fucan structure; Fucan NMR; Anticoagulant activity; Brown seaweed

## 1. Introduction

Fucoidans, a unique class of sulfated fucans isolated from many brown seaweeds, have not been found in other algae or plants [1]. Their composition varies with the species, but they always contain essentially fucose and sulfate, with small proportions of galactose, xylose, mannose and uronic acids. Although various biological activities (anticoagulant, antithrombotic, antiviral, antiproliferative, antifertilizing and antitumoral) are associated with these compounds [2,3], their structure is poorly understood. Percival and McDowell proposed a more or less branched α-(1→2)-linked L-fucose polymer [1], and Patankar et al. [4] a branched α-(1→3)-linked structure (Scheme 1). According to most authors [5,6], the sulfate groups are linked mainly to the 4-position of fucose residues. As a consequence, the relationships between structure and anticoagulant activity are not clearly established. Only the importance of molecular size and sulfate content has been reported [7–11]. However, it is likely that some structural features are re-

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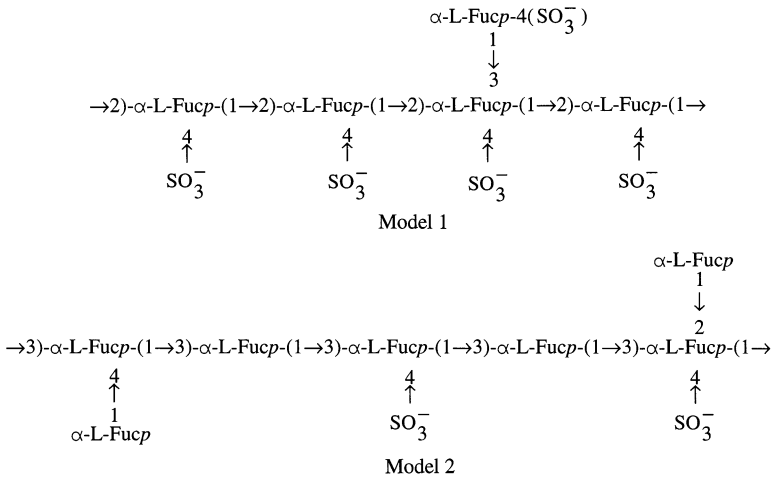



Scheme 1. Fucoidan structures as proposed by Percival and McDowell (Model 1) [1] and by Patankar et al. (Model 2) [4].

quired for anticoagulant activity, especially sulfate clusters to ensure interactions with cationic proteins. In heparin, such clusters are present in Unit III (designated also by F) [12] of the antithrombin binding sequence.

As the fucoidan molecule is too large for use in drugs, low-molecular-weight fucans (LMWFs) were prepared in our laboratory by acid hydrolysis [13,14] or radical cleavage [15]. Such oligosaccharides, some of which retain the anticoagulant properties of the native fucoidan, may be useful as drugs. Moreover, nuclear magnetic resonance (NMR) studies are possible with these relatively small molecules.

This paper provides new structural data concerning the determination of *Ascophyllum nodosum* fucoidans. We obtained 1D and 2D NMR spectra of oligosaccharidic fractions, particularly one purified by centrifugal partition chromatography (CPC). A comparison of several fractions displaying different activated partial thromboplastin times (APTTs) indicated that some of these structural features are related to anticoagulant activity.

## 2. Results and discussion

*Preparation, composition and properties of LMWFs.*—Only enzymatic methods can cleave glycosidic linkages specifically without modifying the structural units composing the original polysaccharide. However, no commercial endofucosidase is available. Though chemical methods cause structural alterations, such as debranching or desulfation, we assumed that two different methods (classical acid hydrolysis [13,14] and a radical depolymerization process [15]) would not produce the same alterations and thus the same mixture of oligosaccharides.

The acid hydrolysis method has been briefly reported elsewhere [13,14] and is fully described in Section 3. After hydrolysis, a purification step by size-exclusion chromatography (SEC) is necessary to obtain highly anticoagulant fractions. Various batches (designated by a superscript number) were produced using this process. The chemical characteristics (MW, sulfate content, sugar composition) and anticoagulant activities (APTT) for five fractions (**$H^1$4**; **$H^2$4**; **$H^3$4**; **$H^3$5**; **$H^4$4**) from four batches are given in Table 1 (normal script numbers refer to SEC fraction numbers). Three additional fractions were prepared either by low-pressure anion-exchange chromatography from **$H^4$4** (**$H^4$4,1** and **$H^4$4,2**) or by CPC from **$H^3$5** (**$H^3$5,p**; see Section 3). As could be expected for oligosaccharides derived from a fucoidan, L-fucose and sulfate groups were by far the most abundant constituents, whereas galactose, xylose (and/or mannose) and uronic acids were present in minor amounts. There was no obvious relation between sugar composition and anticoagulant activity. Fractions (**$H^4$4,1** and **$H^4$4,2**) obtained by low-pressure anion-exchange chromatography from **$H^4$4** displayed very different sulfate contents (18.6 and 35%, respectively) and, not

Table 1
Composition (in %, w/w), molecular weight (MW), and anticoagulant activity (as quantity necessary for doubling APPT) of various LMWFs obtained by partial acid hydrolysis (**H** series) or radical depolymerization (**R** series) [a]

| Fractions [b] | APTT [c] | $MW_w$ [d] | $MW_n$ [e] | Polydispersity | Fucose [f] (%) | $SO_3Na$ [f] (%) | Uronic acid [f] (%) | Galactose [f] (%) | Xylose [f] (%) | $SO_3Na$/fucose [g] | Me/H-1 [h] |
|---|---|---|---|---|---|---|---|---|---|---|---|
| **$H^1$4** | 33 | 4700 | 2900 | 1.6 | 31.5 | 31 | 6.3 | 3 | 2 | 1.4 | 2.8 |
| **$H^2$4** | 68 | 5800 | 4200 | 1.4 | 37 | 31 | 2.6 | 3 | 2 | 1.2 | 2.2 |
| **$H^3$4** | 30 | 5900 | 3700 | 1.6 | 30 | 37 | 8 | 3.5 | 2 | 1.75 | 3.4 |
| **$H^4$4** | 25 | 7700 | 3350 | 2.3 | 29 | 28.5 | 5.7 | 3 | 4 | 1.4 | 2.8 |
| **$H^4$4,1** | 400 | 10 000/2600 | 8750/2250 [i] | 1.15/1.15 | 21.5 | 18.6 | 14.7 | 7 | 8 | 1.2 | 1.9 |
| **$H^4$4,2** | 20 | 11500 | 8100 | 1.4 | 35.5 | 35 | 0.3 | 2 | 0.5 | 1.4 | 2.6 |
| **$H^3$5** | 100 | 5200 | 3700 | 1.4 | 41 | 30.5 | 7.6 | 1.5 | 2 | 1.05 | 2.9 |
| **$H^3$5,p** | 30 | 5500 | 4500 | 1.25 | 40 | 44 | 0 | 0 | 0 | 1.55 | 2.7 |
| **$R^1$** | 25 | 5700 | 4800 | 1.2 | 33.5 | 34 | 3 | 1.3 | 1 | 1.45 | 3.4 |
| **$R^2$** | 30 | 4100 | 1600 | 2.5 | 31 | 36.5 | 5.3 | 1.5 | 0.5 | 1.65 | 2.8 |
| **$R^2$1** | 28 | 3900 | 2000 | 1.9 | 32 | 36 | 4.6 | 1.3 | 0.6 | 1.6 | 3.2 |
| **$R^2$2** | 15 | 8400 | 7600 | 1.1 | 32 | 34 | 3 | 1 | 0.3 | 1.5 | 2.95 |
| **$R^3$** | 20 | 4600 | 2000 | 2.3 | 31 | 37.5 | 5 | 1.5 | 1.3 | 1.7 | 3.2 |

[a] MW was determined by HPSEC using pullulans as standards. As pullulans are neutral glucans and fucans are highly negatively charged polymers, such calibration does not allow an exact measurement of fucan MW. Therefore, these results can only be used for relative comparisons.
[b] Superscript numbers refer to batch numbers.
[c] Concentration required (in μg/mL) to double APTT vs. the reference without fucan. S.D. of 10–20%.
[d] Weighted-average molecular weight.
[e] Number-average molecular weight.
[f] Chemical analysis had a S.D. of 5–15%.
[g] Molar ratio.
[h] Ratio between methyl protons and anomeric protons in 1D $^1$H NMR spectroscopy.
[i] Presence of two peaks.

surprisingly, very different anticoagulant activities (Table 1). This is consistent with previous results showing that anticoagulant activity increases with sulfate content [9,10]. However, **H²4** (from Batch 2) was significantly less anticoagulant than fractions from other batches (**H¹4**; **H³4** and **H⁴4**), even though its sulfate content was not specifically low (31%). The sulfate/fucose ratio was only 1.2 compared with about 1.5 (1.4–1.7) in high anticoagulant fractions, which suggests that the presence of disulfated fucoses is important for anticoagulant activity (see also below).

A different method involving degradation by $OH^{\bullet}$ radicals was used to avoid desulfation during the acidic depolymerization process [15]. The oligosaccharide mixture produced by this reaction did not require further purification to match satisfactory anticoagulant activities. Various samples produced in the same way (**R¹** to **R³**) showed similar characteristics in terms of MW (of the order of 4000–6000 Da), sulfate groups (34–37%) and fucose contents (31–34%) and of the concentrations required to double APTT (of the order of 20–30 μg/mL) (see Table 1). However, neutral sugars (other than fucose) and uronic acid contents were very low. Consequently, it would appear that no sugar other than fucose is necessary for anticoagulant activity. As in previous experiments, two subfractions of several samples were obtained by low-pressure anion-exchange chromatography. The results for one of these samples (**R²**) are reported in Table 1. Both purified fractions (**R²1**; **R²2**) displayed very similar compositions, differing only in molecular size and anticoagulant activity. The higher-MW fraction was more active, which is not surprising since it has been clearly shown that anticoagulant activity increases with MW [7]. The sulfate–fucose ratio was close to 1.5 (or higher), indicating that this method induces less desulfation than acid hydrolysis.

*¹H NMR spectroscopy analysis of a LMWF produced by acid hydrolysis and purified by CPC* (**H³5,p**).—We are currently developing a new method for the purification of sulfated polysaccharides [16], which has resulted in a highly homogeneous anticoagulant fraction (**H³5,p**) purified from **H³5** (see Section 3). The NMR data presented here relate to this fraction, which was used as a reference to facilitate interpretation of other spectra and detect common structural elements present in various fractions with different anticoagulant properties. The $^1$H NMR spectrum of **H³5,p** (Fig. 1) displayed characteristics consistent with the presence of α-L-fucopyranosyl units. The anomeric protons appeared as two broad unresolved multiplets centered at 5.30 and 5.45 ppm, with two additional small resolved doublets at 5.58 and 5.52 ppm. Such chemical shifts (5.3–5.6 ppm) were similar to those observed previously in similar polymers of α-linked L-fucopyranose [17,18]. Methyl signals occurred at 1.15–1.50 ppm, mainly as two peaks (at around 1.43, 1.30 ppm), which together accounted for almost three protons (exactly 2.7, if all anomeric protons count together for one). Methyl signals appeared around 1.35–1.40 ppm only for α-(1→4)-linked L-fucose [19]. Consequently, low-field peaks are probably assignable to the methyl groups of fucoses bearing another fucose at O-4. Patankar et al. [4] also found that 42% of fucose residues were substituted at this position. Important data were deduced from DQF-COSY NMR spectra (Fig. 2). The positions of most H-1–H-2 cross-peaks in Fig. 2(**a**) showed that fucose residues were generally sulfated at O-2 (H-2 at 4.45–4.75 ppm) and that only a few were unsulfated (H-1–H-2 cross-peaks in Fig. 2(**b**), H-2 around 3.95–4.15 ppm). Moreover, these O-2 unsulfated fucose units were sulfated at O-3 position (H-3 between 4.65 and 4.80 ppm; H-2–H-3 cross-peaks are shown in Fig. 2(**d**)). Starting from anomeric protons (Fig. 2(**a**)), connectivities from H-1 to H-4 could be established for some spin systems (Fig. 2), indicating that some fucose residues bore two sulfate groups at O-2 and O-3, respectively (H-3 between 4.70 and 4.90 ppm; H-2–H-3 cross-peaks in Fig. 2(**c**)), while some were only sulfated at O-2 (H-3 proton resonance around 4.10–4.35 ppm; H-2–H-3 cross-peaks in Fig. 2(**e**)). Thus, spin systems can be grouped in four classes (A, B, C, and D), two of which (B and D) correspond to 2-*O*-sulfo-α-L-fucopyranosyl and two (A and C) to 2,3-di-*O*-sulfo-α-L-fucopyranosyl units (Table 2). Obviously, there was little or no sulfation at O-4, the observed

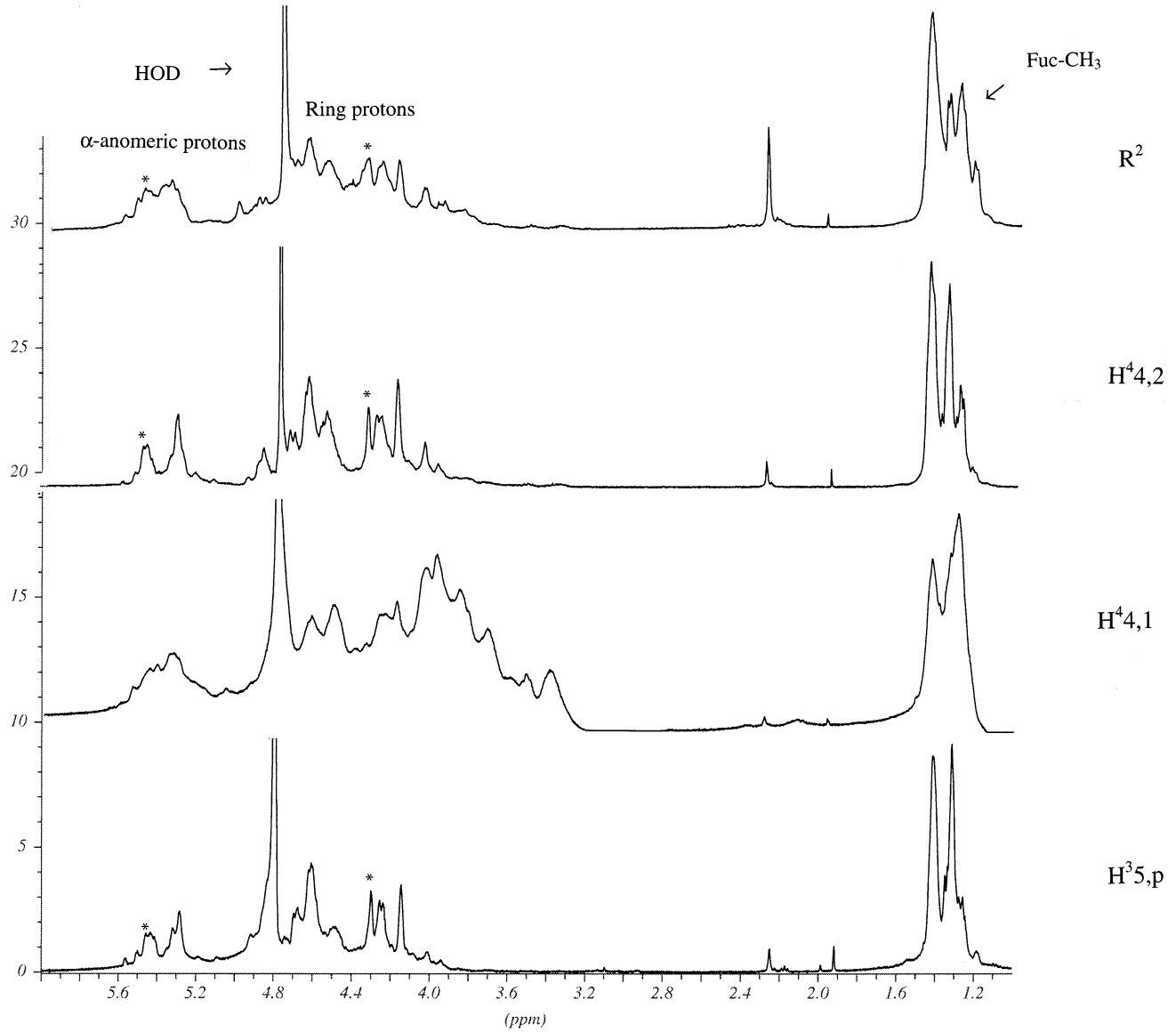



Fig. 1. 1D $^{1}$H NMR spectra of four LMWFs obtained by acid hydrolysis (**H** series) or radical depolymerization (**R$^{2}$**). **H$^{3}$5,p** was a CPC-purified fraction from **H$^{3}$5**, and **H$^{4}$4,1** and **H$^{4}$4,2** were two fractions obtained by low-pressure anion-exchange chromatography that showed very different anticoagulant activity. Asterisks indicate peaks that were always intense for strongly anticoagulant fractions.

values (between 3.95 and 4.35) being inconsistent with O-sulfation (H-4 of fucose 4-*O*-sulfate should be between 4.60 and 4.90 ppm [17,18]). It is noteworthy that the H-4 of fucose sulfated at O-3 appeared at low field (around 4.3 ppm), which is in good agreement with the value observed in the related galactose series for α-D-Gal*p*-OMe-3-$SO_4$ [20]. For fucoses unsulfated at O-3 (spin systems B and D), the chemical shifts of H-4 were 4.18 and 3.97 ppm, respectively. As acid hydrolysis not only cleaves glycosidic linkages but also removes sulfate groups, it is likely that most O-2 and some O-4 were sulfated in the native polymer (additional evidence is provided below). Fucose residues were previously believed to be mainly sulfated at O-4 and sometimes at O-2, but never at O-3. Thus, our report is the first to indicate the presence of 3-*O*-sulfo- and 2,3-di-*O*-sulfo-α-L-fucopyranosyl units in fucoidans.

The heteronuclear $^{13}$C–$^{1}$H HMQC spectrum (Fig. 3) showed that H-1 at 5.58 and 5.52 ppm were correlated with carbons at 93.5 and 93.4 ppm, respectively, both of which corresponded to terminal reducing fucose

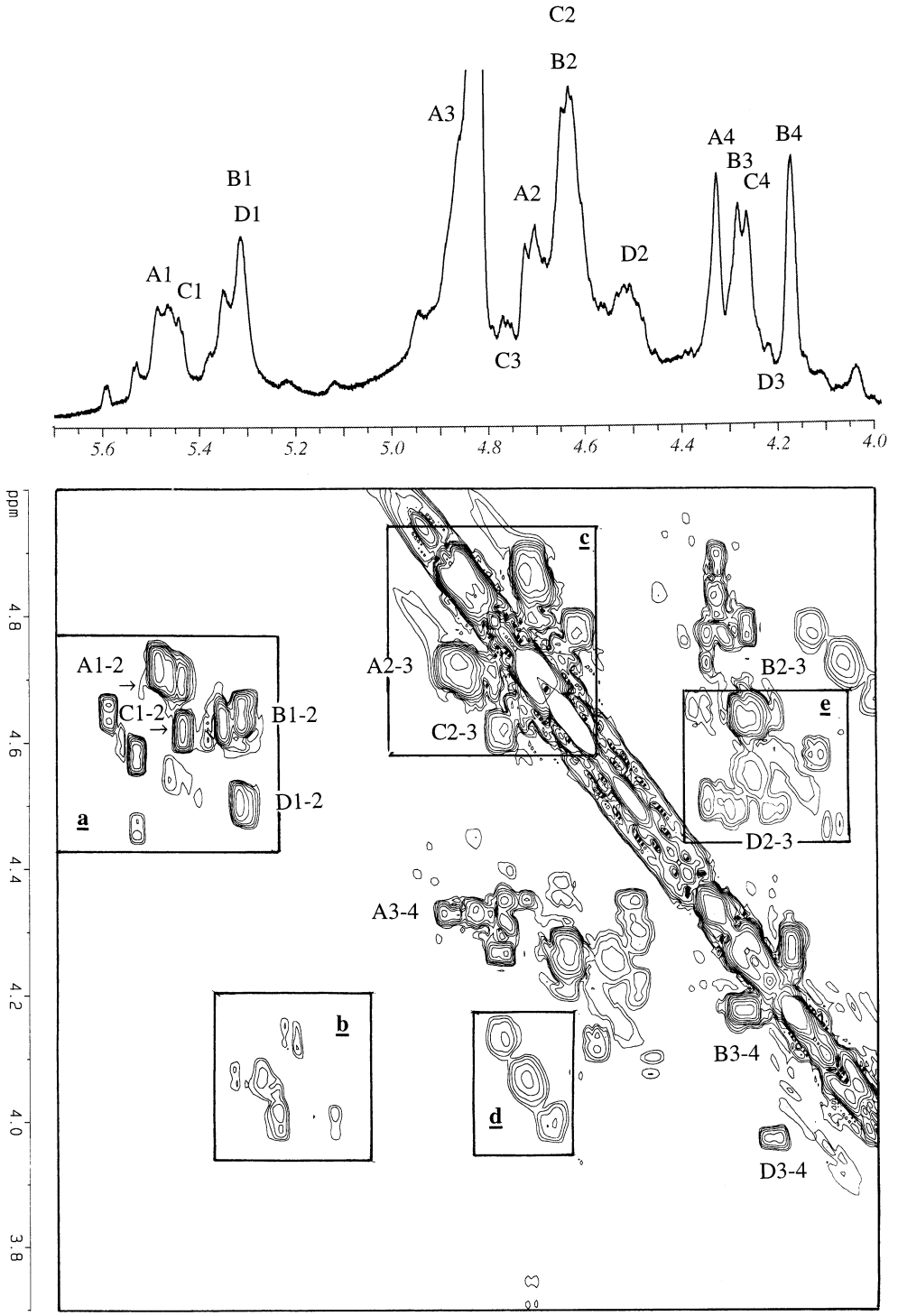



Fig. 2. Expansions of the double quantum filtered COSY spectrum of **H[3]5,p**, a CPC-purified LMWF. (a) H-1–H-2 cross-peaks corresponding to 2-*O*-sulfo-α-L-fucopyranoses and 2,3-*O*-disulfo-α-L-fucopyranoses; (b) H-1–H-2 cross-peaks of fucoses nonsulfated at O-2; (c) H-2–H-3 cross-peaks of 2,3-*O*-disulfo-α-L-fucopyranoses; (d) H-2–H-3 cross-peaks of 3-*O*-sulfo-α-L-fucopyranoses; (e) H-2–H-3 cross-peaks of 2-*O*-sulfo-α-L-fucopyranoses. On the basis of the H-1–H-2 cross-peaks in (a), four spin systems were determined: A, B, C and D.

units (C-1 signal of α-L-fucopyranose at 92.2 ppm [21]). The multiplet at 5.45 was correlated with two carbons at 96.9 and 97.5 ppm (2,3-di-*O*-sulfo-α-L-fucose) and the one at 5.30 ppm with carbons at 101.2 and 101.3 ppm. These data are consistent with the α-L configuration [22] and suggest that two families of fucosyl units (disulfated and monosulfated)

Table 2
NMR data of **H$^3$5,p** (a CPC-purified LMWF)

| Spin systems [a] | $^1$H-1 | $^{13}$C-1 | $^1$H-2 | $^{13}$C-2 | $^1$H-3 | $^{13}$C-3 | $^1$H-4 | $^{13}$C-4 |
|---|---|---|---|---|---|---|---|---|
| A | 5.45–5.50 | 96.9 | 4.70–4.73 | 77.2–75.3 | 4.80–4.90 | 77 | 4.33 | 82.5 |
| B | 5.30–5.35 | 101 | 4.62–4.65 | 76 (70) | 4.25 | 76.1 (73.7) | 4.18 | 72.2 |
| C | 5.45 | 97.5 | 4.61 | 75.3 | 4.78 | 78.1 | 4.27 | 73.7 (76.1) |
| D | 5.27–5.32 | 101 | 4.50 | 78 | 4.20 | 70.2 | 3.97 | 75.3 |

[a] A, B, C and D correspond to four classes of L-fucose residues (see Scheme 2).

were present. As expected, methyl resonances were present at 18.5 ppm. Ring cross-peak resonances fell into four groups: one (inside frame I: $^{13}$C around 70 ppm; $^1$H between 4.25 and 4.70 ppm) corresponded to O-5 or unsubstituted positions [19,22]; one with downfield-shifted protons (inside Frame II: $^{13}$C between 74 and 78 ppm; $^1$H between 4.50 and 4.90 ppm) to O-2 and O-3 sulfated positions; one with downfield-shifted $^{13}$C (around 82 ppm, inside Frame III) to glycosylated 4-positions [19]; and the last one to other unsulfated positions whether substituted or not [22]. An isolated cross-peak at 4.77 and 84 ppm was probably due to a residue sulfated at O-4 [23] (see above). Correlated spots corresponding to A, B, C and D residues (Fig. 3) allowed the corresponding $\delta$ values of $^{13}$C to be deduced, except for two carbons, C-3 and C-4 of B and C residues, respectively, for which corresponding proton chemical shifts were too close (attribution shown on Fig. 3 could be reversed). Partial structures of A, B, C and D are shown in Scheme 2; the C residue was probably not substituted at the 4-O-position since the corresponding C-4 appeared at much higher field (73.7 or 76.1 ppm) than the C-4 of A residue (82.5 ppm). All NMR data are grouped in Table 2. The HMBC spectrum (not shown) corroborated the presence of $\alpha$-(1→3)- and $\alpha$-(1→4)-linked fucoses. Protons of methyl groups downshifted at around 1.40–1.45 ppm were correlated with two groups of carbons at 69–73 ppm (C-5) and 82–86 ppm (substituted C-4 of the A spin system), while those at 1.30 ppm correlated only with carbons at 72–75 ppm (C-5 and non-substituted C-4), thus confirming results deduced from COSY.

*$^1$H NMR spectroscopic analysis of LMWFs produced by acid hydrolysis or radical depolymerization (structure–anticoagulant activity relationships).*—The 1D $^1$H NMR spectra of fractions obtained by acid hydrolysis were similar to those of **H$^3$5,p**. Proton NMR spectra of fractions produced by the radical depolymerization process showed several differences (see **R2** on Fig. 1). For instance, a singlet at 2.30–2.32 ppm (corresponding to an acetyl function) was much more intense. An additional peak due to H-4 of 4-*O*-sulfated residues appeared around 5 ppm. The intensity of this peak was not related to anticoagulant activity. For instance, it was low in the **R$^2$2** spectrum and very strong for **R$^2$1** (data not shown). Nevertheless, obvious differences existed between strongly and slightly anticoagulant fractions, with the latter displaying a less intense peak around 5.45 ppm (Fig. 1) and a methyl H–anomeric H ratio lower than three (Table 1), indicative of a higher percentage of uronic acids and various neutral sugars (mainly xylose and galactose). It was previously reported that only fucoidan-like fractions (homofucans) induce anticoagulant activity [15,24]. Yet DQF-COSY NMR spectra of strongly anticoagulant fractions indicated that almost all fucose residues were sulfated at O-2 (H-2 at 4.45–4.75 ppm), and that only a few were unsulfated (H-2 at 3.80–4.15 ppm). This is especially true for all **R** fractions for which 2-positions were always fully sulfated (spectra not shown). However, this requirement was not sufficient. For example, COSY spectra of **H$^4$4,1** and **H$^4$4,2** showed that almost all 2-positions were sulfated in both samples, despite considerable difference in their anticoagulant activity (see Table 2). By comparing COSY spectra, it was also obvious that disulfated fucoses corresponding to spin system A were more abundant in fractions with higher activity on APTT; consequently this structural feature is important for anticoagulant properties. It is noteworthy that the antithrombin-binding region of heparin

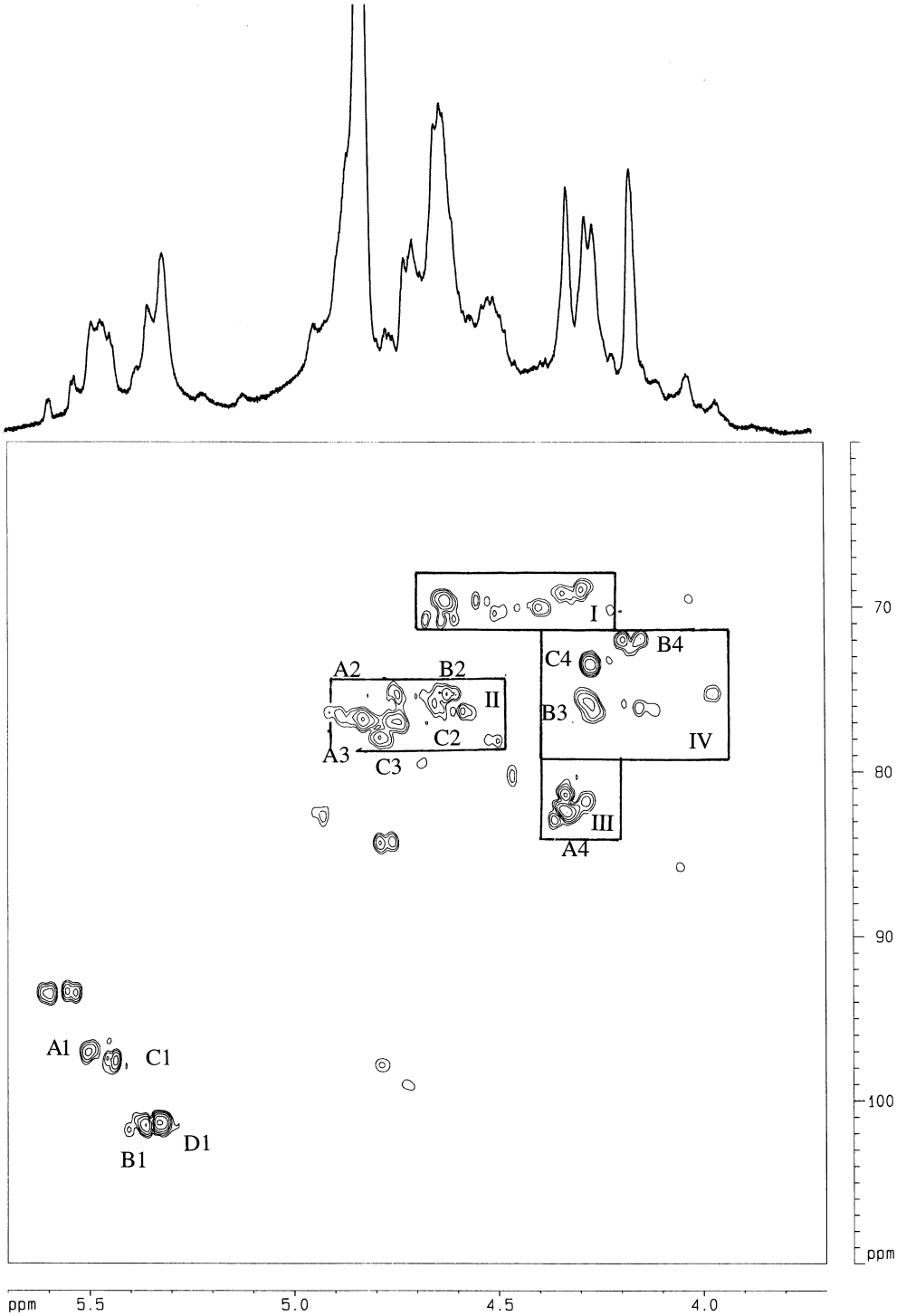
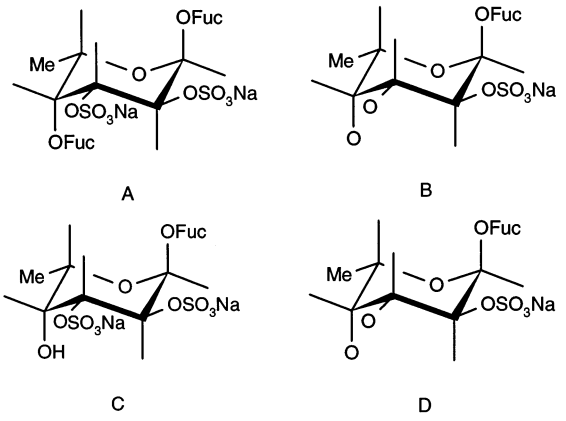



Fig. 3. Expansion of the HMQC spectrum of **H³5,p**. $^{13}C-^{1}H$ correlation spots of A, B, C and D spin systems are indicated.

was also disulfated at the O-2 and O-3 positions of Unit III (or F), and that this sulfation pattern is essential for activity [25]. It has been shown quite recently that enhancement of anticoagulant activity in oversulfated chondroitine sulfate is due to a uronate residue in the $^{1}C_4$ conformation disulfated at both positions 2 and 3 [26].

*IR analysis versus sulfate positions.*—IR spectra of various fractions displayed characteristic absorption bands of sulfated polysaccharides [27]. There were absorptions notably

at 1230–1255 $cm^{-1}$ (S=O stretching) and 845 $cm^{-1}$ (C–O–S bending of sulfates in axial position) [28], with a shoulder at 820 $cm^{-1}$ (C–O–S bending of sulfate in equatorial position) [29]. The 845 $cm^{-1}$ absorption is generally attributed to O-4 sulfates in fucoidan series [4,5]. However, it is debatable whether axial and equatorial sulfates can be distinguished by C–O–S bending vibration bands [30,31]. In fact, these results only indicate that there were at least two kinds of sulfates. Indeed, the exact position of the COS bending band depends on the overall substitution pattern. It has recently been shown for galactose-3-sulfate that C–O–S vibrational bands vary with the anomeric configuration and the hydration level [32], and crowded molecules (such as disulfated ones) exist as a mixture of various conformers [26]. Thus, IR data are hardly a reliable means for determining sulfate positions (except for a very well-known series of compounds). In fact, sulfate positions have never been clearly determined in brown algae fucoidan. In studies of fucoidans extracted from *E. kurome*, Nishino et al. [6] deduced the position of sulfates from those of deuterated methyl after desulfation and methylation (by $CD_3I$). When desulfation is performed by methanolic hydrogen chloride, glycoside linkages, as well as sulfates, can be cleaved. In fact, this indicates that most O-4 positions are substituted in the fucoidan of *E. kurome*. Percival and McDowell [1] deduced sulfation at O-4 only on the basis of negative evidence (non-alkali lability of sulfate groups). However, sulfation at O-4 can be higher in native polymer since some desulfation may occur during the degradation process, especially during acid hydrolysis.




Scheme 2. Partial structure for A, B, C and D units present in **H[3]5,p**. Such units were present in other LMWFs in various proportions.

*Conclusions.*—It has been shown that anticoagulant activity increases with MW and the sulfation content [7–11]. Nishino and Nagumo [10] found that only fucans with a sulfate–total sugar residue ratio greater than one displayed significant anticoagulant activity. This is consistent with our results indicating that anticoagulant activity requires sulfation at all O-2 and some O-3 positions, whereas sulfation at O-4 seems unnecessary. The minimum-length chain requirement is not clear, since side reactions cannot be avoided regardless of the process used to reduce the molecular size of the native polymer. It is not impossible that shorter anticoagulant molecules could be obtained by another depolymerization process (e.g., an enzymatic one).

## 3. Experimental

*Preparation of LMWFs.*—Crude fucoidan was extracted as described previously [33]. The acid hydrolysis procedure has already been briefly reported in Ref. [13]. Freeze-dried crude fucoidan was dissolved at a concentration of 10 mg/mL in 1 N $H_2SO_4$, and hydrolysis was performed at 60 °C for 1.5 h. After neutralization with NaOH, the resulting hydrolysates were desalted by tangential ultrafiltration (5000 Da cut-off membrane; Filtron, Clinton, MA, USA) and freeze-dried. The lyophilisate (500 mg) was then redissolved in 5 mL of 0.2 M NaCl, deposited on a Sephacryl S-300 HR column (35–45 × 4.8 cm; Pharmacia, Uppsala, Sweden) and eluted with the same solvent. Five fractions were obtained, desalted as previously described and freeze-dried (**H4** and **H5** were the fourth and fifth fractions, respectively). **H4** yields (calculated from crude fucoidan) were usually low (around 15%). Radical depolymerizations were performed on fucoidan, as previously described [15]. Briefly, fucoidan was depolymerized by $OH^{\bullet}$ radicals generated from $Cu^{2+}$-catalyzed decomposition of $H_2O_2$. After Chelex filtration and ultrafiltration (1000 Da cut-off membrane), **R** fractions were obtained in better yields (around 40–50%).

*Anion-exchange chromatography*.—The previously obtained LMWF (**H⁴4** or **R²**, 2.5 g) was dissolved in water and applied to a 200 mL DEAE Sepharose fast-flow column (Pharmacia, Uppsala, Sweden) at a flow rate of 2 mL/min (with water as eluent). Step-gradient elution of LMWF was then performed at 5 mL/min. The first fractions (**H⁴4,1** or **R²1**) were eluted with NaCl 0.8 M (600 mL), and the next fractions (**H⁴4,2** or **R²2**) with 2 M NaCl (600 mL). NaCl was eliminated by ultrafiltration with a 1000 Da cut-off membrane.

*Centrifugal partition chromatography (CPC)*.—The HPCPC Series 1000 apparatus (Sanki Engineering, Nagaokakyo, Kyoto, Japan) was operated as described previously [16]. The organic stationary phase was a 10% (v/v) solution of Amberlite LA2 protonated by HCl (Amberlite LA2 is an oil-soluble secondary fatty amine manufactured by Rohm&Haas, Philadelphia, PA, USA) in methylisobutylketone (MiBK). An aliquot of Fraction **H³5** (1.5 g) was injected into the chromatograph and eluted in displacement mode, with an aq mobile phase of 0.05 M NaOH saturated with a 10% solution of unprotonated LA2 in MiBK. The effluent (1 mL/min flow) was monitored with a microflow pH sensor (Broadley-James Corp., Santa Ana, CA, USA). A third of the injected compound was unretained and appeared in the first 20 mL. The following 600 mL contained only NaCl before retained sulfated fucans appeared. Fraction **H³5,p**, the final one just before the pH became alkaline when $OH^-$ appeared, had the highest anticoagulant activity.

*Monosaccharide analysis*.—Monosaccharide content was determined after acid hydrolysis [34] ($H_2SO_4$, 1 M, 100 °C, 3.5 h). Hydrolysates were diluted 100 times and then neutralized with Dowex resin ($HCO_3^-$ form) after addition of the reference product (2-deoxy-D-*lyxo*-hexose) for correction (usually slight and unnecessary) of possible dilution due to the resin. If required, additional dilutions were performed to obtain a final concentration of around 10 μM. Monosaccharide concentrations were then measured by high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD): CarboPac PA1 column (Dionex, Sunnyvale, CA, USA); NaOH 17.6 mM as eluent, prepared by dilution of a 50% w/w solution (J.T. Baker, Deventer, The Netherlands) in pure water (Milli-RO 4 purification system, Millipore); flow rate 1 mL/min; model 400 amperometric detector (EG&G, Princeton, NJ, USA) working in pulse mode; electrode settings: $E_1 = 40$ mV, $T_1 = 0.48$ s, $E_2 = 600$ mV, $T_2 = 0.2$ s, $E_3 = -150$ mV, $T_3 = 0.24$ s. $E_3$ was adjusted to minimize baseline drift. The complete system has been described elsewhere [34]. Quantification was performed by external calibration with standard solutions of fucose, galactose and xylose (all obtained from Sigma, St. Louis, MO, USA). In these chromatographic conditions, xylose and mannose were eluted together, and the corresponding peak was quantified as pure xylose. Data were collected and processed on a microcomputer equipped with Data System 450 MT software (Kontron, Milan, Italy).

*Molecular weight determination*.—Each fraction was analyzed by high-performance size-exclusion chromatography (HPSEC) in NaCl 0.2 M using a $30 \times 0.32$ cm i.d. Superdex column (Pharmacia PC 3.2/30, Uppsala, Sweden) at a flow rate of 0.1 mL/min. Column calibration was performed with standard pullulans[1]. Area measurements and calculations of molecular mass and polydispersity were carried out using Turbochrom software (Perkin–Elmer, Norwalk, CT, USA).

*Sulfate content*.—Sulfate content was deduced from sulfur elemental analysis performed for each fraction by the Central Microanalysis Department of the CNRS (Gif sur Yvette, France).

*Uronic acid content*.—Uronic acid content was determined using the modified *m*-hydroxydiphenyl–sulfuric acid method of Filisetti-Cozzi and Carpitta [35], with D-glucuronic acid as standard (Sigma).

[1] Pullulans are neutral glucans, whereas fucans are highly negatively charged oligosaccharides. Consequently, such calibration does not allow exact measurement of fucan molecular mass, and was only used for relative comparisons.

*Clotting assays.*—Activated partial thromboplastin time (APTT) was determined as described previously [36]. The results shown in Table 1 are expressed as the fucan concentrations required to double the control APTT in human platelet-poor plasma.

*$^1H$ NMR spectroscopy.*—1D $^1H$ NMR spectra were recorded on a Bruker DRX-400 spectrometer, equipped with an indirect 5 mm $^1H\{BB\}$ gradient probehead, at a probe temperature of 298 K. Prior to analysis, samples were exchanged twice in $D_2O$ (99.9 atom% $D_2O$, Euriso-top, Gif sur Yvette, France), with intermediate freeze-drying or evaporation (Speed-Vac), and then redissolved in 99.96 atom% $D_2O$ (E. Merck). Chemical shifts are expressed in ppm by reference to an external standard (trimethylsilylpropionic acid). The 1D spectra were recorded with a spectral width of 4111 Hz in a 32K dataset. No suppression of the HOD signal was performed. Double-quantum-filtered COSY spectra (DQF-COSY) were recorded by collecting 512 (F2) × 256–300 (F1) data points zero-filled to 512 (F1) using a spectral width of 2100 Hz (400 MHz), with a repetition time of 2 s. Heteronuclear multiple quantum coherence (HMQC) with inverse detection and heteronuclear multiple band coherence (HMBC) were carried out using the pulse program supplied with the apparatus.

*IR spectroscopy.*—IR spectra were recorded in KBr after preparation of KBr pellets by dissolving 200 mg KBr in 1.0 mL of LMWF solution (1.0 mg/mL) in distilled water, filtering on an 0.45 μm microfilter and freeze-drying to obtain a white powder. Spectra were recorded on a Perkin–Elmer 1600 FTIR device from 600–4000 $cm^{-1}$. Data were processed with IRDM software (Perkin–Elmer).

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