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Note

A disaccharide repeat unit is the major structure in fucoidans from two species of brown algae

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Abstract

The predominant repeating structure of a fraction of the fucoidan from *Ascophyllum nodosum* prepared by acid hydrolysis and centrifugal partition chromatography (LMWF) was established as:

$[\rightarrow 3)$-α-L-Fuc$(2SO_3^-)$-$(1\rightarrow 4)$-α-L-Fuc$(2,3diSO_3^-)$-$(1]_n$

by NMR spectroscopy and methylation analysis. The proton and carbon NMR spectra of this unit have been assigned and found to correspond with features in the spectra of the whole purified fucan from *A. nodosum* which account for most of the integrated intensity. The same structure has also been recognised in the fucoidan of *Fucus vesiculosus*. The fraction LMWF has in vitro anticoagulant activity, indicating that the above structure may be partly responsible for biological activity in the native fucoidan.

Keywords: Fucoidan; Fucan structures; Fucan NMR; Brown algae

Fucoidans, first isolated by Kylin[1] almost one century ago, are sulphated fucans extracted from Phaeophycophyta (or brown algae) such as *Fucus vesiculosus*, or *Ascophyllum nodosum*. The fucoidan of *F. vesiculosus* has been the most comprehensively studied, and this has been described as consisting of L-fucopyranose units with either α-$(1\rightarrow 2)$[2] or α-$(1\rightarrow 3)$ glycosidic linkages.[3] A commercially available preparation of *F. vesiculosus* fucoidan is in common use as a reagent in studies of mammalian fertilisation,[4] and for its ability to act as a ligand for selectins[5] and macrophage scavenger receptors.[6]

The structures of fucoidans are heterogeneous and branched, so detailed descriptions

Abbreviations: HMBC, heteronuclear multiple bond correlated spectroscopy; HSQC, heteronuclear single quantum correlated spectroscopy; LMWF, low molecular weight fucan; LMWH, low molecular weight heparin; ROE, rotating frame nuclear Overhauser enhancement; ROESY, rotating frame nuclear Overhauser enhancement spectroscopy.

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cannot be achieved by the study of the whole polysaccharides alone; however, highly purified fractions obtained by a variety of methods have been studied. One fraction in particular has recently been described as having anticoagulant activity in vitro, moderate molecular weight, and a relatively simple NMR spectrum.[7] The present study seeks to establish that this fraction, LMWF (fraction $H^3$5,p)[7], consists chiefly of oligosaccharides of about 8–14 fucose residues with a regular repeating disaccharide structure. Heterogeneity seen in the NMR spectra arises from terminal and near-terminal residues in these short oligosaccharides. As LMWF has anticoagulant activity in vitro, it is possible that the activity of the whole fucoidan is due to this predominant regular structure.

The main characteristics of LMWF have been already described.[7] In the previous paper, molecular weight (M_W) was determined with uncharged pullulans as standards. When a low molecular weight heparin (LMWH) was used as mass standard, LMWF appeared smaller ($M_W = 3090$), consisting of approximately 8–14 residue oligofucans. In both heparin and LMWF, each monosaccharide residue bears on average 1.5 sulphates. The MW profiles of LMWF and of reference material are shown superimposed in Fig. 1.

It has been shown previously that oligofucans of LMWF consist mainly of two fucose residue types A (2,3-disulphated fucose) and B (2-sulphated fucose), with two additional less abundant units; C (another 2,3-disulphated fucose residue) and D (another 2-monosulphated fucose residue), glycosidic linkages being α-(1→4) and α-(1→3).[7] In the present paper, these results are confirmed and extended by the use of ROESY spectra (Fig. 2(b)). ROE connectivity completes the assignment of fucose residues through strong ROE cross-peaks between H-4 and H-5 of both A and B. ROESY peaks also connect the two H-6 methyl resonances with corresponding H-5 and H-4 signals. Assignment of the two major types of fucose residue, A and B, is summarised in Table 1. Carbon chemical shifts assignments for residues A and B were determined from an HSQC spectrum (not shown). Chemical shifts varied a little with the recording temperature and the spectrometer. The data shown in Table 1 were measured with the 500 MHz spectrometer at 45 °C and

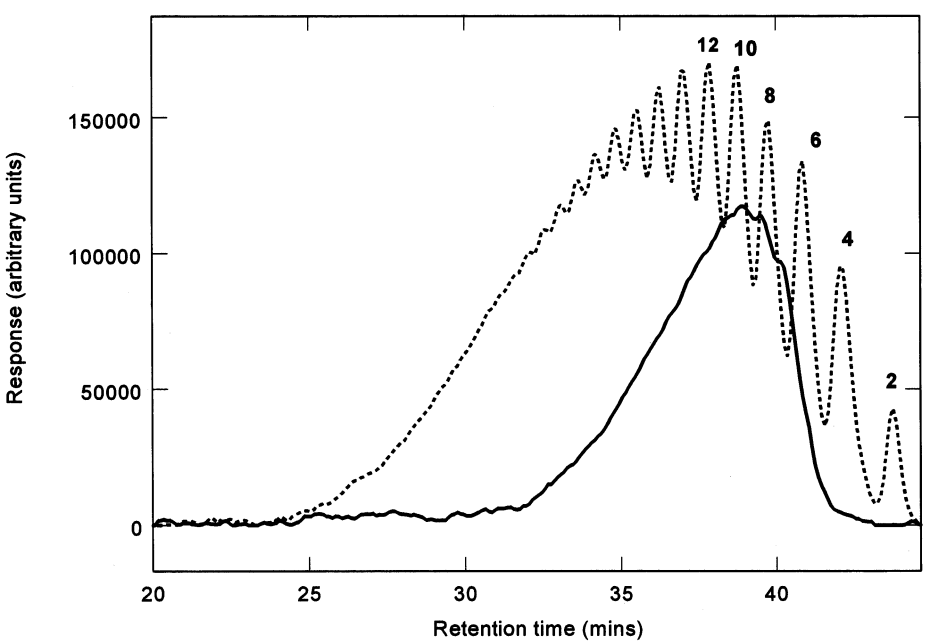

Fig. 1. Superimposed gel permeation chromatograms of 1st IRP LMWH for MW calibration (---) and of LMWF (—), according to the method of Mulloy et al.[17] The low molecular weight heparin calibrant consists of a series of even-numbered oligosaccharides, some of which can be resolved in the system. These are marked with their degree of polymerisation. As the fucan fraction is sulphated to the same level as heparin, similarly sized oligosaccharides of both structures are likely to run with the same retention times; on this basis most of the fucan fraction appears to be in the 8–14 residue range.

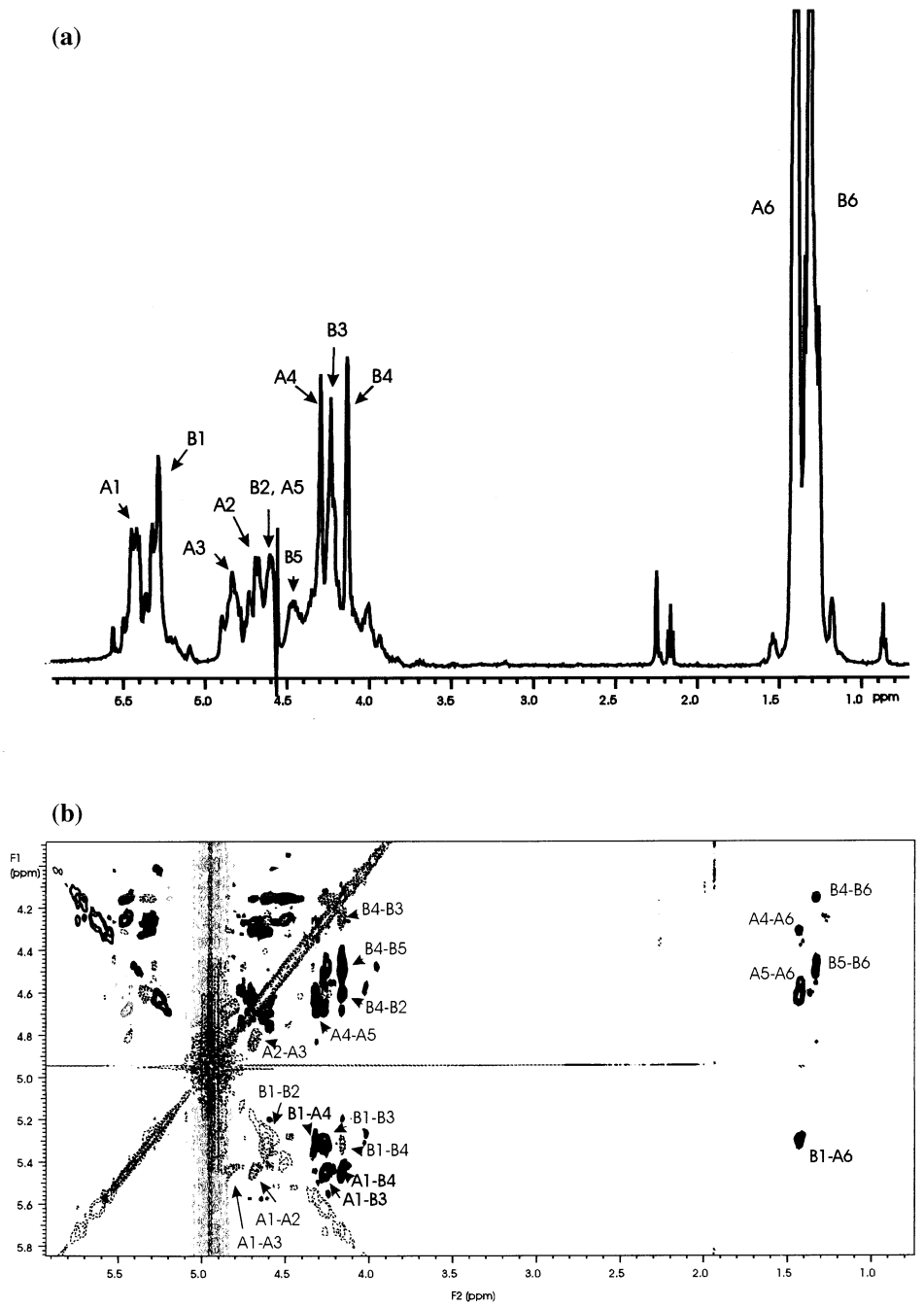

Fig. 2. ^{1}H NMR spectra of the fraction LMWF, recorded at 500 MHz in D_2O. (a) 1D spectrum recorded at 45 °C. (b) ROESY spectrum recorded at 10 °C. Both TOCSY cross-peaks (—) and ROESY cross-peaks (...) appear in the spectrum. Chemical shifts are relative to TSP at 0 ppm.

display slight differences from the previously published ones recorded at 25 °C (0.02 ppm in proton, 0.5 ppm in ^{13}C, or less).[7]

The same ROESY spectrum (Fig. 2(b)) provides evidence that the two residues, A and B, alternate to form a repeating disaccharide. Inter-residue cross-peaks can be seen between H-1 of residue B and both H-4 and H-6 of residue A, providing strong evidence for a $1\rightarrow4$ linkage between residue B and the 2,3 disulphated residue A. Inter-residue ROESY cross-peaks from H-1 of A can be seen to H-3 and H-4 of B, a pattern characteristic of a $1\rightarrow3$ A–B linkage. Similar inter-residue NOE patterns have also been seen in uniformly 4-linked and uniformly 3-linked linear sul-

Table 1
NMR data for the various fucose residues constitutive of oligofucans present in fraction LMWF

Residues	H-1	C-1	H-2	C-2	H-3	C-3	H-4	C-4	H-5	C-5	H-6	C-6
A [a]	≈5.45	96.9–97.9	≈4.69	75.2	4.86	76.9	4.32	≈82.1	4.61	70.6	1.40	18.4
A [b]	5.45		4.68		4.85		4.29					
B [a]	5.30 and 5.34	≈101.4	4.62–4.65	76	4.26	76	4.17	71.8–72.1	4.50	69.4	1.31	18.2
B [b]	5.29		4.61		4.20		4.13					
C [c]	5.43	97.5	4.61	75.3	4.78	78.1	4.27	73.7				
D [c]	5.30	101	4.50	78	4.20	70.2	3.97	75.3				
E [d]	5.58	93.4	4.64		4.73		4.34					
F [d]	5.52	93.4	4.57		4.11		4.14					

[a] A and B are internal residues.
[b] Values taken from spectra of native fucoidans in Ref. 10.
[c] C and D are the corresponding non-reducing end units to A and B, respectively.
[d] E and F are the corresponding reducing end α-anomeric units to A and B, respectively.

phated fucans.[8,9] Because the oligofucans in this sample are relatively short (8–14 fucose units), terminal fucose residues, both at the reducing and the non-reducing ends, make significant contributions to the NMR spectra. The two spin systems C and D were previously identified as fucose residues bearing the same pattern of sulphation as A and B, respectively.[7] Unambiguous NMR assignments are summarised in Table 1. C differs from A most noticeably in the chemical shifts of the H-4 (in A: 4.32 ppm; in C: 4.27 ppm) and C-4 (in A: 82.1 ppm; in C: 73.7 ppm) resonances. C may therefore be a non-reducing end terminal A residue, lacking the downfield glycosidation shifts at H-4 and C-4 of A. Similarly, D differs from B chiefly at H-3 (in B: 4.26 ppm; in D: 4.20 ppm) and C-3 (in B: 76 ppm; in D: 70.2 ppm), and by the same reasoning may be a non-reducing terminal residue. In both cases, the high field shift of terminal residue H-2 (see Table 2) is due to the disappearance of interactions across the glycosidic linkage with the sulphate substituent at O-3 or O-2 of the following residue A or B, respectively. Similarly, the low-field shift of C-4 of D (versus B) results from the elimination of the 3-*O*-substituent which shifts the C-4 upfield in the galactose series. It was not possible to extend the assignment of spin systems C and D to positions 5 and 6 without ambiguity.

In the earlier study[7] two small doublets at 5.58 and 5.52 ppm were attributed to H-1 of reducing end α-fucose residues on the basis of ^{1}H and ^{13}C chemical shifts. By careful examination of the COSY spectrum, these doublets have been attributed to A-type and B-type reductive terminal ends, (E and F residues in Table 1, respectively). The area ratios (all H-1)/(E H-1) and (all H-1)/(F H-1) were around 40 and 25, respectively, which corresponds to an oligofucan average length of 15 residues, roughly in concordance with SEC results. This calculation probably overestimates the true molecular size; although no H-1 − H-2 cross-peaks corresponding to β-forms were visible in the spectrum, weak H-6 − H-5 cross-peaks (at 1.44–3.94 ppm and 1.30–3.84 ppm) were present with H-5 chemical shift characteristic of β-reducing end residues.

Table 2
Percentage (in total ionic current %) of different kinds of substituted fucose present in LMWF, before and after solvolytic desulphation

Derivative as PMFA [a]	Percentage [b] in LMWF	Percentage [b] in desulphated LMWF
2,3,5-Tri-*O*-methyl-L-fucitol	<1	0
2,3,4-Tri-*O*-methyl-L-fucitol	<1	5
3,4-Di-*O*-methyl-L-fucitol	<1	0
2,4-Di-*O*-methyl-L-fucitol	18	56
2,3-Di-*O*-methyl-L-fucitol	27	35
4-*O*-Methyl-L-fucitol	41	≈3
3-*O*-Methyl-L-fucitol		0
2-*O*-Methyl-L-fucitol	11	<2
L-Fucitol	3	<2

[a] Partially methylated fucitol acetate.
[b] Percentages were based on the peak areas.

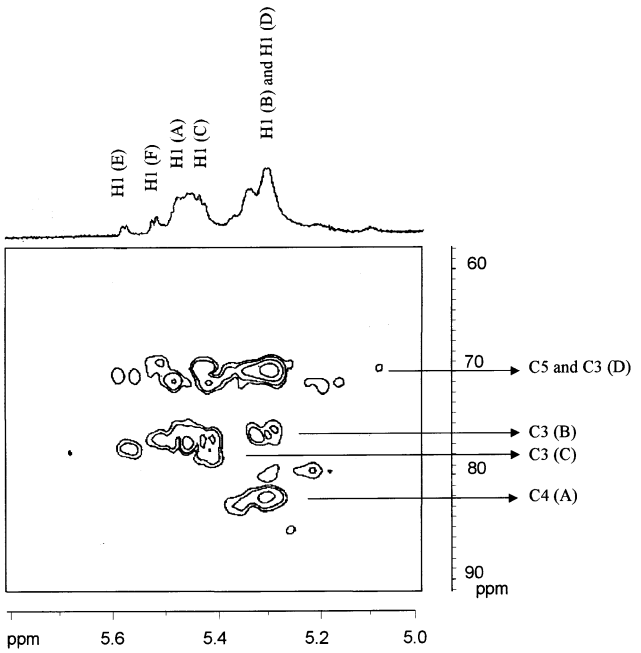

Fig. 3. Anomeric region of the HMBC spectrum of LMWF, showing inter- and intra-residue correlations between anomeric protons and ring carbons.

The 1D-^{1}H and COSY spectra[7] showed that there were at least three kinds of A residues differing slightly in their H-1 chemical shifts (between 5.42 and 5.48 ppm) and two kinds of B units giving two separate peaks at 5.3 and 5.34 ppm. This heterogeneity is probably due to the exact position of residues inside chains of variable lengths.

The HMBC spectrum was complex and complete assignment difficult. However, results deduced from it are in accord with the proposed structure and assignment. Firstly, the signal at 1.40 ppm (H-6 of A) is connected with carbons at 70.6 (C-5 of A) and 82.1 ppm (C-4 of A), while that at 1.31 ppm (H-6 of B) is correlated with carbons at 70–75.5 ppm (C-5 and C-4). In addition (see Fig. 3), it is clear that B and D anomeric protons are correlated to carbons at ≈ 70 (C-5 and C-3 of D), 76 (C-3 of B) and 82 (C-4 of A) ppm. In this way, the proposed α-(1 → 4) linkage B → A is corroborated. A and C anomeric protons are connected only with carbons at ≈ 70 (C-5) and 76–78 ppm (C-3 of A, B and C) in agreement with an α-(1 → 3) linkage A → B (Fig. 4).

GCMS analysis of partially methylated fucitol acetates obtained before and after desulphation of LMWF confirmed these results (see Table 2). Before desulphation, the major peak was attributed to 2,3-*O*- and 2,4-*O*-disubstituted fucose residues. Other abundant deriva-

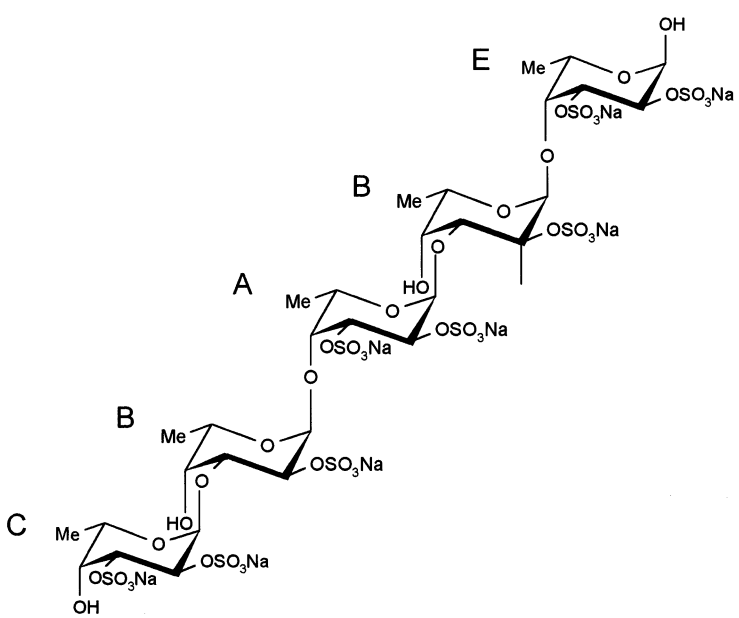

Fig. 4. Structure of sulphated oligofucans constitutive of LMWF, shown as the sodium salt. Reducing and non-reducing end terminal residues are of type A or B (called respectively C and D (non-reducing), E and F (reducing) in the text). The number of repetitive BA disaccharidic units is mainly between four and seven. Available data indicate that this structure constitutes the backbone of the native fucoidan, which also bears additional branches (sulphate, xylose, fucose and sulphated fucose) at position-4 of residues B.

tives were due to 3,4-*O*-disubstituted fucose as well as 3-*O*- and 4-*O*-monosubstituted fucose residues. There was no peak corresponding to unsubstituted non-reducing terminal fucose, and only a very low peak attributable to a fully substituted fucose residue. These results are not completely consistent with NMR data from which more di and trisubstituted residues were expected. This could result from some desulphation occurring during methylation in a very strongly basic medium; or this could be due to a selective retention of less sulphated (less hydrophilic) oligofucoses on the reverse-phase column. After solvolytic desulphation, all derivatives with substitution at O-2 disappeared, showing the absence of $1\rightarrow2$ intra-residue linkages. There was almost no terminal fucose linkage ($\approx 5\%$) and consequently only few branches if any. The main peaks were 1,3,5-tri-*O*-acetyl-2,4-di-*O*-methyl-L-fucitol and 1,4,5-tri-*O*-acetyl-2,3-di-*O*-methyl-L-fucitol showing that the fucan framework is constituted by the disaccharide $[\rightarrow3)$-α-L-Fuc-$(1\rightarrow4)$-α-L-Fuc-$(1\rightarrow]$. The alternative hypothesis assuming that fucans are constituted with blocks of $[\rightarrow3)$-α-L-Fuc-] and $[\rightarrow4)$-α-L-Fuc-] residues is inconsistent with NMR results and cannot be retained.

It has previously been shown that 1D NMR spectra of purified native fucoidans from *A. nodosum* and *F. vesiculosus* are very similar.[10] Most of the intensity in both spectra arises from two fucose residues which bear a close similarity to A and B in this study (Table 2).

The main difference between LMWF and native fucoidan 1D proton spectra is the presence in the latter of many resonances in the region 4.1–3.0 ppm, due to protons of unsubstituted positions of fucose residues (side-chain fucoses or desulphated backbone fucoses) or other monosaccharides such as xylose. Methylation studies of native fucoidan from *A. nodosum* and *F. vesiculosus*[3,10] give results concordant with a structure of 3- and 4-linked fucose residues; together these residues are always among the most abundant in desulphated[10] or low sulphated samples.[3] Methylation analysis demonstrates that native fucoidan contains many side-chains no longer present in LMWF after acid hydrolysis and purification steps.

Consequently, because of its size (around ten monosaccharide residues), the LMWF fraction must be a fragment of the backbone (rather than a side-chain) of *A. nodosum* fucan, which is constituted of the B-A disaccharide repeating unit

$[\rightarrow3)$-α-L-Fuc$(2SO_3^-)$-$(1\rightarrow4)$-α-L-Fuc$(2,3diSO_3^-)$-$(1]_n$.

This conclusion is not compromised by the relatively low yield of LMWF. A homogeneous oligosaccharide of this length cannot be obtained in high quantity from a strongly degradative procedure producing many different fragments. An overall yield of 2% was a satisfactory result after two purification steps.[7] In the native fucoidan, the B residues bear (more or less regularly) a substituent at O-4 (sulphate, xylose, fucose or sulphated fucose). Because of its similarity, *Fucus vesiculosus* fucoidan probably displays a very similar (if not identical) structure. Nevertheless, this structure is not common to all Phaeophycophyta fucoidans, and may be restricted only to those of Fucales. In particular, it appears that $1\rightarrow2$ linkages (not found in this study) are present in anticoagulant fucoidans of some Laminariales as *L. brasiliensis*,[10] *E. kurome*[11] and *Chorda filum*.[12] The demonstrable activity of LMWF in the APTT assay[7] shows that branched structures are not always necessary for anticoagulant activity.

The degree of sulphation of this fucoidan fraction, at three sulphates per disaccharide, is exactly the same as that of the major repeating unit of heparin,

[4)-α-L-IdoA$(2SO_3^-)$-$(1\rightarrow4)$-β-D-Glc-$(NSO_3^-,6SO_3^-)$-$(1\rightarrow]_n$

though the structure and linkage of the monosaccharide units and the positions of sulphation are different. The 2,3-disulphated residue is a common feature found in anticoagulant fucoidan fractions, as well as in anticoagulantly active oversulphated chondroitin sulphate[13] and in pentosan polysulphate.[14,15] Leaving out of consideration, those properties of heparin expressed through high affinity for antithrombin, which depend on the presence of a specific and unusual oligosaccharide sequence,[16] the spectrum of biological activities of fucoidans and heparin are sufficiently simi-

lar to suggest some common mechanisms of action. Fucoidan fractions of regular and defined sequence will be invaluable in comparative investigations of the relationship of structure to function of sulphated polysaccharides.

1. Experimental

Preparation, purification and characterisation of the fucan fraction.—LMWF was prepared as previously described (fraction $H^3$5,p).[7] Molecular weight was also characterised as previously described[17] using as calibrant the 1st International Reference Preparation Low Molecular Weight Heparin for Molecular Weight Calibration, NIBSC 90/686.

Solvolytic desulphation.—LMWF (3 mg) was dissolved in a mixture of DMSO, MeOH, pyridine (89:10:1, v/v/v) and heated to 100 °C for 2 h. After cooling, deionised water (9 mL) was added and the resulting solution neutralised, dialysed against water and freeze-dried. The residue was used for methylation analysis.

Methylation analysis.—Methylation analyses were performed using a modification of the Hakomori procedure[18] as previously described.[19]

NMR spectroscopy.—NMR spectra were recorded using a Varian Unity 500 or a Bruker DRX-400 spectrometer. Pulse sequences for two-dimensional techniques were supplied by the spectrometer manufacturers. The ROESY spectrum was recorded at 10 °C with a 150 ms mixing time, and the pulse sequence was modified to include a spin-echo sequence in order to improve the baseline (spectrum recorded only on the 500 MHz apparatus). The HSQC spectrum (run at a temperature of 45 °C) was optimised for coupling constant $^1J_{CH}$ of 150 Hz, and the HMBC spectrum was run at a temperature of 25 °C on the DRX-400 spectrometer using the conventional pulse program provided by Bruker, with a delay of 60 ms for evolution of long range couplings. Chemical shifts in Table 1 were determined at 45 °C.

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