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Carbohydrate Research 314 (1998) 1–12

CARBOHYDRATE RESEARCH

# NMR spectroscopic characterisation of oligosaccharides from two *Ulva rigida* ulvan samples (Ulvales, Chlorophyta) degraded by a lyase

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Received 18 September 1998; accepted 2 November 1998

**Abstract**

The chemical structure and the sequence of repeating units in ulvans of similar compositions from two different *Ulva rigida* samples collected in the Canary Islands and in Brittany were studied after ulvan-lyase degradation and NMR spectroscopic analysis of the reaction products. Both ulvans were composed of ulvanobiuronic acid 3-sulfate type A [→4)-β-D-GlcA-(1→4)-α-L-Rha 3-sulfate-(1→] (symbolised as $A_{3s}$) and contained disaccharides composed of [→4)-β-D-Xyl-(1→4)-α-L-Rha 3-sulfate-(1→] and [→4)-β-D-Xyl 2-sulfate-(1→4)-α-L-Rha 3-sulfate], respectively referred to as ulvanobiose 3-sulfate ($U_{3s}$) and ulvanobiose 2′,3-disulfate ($U_{2's,3s}$). In the Canary Islands sample, these $U_{3s}$ and $U_{2's,3s}$ occurred dispersed among $A_{3s}$ sequences and as short blocks of two or three units. In contrast, in the Brittany samples, these units were dispersed among $A_{3s}$ structures and next to $A_{3s}$ units branched at O-2 of α-L-Rha 3-sulfate by a terminal β-D-GlcA and symbolised as $A_{2g,3s}$. However, more complex structures are likely to occur in the enzyme resistant fraction remaining from this ulvan. An average structure sequence of these two ulvans was proposed. The transposition of the $^{13}C$ NMR data of the new identified structures to the parent polysaccharides was not possible, probably due to the different sequence distributions affecting the carbons chemical shifts. 

*Keywords:* Ulvan; *Ulva rigida*; Lyase; Oligosaccharides; NMR

## 1. Introduction

Green algae belonging to Ulvales are used as food or feed [1] and are frequently involved in algal proliferation in eutrophicated coastal and lagoon waters [2]. Ways to use the latter biomass besides compost [3,4], methane production [5] or paper making [6] could be based on specific properties of their cell-wall polysaccharides. These macromolecules retain heavy metals and several *Ulva* and *Enteromorpha* species are used as bioindicators of pollution [7,8]. In edible Ulvales, cell-wall polysaccharides play a nutritional role as dietary fibre [9–13] and, from different genera, they demonstrate biological activities [14–18] or gelling abilities [19–21].

A better description of the chemical structure of Ulvales cell-wall polysaccharides can lead to a better understanding of their different biological, physico-chemical and rheological properties. Several studies on *Ulva* sp. allowed water-soluble sulfated xylorhamnoglycuronans [22,23] named ulvan, alkali-soluble linear β-(1→4)-D-xyloglucans and a β-(1→4)-D-glucuronan [21,23] and insoluble amorphous cellulose [24,25] to be identified.

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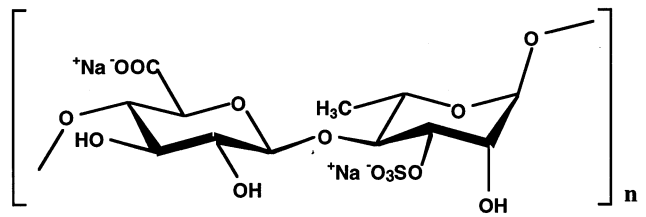
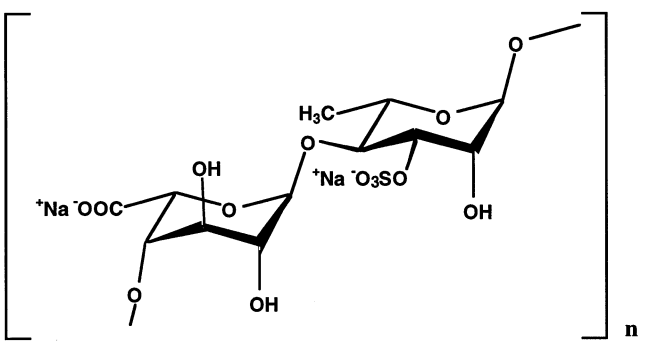
0008-6215/98/$ - see front matter 
PII: S0008-6215(98)00293-6

Various distributions and molecular associations of these polysaccharides in the cell wall of *Ulva* sp. have been proposed [26]. The complexity and the different biological and functional properties of ulvan stimulated more detailed studies on the chemistry of *Ulva* sp. ulvan. It is essentially composed of rhamnose 3-sulfate and glucuronic acid or iduronic acid existing as aldobiuronic acids called ulvanobiuronic acid 3-sulfate A or B and symbolised by $A_{3s}$ or $B_{3s}$, respectively (Scheme 1) [27–32]. Xylose can also occur as partially sulfated residues at O-2 and glucuronic acid as a branch at O-2 of rhamnose 3-sulfate [27]. Nuclear magnetic resonance (NMR) spectroscopic characterisation of several ulvan oligosaccharides indicated that xylose replaces uronic acids in the disaccharide repeating structures and that (1→4)-linked β-D-glucuronic acid can occur as contiguous residues [28–30]. The NMR data obtained from the latter analyses permitted the attribution of the signals of the main repeating structures of ulvan $^1$H and $^{13}$C spectra [30,31]. This paper reports on an approach to sequencing of ulvan from two *Ulva* samples designated as *U. rigida* [23,33] by NMR identification of oligosaccharides obtained after ulvan-lyase degradation.




**Sodium ulvanobiuronate 3-sulfate A ($A_{3s}$)**




**Sodium ulvanobiuronate 3-sulfate B ($B_{3s}$)**

Scheme 1. Chemical structure for two main repeating disaccharides in ulvan from *Ulva* sp.

## 2. Materials and methods

*Materials.*—Ulvans used were samples extracted from *U. rigida* collected in Las Palmas (Canary Islands, Spain) and in Brittany (Pointe Saint Gildas, France) in previous studies on *Ulva* dietary fibres [23,33]. The ulvan-lyase was prepared according to [29] and due to the limited amount of activity, no attempt was made to purify it. Its activity, given in nkat/mL, corresponds to the amount of enzyme necessary to produce 1 nmol of reducing rhamnose per second of reaction using 'sea-lettuce' ulvan as substrate [29]. The reducing sugar assay was performed according to the Nelson–Somogyi method [34].

*Enzymatic degradation.*—Ulvans (4% in Tris–HCl 0.1 mol/L, pH 9.0) were degraded over about 3 days at 40 °C by successive addition of lyase activity: 5.1 nkat for 16 h, 5.1 nkat for 24 h and 9.0 nkat for 24 h. The solutions were then boiled in a water-bath for 10 min.

*Gel permeation chromatography.*—Freeze-dried ulvan enzymatic degradation products were redissolved in a small amount of $NaNO_3$ (0.050 mol/L) and chromatographed through columns of Bio-Gel P2 or P4 (97 × 2.6 cm, Bio-Rad) eluted by $NaNO_3$ (0.05 mol/L, 25–27 mL/h). Peaks detected by differential refractometry were pooled, concentrated by rotary-evaporation and desalted on a Sephadex G10 column (100 × 1.6 cm, Pharmacia) eluted with deionized water and freeze-dried. Elution volumes are expressed as $K_{av}$, with $K_{av} = (V_e - V_0)/(V_t - V_0)$ where $V_t$ and $V_0$ are the total and the void volume of the column and $V_e$ the elution volume of the sample.

*Nuclear magnetic resonance spectroscopy.*—$^1$H and $^{13}$C NMR spectra were recorded on a Bruker ARX 400 spectrometer on deuterium exchanged (twice in $D_2O$ 99.9% D) oligosaccharides in 100% D deuterium oxide (Aldrich). Chemical shifts were calculated from internal acetone resonance ($\delta^1$H 2.225, $\delta^{13}$C 31.45 ppm). Polysaccharides were solubi-

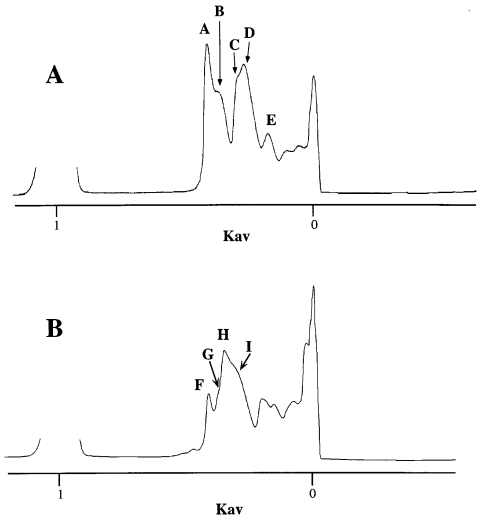



Fig. 1. Bio-Gel P4 chromatograms of oligosaccharides recovered after ulvan-lyase degradation of *U. rigida* ulvan from (A) Canary Islands and (B) from Brittany. Letters correspond to fractions collected.

lized in 99.9% D deuterium oxide and spectra were recorded at 333 K. Carbon chemical shifts are expressed from $Me_2SO$ at 39.6 ppm. Non-exchangeable proton and carbon assignments and sugar sequences of oligosaccharides were determined from 2D experiments (COSY90, NOESY, $^1H-^{13}C$ HMQC and $^1H-^{13}C$ HMBC) using the conventional pulse programs provided by Bruker.

## 3. Results

*Ulvan oligosaccharides from* U. rigida *collected on the Canary Islands.*—Ulvan extracted as water-soluble dietary fibers in a previous study [33] from *U. rigida* grown in the Canary Islands and composed of rhamnose (26.9 mol%), xylose (10.6 mol%), uronic acid (21.5 mol%) and sulfate (36.7 mol%) was degraded by the ulvan-lyase preparation isolated from a marine bacterium [29]. The degradation products were chromatographed on Bio-Gel P2 and the material eluting near the void volume ($K_{av}$ 0–0.21) was chromatographed on Bio-Gel P4 (Fig. 1(A)). The main peak eluting from Bio-Gel P2 ($K_{av}$ 0.42) was identified by $^1H$ NMR spectroscopy as being composed mainly of Δ-(1→4)-L-Rha 3-sulfate (Δ refers to the unsaturated uronic acid, 4-deoxy-L-threo-hex-4-enopyranosiduronic acid), as the chemical shifts were those reported for this disaccharide [29]. The oligosaccharide fractions (B–E, Fig. 2) isolated by Bio-Gel P4 chromatography were identified by NMR spectroscopy (Figs. 2 and 3). Fraction A had a $^1H$ NMR spectrum identical to that of Δ-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl-(1→4)-L-Rha 3-sulfate [29].

Fraction B was identified after complete attribution of the $^1H$ and $^{13}C$ NMR signals by COSY and HMQC 2D-NMR experiments (Table 1) and by comparison with the chemical shifts of Fraction A and those of P2B in [29] (Fig. 3). The intensity of the anomeric carbon resonances and the amount of different spin systems identified indicated that Frac-

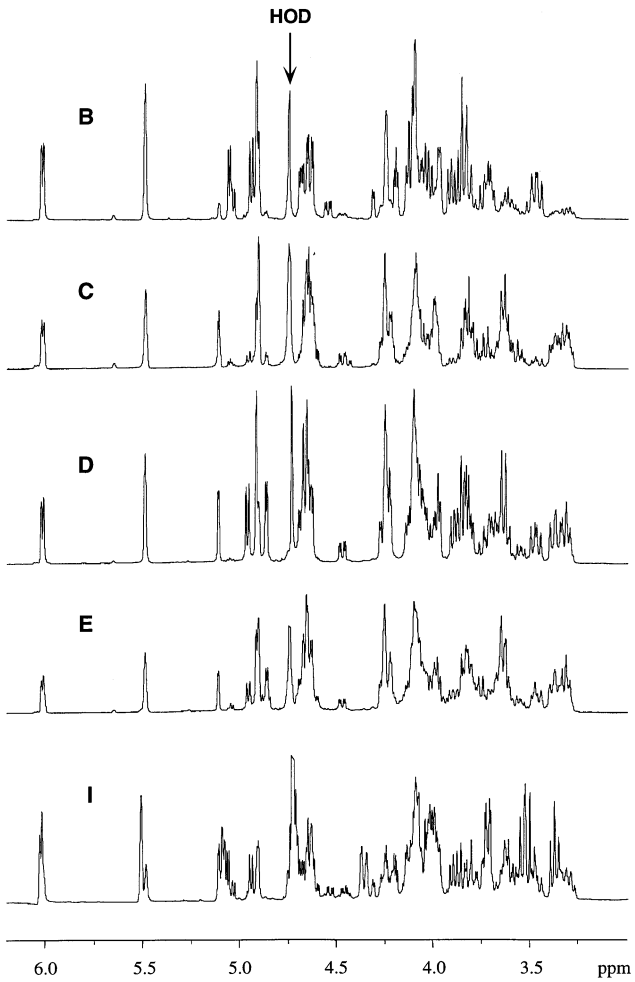



Fig. 2. $^1H$ NMR spectra of oligosaccharide Fractions B–E collected after Bio-Gel P4 chromatography of the ulvan-lyase degradation products of *U. rigida* ulvan from the Canary Islands and of Fraction I isolated from the Brittany sample (300 K, acetone $\delta^1H$: 2.225 ppm).

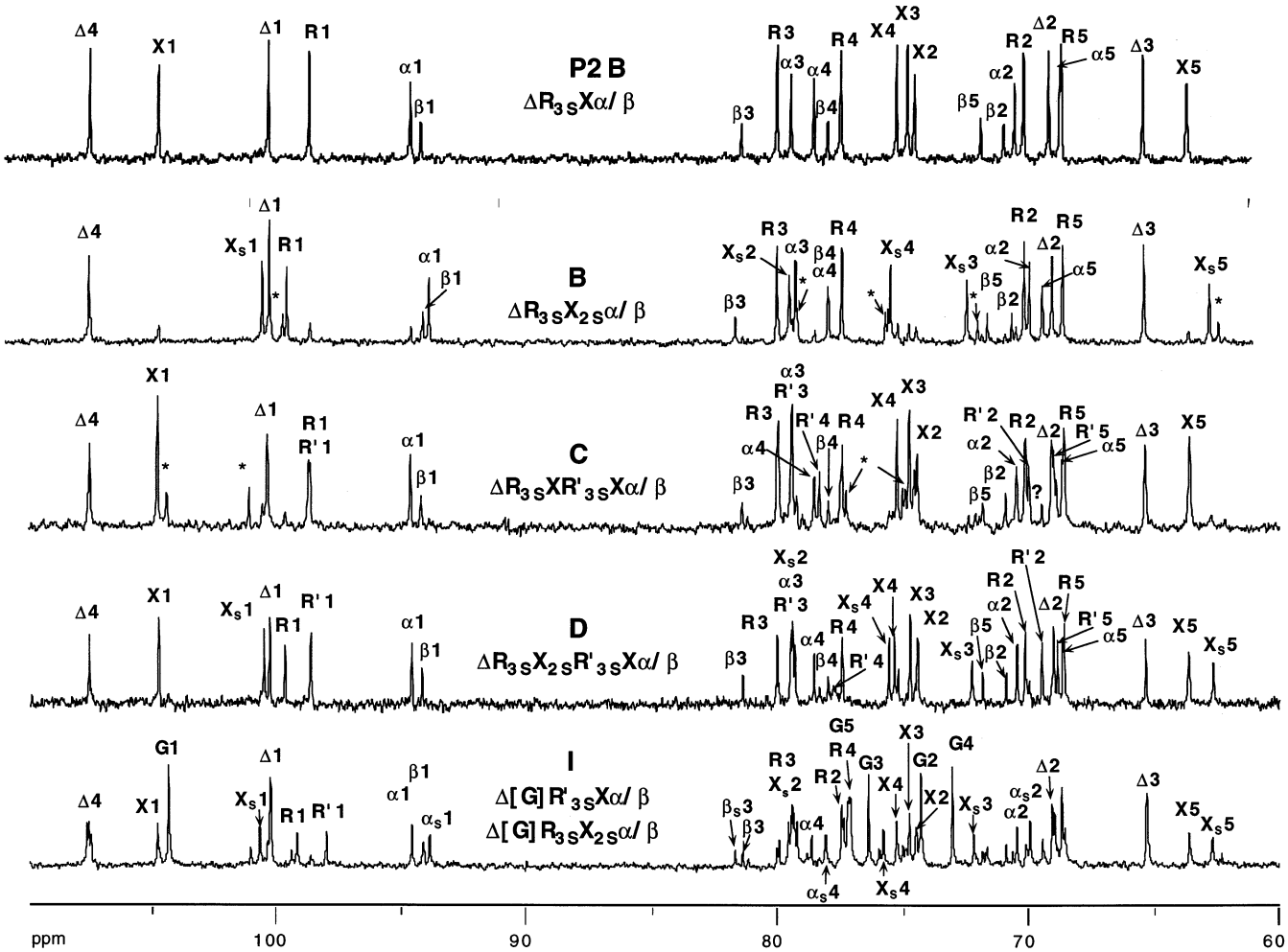



Fig. 3. $^{13}C$ NMR spectra of oligosaccharide Fraction P2B from Ref. [29], Fractions B, C, D and I of oligosaccharides recovered after Bio-Gel P4 chromatography of the ulvan-lyase degradation products of ulvan from the two *U. rigida* samples (300 K, acetone $\delta^{13}C$: 31.45 ppm; question mark: unattributed signal). Letters and numbers correspond to carbons in sugar residues of oligosaccharides depicted in the Figure: Δ, G, R and R′, X, α/β refer to 4-deoxy-L-*threo*-hex-4-enopyranosiduronic acid, 4-linked β-D-GlcA, 4-linked α-L-Rha, 4-linked β-D-Xyl and 4-linked reducing end L-Rha in α or β configuration, respectively; [G] refers to terminal β-D-GlcA linked to O-2 of α-L-Rha; the subscripts 2s and 3s refer to positions of sulfate groups.

tion B was a tetrasaccharide. Major chemical shifts differences were observed for all the protons and carbons of the xylose residue. The carbons most affected were C-2 ( $\sim +5.8$ ppm), C-1 ( $\sim -4.2/-4.4$ ppm) and C-3 ( $\sim -2.3$ ppm, Table 1, Fig. 3(B)) and the shifts are characteristic of a substitution at O-2. As xylose residues in ulvans have been shown to be partially sulfated at O-2 [22,27], the substitution observed on this sugar residue was attributed to sulfation. Both $^{1}H$ and $^{13}C$ chemical shifts of the reducing rhamnose 3-sulfate were affected by the substituted xylose residue and, unlike oligosaccharide A, the latter residue was also affected by the anomeric configuration of the reducing sugar. Small chemical shifts differences were observed for all the $^{1}H$ and $^{13}C$ signals (Table 1, Fig. 3(B)) and tentative assignments of the xylose carbons linked to the reducing end β-rhamnose 3-sulfate (asterisk in Fig. 3(B)) are given in Table 1. The sensitivity of the 2D-NMR experiments was insufficient to allow for their unambiguous attribution. The sugar sequence and linkages were the same as oligosaccharide A, as deduced from the close carbon chemical shifts values for C-4 of the rhamnose 3-sulfate and xylose residues with those of P2B (Fig. 3). Further evidence for these linkages came from 2D-NMR experiments performed on Fraction D (see below). Fraction B was thus identified as Δ-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl

Table 1
$^{1}H$ and $^{13}C$ chemical shifts and coupling constants (in parentheses) of oligosaccharide Fraction B (acetone $\delta^{1}H$: 2.225 and $\delta^{13}C$: 31.45; 300 K for $^{1}H$ and 320 K for $^{13}C$)

| | | 1 | 2 | 3 | 4 | 5 | 5′ | 6 |
|---|---|---|---|---|---|---|---|---|
| Rha α | $^{1}H$ | 5.06 (4.3) | 4.19 | 4.69 | 3.91 | 4.03 | | 1.34 |
| | $^{13}C$ | 93.91 | 69.96 | 79.56 | 78.02 | 69.43 | | 18.53 |
| Rha β | $^{1}H$ | 4.92 (~1.9) | 4.32 | 4.55 | 3.77 | 3.61 | | 1.33 |
| | $^{13}C$ | 94.15 | 70.67 | 81.70 | 78.02 | 71.63 | | 18.53 |
| Xyl 2S α | $^{1}H$ | 4.95 (6.1) | 4.12 | 3.86 | 3.72 | 3.476 | 4.13 | |
| | $^{13}C$ | 100.62 | 79.32 | 72.46 | 75.53 | 62.72 | | |
| Xyl 2S β | $^{1}H$ | 5.04 (5.5) | 4.14 | 3.88 | 3.72 | 3.50 | 4.13 | |
| | $^{13}C$ | 100.28 | 79.25 | 72.46 | 75.53 | 62.72 | | |
| Rha | $^{1}H$ | 4.92 (2.4) | 4.26 | 4.65 | 3.84 | 4.07 | | 1.18 |
| | $^{13}C$ | 99.63 | 70.18 | 80.03 | 77.48 | 68.63 | | 17.82 |
| Δ | $^{1}H$ | 5.49 | 4.11 | 3.99 | 6.02 | | | |
| | $^{13}C$ | 100.34 | 69.05 | 65.37 | 107.60 | 145.42 | | 170.49 |

2-sulfate-(1→4)-L-Rha 3-sulfate, but minor quantities of other oligosaccharides, such as one containing unsulfated xylose linked to reducing rhamnose 3-sulfate were apparent from small signals observed on both $^{1}H$ and $^{13}C$ NMR spectra (Fig. 2(B) and Fig. 3(B)).

The $^{1}H$ and $^{13}C$ NMR spectra of Fraction C was very similar to that of oligosaccharide A but with lower intensities for the non-reducing and reducing ends signals (Fig. 2(C) and Fig. 3(C)). Based on the integral of the H-2 resonances of the rhamnose 3-sulfate residues (4.23–4.27 ppm) and that of H-1 of the unsaturated uronic acid (5.49 ppm), three rhamnose residues were inferred. By analogy with Fraction A, this oligosaccharide was identified as the hexasaccharide Δ-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl-(1→4)-L-Rha 3-sulfate. The complete attribution of the $^{1}H$ and $^{13}C$ NMR signals was achieved by COSY and HMQC 2D-NMR experiments and by comparison of the chemical shifts with those of oligosaccharide P2B, and particularly with those of the reducing end rhamnose 3-sulfate in the α-configuration for the additional internal rhamnosyl residue (Table 2). Of interest is the splitting of the xylose C-2 signal which may be due to the slightly different environment of the two sugar residues. Other minor signals were observed in the NMR spectra of Fraction C (noted by asterisk in Fig. 3(C)) and were attributed to a small proportion of ulvanobiuronic acid 3-sulfate type A repeating units.

Oligosaccharide D showed $^{1}H$ and $^{13}C$ NMR spectra with signals common to the major oligosaccharide in Fractions A, C and B (Fig. 2(C) and Fig. 3(C)). All the $^{1}H$ and $^{13}C$ NMR signals were attributed by a 2D COSY and HMQC experiments (Table 3) and by comparison with those in oligosaccharide A, P2B in [29] and B (Table 1). In the light of the various spin systems identified, this oligosaccharide is composed of six sugars: one 4-deoxy-L-*threo*-hex-4-enopyranosiduronic acid at the non-reducing end, one reducing end rhamnose 3-sulfate, one xylose and one xylose 2-sulfate and two different internal rhamnose 3-sulfate residues. The sugar sequence established from the 2D HMBC experiment (Fig. 4) confirmed that all were (1→4)-linked in the following order: Δ-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl 2-sulfate-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl-(1→4)-L-Rha 3-sulfate. The conformation of rhamnose 3-sulfate between the two xylose residue is most likely different from that at the reducing-end, close to the unsaturated uronic acid or next to an internal glucuronic acid residue [29] as shown from the modifications in the chemical shifts and particularly from the coupling constants such as the $J_{1,2}$ (Table 3). This probably results from the 2-*O*-sulfation of xylose and was also observed on the reducing end rhamnose 3-sulfate of oligosaccharide B. The consequence of such sulfation on xylose may affect the overall regularity of the polysaccharide chain conformation.

Table 2
$^{1}H$ and $^{13}C$ chemical shifts of oligosaccharide Fraction C (acetone $\delta^{1}H$: 2.225 and $\delta^{13}C$: 31.45 ppm, 300 K)

| | | 1 | 2 | 3 | 4 | 5 | 5′ | 6 |
|---|---|---|---|---|---|---|---|---|
| Rha α | $^{1}H$ | 5.11 | ~4.23 | ~4.63 | ~3.81 | 3.99 | | 1.32 |
| | $^{13}C$ | 94.61 | 70.57 | 79.48[a] | 78.60 | 68.73 | | 18.32 |
| Rha β | $^{1}H$ | 4.90 | 4.27 | 4.46 | 3.73 | 3.54 | | 1.32 |
| | $^{13}C$ | 94.21 | 70.97 | 81.43 | 77.99 | 71.86 | | 18.32 |
| Xyl | $^{1}H$ | 4.65 | 3.31 | 3.61 | 3.66 | 3.37 | 4.09 | |
| | $^{13}C$ | 104.68 | 74.59[a] | 74.84 | 75.46 | 63.60 | | |
| Rha′ | $^{1}H$ | 4.90 | ~4.23 | ~4.63 | ~3.81 | 4.05 | | 1.32 |
| | $^{13}C$ | 98.78[a] | 70.07 | 79.57[a] | 78.39 | 69.07 | | 18.12 |
| Xyl′ | $^{1}H$ | 4.65 | 3.31 | 3.61 | 3.66 | 3.37 | 4.09 | |
| | $^{13}C$ | 104.68 | 74.51[a] | 74.84 | 75.46 | 63.60 | | |
| Rha | $^{1}H$ | 4.90 | ~4.23 | ~4.63 | ~3.85 | 4.02 | | 1.19 |
| | $^{13}C$ | 98.70[a] | 70.22 | 80.01 | 77.55 | 68.70 | | 17.85 |
| Δ | $^{1}H$ | 5.47 | 4.07 | 3.99 | 5.99 | | | |
| | $^{13}C$ | 100.52 | 69.31 | 65.63 | 107.37 | 145.68 | | ~170.10 |

[a] Signals may be interchanged in the same column.

Fraction E had a $^{1}H$ NMR spectrum similar to that of oligosaccharide D except that the intensity of the signals for the sugar at both ends and that of β-D-xylose 2-sulfate-(1→4)-α-L-rhamnose 3-sulfate were lower (Fig. 2(E)). An additional resonance for H-1 of rhamnose 3-sulfate was observed at 4.92 ppm and the intensity of the xylose H-1 resonance at 4.66 ppm increased. Based on the integrals of the different anomeric protons and those of rhamnose 3-sulfate H-3, this fraction was an octasaccharide. The sugar sequence Δ-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl 2-sulfate-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl-(1→4)-L-Rha 3-sulfate was thus assumed from the similarity of the $^{1}H$ NMR chemical shifts with those in oligosacharide D (Fig. 2(D,E)). However, other positions for the β-D-xylose 2-sulfate 1→4 α-L-rhamnose 3-sulfate disaccharide in this oligosaccharide cannot be ruled out, particularly if we take into account the minor signals flanking that of H-1 of rhamnose 3-sulfate at 4.87 ppm and those near 5.05 ppm (Fig. 2(E)).

*Ulvan oligosaccharides from* U. rigida *collected in Brittany*.—The ulvan from the seaweed refered to as *U. rigida*, and composed of rhamnose (23.3 mol%), xylose (10.9 mol%), uronic acid (24.9 mol%) and sulfate (35.4 mol%) [27], was treated with ulvan-lyase as for the above ulvan sample. The degradation products were also chromatographed on Bio-Gel P2 and the material eluting at $K_{av}$ 0–0.24 was chromatographed on Bio-Gel P4 (Fig. 1(B)). The main fraction from Bio-Gel P2 eluting at $K_{av}$ 0.38 was also identified as mainly composed of the disaccharide Δ-(1→4)-L-Rha 3-sulfate. Oligosaccharide Fractions F, G and H eluted from Bio-Gel P4 were Δ-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl-(1→4)-L-Rha 3-sulfate, Δ-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl 2-sulfate-(1→4)-L-Rha 3-sulfate and Δ-(1→4)-α-L-Rha 3-sulfate-(1→4)-β-D-GlcA-(1→4)-L-Rha 3-sulfate, respectively, based on the similar chemical shifts observed for the signals on their $^{1}H$ NMR spectrum compared to those already described (oligosaccharides A, B and P4B in [29]).

Oligosaccharide Fraction I was more complex (Fig. 2(I)) and the structure of the major oligosaccharide was determined from COSY, HMQC and HMBC 2D NMR experiments and by comparison of its $^{13}C$ NMR spectrum (Fig. 3(I)) with that of other ulvan oligosaccharides. Twelve different spin systems were identified in the COSY map and the major $^{1}H$ and $^{13}C$ NMR signals assignments are reported in Table 4. The structures depicted in Fig. 5 were identified from the chemical shifts and by comparison with the NMR data of structures previously described [28,29]. Based on the anomeric carbons and protons intensity, two major oligosaccharides were present in approximately equal amounts. Both were

Table 3
$^{1}H$ and $^{13}C$ chemical shifts and coupling constants (in parentheses) of oligosaccharide Fraction D (acetone $\delta^{1}H$: 2.225 and $\delta^{13}C$: 31.45, 300 K)

| | | 1 | 2 | 3 | 4 | 5 | 5′ | 6 |
|---|---|---|---|---|---|---|---|---|
| Rha α | $^{1}H$ | 5.11 (1.2) | 4.25 | 4.65 | 3.82 | 4.00 | | 1.32 |
| | $^{13}C$ | 94.60 | 70.48[a] | 79.44 | 78.58 | 68.62[a] | | 18.48[a] |
| Rha β | $^{1}H$ | 4.92 (~0.9) | 4.27 | 4.47 | 3.74 | 3.55 | | 1.33 |
| | $^{13}C$ | 94.19 | 70.91 | 81.41 | 78.02 | 71.84 | | 18.34[a] |
| Xyl | $^{1}H$ | 4.66 | 3.37 | 3.63 | 3.68 | 3.37 | 4.09 | |
| | $^{13}C$ | 104.77 | 74.45 | 74.75 | 75.37 | 63.62 | | |
| Rha′ | $^{1}H$ | 4.87 (3.5) | 4.23 | 4.69 | 3.89 | 4.05 | | 1.32 |
| | $^{13}C$ | 98.67 | 69.49 | 79.49 | 77.80 | 68.87[a] | | 18.34[a] |
| Xyl 2S | $^{1}H$ | 4.96 (6.0) | 4.11 | 3.86 | 3.70 | 3.47 | 4.12 | |
| | $^{13}C$ | 100.55 | 79.35 | 72.27 | 75.59 | 62.63 | | |
| Rha | $^{1}H$ | 4.92 (~1.0) | 4.25 | 4.64 | 3.83 | 4.06 | | 1.17 |
| | $^{13}C$ | 99.70 | 70.16[a] | 80.04 | 77.46 | 68.62[a] | | 17.80 |
| D | $^{1}H$ | 5.49 | 4.10 | 3.97 | 6.02 | | | |
| | $^{13}C$ | 100.32 | 69.02 | 65.34 | 107.60 | 145.42 | | 170.50 |

[a] Signals may be interchanged in the same column.

identified as pentasaccharides with different sugar sequences determined by the NOESY and HMBC experiments (Fig. 5). In particular, a non-reducing terminal glucuronic acid residue was identified by comparison with the chemical shifts of branched oligosaccharides D2 and D3 reported in Ref. [28]. The two major oligomers in Fraction I differed in the presence or absence of sulfate at O-2 of the xylose residue and their structures were identified as Δ-(1→4)-[β-D-GlcA-1→2]-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl-(1→4)-L-Rha 3-sulfate and Δ-(1→4)-[β-D-GlcA-1→2]-α-L-Rha 3-sulfate-(1→4)-β-D-Xyl 2-sulfate-(1→4)-L-Rha 3-sulfate. The presence of the glucuronic acid branch at O-2 of rhamnose 3-sulfate, next to the unsaturated uronic acid at the non-reducing end, affected the chemical shift of the latter sugar anomeric proton and, most pronounced, that of rhamnose C-2 (+7.32/7.01 ppm). Minor amounts of other structures were also present such as $A_{3s}$ observed on the COSY map and on Fig. 3(I). However, the complete assignment of these structures was not possible because of the sensitivity and resolution limits of the NMR spectrometer.

*Comparison between the $^{13}C$ NMR spectra of the parent polysaccharides and that of oligosaccharides.*—The $^{13}C$ NMR spectra of the parent polysaccharides used to generate the various oligosaccharides are given in Fig. 6. The major signals were attributed to the repeating $A_{3s}$ disaccharide based on their chemical shift (G1: 104.4–104.6; G2: 74.8–74.9; G3: 75.3–75.6; G4: 80.2–80.4; G5: 77.7–77.9; G6: 176.4–176.5; R1: 101.0–101.1; R2: 70.3–70.5; R4: 79.5–79.7: R5: 69.4–69.6; R6: ~18.4 ppm) [30]. The great complexity of the spectra and the splitting of the major signals probably reflects the hybrid nature of these polysaccharides, as different repeating structures containing, for example, sulfated, non-sulfated xylose or contiguous glucuronic acid residues (Gg4 at ~82.1 ppm Fig. 6(B)) [29] may be present, and these repeating units may be distributed differently. Based on the carbon chemical shifts of the oligosaccharides described above, signals in the region A between 98.8 and 100.4 ppm in Fig. 6 assigned as arising from C-1 of Rha 3-sulfate indicate the presence of branched and/or unbranched rhamnose 3-sulfate linked to xylose and/or xylose 2-sulfate. These assignments may be confirmed by signals in the region B between 62.5 and 64.0 ppm in Fig. 6 which are attributed to C-5 of xylose and xylose 2-sulfate. Further evidence for the presence of xylose 2-sulfate can be obtained from the signals at 78.5–78.8 ppm attributed to C-2 of this residue (Xs2, Fig. 6(A)). The presence of glucuronic acid branching is indicated by higher intensity for the G1 signal (104.4–104.6 ppm) relative to the rhamnose anomeric signals and from the presence of signals at 72.8–73.1 ppm attributed to C-4

of the terminal glucuronic acid (G′4, Fig. 6(B)).

## 4. Discussion

Cell-wall polysaccharides from *Ulva* species are particularly resistant to degradation by microorganisms when part of the dietary fibres of the edible 'sea-lettuce' and some components have long retention time during methanisation of the proliferating biomass [5,10–13]. This resistance to biodegradation is due in part to the particular complexity of the chemical structure of ulvan and it is likely that its biodegradation proceeds in nature through

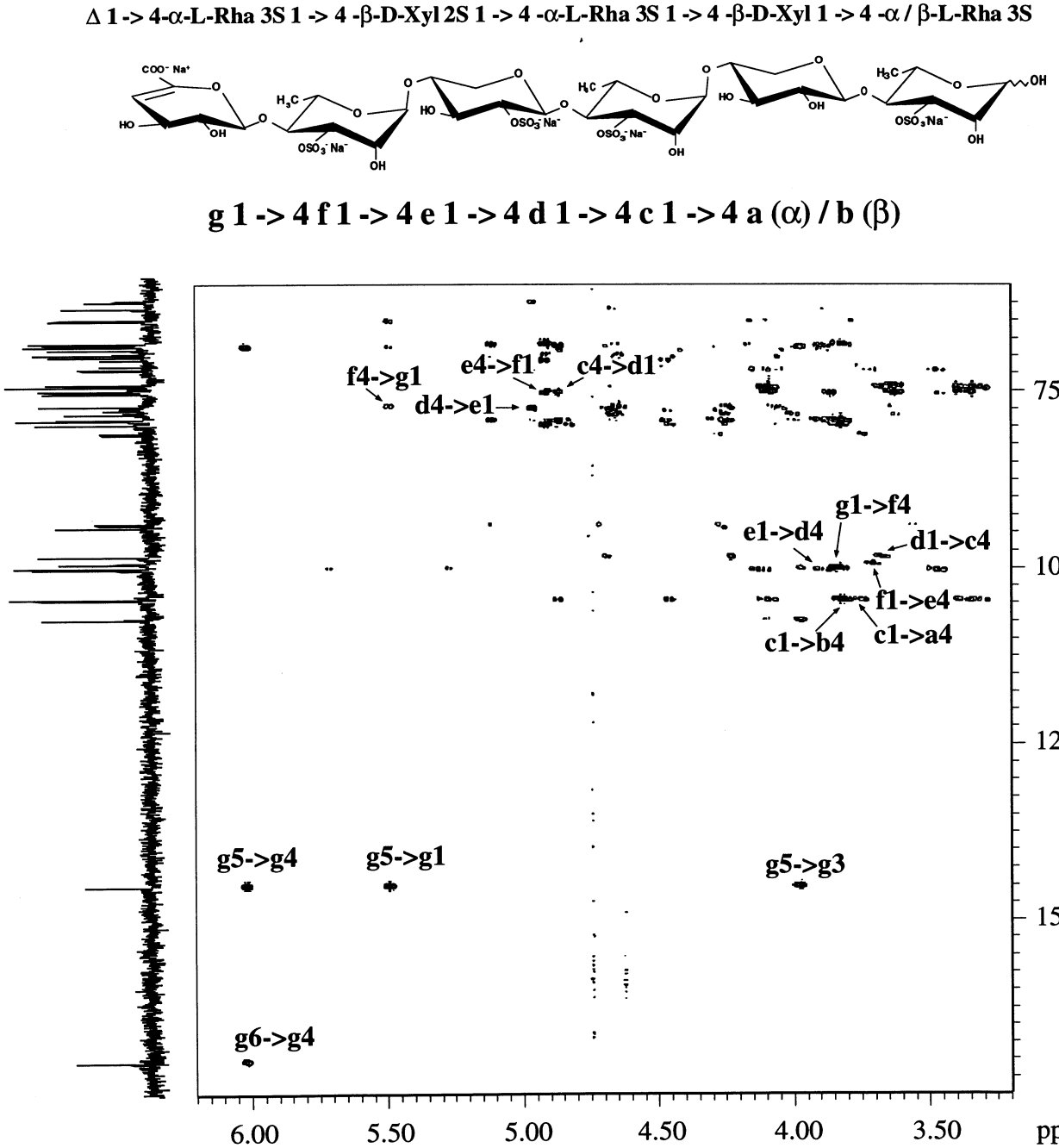



Fig. 4. HMBC map and chemical structure of oligosaccharide in Fraction D. Letters correspond to the sugar residues depicted and numbers to the carbon or proton ($^{13}C \rightarrow {}^{1}H$) of the different correlating residues; 512 experiments of 2 K data points and 64 transients each with recycling time of 1.0 s were recorded at 300 K and transformed to $1 \times 2$ K data points with unshifted sine bell multiplication in the F2 dimension and an exponential multiplication (line broadening of 0.1 Hz) in the F1 dimension. The delay for the long range coupling evolution was 75 ms.

Table 4
$^{1}H$ and $^{13}C$ chemical shifts and coupling constants (in parentheses) of oligosaccharide Fraction I (acetone $\delta^{1}H$: 2.225 and $\delta^{13}C$: 31.45, 300 K; the letters correspond to the residues depicted in Fig. 5)

| Residue | | 1 | 2 | 3 | 4 | 5 | 5′ | 6 |
|---|---|---|---|---|---|---|---|---|
| **a** | $^{1}H$ | 5.11 (2.1) | 4.24 | 4.64 | 3.80 | 3.98 | | ~1.31 |
| | $^{13}C$ | 94.59 | 70.47 | 79.36 | 78.67 | ~68.7 | | 18.46 |
| **b** | $^{1}H$ | 5.09 (2.2) | 4.36 | 4.71 | 4.05 | ~4.05 | | ~1.17 |
| | $^{13}C$ | 99.21 | 77.48 | 79.58 | 77.21 | 69.08 | | 17.72 |
| **c** | $^{1}H$ | 5.08 (2.2) | 4.34 | 4.71 | 3.99 | ~4.05 | | ~1.17 |
| | $^{13}C$ | 98.03 | 77.40 | 79.58 | 77.21 | 69.08 | | 17.72 |
| **d** | $^{1}H$ | 5.06 (~1.9) | 4.18 | 4.68 | 3.90 | 4.02 | | ~1.33 |
| | $^{13}C$ | 93.87 | 69.95 | 79.44 | 78.11 | 69.45 | | 18.32 |
| **e** | $^{1}H$ | 5.03 (5.3) | 4.14 | 3.88 | ~3.73 | ~4.12 | ~3.47 | |
| | $^{13}C$ | | | | | 62.30 | | |
| **f** | $^{1}H$ | 4.93 (5.9) | 4.12 | 3.85 | 3.73 | 4.12 | 3.47 | |
| | $^{13}C$ | 100.70 | 79.36 | 72.19 | 75.82 | 62.67 | | |
| **g** | $^{1}H$ | 4.90 (1.1) | 4.26 | 4.45 | 3.72 | nd | 4.13 | ~1.32 |
| | $^{13}C$ | 94.14 | 78.11 | 81.39 | 78.11 | 71.85 | | 18.46 |
| **h** | $^{1}H$ | 4.77 (1.8) | 4.21 | 4.60 | 3.81 | ~4.12 | | ~1.32 |
| | $^{13}C$ | 101.07 | 70.47 | 79.95 | 77.4 | 68.56 | | ~18.46 |
| **i** | $^{1}H$ | 4.72 | 3.37 | ~3.55 | ~3.50 | ~3.71 | | |
| | $^{13}C$ | 104.38 | 74.31 | 76.41 | 73.05 | 77.21 | | 176.96 |
| **j** | $^{1}H$ | 4.64 | 3.30 | ~3.62 | 3.65 | 4.08 | 3.35 | |
| | $^{13}C$ | 104.82 | 74.50 | 74.77 | 75.28 | 63.60 | | |
| **k** | $^{1}H$ | 5.47 | 4.08 | 3.99 | 6.00 | | | |
| | $^{13}C$ | 100.28 | 68.98 | 65.30 | 107.6 | 145.35 | | 170.47 |
| **l** | $^{1}H$ | 5.50 | 4.08 | 3.99 | 6.01 | | | |
| | $^{13}C$ | 100.28 | 68.98 | 65.30 | 107.6 | 145.35 | | 170.47 |

the simultaneous action of several enzymes. One of them has been identified from a marine bacterium as an ulvan-lyase. A crude preparation of this enzyme was able to cleave the α-L-Rha 3-sulfate-(1→4)-β-D-GlcA linkage in 'sea-lettuce' ulvan [29]. Applied to two *U. rigida* ulvan samples in the present study, it allowed the presence of $A_{3s}$ sequences in these polysaccharides to be confirmed and provided a better insight into the distribution of the xylose and branched glucuronic acid residues. In both ulvan samples and as in 'sea-lettuce' ulvan, xylose residues occur in the disaccharidic repeating sequence [→4)-β-D-Xyl-(1→4)-α-L-Rha 3s-(1→] isolated among $A_{3s}$ structures in the ulvan chain. However, in the Canary Islands *U. rigida* sample they also occur as short blocks of 2 or 3 of these disaccharide structures, and sometimes with sulfation at O-2 of the xylosyl residue. Thus, this xylosylated disaccharides are major building blocks of this ulvan and the trivial names (and symbols) ulvanobiose 3-sulfate ($U_{3s}$) and ulvanobiose 2′,3-disulfate ($U_{2's,3s}$) are proposed for them. In this notation, the number corresponds to the carbon bearing the sulfate and prime (′) refers to the xylosyl residue. Considering the structure of the oligosaccharides recovered in this and in the previous study and the low degradability of ulvan enriched in $B_{3s}$ repeating units [29], it is likely that at least two or three contiguous $A_{3s}$ units are required for the enzymatic cleavage to occur. Assuming this mode of action, an average sequence of ulvan from *U. rigida* from the Canary Islands could be as follows, the proportion of each repeating structures being not representative due to the lack of quantitative data:

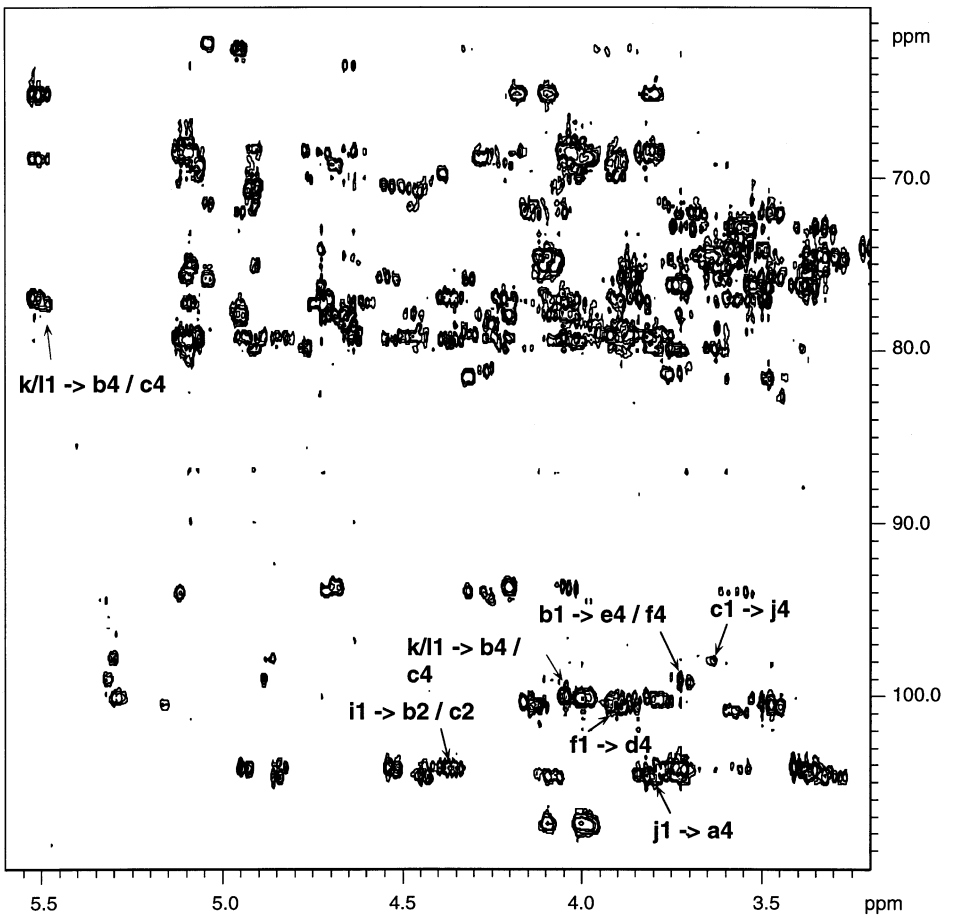
-$A_{3s}$-$A_{3s}$-$U_{3s}$-$A_{3s}$-$A_{3s}$-$U_{2's,3s}$-$A_{3s}$-$A_{3s}$-$U_{3s}$-$A_{3s}$-$U_{3s}$-$A_{3s}$-$A_{3s}$-$U_{2's,3s}$-$A_{3\ s}$-$U_{3s}$-$A_{3s}$-$A_{3s}$-$U_{(2's),3s}$-$A_{3s}$-$U_{(2's)3s}$-$A_{3s}$-$U_{3s}$-$A_{3s}$- (the parentheses refer to possible sites for one sulfate)

In contrast to 'sea-lettuce' ulvan and to the Brittany *U. rigida* ulvan sample, it may not contain long $A_{3s}$ sequences ($\geq 3$) as no oligosaccharide with the structure of that isolated in Fraction H from the Brittany *U.*

*rigida* ulvan sample was obtained. However, as for the other *U. rigida* ulvan, it did not contain short segments of two contiguous glucuronic acids nor iduronic acid containing disaccharides ($B_{3s}$), thought to be biosynthetically related to $A_{3s}$ units [30]. On the other hand, the ulvan from the Brittany *U. rigida* sample did not contain contiguous $U_{3s}$ or $U_{2's,3s}$ disaccharide units but instead, $A_{3s}$ branched structures containing β-D-GlcA linked at O-2 of α-L-Rha 3-sulfate next to $U_{3s}$ or $U_{2's,3s}$ units. A proposed short hand notation for this branched structure is $A_{2g,3s}$, the subscript 2g referring to the terminal branched glucuronic acid. Considering the assumed mode of action of the crude enzyme preparation and the oligosaccharides identified, an average sequence for this ulvan irrespective of exact proportions of the different repeating structures could be:

$$-A_{3s}-A_{3s}-A_{3s}-U_{3s}-A_{3s}-A_{3s}-U_{2's,3s}-A_{3s}-A_{3s}-A_{3s}-A_{3s}-A_{2g,3s}-U_{3s}-A_{3s}-A_{2g,\ 3s}-U_{2's,3s}-A_{3s}-$$

However, other complex structures cannot be excluded as there were unidentified fragments and an enzyme-resistant fraction eluting from the Bio-Gel P4 column (Fig. 1(B)). They probably contain sequences with contiguous glucuronic acids since these were observed on the $^{13}C$ NMR spectrum of the parent polymer (Fig. 6(B)). The presence of a branched oligosaccharide confirmed the methylation




**Δ 1 -> 4 - [GlcA 1 -> 2-] -α-L-Rha 3S 1 -> 4 -β-D-Xyl 1 -> 4 -α / β-L-Rha 3S**

**k/l1 -> 4 [i 1 - > 2] - c 1 -> 4 j 1 -> 4 a / g**

**Δ 1 -> 4 - [GlcA 1 -> 2-] -α-L-Rha 3S 1 -> 4 -β-D-Xyl 2S 1 -> 4 -α / β-L-Rha 3S**

**k/l 1 -> 4 [i 1 - > 2] - b 1 -> 4 e / f 1 -> 4 d / h**

Fig. 5. HMBC map and chemical structure of oligosaccharide in Fraction I. Letters correspond to the sugar residues depicted and numbers to the carbon or proton of the different correlating residues ($^{13}C \rightarrow {}^{1}H$); conditions of acquisition and processing were as given in the legend of Fig. 4.

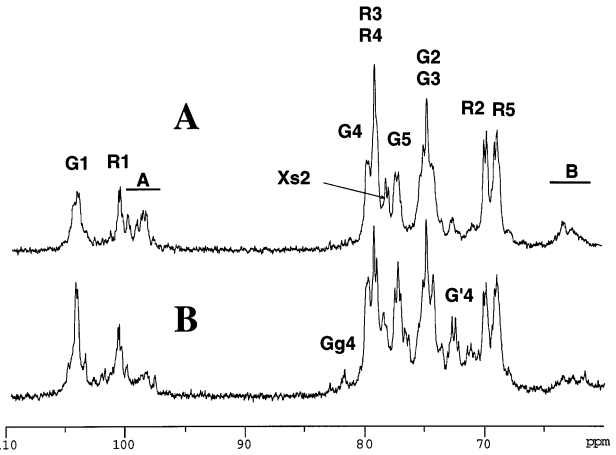



Fig. 6. $^{13}C$ NMR spectra of ulvan from *U. rigida* collected in (A) Canary Islands and (B) Brittany. Spectra were recorded at 333 K; chemical shifts are calculated from internal $Me_2SO$ ($\delta^{13}C$: 39.6 ppm). G and R refer to glucuronic acid and rhamnose 3-sulfate carbons, respectively, in the $A_{3s}$ repeating structure; X2s refers to C-2 of xylose 2-sulfate, G′4 to C-4 of branched glucuronic acid, Gg4 to C-4 of contiguous 4-linked glucuronic acid; regions A and B are discussed in the text.

analysis data [27] and the NMR data on oligosaccharides from acid hydrolysis of desulfated ulvan [28]. Thus, this ulvan is clearly branched by a single glucuronic acid residue and the latter is not the starting point of a long side chain. Indeed, in the latter case, the glucuronic acid would probably have appeared as 4-deoxy-L-*threo*-hex-4-enopyranosiduronic acid. In addition, the branched $A_{2g,3s}$ structure does not appear to occur as an isolated trisaccharide among $A_{3s}$ repeating units, as the smallest oligomers isolated were always containing $U_{3s}$ or $U_{2's,3s}$ disaccharide.

The $^{13}C$ NMR data obtained from these ulvan oligosaccharides cannot readily be transposed to identify the $^{13}C$ NMR chemical shifts for the different xylosyl or branched glucuronyl-containing structures on the spectra of the parent polymers. One reason may be the heterogeneous distribution of these fragments within ulvan chains but not between chains. Indeed, as the Brittany *U. rigida* ulvan yielded a single sharp fraction on anion exchange chromatography [23] and Percival observed that the different fractions recovered from DEAE-cellulose chromatography of *U. lactuca* ulvan had very similar compositions [22], it appears that these different sequences are homogeneously distributed among all ulvan chains. Other factors potentially involved in the poor resolution of the $^{13}C$ NMR spectrum of the parent polysaccharides are interactions between ulvan chains and between ulvan and the small amounts of contaminating proteins in these extracts (13.9–14.7%) [23,33].

The difference in the fine chemical structures between these two ulvans and their ulvan-lyase degradation products reflect either different ecophysiological growth conditions of the algae or different species. In this respect, oligosaccharide structures and chromatographic profiles may be relevant chemotaxonomic markers to complement morphological and/or molecular biological markers. It would now be interesting to relate the fine structural differences observed in these two ulvans with similar sugar compositions and sulfate content with their functional and biological properties.

## Acknowledgements

The excellent technical assistance of J. Vigouroux is acknowledged. This work was supported in part by the VANAM Programme from the Region Pays de la Loire.

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