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Structural determination of the exopolysaccharide of *Pseudoalteromonas* strain HYD 721 isolated from a deep-sea hydrothermal vent

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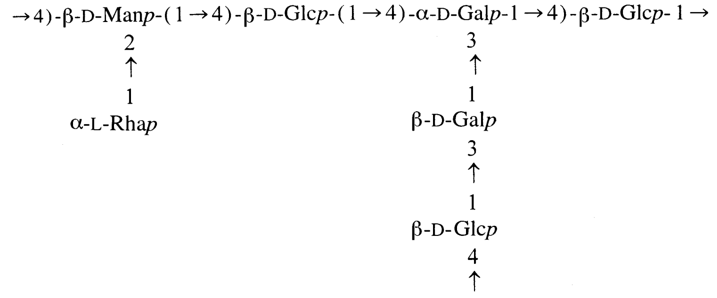
Abstract

The structure of the exopolysaccharide produced by *Pseudoalteromonas* reference strain HYD 721 recovered from a deep-sea hydrothermal vent has been investigated. By means of methylation and β-elimination analysis, selective degradation of the uronic acids, partial depolymerization and NMR studies, the repeating unit of the polymer was deduced to be a branched octasaccharide with the structure shown. © 1999 Elsevier Science Ltd. All rights reserved.



$$\begin{array}{cccc}
\rightarrow 4)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow & 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow & 4)\text{-}\alpha\text{-D-Gal}p\text{-}1\rightarrow & 4)\text{-}\beta\text{-D-Glc}p\text{-}1\rightarrow \\
2 & & 3 & \\
\uparrow & & \uparrow & \\
1 & & 1 & \\
\alpha\text{-L-Rha}p & & \beta\text{-D-Gal}p & \\
 & & 3 & \\
 & & \uparrow & \\
 & & 1 & \\
 & & \beta\text{-D-Glc}p & \\
 & & 4 & \\
 & & \uparrow & \\
 & & 1 & \\
 & & [SO_3H]\rightarrow 3\beta\text{-D-Man}p &
\end{array}$$

Keywords: Bacterial polysaccharide; Hydrothermal vent; Uronic acid

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1. Introduction

Bacterial exopolysaccharide producers were found in 1987 among isolates from a deep-sea hydrothermal environment [1]. Because of the extreme conditions (high pressure, high temperature gradient, and high concentrations of toxic elements) observed near deep-sea hydrothermal vents, polysaccharides of unusual

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properties were expected to be produced by these bacteria. The characterization of several polysaccharides obtained from laboratory-grown hydrothermal bacterial isolates showed very different chemical and rheological properties compared to other bacterial polysaccharides [2,3]. The structures of three of these polysaccharides have been investigated. The repeating unit of the exopolysaccharides produced by strain HYD 1545 [4] and strain HYD 1644 [5] were partially determined. Each repeating unit contains at least three uronic acid residues and HYD 1644 is characterized by the presence of a novel acid residue, a 3-*O*-[(*R*)-1-carboxyethyl]-β-D-Glc*p*A [5]. Recently, the complete structure of an exopolysaccharide secreted by *Alteromonas macleodii* subsp. *fijiensis* has been elucidated [6]. Its repeating unit was deduced to be a hexasaccharide consisting of three uronosyl residues with a branch point at a galacturonosyl residue and a side chain terminated by a 4,6-*O*-(1-carboxyethylidene)-β-D-Man*p*.

Table 1
Analysis of the alditol acetates derived from alkylation of *Pseudoalteromonas* strain HYD 721 exopolysaccharide

Alkylated sugars (as alditol acetates)	t_R [a]	Detector response (%)		
		(I) [b]	(II) [b]	(III) [b]
2,3,4-Me_3Rha [c]	0.397	9	7	7
3-Et-2,4,6-Me_3Gal	0.502			15
2,3,4,6-Me_4Man	0.506	9	15	7
2,4,6-Me_3Man	0.617	7	3	5
2,4,6-Me_3Gal	0.625	15	13	2
2,3,6-Me_3Man	0.633	3	4	5
2,3,6-Me_3Gal	0.655	1	2	5
2,3,6-Me_3Glc	0.666	23	21	27
2,6-Me_2Gal	0.707	15	16	10
3,6-Me_2Man	0.736	13	10	9
2,3-Me_2Glc-*6-d_2* [d]	0.787	5	9	3 [e]

[a] t_R = Retention time for the corresponding alditol acetate relative to that of *myo*-inositol hexaacetate (t_R = 1.000).

[b] I, Methylated and carboxyl reduced polysaccharide; II, desulfated, methylated and carboxyl-reduced polysaccharide; III, methylated, β-eliminated and ethylated polysaccharide.

[c] 2,3,4-Me_3Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc.

[d] 2,3-Me_2Glc-*6-d_2* = 1,4,5,6-tetra-*O*-acetyl-6,6-dideutero-2,3-di-*O*-methylglucitol.

[e] Due to incomplete degradation of the uronic acids.

The exopolysaccharide discussed in this work is produced by *Pseudoalteromonas* strain HYD 721 and seems very different in composition from the exopolysaccharides described above in that it contains 57% neutral sugars and 11% uronic acids [2]. This report describes structural studies conducted on this polysaccharide, using monosaccharide analysis, methylation analysis, β-elimination, and NMR spectroscopy after selective degradation of the uronic acids and after partial depolymerization.

2. Results and discussion

Isolation, purification and composition of the polysaccharide.—A crude preparation of the polysaccharide was obtained by precipitation of the culture supernatant of *Pseudoalteromonas* strain HYD 721 with ethanol at 40% (v/v). The polymer was purified by anion-exchange chromatography, and fractions corresponding to the main peak, which were eluted with 0.65 M NaCl, were combined, dialyzed and lyophilized. The yield was 84%. A molecular weight of 8.7×10^5 g mol^{-1} was determined by gel-filtration chromatography.

The neutral glycosyl residue composition of the polysaccharide was determined by acid hydrolysis and by the preparation and analysis of alditol acetates, which indicated that it was composed of glucose, galactose, mannose and rhamnose in the molar ratio 2:2:2:0.8. Glucuronic acid was identified by GLC analysis of the per-*O*-trimethylsilylated methyl glycoside derivatives. Using this method, the relative proportions of glucose, galactose, mannose, rhamnose and glucuronic acid were determined to be 2:2:2:0.8:1. Each glycosyl residue was shown to be in the D-configuration, with the exception of the rhamnose residue that was in L-configuration [11].

Glycosyl-linkage analysis of the native polysaccharide.—Methylation analysis of the polysaccharide (Table 1) showed non-substituted Rha*p* and 4-substituted Glc*p*. Since composition results showed two glycosyl residues in the repeating unit, and methylation data indicated that the only type of glycosyl residue present was 4-linked, we could reasonably conclude that each repeating unit contained two

4-substituted Glc*p* residues. Three galactopyranosyl residues were observed: 3-substituted Gal*p* (detector response of 15%; Table 1), 4-substituted Gal*p* (detector response of 1%; Table 1), and 3,4-disubstituted Gal*p* (detector response of 15%; Table 1). The low value obtained for the detector response of the PMAA corresponding to the 4-substituted Gal*p*, and the fact that only two Gal residues are present in the repeating units according to composition results, suggested that the group linked to the 3-position of the 3,4-disubstituted Gal*p* residue is sometimes absent ($<10\%$). GLC–MS analysis revealed four mannopyranosyl residues: non-substituted Man*p*, 3-substituted Man*p*, 4-substituted Man*p* (detector response of 3%; Table 1) and 2,4-disubstituted Man*p* (detector response of 13%; Table 1). The low value obtained for the detector response of the PMAA corresponding to the 4-substituted Man*p* suggested that this compound and the 2,4-disubstituted Man*p* derived from the same residue, with the group linked to the 4-position of the 2,4-disubstituted Man*p* sometimes absent ($<20\%$).

When the polysaccharide was methylated and then reduced with 'Superdeuteride' [$LiB(C_2H_5)_3D$], subsequent analysis revealed one additional component, 4,6-disubstitued Glc*p*, containing two deuterium atoms on C-6. Thus, this residue exists in the polysaccharide as 4-substituted Glc*p*A. These results are consistent with a repeating unit containing two branch points, one at a Gal*p* residue and a second at a Man*p* residue. One of the two side chains is terminated by a Rha*p*, and the second one is ended by a Man*p*.

Location of the sulfate ester groups.—Methylation analysis carried out on the polysaccharide after desulfation showed a reduced level of the PMAA derived from the 3-substituted Man*p*, as well as an increase in the PMAA derived from non-substituted Man*p* (Table 1). These results are consistent with a side chain that is terminated by a Man*p* residue that is partially substituted in the 3-position by a sulfate ester group. Relative proportions observed for the 3-substituted Manp and for the non-substituted Man*p* suggested that about 50% of the mannosyl residues of the side chain are sulfated.

Position of the uronic acid residue.—In order to determine the linkage position of the GlcA units in the polysaccharide, the per-*O*-methylated polysaccharide was subjected to β-elimination. This was followed by ethylation, and GC–MS analysis of the partially methylated and ethylated alditol acetates showed 3-ethyl-2,4,6-tri-*O*-methyl-Gal*p* as the only ethylated alditol, derived from the 3-substituted Gal*p* (Table 1), whose O-3 was converted to a hydroxyl group on degradation of the glucuronic acid. This result indicated that the glucuronic acid residue was linked to the 3-position of the monosubstituted galactopyranosyl residue.

Composition and glycosyl-linkage analysis of a glycosyluronic acid-degraded polysaccharide fraction HYD 721 Li.—In order to determine the constituent sugar composition and glycosyl linkage pattern of the polysaccharide after glucuronic acid degradation, a larger sample (HYD 721 Li) was prepared by treatment of the native polysaccharide with lithium metal in ethylenediamine. The main fraction was eluted in the void volume of the chromatographic column of Bio Gel P-10 indicating that this polysaccharide has a molecular weight higher than 20,000 g mol^{-1}. The glycosyl composition of HYD 721 Li determined by methanolysis and the preparation and analysis of the per-*O*-trimethylsilylated methyl glycoside derivatives indicated that it is composed of glucose, galactose, mannose and rhamnose in the molar ratio 2:2:1:0.7. No glucuronic acid was detected by GC analysis. These results suggest that both a 4-substituted Glc*p*A and a Man*p* residue belong to a side chain of the repeating unit, in accordance with the low yield (60%) due to partial loss of this branch.

Methylation analysis of the polysaccharide HYD 721 Li (Table 2) indicated only traces of both 4-substituted Glc*p*A and non-substituted Man*p*, along with the disappearance of the 3-substituted Man*p* residue (compare with the results shown for sample I in Table 1). Table 2 revealed the presence of non-substituted Gal*p*, which was derived from degradation of the Glc*p*A residue that had been attached to the Gal*p* O-3. This result is consistent with the β-elimination data described above. These results confirmed the disappearance of both the glucuronic acid residue and the sulfated mannopyranosyl residue from the polysacchar-

Table 2
Analysis of the alditol acetates derived from alkylation of the HYD 721 Li and HYD 721 DP exopolysaccharides

Alkylated sugars (as alditol acetates)	t_R [a]	Detector response (%)	
		(IV)[b]	(V)[b]
2,3,4-Me_3Rha[c]	0.397	8	5
2,3,4,6-Me_4Man	0.506	3	7
2,3,4,6-Me_4Gal	0.537	15	
2,4,6-Me_3Man	0.617		8
2,4,6-Me_3Gal	0.625	3	14
2,3,6-Me_3Man	0.633	7	7
2,3,6-Me_3Gal	0.655	6	1
2,3,6-Me_3Glc	0.666	34	27
2,6-Me_2Gal	0.707	11	20
3,6-Me_2Man	0.736	12	8
2,3-Me_2Glc-*6-d_2*[d]	0.787	1	3

[a] t_R = Retention time for the corresponding alditol acetate relative to that of *myo*-inositol hexaacetate (t_R = 1.000).

[b] IV, Methylated and carboxyl reduced polysaccharide HYD 721 Li; V, methylated and carboxyl-reduced polysaccharide HYD 721 DP.

[c] 2,3,4-Me_3Rha = 1,5-di-*O*-acetyl-2,3,4-tri-*O*-methylrhamnitol, etc.

[d] 2,3-Me_2Glc-*6-d_2* = 1,4,5,6-tetra-*O*-acetyl-6,6-dideutero-2,3-di-*O*-methylglucitol.

ide HYD 721 Li. Furthermore, they suggest that the disaccharide [SO_3H](3-D-Man*p*-(1 → 4)-D-Glc*p*A is linked to the 3-position of the galactopyranosyl residue and is part of one of the side chain of the repeating unit.

NMR spectral studies of HYD 721 Li.—The ^{1}H NMR spectrum (Fig. 1) of the polysaccharide HYD 721 Li showed a complex anomeric region, but the analysis of the HSQC spectrum (Fig. 2) indicated nine signals for H-1/C-1, three α-linked at δ 5.46/101.8, 5.42/101.8, 5.00/103.2, and six β-linked at δ 4.92/104.1, 4.81/102.6, 4.76/101.9, 4.67/105.6, 4.61/106.7 and 4.51/104.5. No signals were observed in the region corresponding to the reducing-end carbon resonances (δ 90–98), confirming that HYD 721 Li was not an oligosaccharide. The residues were labeled A–G, I and K in decreasing order of the chemical shifts for the H-1 resonances.

COSY and TOCSY experiments were undertaken to make the proton assignments, and the HSQC spectrum was used to obtain the corresponding carbon resonances. Due to the poor resolution of the spectra (not shown) and severe overlaps, it was difficult to extract, without any ambiguities, all the proton assignments. However, values of some chemical shifts in combination with the data of the methylation analysis allowed us to identify the different residues (Table 3). Residues A and B showed downfield chemical shifts of the H-2 signals, as expected for sugars with the α-*galacto* configuration. Residue A was identified as the 3,4-disubstituted-α-D-Gal*p* residue due to the downfield C-3 and C-4 signals (δ 80.8 and 78.5, respectively), along with the

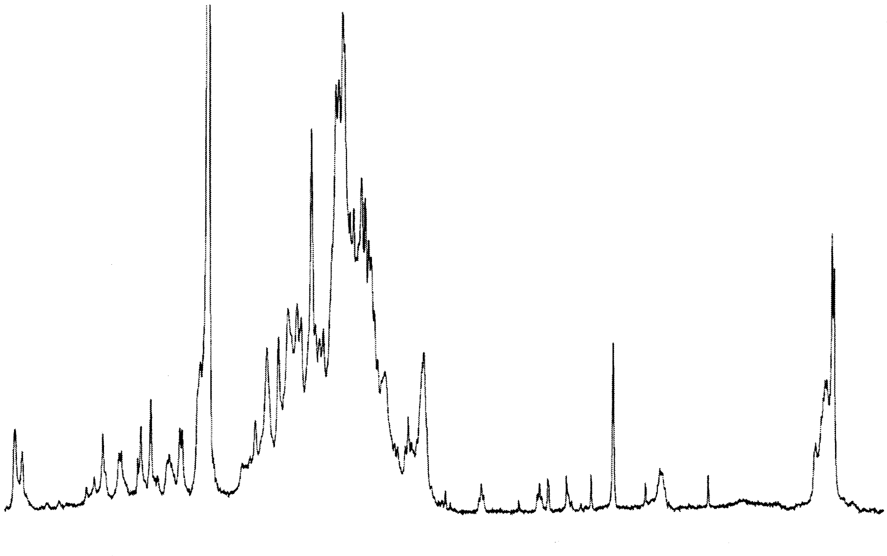


Fig. 1. ^{1}H NMR spectrum (600 MHz) recorded at 320 K in D_2O of the HYD 721 Li polysaccharide.

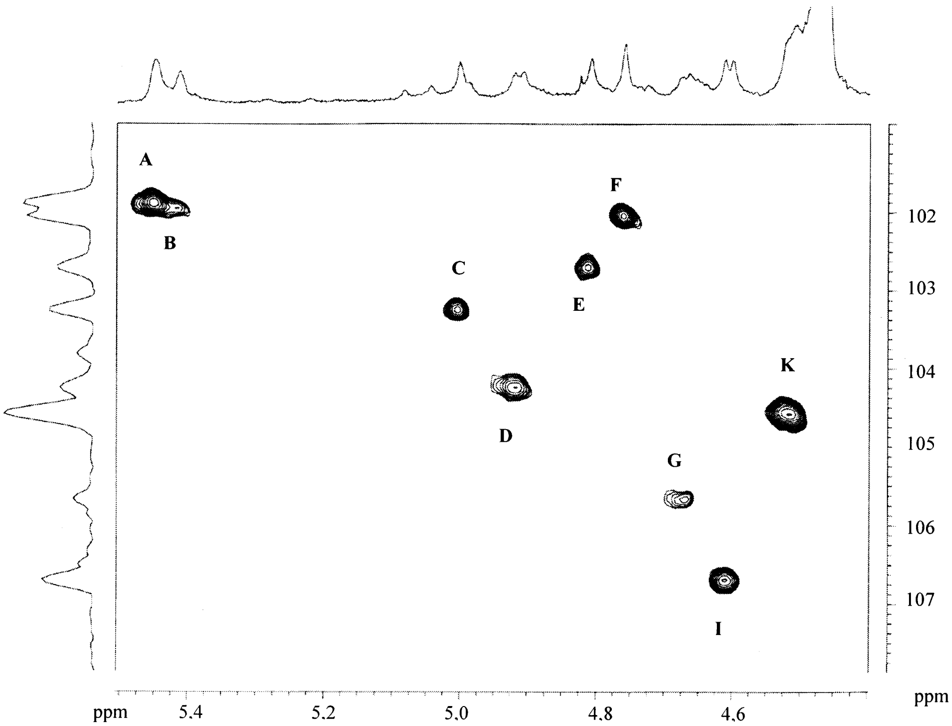


Fig. 2. Anomeric region of the 600 MHz HSQC spectrum recorded at 320 K in D_2O of the HYD 721 Li polysaccharide.

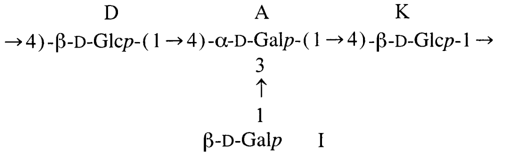
Table 3
Chemical shift (δ, ppm) of the signals in the ^1H and ^{13}C NMR spectra of the HYD 721 Li exopolysaccharide

Residue	$^1H/^{13}C$[a]					
	1	2	3	4	5	6
(3,4)-α-D-Gal*p*-(1 →	5.46	4.18	4.07	4.46		
A	101.8	70.3	80.8	78.5		
→ 4)-α-D-Gal*p*-(1 →	5.42	3.94	3.95	4.23		
B	101.8	71.4	71.8	80.5		
α-L-Rha*p*-(1 →	5.00	4.06	3.83	3.44	4.10	1.26
C	103.2	72.3	72.2	73.8	71.0	18.5
→ 4)-β-D-Glc*p*-(1 →	4.92	3.36	3.70	3.65	3.55	
D	104.1	75.5	75.9	78.8	76.5	
(2.4)-β-D-Man*p*-(1 →	4.81	4.17				
E	102.6	78.0				
→ 4)-β-D-Man*p*-(1 →	4.76	4.11				
F	101.9	72.3				
→ 4)-β-D-Glc*p*-(1 →	4.67	3.40	3.68	3.60		
G	105.6	75.5	77.1	77.5		
β-D-Gal*p*-(1 →	4.61	3.62	3.67	3.94		
I	106.7	73.4	74.1	71.8		
→ 4)-β-D-Glc*p*-(1 →	4.51	3.35	3.79	3.67		
K	104.5	75.0	77.9	81.0		

[a] Carbons involved in interglycosidic linkages are underlined.

downfield position of its H-3 and H-4 resonances (δ 4.07 and 4.46, respectively). Residue B was identified as 4-substituted α-D-Gal*p* due to the downfield resonance of C-4 (δ 80.5). The complete assignment of the resonances of residue C identified it as the α-L-Rha*p* due to the presence of the upfield signals of H-6 at δ 1.26 and C-6 at δ 18.5. The β-*gluco* configuration, attributed to the residues D, G and K, was in complete agreement with the upfield position of the H-2 signals (δ 3.36, 3.40 and 3.35, respectively). Each of these residues were identified as 4-substituted-β-D-Glc*p* as supported by the downfield location of their C-4 resonances at δ 78.8, 77.5 and 81.0, respectively [6]. The downfield chemical shifts of the H-2 signals of E (δ 4.17) and F (δ 4.11) were in agreement with their assignment to residues with a β-*manno* configuration. Residue E was identified as the 2,4-disubstituted-β-D-Man*p* on the basis of the downfield location of its C-2 resonance (δ 78.0). Residue F was, therefore, identified as the 4-substituted-β-D-Man*p* residue. The only remaining glycosyl residue, I, was identified as non-substituted β-D-Galp, in complete agreement with the downfield chemical shift of its H-2 signal at δ 3.62, along with the absence of downfield carbon signals corresponding to a substituted residue.

Glycosyl sequence information was obtained from a NOESY experiment (Fig. 3). The spectrum showed a clear inter-residue contact between H-1 of I and H-3 of A. The H-1 of residue D gave a NOE contact with H-4 of residue A, as well as a contact of lower intensity with H-3 of A. An intense NOE correlation was observed between H-1 of A and H-4 of K, along with weaker contacts with H-3 and H-2 of K, demonstrating that A is linked to the 4-position of K. Therefore, the repeating unit of the polysaccharide HYD 721 Li contained the tetrasaccharide unit **1**:



$$\begin{array}{ccc} \text{D} & \text{A} & \text{K} \\ \rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow & 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow & 4)\text{-}\beta\text{-D-Glc}p\text{-}1\rightarrow \\ & 3 & \\ & \uparrow & \\ & 1 & \\ & \beta\text{-D-Gal}p\quad\text{I} & \end{array}$$

1

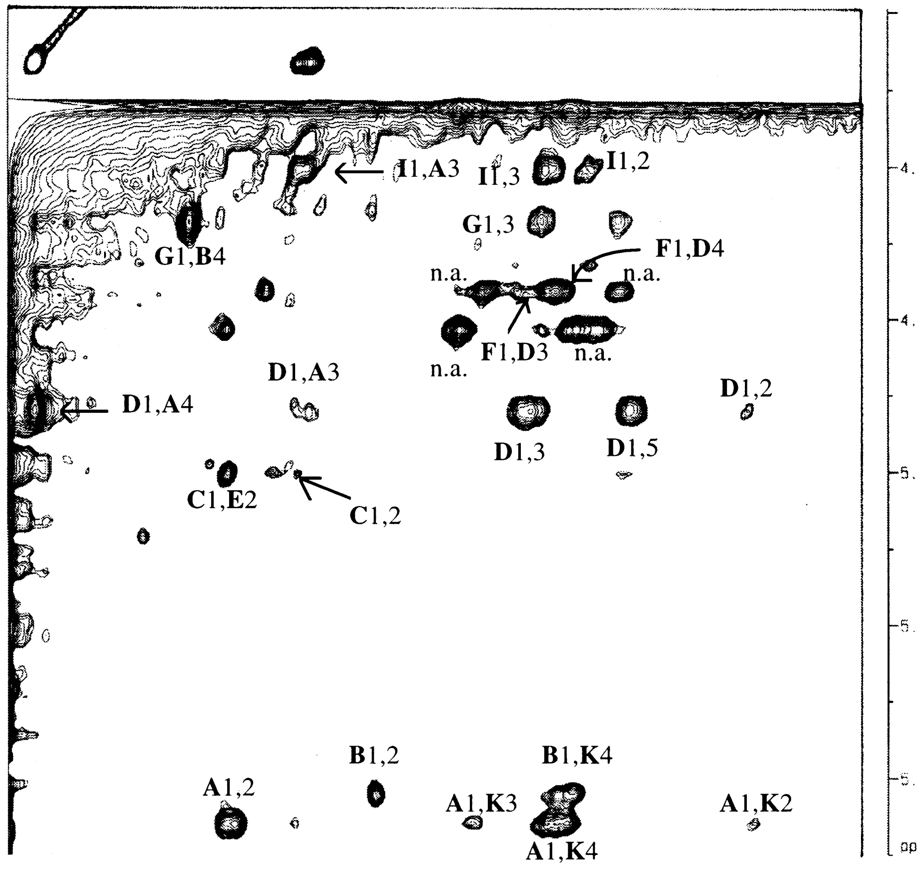
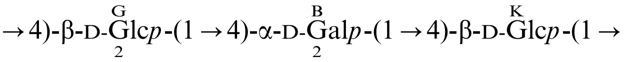


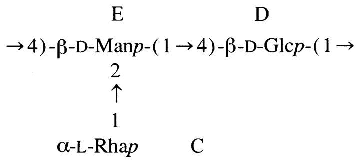
Fig. 3. Anomeric region of the 600 MHz NOESY spectrum recorded at 320 K in D_2O of the HYD 721 Li polysaccharide.

Long-range NOE correlations observed between H-1 of G and H-4 of B, and between H-1 of B and H-4 of K, defined the trisaccharide unit **2**, and showed that D and G correspond to the same residue in which the resonance of the anomeric proton depends on the presence or absence, respectively, of the side chain at O-3 of the galactopyranosyl residue; i.e., residue A in tetrasaccharide **1** and residue B in trisaccharide **2**.



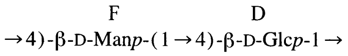
$$\rightarrow 4)\text{-}\beta\text{-D-}\overset{\text{G}}{\underset{2}{\text{Glc}}}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-D-}\overset{\text{B}}{\underset{2}{\text{Gal}}}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-}\overset{\text{K}}{\text{Glc}}p\text{-}(1\rightarrow$$

The H-1 of residue C presented a clear NOE contact with H-2 of E, but the H-1 of E did not provide conclusive structural information. Correlations established between H-1 of F and H-3 and H-4 of D showed that residue D is substituted in the 4-position by residue F. Since E and F were both identified as the 4-substituted-β-D-Man*p* by methylation analysis, with the only difference being a second substitution on the 2-position of E, it was concluded that the repeating unit of the polysaccharide HYD 721 Li also contained the trisaccharide unit **3**, and the disaccharide **3a** when the rhamnose side chain is absent.



$$\begin{array}{ll} \text{E} & \text{D} \\ \rightarrow 4)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow 4) & \text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \\ \quad 2 & \\ \quad \uparrow & \\ \quad 1 & \\ \alpha\text{-L-Rha}p & \text{C} \end{array}$$

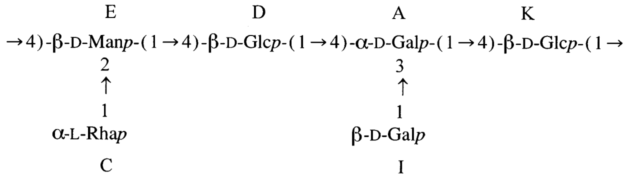
3



$$\begin{array}{ll} \text{F} & \text{D} \\ \rightarrow 4)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow 4) & \text{-}\beta\text{-D-Glc}p\text{-}1\rightarrow \end{array}$$

3a

Because of the poor resolution, the NOESY spectrum did not provide structural information about the interglycosidic linkage involving H-1 of K. However, the only hypothesis that is in agreement with all the data is a glycosidic linkage between K and the 4-position of the 2,4-disubstituted-β-D-Man*p* residue (E) in the main chain of the repeating unit. Therefore, structure **4** could be suggested for the repeating unit of the polysaccharide HYD 721 Li, with some irregularities resulting from the partial presence of the side chains:



$$\begin{array}{llll} \text{E} & \text{D} & \text{A} & \text{K} \\ \rightarrow 4)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow 4) & \text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4) & \text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow 4) & \text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \\ \quad 2 & & \quad 3 & \\ \quad \uparrow & & \quad \uparrow & \\ \quad 1 & & \quad 1 & \\ \alpha\text{-L-Rha}p & & \beta\text{-D-Gal}p & \\ \text{C} & & \text{I} & \end{array}$$

4

Composition and glycosyl-linkage analysis of the partially depolymerized polysaccharide HYD 721 DP.—The polysaccharide HYD 721 DP was obtained after partial depolymerization and purification from compounds of low molecular weight by ultrafiltration. GLC analysis of the per-*O*-trimethylsilylated methyl glycosides revealed that this polymer was composed of glucose, galactose, mannose, rhamnose and glucuronic acid with a molar ratio of 2:2:2:0.5:1, suggesting that hydrolysis led to a loss of rhamnose (about 30%). In agreement with this result, methylation analysis (Table 2) showed an increase in the amount of 4-substituted Man*p* along with a decrease in the response of the 2,4-disubstituted Man*p* (compare with the results for sample I shown in Table 1). Partially methylated alditol acetates corresponding to non-substituted Man*p* and to the 3-substituted Man*p* were present in equal amounts revealing that sulfate ester groups resisted hydrolysis under the conditions used.

NMR spectral studies of HYD 721 DP.—The ^{1}H NMR spectrum of the polysaccharide HYD 721 DP (Fig. 4) showed a complex anomeric region suggesting the presence of new residues in the repeating unit when compared to HYD 721 Li. In addition to residues A, B, C, D, E, F, I and K, the HMQC spectrum (Fig. 5) revealed three new H-1/C-1 cross-peaks for β-linked residues, at δ 4.76/100.6, 4.75/105.5 and 4.66/102.1, and labeled L, M and N, respectively (Table 4). The disappearance of the H-1/C-1 signal corresponding to G, along with the important decrease of intensity resulting in the lack of a distinct H-1/C-1 signal of B confirmed the interglycosidic linkage established for structure **2**. Most of the proton and carbon assignments resulting from NMR studies of HYD 721 Li were also observed for HYD 721 DP (Table 5). Some differences were observed. Residue I showed a downfield location of its C-3 signal at δ 84.3, indicating

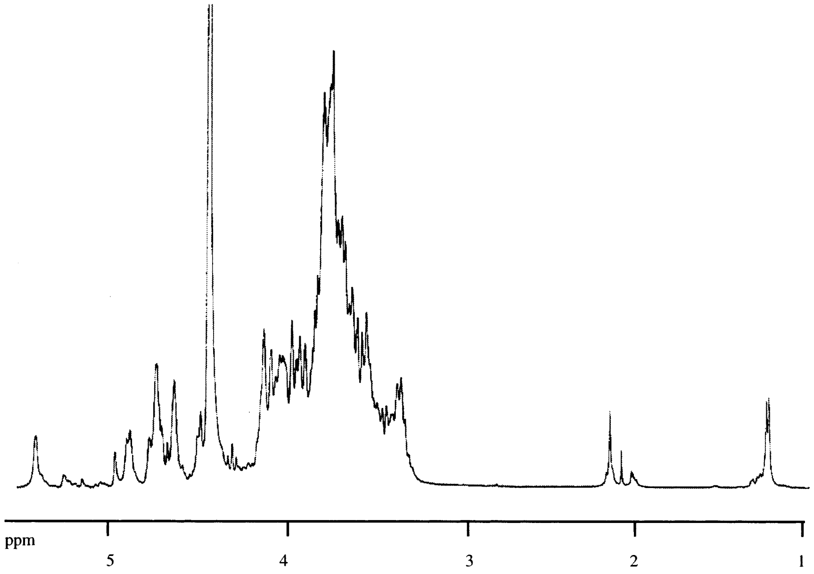


Fig. 4. ^{1}H NMR spectrum (400 MHz) recorded at 328 K in D_2O of the HYD 721 DP polysaccharide.

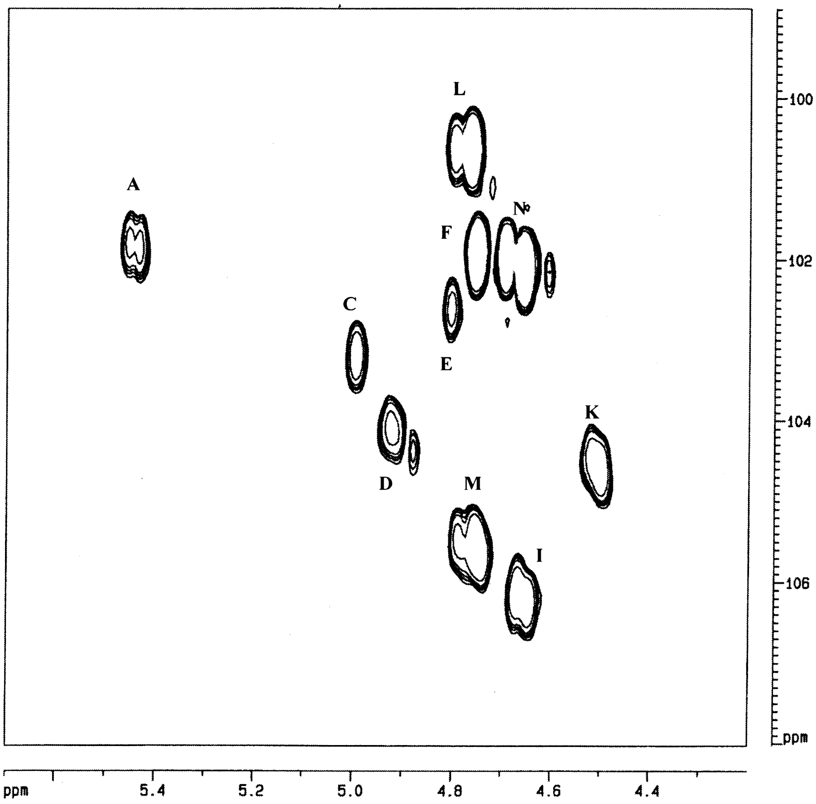
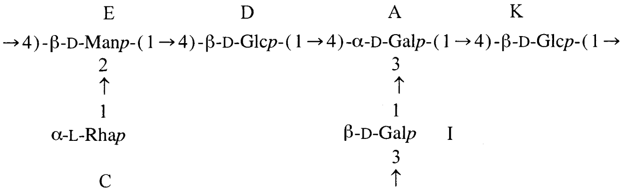


Fig. 5. Anomeric region of the 400 MHz HMQC spectrum recorded at 328 K in D_2O of the HYD 721 DP polysaccharide.

the substitution at the Gal*p* 3-position by the remainder of a side chain not present in HYD 721 Li. Residue M was identified as the 4-substituted-β-D-Glc*p*A residue as supported by the upfield position of its H-2 signal at δ 3.44 and the downfield chemical shift of the C-4 resonance at δ 81.8. The β-*manno* configuration attributed to residues L and N was in complete agreement with the downfield position of their H-2 resonances (δ 3.94 and 3.98, respectively). L was identified as the [SO_3H](3-β-D-Man*p* due to the presence of downfield chemical shifts of the H-3 and C-3, at δ 4.33 and 76.6. The remaining mannosyl component, N, could consequently be assigned to the non-substituted β-D-Man*p* residue.

NOESY (Fig. 6) and HMBC (Table 5) experiments were employed in order to obtain further information on the glycosyl sequence. The observation of NOE contacts observed between I and A, D and A, and A and K corroborated earlier observations and is consistent with structure **1**. This glycosyl sequence was confirmed by the presence of C-1–H-3′ and H-1–C-3′ correlations between residues I and A, and by the presence of C-1–H-4′ and H-1–C-4′ correlations between D and A as well as between A and K. Furthermore, C-1–H-2′ and H-1–C-2′ inter-residue contacts were observed between C and E, confirming the α-(1→2) linkage. Due to severe overlaps, the NOESY spectrum did not provide conclusive information involving anomeric protons of residues E and F. However, interpretation of the HMBC spectrum supplied indications about the location of these two residues in the repeating unit. Atoms H-1 and C-1 of F gave contacts with C-4 and H-4 of D, respectively. Residue E only differs from F by the presence of the substitution at the 2-position by the α-L-Rha*p*. The H-1 of K showed NOE contacts with an unassigned signal at δ 3.80, and, on the HMBC spectrum a correlation was observed between H-1 of K and a carbon signal at δ 78.6. Since a cross-peak at δ 3.80/78.6 exists on the HSQC spectrum and could be attributed to residue E, these results allowed us to tentatively define structure **5** as the following:



$$\begin{array}{cccc} \text{E} & \text{D} & \text{A} & \text{K} \\ \rightarrow 4)\text{-}\beta\text{-D-Man}p\text{-}(1\rightarrow & 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow & 4)\text{-}\alpha\text{-D-Gal}p\text{-}(1\rightarrow & 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow \\ 2 & & 3 & \\ \uparrow & & \uparrow & \\ 1 & & 1 & \\ \alpha\text{-L-Rha}p & & \beta\text{-D-Gal}p\ \ \text{I} & \\ & & 3 & \\ \text{C} & & \uparrow & \end{array}$$

5

NOE contacts were observed between H-1 of M and H-3 of I, along with connectivities observed on the HMBC spectrum from H-1

Table 4

Chemical shift (δ, ppm) of the signals in the ^{1}H and ^{13}C NMR spectra of the HYD 721 DP exopolysaccharide

Residue	^{1}H/^{13}C[a]					
	1	2	3	4	5	6
→(3,4)-α-D-Gal*p*-(1→	5.45	4.17	4.06	4.45	4.03	3.70–3.97
A	101.8	70.1	$\underline{80.7}$	$\underline{78.2}$	73.0	62.6
→4)-α-D-Gal*p*-(1→	5.40	3.93				
B	101.8	71.5				
α-L-Rha*p*-(1→	5.00	4.05	3.82	3.44	4.10	1.27
C	103.2	72.1	72.5	74.0	70.5	18.5
→4)-β-D-Glc*p*-(1→	4.92	3.36	3.71	3.64	3.54	3.89–4.00
D	104.1	75.5	76.1	$\underline{79.0}$	77.1	62.4
→2,4)-β-D-Man*p*-(1→	4.80	4.18				
E	102.6	$\underline{78.2}$				
[SO_3H]→3-β-D-Man*p*-(1→	4.76	3.94	4.33	3.84		
L	100.6	76.6	76.8	73.7		
→4)-β-D-Man*p*-(1→	4.75	4.10				
F	101.9	72.1				
→4)-β-D-Glc*p*A-(1→	4.75	3.44	3.60	3.85	3.79	
M	105.5	74.6	72.1	$\underline{81.8}$	77.9	174.6
β-D-Man*p*-(1→	4.66	3.98				
N	102.1	72.5				
→3)-β-D-Gal*p*-(1→	4.66	3.78	3.81	4.13	3.68	3.67–3.90
I	106.2	72.3	$\underline{84.3}$	75.3	76.8	63.0
→4)-β-D-Glc*p*-(1→	4.51	3.36	3.80	3.65	3.93	3.72–3.78
K	104.5	75.1	77.9	$\underline{80.8}$	76.6	62.8

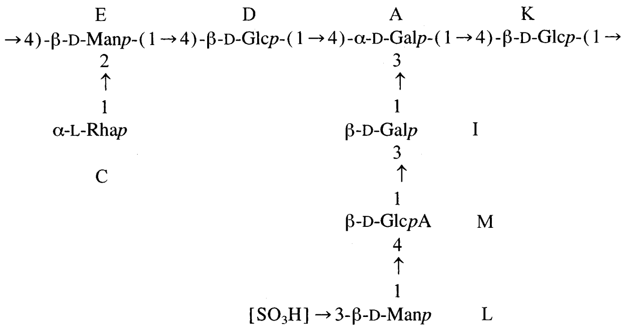
[a] Carbons involved in interglycosidic linkages are underlined.

Table 5
Correlations observed in the 400 MHz HMBC spectrum recorded at 328 K in D_2O of the HYD 721 DP exopolysaccharide

Residue	Anomeric atoms		$J_{H.C}$ correlations		Residue, atom
	δ_H	δ_C	δ_C	δ_H	
→3,4)-α-D-Gal*p*-(1→	5.45		73.0		A, C-5
A			80.8		K, C-4
		101.8		3.65	K, H-4
				4.03	A, H-5
→4)-α-D-Gal*p*-(1→	5.40				
B		101.8			
α-L-Rha*p*-(1→	5.00		70.5		C, C-5
C			72.1		C, C-2
			78.2		E, C-2
		103.2		4.18	E, H-2
→4)-β-D-Glc*p*-(1→	4.92		78.2		A, C-4
D		104.1		3.36	D, H-2
				4.45	A, H-4
→2,4)-β-D-Man*p*-(1→	4.80				
E		102.6			
[SO_3H]→3-β-D-Man*p*-(1→	4.76		81.8		M, C-4
L		100.6		3.85	M, H-4
→4)-β-D-Man*p*-(1→	4.75		79.0		D, C-4
F		101.9		3.64	D, H-4
→4)-β-D-Glc*p*A-(1→	4.75		84.3		I, C-3
M		105.5		3.44	M, H-2
				3.81	I, H-3
				4.13	I, H-4
β-D-Man*p*-(1→	4.66		72.5		N, C-2
N			81.8		M, C-4
		102.1		3.85	M, H-4
β-D-Gal*p*-(1→	4.66		80.7		A, C-3
I		106.2		3.68	I, H-5
				3.81	I, H-3
				4.06	A, H-3
→4)-β-D-Glc*p*-(1→	4.51		78.6		na[a]
K		104.5		3.36	K, H-2
				3.80	na[a]

[a] The signal at δ 3.80/78.6 is present in the HMQC spectrum.

and C-1 of M to C-3 and H-3 of I, respectively. These results agree with the β-elimination data and establish the substitution of the 3-position of the Gal*p* by the Glc*p*A. Anomeric atoms of L and N showed clear correlations with H-4 and C-4 of M, suggesting the β-(1→4) linkage between the terminal Man*p* and its 3-sulfated equivalent and the 4-β-D-Glc*p*A. From the combined evidence of the NOE and the HMBC C-H correlations, the following repeating unit structure **6** can be postulated for the polysaccharide HYD 721 DP:



6

Regarding the composition analysis along with methylation data, the only difference between HYD 721 DP and the native polysaccharide is the lower rhamnosylation frequency of the HYD 721 DP polysaccharide. Thus it can be concluded that the structure **6** represents the repeating unit of the polysaccharide HYD 721, with about 80% of the mannosyl residues of the main chain substituted at O-2 by a rhamnosyl residue, and about 50% of terminal mannosyl residues located on the second side chain carrying a sulfate ester group.

3. Experimental

Bacterial strain.—*Pseudoalteromonas* sp. HYD 721 was isolated from the polychaete annelid *Alvinella pompejana* near the active hydrothermal vent located at 13°N on the East Pacific Rise, during the 'Hydronaut' oceanographic cruise in 1987.

Production and isolation of the polysaccharide.—The polysaccharide was produced as previously described [4,1] using a 2-L fermentor containing 1 L of 2216E medium supplemented with glucose, at atmospheric pressure, a temperature of 25 °C and at pH 7.2. After 30 h, the viscosity of the medium reached a stable value of 120 Pa. Cells were removed from the medium by high-speed centrifugation at 20,000 *g* for 2 h. The exopolysaccharide was precipitated from the supernatant with ethanol at 40% (v/v) and washed with ethanol/water (70–100%, v/v). The yield was 5 g of polysaccharide, which was stored at room temperature.

Purification of the polysaccharide.—The crude polysaccharide was dissolved in 50 mM Tris solution (pH 7.5) and applied to an anion-exchange chromatographic column (35 × 2.6 cm) of Sepharose CL-6B. Elution was performed using the same buffer and a 0.1–1 M NaCl gradient. Fractions were collected, assayed for uronic acid [7] and for neutral sugars [8], dialyzed and lyophilized.

Determination of the molecular weight.—The polysaccharide (5 mg) was dissolved in 0.15 M NaCl, applied to a gel-filtration chromatographic column (42 × 1.2 cm) of Sephacryl S400HR and eluted with the same buffer. Elution was followed with assay of fractions for neutral sugars [8]. Preliminary calibration of the column was conducted using dextrans of different molecular weights.

General methods.—GLC–MS was carried out on alditol acetates and partially methylated alditol acetates on a Hewlett–Packard model

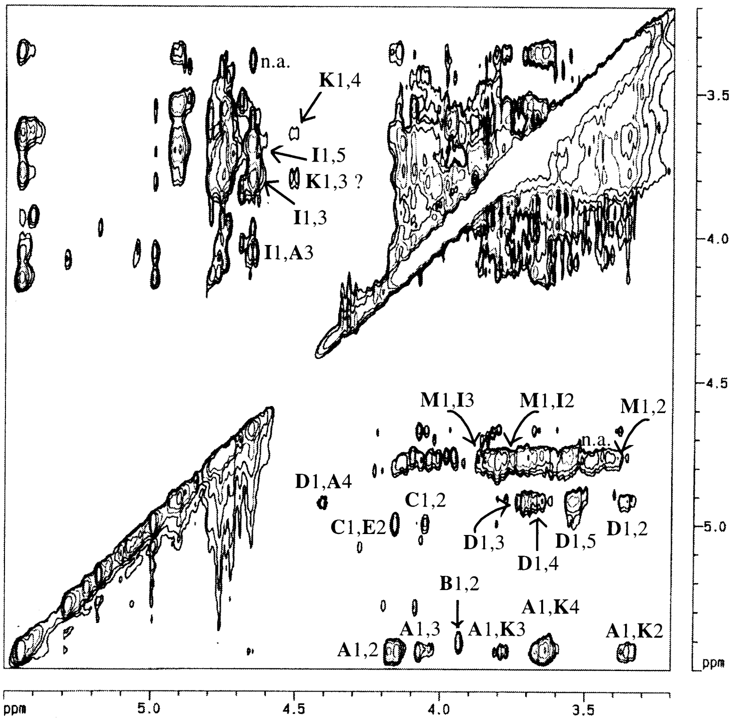


Fig. 6. NOESY spectrum (400 MHz) recorded at 328 K in D_2O of the HYD 721 DP polysaccharide.

5890 system or a HRGC-5160 system using an SP 2330 fused silica column (0.25 mm × 30 m) and a temperature program of 170–240 °C at 4 °C min^{-1} followed by 10 min at 240 °C. GC on the per-*O*-trimethylsilylated methyl glycosides was performed on a GC-8000 system using a CP-Sil-5CB fused silica column (0.25 mm × 50 m) and a temperature program of 120–240 °C at 2 °C min^{-1}.

Constituent analysis.—Methanolysis was performed in 2 M MeOH–HCl, and the methyl glycosides were converted to the corresponding per-*O*-trimethylsilylated derivatives as described by Montreuil et al. [9]. Hydrolysis was performed in 1 M TFA, and the monosaccharides were reduced and converted in the corresponding alditol acetates as described by Blakeney et al. [10]. The absolute configuration of the sugars was determined as devised by Gerwig et al. [11].

Methylation analysis.—Glycosyl-linkage positions were determined using a modification of the Hakomori procedure [12] using lithium methylsulfinylmethanide [13,14] and MeI in dimethyl sulfoxide. The methylated compounds were recovered by use of SepPak C_{18} cartridges (Millipore) [15]. Reduction of ester groups with 'Superdeuteride' $LiB(C_2H_5)_3D$ was carried out according to York et al. [16]. The methylated product was then hydrolyzed in 2 M TFA (2 h, 120 °C), reduced and acetylated. PMAA were identified by reference to authentic standards prepared according to Doares et al. [17].

β-Elimination.—The sample was β-eliminated according to the procedure of Aspinall and Rosell [18]. Briefly, the methylated polysaccharide was dissolved in dimethyl sulfoxide, BuLi was added to form lithium methylsulfinylmethanide, and the sample was then ethylated as described [18], hydrolyzed, and converted to alditol acetates by conventional methods.

Location of the sulfate ester groups.—The position of the sulfate esters was determined using the method described by Kovensky et al. [19]. Desulfation was accomplished on the pyridinium salts of the polymer using 9:1 Me_2SO–H_2O (v/v) at 80 °C during 5 h [20]. Then the desulfated polysaccharide was subjected to the methylation analysis, and the resultant alditol acetates gave additional structural information in that new methylated hydroxyl groups appeared.

Selective degradation of the glycosyluronic acid residues.—The glycosyluronic acid residues of the polysaccharide (20 mg) were degraded by a treatment with lithium metal dissolved in ethylenediamine as described by Lau et al. [21]. The residue isolated from the treatment of the exopolysaccharide with lithium was dissolved in water and chromatographed on a column of Bio-Gel P-10 (1.5 × 74 cm). Fractions of 3 mL were collected, assayed for neutral sugars [8], dialyzed and lyophilized. The yield was 12 mg of polysaccharide HYD 721 Li, which eluted in the void volume.

Partial hydrolysis.—Polysaccharide (100 mg) was treated with 0.05 M TFA at 100 °C for 1 h, diluted with water. The HYD 721 DP solution was subjected to ultrafiltration using a Millipore system with PM-10 membrane (10,000 Da cut-off), washed with water, concentrated, and finally lyophilized. The yield was 23 mg.

NMR spectral studies.—NMR spectra were recorded at 320 K on a solution of HYD 721 Li in D_2O containing acetone as internal standard (δ_H 2.225 ppm, δ_{CH_3} 31.45 ppm) on Bruker AMX-500 and AMX-600 spectrometers using UXNMR software. ^1H–^1H correlated spectroscopy (COSY), total correlation spectroscopy (TOCSY) with a mixing time of 20 ms, and heteronuclear single quantum coherence (HSQC) were employed to assign signals and were performed according to standard pulse sequences. For inter-residue correlation, a two-dimensional nuclear Overhauser effect spectroscopy (NOESY) experiment was used with a mixing time of 160 ms.

NMR spectra were recorded at 328 K on solution of HYD 721 DP on a Bruker DRX-400 spectrometer using UXNMR software. 2D (COSY, TOCSY with a mixing-time of 60 ms and HMQC) experiments were performed according to standard pulse sequences. For inter-residue correlations, a two-dimensional NOESY experiment with a mixing time of 750 ms and a heteronuclear multiple bond correlation (HMBC) with a delay of 60 ms were used. ^1H and ^{13}C NMR chemical shifts were expressed in ppm relative to sodium 2,2,3,3-tetradeuterio-4,4-dimethyl-4-silapentanoate.

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