Carbohydrate Research, 156 (1986) 165–172
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL STUDIES OF A POLYSACCHARIDE (S-88) ELABORATED BY *Pseudomonas* ATCC 31554

PER-ERIK JANSSON, N. SAVITRI KUMAR*, AND BENGT LINDBERG
Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

(Received May 3rd, 1986; accepted for publication, May 28th, 1986)

ABSTRACT

The structure of the extracellular polysaccharide elaborated by *Pseudomonas* ATCC 31554 has been investigated, methylation analyses, specific degradations, and ^{1}H-n.m.r. spectroscopy being the main methods used. It is concluded that the polysaccharide is composed of pentasaccharide repeating-units with the structure:

$$\rightarrow 3)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{A-}(1\rightarrow 4)\text{-}\beta\text{-D-Glc}p\text{-}(1\rightarrow 4)\text{-}\alpha\text{-L-[Rha or Man]-}(1\rightarrow$$
$$\begin{array}{c} 3 \\ \uparrow \\ 1 \\ \alpha\text{-L-Rha} \end{array}$$

An unusual feature is that a sugar residue in the chain may be either L-rhamnose or L-mannose. The polysaccharide also contains *O*-acetyl groups (~5%) which have not been located.

INTRODUCTION

Several extracellular polysaccharides have been isolated which have potential commercial application as gelling or thickening agents[1]. The first of these, gellan gum or PS-60, is a linear polysaccharide with a tetrasaccharide repeating-unit[2,3]. Welan gum (S-130) was subsequently shown[4] to have the same repeat structure as gellan gum, but with a single sugar side-chain which is L-rhamnose or L-mannose in the ratio ~2:1. It is interesting to note that the presence of this single unit side-chain transforms the properties from a gelling polysaccharide (upon heating above 85° and cooling in the presence of cations) to a polysaccharide which has excellent compatibility with divalent cations at temperatures up to 135°. A third polysaccharide in this series, rhamsan gum or S-194[5], also has the same backbone structure as gellan and welan gum, but carries a disaccharide side-chain[6]. This poly-

*On leave from: Department of Chemistry, University of Peradeniya, Peradeniya, Sri Lanka.

0008-6215/86/$ 03.50

saccharide has rheological properties similar to those of welan gum, but there are some differences in stability and compatibility which lead to different industrial applications[7,8].

We now report on the structure of S-88, a fourth member of this series of polysaccharides[9]. It is derived from a *Pseudomonas* sp. (ATCC 31554) and has properties similar to those of welan and rhamsan gum, which are derived from *Alcaligenes* sp.

RESULTS AND DISCUSSION

The polysaccharide (PS) on acid hydrolysis yielded a mixture of L-rhamnose, L-mannose, and D-glucose in the relative proportions 44:15:41. It also contained D-glucuronic acid and *O*-acetyl groups (~5%). The absolute configurations of the sugars were determined by the procedure devised by Gerwig *et al.*[10].

The PS formed highly viscous, aqueous solutions and therefore gave poor n.m.r. spectra. In order to obtain better spectra, the PS was deacetylated by treatment with base and then treated with acid under mild conditions. Sugar analysis of the modified PS gave L-rhamnose, L-mannose, and D-glucose in the relative proportions 31:25:44. The ^{1}H-n.m.r. spectrum of this modified PS contained, *inter alia*, signals for anomeric protons at δ 5.24 (not resolved), 5.22 (not resolved), 5.15 ($J_{1,2}$ small) (1.4 H together), 4.72 (1 H, $J_{1,2}$ 7.8 Hz), 4.56 (1 H, $J_{1,2}$ 7.8 Hz), and 4.54 (1 H, $J_{1,2}$ 8.1 Hz), and for H-6 of L-rhamnosyl residues at 1.33 ($J_{5,6}$ 6.5 Hz) and 1.27 ($J_{5,6}$ 6.4 Hz) (4 H together). From the values of the coupling constants, it may be concluded that all D-glucosyl and D-glucosyluronic acid residues are pyranosidic and β-linked. Assuming that the L-rhamnosyl and L-mannosyl residues are pyranosidic, as will be shown below, they should be α-linked.

Methylation analysis of the PS, with and without carboxyl-reduction of the methylated product, gave the values listed in Table I, columns *A* and *B*. The stoichiometry is not very good, but the results, in conjunction with those discussed below, suggest that the PS contains terminal α-L-rhamnopyranosyl groups, β-D-glucopyranosyl residues linked through O-3, β-D-glucopyranosyl residues linked through O-3 and O-4, β-D-glucopyranosyluronic acid residues linked through O-4, and α-L-rhamnopyranosyl and α-L-mannopyranosyl residues linked through O-4. That the last two residues are not furanosidic and linked through O-5 is evident from results discussed below. The methylation analysis of the modified PS, with carboxyl-reduction of the methylated product (Table I, column *C*), shows that some terminal residues had been hydrolysed off and that some glycosidic linkages in the main chain had been cleaved.

The fully methylated PS was treated with lithium methylsulfinylmethanide in dimethyl sulfoxide, followed by trideuteriomethylation. A hydrolysate of the product contained the sugars listed in Table I, column *D*. The course of the degradation is indicated in Scheme 1. The formation of 2,6-di-*O*-methyl-4-*O*-tri-

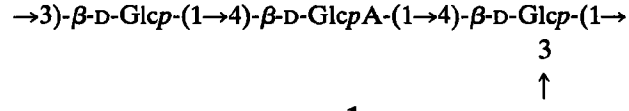
TABLE I

METHYLATION ANALYSIS OF S-88 AND SOME DEGRADATION PRODUCTS[a]

Sugar[b]	T[c]	*Mole %*							
		A	B	C	D	E	F	G	H
1,2,3,5-Rhamnitol	0.31							24.1	4.6
1,2,3,5,6-Mannitol	0.49						4.0	2.9	3.7
2,3,4-Rha	0.55	23.7	30.0	14.3	38.3				
2,3-Rha	0.95	18.2	17.7	6.6	16.0				6.7
2,3,4,6-Glc	1.00			5.4			23.0	26.3	12.0
2,4,6-Glc	1.65	30.4	22.0	18.0	29.8[d]				8.6
2,3,6-Man	1.79	14.6	9.4	16.8	15.8				15.9
2,3,6-Glc	2.00			13.5		49.5	41.8	30.7	35.1
2,3,4-Glc	2.04					50.5			
2,6-Glc	2.78	13.1	9.8	5.6					
2,3-Glc	3.56		11.5	19.8			31.1	16.1	13.3

[a]Key: *A*, methylated polysaccharide; *B*, methylated and carboxyl-reduced polysaccharide; *C*, modified polysaccharide; *D*, product from uronic acid degradation; *E*, acidic trisaccharide **2**; *F*, acidic tetrasaccharide **3**; *G*, the same as *F*, enriched in **3a**; *H*, octasaccharide **4**. [b]2,3,4-Rha = 2,3,4-tri-*O*-methyl-L-rhamnose, *etc.* [c]Retention time of the derived alditol acetate, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol, on a DB-225 column at 190°. [d]Trideuteriomethyl at O-4.

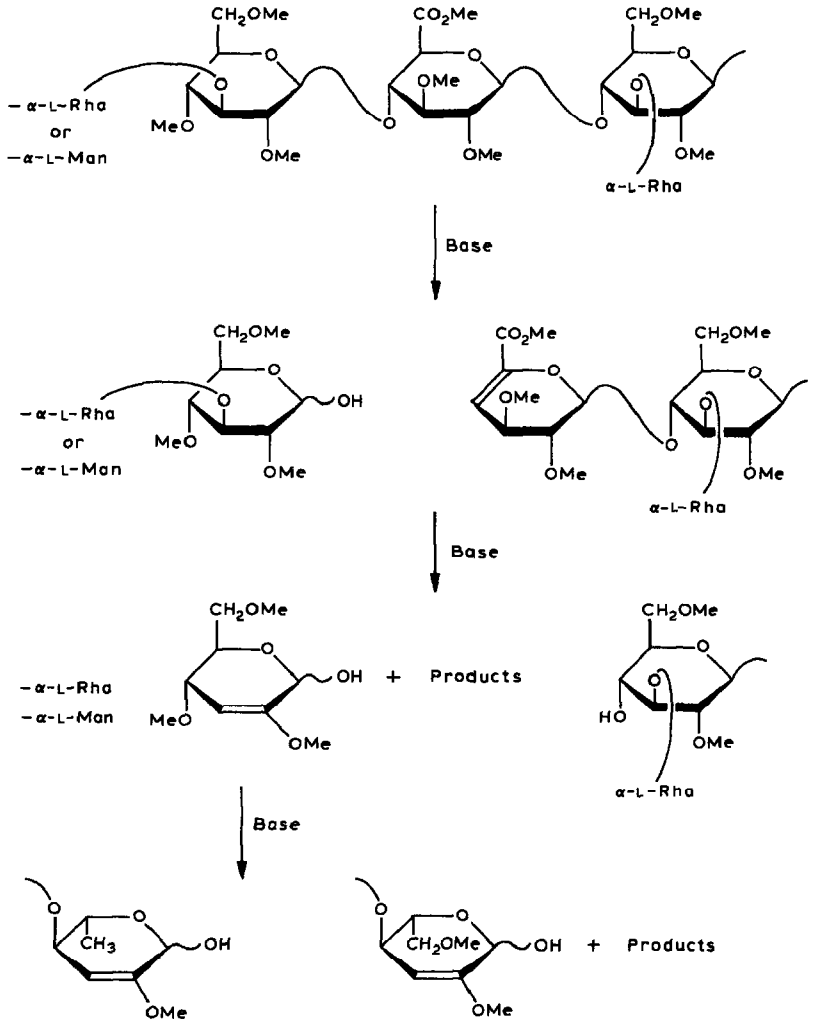
deuteriomethyl-D-glucose demonstrates that the glucuronic acid was linked to O-4 of the branching D-glucopyranosyl residue. The complete disappearance of unlabelled 2,4,6-tri-*O*-methyl-D-glucose demonstrates that this sugar was linked to O-4 of the D-glucopyranosyluronic acid residue. The survival of all the α-L-rhamnopyranosyl and α-L-mannopyranosyl residues is surprising, as at least one of these should be linked to O-3 of the 3-substituted β-D-glucopyranosyl residue. When, however, the reaction time for the uronic acid degradation was prolonged, most of the 4-linked α-L-rhamnopyranosyl and α-L-mannopyranosyl residues were degraded. As a result of the uronic acid degradation, the partial structure **1** is established.



1

In order to obtain more information, the deacetylated PS was hydrolysed to a mixture of mono- and oligo-saccharides, which were fractionated by ion-exchange chromatography on DEAE-Trisacryl M. The oligosaccharides obtained were then desalted and further purified by chromatography on Bio-Gel P-2.

One of the fractions, eluted in the trisaccharide region on the P-2 column, on acid hydrolysis yielded L-rhamnose, L-mannose, and D-glucose in the relative proportions 70:8:22. The ^{1}H-n.m.r. spectrum (100 MHz) contained, *inter alia*,



Scheme 1. Uronic acid degradation of S-88.

signals at δ 5.18 (0.5 H, not resolved), 4.83 (0.5 H, not resolved), 4.70 (1 H, $J_{1,2}$ 8.0 Hz), 4.50 (1 H, $J_{1,2}$ 8.0 Hz), and 1.33 (3 H, $J_{5,6}$ 6.0 Hz). The first two signals and the last signal could be assigned to reducing L-rhamnopyranose residues. Small signals which may be assigned to α- and β-L-mannopyranose residues were also observed. In the methylation analysis of the derived alditol, with carboxyl-reduction of the methylated product (Table I, column *E*), the volatile tetra-*O*-methyl-L-rhamnitol and penta-*O*-methyl-L-mannitol derivatives were lost. The combined results, however, demonstrate that the fraction was a mixture of the aldotriouronic acids **2a** and **2b**, in which the former preponderated. It therefore seems that α-L-rhamnopyranosyl and α-L-mannopyranosyl residues can replace each other in the PS.

β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Sug
2a Sug = Rha
2b Sug = Man

Another fraction, eluted somewhat earlier from the P-2 column, on sugar analysis gave L-rhamnose, L-mannose, and D-glucose in the relative proportions 47:13:40. The ^{1}H-n.m.r. spectrum contained, *inter alia*, signals at δ 5.18 (0.2 H, $J_{1,2}$ 1.9 Hz, α-Man), 5.11 (0.4 H, $J_{1,2}$ 1.9 Hz, α-Rha), 4.89 (0.1 H, $J_{1,2}$ 1.2 Hz, β-Man), 4.86 (0.3 H, $J_{1,2}$ 1.2 Hz, β-Rha), 4.73 (1 H, $J_{1,2}$ 8.0 Hz), 4.60 (1 H, $J_{1,2}$ 8.0 Hz), 4.54 (1 H, $J_{1,2}$ 7.8 Hz), and 1.34 and 1.36 (2 H together, $J_{5,6}$ ~6 Hz). This, in conjunction with the methylation analysis of the derived alditol, with carboxyl-reduction of the methylated product (Table I, column *F*), and the structures of **2α** and **2b**, demonstrates that the fraction is a mixture of two aldotetraouronic acids, **3a** and **3b**. Again, the volatile L-rhamnose derivative was lost in the methylation analysis. The presence of 1,2,3,5,6-penta-*O*-methyl-L-mannitol demonstrates that the L-mannose residues are pyranosidic and linked through O-4.

β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Sug-(1→
3a Sug = Rha
3b Sug = Man

In a separate experiment, when the deacetylated PS was hydrolysed under conditions milder than those used above, **3a** was the preponderant aldotetraouronic acid formed. A hydrolysate contained L-rhamnose (42%), L-mannose (5%), and D-glucose (53%) as the neutral sugars. In the methylation analysis (Table I, column *G*), 1,2,3,5-tetra-*O*-methyl-L-rhamnitol was obtained, demonstrating that the corresponding L-rhamnosyl residue in the PS is pyranosidic. The ^{1}H-n.m.r. spectrum showed, *inter alia*, all the signals given above by the tetrasaccharide mixture except those for the mannose residue.

A product that was eluted late from the ion-exchange column, but early in gel filtration, proved to be a mixture of octasaccharides. A hydrolysate contained L-rhamnose, L-mannose, and D-glucose in the relative proportions 20:24:56. Methylation analysis of the derived alditol and carboxyl-reduction of the methylated product (Table I, column *H*) showed that the octasaccharide was linear and that L-rhamnose and L-mannose could replace each other both as chain residues and as reducing terminals. This was also evident from the ^{1}H-n.m.r. spectrum of the alditol mixtures, which contained double signals for both the chain and the terminal L sugars (Table II). From the combined evidence, the fraction should therefore be a mixture of the oligosaccharides **4**.

β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Sug*p*-(1→3)-β-D-
Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-L-Sug
4

Sug = Rha or Man

TABLE II

^{1}H-N.M.R. DATA FOR THE OCTASACCHARIDE ALDITOL MIXTURE

δ (p.p.m.)	*J (Hz)*	*Integral*
1.27	6.4	2.6 H
1.31	6.3	
4.53	8.0	
4.54	8.0	
4.56	8.0	5 H
4.60	8.0	
4.62	8.0	
4.72	8.0	H
5.14	n.r.	H
5.21	n.r.	

The results of the partial hydrolysis studies therefore suggest that the PS is composed of pentasaccharide repeating-units (**5**), in which α-L-rhamnopyranosyl and α-L-mannopyranosyl residues may replace each other.

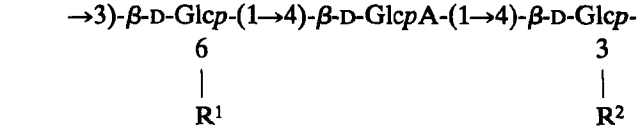
→3)-β-D-Glc*p*-(1→4)-β-D-Glc*p*A-(1→4)-β-D-Glc*p*-(1→4)-α-L-Sug*p*-(1→
3
↑
1
α-L-Rha*p*

5

Sug = Rha or Man

As these residues occur in comparable amounts, it might be argued that the PS is actually composed of decasaccharide repeating-units with a completely regular structure. However, this possibility seems less likely because such large repeating-units are rare. During the acid hydrolysis, α-L-rhamnosidic and α-L-mannosidic linkages should be preferentially hydrolysed, the former being the more readily hydrolysed. The fact that aldotetraouronic acid **3a**, but only little **3b**, was formed under very mild hydrolytic conditions indicates that the former was formed by hydrolysis of two α-L-rhamnopyranosidic linkages and that the distribution between α-L-rhamnopyranosyl and α-L-mannopyranosyl residues is more or less random. If the polysaccharide had been composed of regular decasaccharide repeating-units, comparable amounts of **3a** and **3b** would have been expected, independent of the hydrolytic conditions.

There is a remarkable similarity between S-88, gellan gum[2], S-130[3], and S-194[4], in that their repeating units all contain the same linear tetrasaccharide, unsubstituted for gellan gum but substituted with mono- or di-saccharide side-chains for the others (**6**).



6

Gellan gum Sug = Rha, $R^1 = R^2 = H$
S-130 (welan gum) Sug = Rha, $R^1 = H$, R^2 = α-L-Rha-(1→ or α-L-Man-(1→
S-194 (rhamsan gum) Sug = Rha, R^1 = β-D-Glcp-(1→6)-α-D-Glcp-(1→
S-88 Sug = Rha or Man, $R^1 = H$, R^2 = α-L-Rha-(1→

S-88 and S-130 have almost the same structure, the difference being that L-rhamnose and L-mannose may replace each other as chain residues in the former, but as terminal groups in the latter, polysaccharide.

EXPERIMENTAL

General methods. — Concentrations were performed under diminished pressure at <40° (bath), or at room temperature by flushing with air. For g.l.c., a Hewlett–Packard 5830A instrument fitted with a flame-ionisation detector was used. Separations were performed on an SE-54 fused-silica capillary column. The temperature programme for alditol acetates was 150°, 2 min; 150–230°, 2°/min; and for partially methylated alditol acetates was 130°, 2 min; 130–230°, 2°/min. G.l.c.–m.s. was performed on a Hewlett–Packard 5790-5970 system. Separations were performed on a DB-225 (Durabond) capillary column at 190°. All identifications of mass spectra were unambiguous and are not discussed. Absolute configurations of the sugars were determined by the method of Gerwig *et al.*[10].

Methylation analyses were performed essentially as previously described, using lithium methylsulfinylmethanide. Methylated products were purified on Sep-Pak C_{18} cartridges, using the procedure described by Waeghe *et al.*[11].

Carboxyl-reduction of the methylated polysaccharide was carried out with lithium borohydride in tetrahydrofuran at 70° for 4 h in a screw-cap test tube (13 × 100 mm). Excess of borohydride was decomposed with M acetic acid, and the solution was evaporated to dryness by flushing with air at room temperature. The contents of the tube were partitioned between dichloromethane and water. The dichloromethane layer was washed twice with water and then evaporated to dryness.

^{1}H-N.m.r. spectra of solutions in D_2O were recorded at 85° with a JEOL FX-100 or GX-400 instrument. Chemical shifts are reported in p.p.m. downfield from that of the signal for internal sodium 2,2,3,3-tetradeuterio-3-trimethylsilyl-propionate.

Deacetylation of S-88. — This was effected by stirring S-88 (200 mg) in hot water (200 mL) until an almost homogeneous solution was obtained. The solution

was cooled to room temperature, M sodium hydroxide (22 mL) was added, and the solution was stirred at 70° overnight (16 h). The solution was then cooled, neutralised (pH 7), dialysed (2 days), concentrated, and freeze-dried.

Partial hydrolysis of S-88. — (*a*) Trifluoroacetic acid (2M) was added to a concentration of 0.1M to *O*-deacetylated S-88 (30 mg) in water (30 mL) at 100°. After 30 min, the solution was cooled in an ice bath, concentrated, and freeze-dried. The sample was used for ^{1}H-n.m.r. spectroscopy.

(*b*) Samples of deacetylated S-88 (150 mg) were hydrolysed at 100° for 3 h and 4 h, respectively, in 0.1M trifluoroacetic acid. The resulting solutions were concentrated and freeze-dried.

Fractionation of oligosaccharides. — The mixtures of oligosaccharides were fractionated on a column (350 mm × 25 mm) of DEAE-Trisacryl M (LKB-Products, Sweden) by elution with 0.01M sodium dihydrogenphosphate followed by a gradient of sodium chloride (0 → 0.5M) in the same phosphate solution (1 L). The eluates were monitored with the anthrone reagent. Each oligosaccharide fraction was desalted by gel filtration on Bio-Gel P-2.

The first fraction to be eluted consisted entirely of rhamnose. Mixtures of tetrasaccharides, trisaccharides, and octasaccharides were eluted when the concentration of sodium chloride in the phosphate buffer was approximately 0.19M, 0.23M, and 0.26M, respectively.

Uronic acid degradation of S-88. — This was carried out as described previously[4].

ACKNOWLEDGMENTS

We thank Dr. John Baird (Kelco Division, San Diego) for supplying the polysaccharide and for his interest. We also thank Dr. Ulf Lindquist for valuable discussions. This work was supported by a maintenance grant from the International Seminar in Chemistry, University of Uppsala (to N. S. Kumar), and by a grant from the National Swedish Board for Technical Development.

REFERENCES

1 J. K. Baird, P. A. Sandford, and I. W. Cottrell, *Biotechnology*, 1 (1983) 778–783.
2 P.-E. Jansson, B. Lindberg, and P. A. Sandford, *Carbohydr. Res.*, 124 (1983) 135–139.
3 M. A. O'Neill, R. R. Selvendran, and V. J. Morris, *Carbohydr. Res.*, 124 (1983) 123–133.
4 P.-E. Jansson, B. Lindberg, G. Widmalm, and P. A. Sandford, *Carbohydr. Res.*, 139 (1985) 217–223.
5 J. A. Peik, S. M. Steenbergen, and H. R. Hayden, U.S. Pat. 4,401,760 (1983); *Chem. Abstr.*, 99 (1983) 51,864m.
6 P.-E. Jansson, B. Lindberg, J. Lindberg, E. Maekawa, and P. A. Sandford, *Carbohydr. Res.*, 156 (1986) 157–163.
7 P. A. Sandford, I. W. Cottrell, and D. J. Pettitt, *Pure Appl. Chem.*, 56 (1984) 879–892.
8 G. T. Colegrove, *I & EC Product Research and Development*, 22 (1983) 456–460.
9 K. S. Kang and G. T. Veeder, U.S. Pat. 4,535,153 (1985); *Chem. Abstr.*, 95 (1981) 185,579y.
10 G. J. Gerwig, J. P. Kamerling, and J. F. G. Vliegenthart, *Carbohydr. Res.*, 77 (1979) 1–7.
11 T. J. Waeghe, A. G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.