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Structural studies of the capsular polysaccharide from *Aerococcus viridans* var. *homari*.

Kerstin Hermansson, Lennart Kenne, Bengt Lindberg,
Department of Organic Chemistry, Arrhenius Laboratory, University of Stockholm, S-106 91 Stockholm (Sweden)

B. Arie, Robert G. Brown, and James E. Stewart
Biological Sciences Branch, Fisheries and Ocean Canada, Box 1006, Dartmouth, N.S. B2Y 4A2, and Dept. of Biology, Dalhousie University, Halifax, N.S. B3H 4J1 (Canada)

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ABSTRACT

The capsular polysaccharide from *Aerococcus viridans* var. *homari* has been investigated, using n.m.r. spectroscopy, methylation analysis, and specific degradations as the main methods. The polysaccharide is composed of tetrasaccharide repeating-units having the following structure.

→3)-β-D-Qui*p*NAc-(1→3)-β-D-Glc*p*A-(1→3)-α-L-Alt*p*A-(1→
4
↑
1
4-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*

In this structure, D-QuiN stands for 2-amino-2,6-dideoxy-D-glucose (quinovosamine). Two of the three acidic sugars found, namely, L-altruronic acid and 4-*O*-[(*S*)-1-carboxyethyl]-D-glucose, have not been found in any other natural source. As evident from the n.m.r. spectra, the L-altruronic acid is not present in the $^1C_4$ conformation, but flips to a conformation close to this on carboxyl reduction.

INTRODUCTION

The Gram-positive bacterium *Aerococcus viridans* var. *homari* (formerly *Gaffkya homari*) is highly pathogenic to lobsters. It causes a disease that is commercially costly with a high fatality rate when the lobsters are kept in corfs. It has been found that virulent strains are heavily encapsuled, whereas avirulent strains have minimal capsular material[1]. We have previously demonstrated that the capsular material is an acidic polysaccharide, and have isolated and identified one of its sugar components as 4-*O*-[(*S*)-1-carboxyethyl]-D-glucose[2]. This sugar has not been found elsewhere in Nature, but the corresponding 4-*O*-[(*R*)-1-carboxyethyl]-D-glucose is a component of the O-antigen from *Shigella dysenteriae* type 3 (ref. 3) and the *Klebsiella* type 66 capsular polysaccharide[4]. We now report on further structural studies of the capsular polysaccharide from *A. viridans* var. *homari*.

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RESULTS AND DISCUSSION

The crude capsular material was prepared as described[2], treated with DNAse and RNAse, followed by proteinase K, and then further fractionated by anion-exchange chromatography to yield the pure polysaccharide (PS).

The ^{13}C-n.m.r. spectrum (Fig. 1) of the PS contained, *inter alia*, signals for four anomeric carbons (Table I), indicating a tetrasaccharide repeating-unit. The spectrum also contained four signals for carbonyl carbons at δ 175–180, indicating the possible presence of uronic acids, and three signals for methyl groups, one of which could be assigned to an *N*-acetyl group.

The ^{1}H-n.m.r. spectrum (Fig. 2) contained, *inter alia*, signals for four anomeric protons (Table I). The spectrum also contained signals for one *N*-acetyl group and two methyl groups at δ 1.38 and 1.36. The former methyl signal was coupled to a proton signal at δ 4.04. This spin system could be assigned to the carboxyethyl group of the 4-*O*-[(*S*)-1-carboxyethyl]-β-D-glucopyranosyl residue.

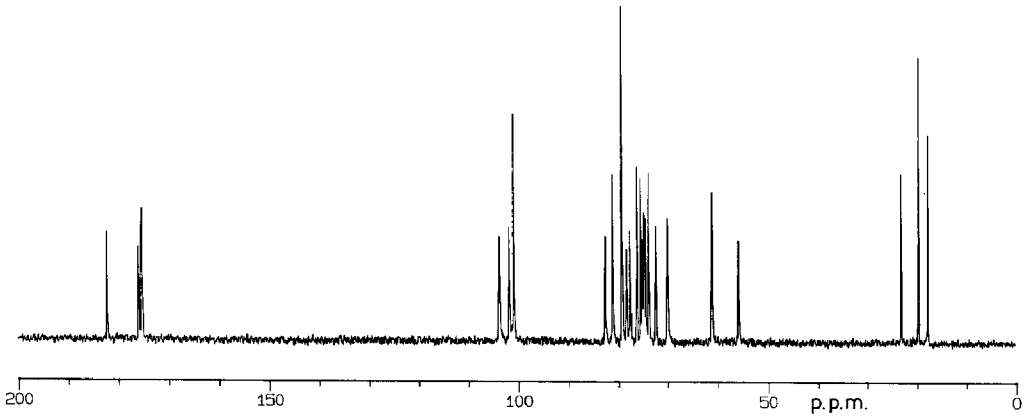


Fig. 1. 67.8-MHz ^{13}C-n.m.r. spectrum of the capsular polysaccharide from *A. viridans*.

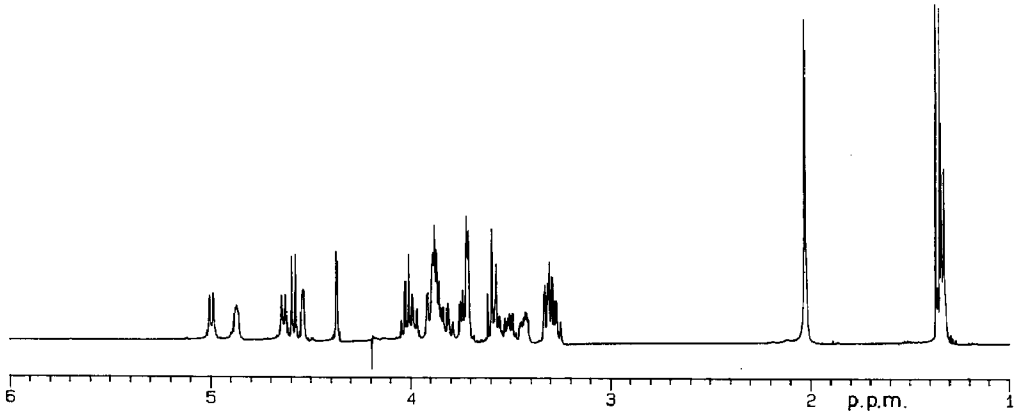


Fig. 2. 400-MHz ^{1}H-n.m.r. spectrum of the capsular polysaccharide from *A. viridans*.

TABLE I

^{1}H- And ^{13}C-n.m.r. data of native and carboxyl-reduced polysaccharide from A. *viridans*

Polysaccharide	*H-1*	*H-1'*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Native														
→3)-β-D-Quip*N*Ac(1→	4.99		3.92	3.84	3.29	3.54	1.36		101.07	55.98	82.60	74.83	72.44	17.92
	(*7.9*)[a]		(*10.7*)	(*8.5*)	(*10.4*)	(*6.1*)			(*163*)[a]					
→3)-α-L-Alt*p*A(→[b]	4.89[c]		3.73	3.74	4.55	4.41			101.88	70.12	81.01	69.97	78.10	
	(*7.5*)		(*4.5*)	(*4.4*)	(*5.3*)				(*168*)					
→3,4)-β-D-Glc*p*A(1→[b]	4.65		3.58	3.86	4.01	3.89			103.87	74.62	81.20	75.09	77.62	
	(*7.8*)		(*10*)	(*7.5*)	(*6.8*)				(*165*)					
4-*O*-*S*)-β-D-Glc*p*(1→[d]	4.60		3.36	3.62	3.33	3.45	3.74	3.92	101.07	73.92	75.48	79.26	76.26	61.23
	(*7.8*)		(*9.5*)	(*9.5*)	(*9.5*)	(*6.0,2.8*)	(*11.8*)		(*165*)					
(*S*)-1-Carboxyethyl[b]			4.04	1.38						79.26	19.75			
			(*7.5*)											
Carboxyl-reduced														
→3)-β-D-Quip*N*Ac	5.04		3.92	3.71	3.28	3.54	1.37		100.94	56.42	82.23	74.82	72.66	17.99
	(*8.4*)								(*165*)					
→3)-α-L-Alt*p*	4.88		3.91	4.01	4.05	4.14	3.79	3.79	102.26	69.64	78.93	65.67	72.86	61.60
	(*4.0*)		(*5.8*)	(*4.0*)	(*7.5*)	(*5.5*)			(*169*)					
→3,4)-β-D-Glc*p*	4.61		3.54	3.97	3.87	3.63	3.85	3.89	102.68	75.05	80.32	73.20	76.20	61.40
	(*7.9*)								(*161*)					
4-*O*-*S*)-β-D-Glc*p*[e]	4.68		3.41	3.60	3.49	3.46	3.79	3.94	101.10	73.88	76.71	77.55	76.10	61.28
	(*7.9*)								(*161*)					
(*S*)-2-(1-Hydroxy)propyl	3.57	3.68	3.91	1.18					66.27	79.15	17.38			

[a] $^{3}J_{H,H}$ and $^{1}J_{C,H}$ values in parenthesis. The $^{3}J_{H,H}$ values were obtained from the phase-sensitive COSY spectrum. [b] The signals of the carbonyl carbons at δ 175.64, 176.08, and 182.40 could not be assigned. [c] The signal in the 1D-spectrum was not resolved due to the small chemical shift difference between signals from H-2 and H-3. [d] 4-*O*-[(*S*)-1-Carboxyethyl]-β-D-Glc*p*. [e] 4-*O*-[(*S*)-2-(1-Hydroxy)propyl]-β-D-Glc*p*.

The signals from all protons and carbons (Table I), except those given by the carboxyl acid carbons, could be assigned by 2D-n.m.r. methods. The spin system containing an H-1 signal at δ 4.99 showed large coupling constants for all ring proton signals, and coupling between H-5 and a methyl group. These data, together with the C-2 signal at δ 55.98, demonstrate that the signals are derived from a 2-acetamido-2,6-dideoxy-β-glucopyranosyl residue. Another spin system, containing an H-1 signal at δ 4.60, also showed large coupling constants for the ring proton signals and also coupling between H-5 and two H-6 protons. The chemical shifts of the proton and carbon signals agreed well with those given by a 4-substituted β-glucopyranosyl residue, and, consequently, the signals could be assigned to a 4-*O*-[(*S*)-1-carboxyethyl]-β-D-glucopyranosyl group. The two remaining sugar residues gave no H-6 signals and were tentatively identified as uronic acid residues.

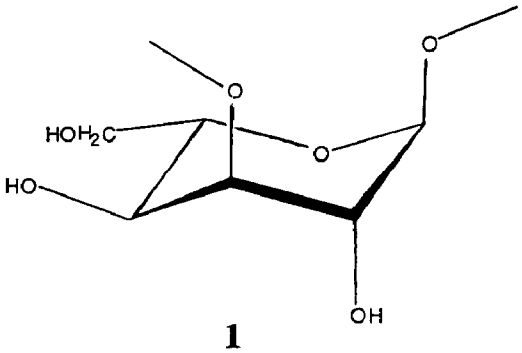
In order to facilitate the sugar and methylation analysis, the PS was carboxyl-reduced. Hydrolysis of the product, reduction with sodium borohydride, and acetylation yielded the acetates of a 1,6-anhydrohexopyranose, glucitol, another hexitol, 2-amino-2,6-dideoxyglucitol, and 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucitol in the relative proportions 0.36:1:0.47:0.41:0.92, as revealed by g.l.c–m.s. The retention times for the acetylated 1,6-anhydrohexopyranose and the hexitol agreed with those given by 1,6-anhydroaltropyranose triacetate and altritol hexa-acetate. An equilibrium between altrose and its 1,6-anhydropyranose derivative was established in dilute aqueous acid, which yielded $\sim$57% of the latter sugar[5].

The absolute configurations of the sugars were determined by the method of Gerwig *et al.*[6], and were D-glucose, L-altrose, and 2-amino-2,6-dideoxy-D-glucose. The fourth sugar had already been identified as 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-D-glucose[2].

On hydrolysis of the native PS and analysis as above, 2-amino-2,6-dideoxyglucitol and glucitol were present in the ratio 1.8:1. Glucitol was formed from glucofuranurono-6,3-lactone by reduction with sodium borohydride. Because altruronic acid does not lactonise, no altritol was formed.

Methylation analysis of the carboxyl-reduced PS demonstrated that, in the native PS, one of the hexuronic acids was linked through O-3 and the other through O-3 and O-4, and the quinovosamine through O-3, and that the 4-*O*-[(*S*)-1-carboxyethyl]-D-glucose was terminal.

The ^{13}C-n.m.r. spectrum of carboxyl-reduced material showed, *inter alia*, signals for four anomeric carbons (Table I). Three of these had $^{1}J_{C,H}$ values between 161 and 165 Hz, and the fourth had $^{1}J_{C,H}$ 169 Hz. The only carboxyl signal, δ 175.01, was assigned to the *N*-acetyl group of the QuiNAc residue. The ^{1}H-n.m.r. spectrum of the carboxyl-reduced PS contained, *inter alia*, signals for four anomeric protons, three with large coupling constants, $^{3}J_{H,H}$ 7.9–8.4 Hz, and one with $^{3}J_{H,H}$ 4.0 Hz. The three former signals could be assigned to the 2-acetamido-2-deoxy-β-D-glucopyranosyl residue, the β-D-glucopyranosyl residue, and the 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-β-D-glucopyranosyl group. The fourth could consequently be assigned to the L-altropyranosyl residue, and analysis of the spin system showed that it was α-linked and present in a conformation close to $^{1}C_{4}$ (**1**).



1

The sequence of the sugars was determined by NOESY spectra of the native PS (Table II) and of the carboxyl-reduced PS. Cross-peaks were observed between the H-1 signal of α-L-Alt*p*A and the H-3 signal of β-D-Qui*p*NAc, and between the H-1 signal of β-D-Glc*p*A and the H-3 signal of α-L-Alt*p*A. A cross-peak of low intensity was observed between the H-1 signal of β-D-Qui*p*NAc and the H-3 signal of β-D-Glc*p*A. That β-D-Glc*p*A is linked to O-3 of α-L-Alt*p*A was further confirmed by a corresponding weak signal in the NOESY spectrum of the carboxyl-reduced PS. These results establish the partial structure **2**, which represents the linear part of the PS.

→3)-β-D-Qui*p*NAc-(1→3)-β-D-Glc*p*A-(1→3)-α-L-Alt*p*A(1→

2

That the 4-*O*-[(*S*)-1-carboxyethyl]-β-D-glucopyranosyl group is linked to O-4 of one of the hexuronic acids was established by results from NOESY experiments in conjunction with the methylation analysis. A weak n.O.e. between the H-1 of this group and the H-4 of β-D-Glc*p*A indicated linkage to O-4 of the latter residue, and this inference was confirmed by a corresponding n.O.e. observed for the carboxyl-reduced PS. The considerable downfield shift (δ 75.09) of the C-4 signal for the β-D-Glc*p*A residue, compared to the chemical shift of the corresponding signal[7] (δ 72.3) for methyl β-D-glucopyranosiduronic acid, is also in agreement with this conclusion. It is concluded from the combined results presented above that the capsular polysaccharide elaborated by *A. viridans* var. *homari* is composed of tetrasaccharide repeating-units having the structure **3**.

→3)-β-D-Qui*p*NAc-(1→3)-β-D-Glc*p*A-(1→3)-α-L-Alt*p*A-(1→
4
↑
1
4-*O*-[(*S*)-1-carboxyethyl]-β-D-Glc*p*

3

TABLE II

Observed inter-residue n.O.e. contacts for anomeric protons in the native polysaccharide

Anomeric proton	*N.O.e. contact*
4.99 (β-D-Qui*p*NAc)	3.86 (β-D-Glc*p*A, H-3, weak[a])
4.89 (α-L-Alt*p*A)	3.84 (β-D-Qui*p*NAc, H-3)
4.65 (β-D-Glc*p*A)	3.74 (α-L-Alt*p*A, H-3)
4.60 (4-*O*-[(*S*)-1-Carboxyethyl]-β-D-Glc*p*)	4.01 (β-D-Glc*p*A, H-4, weak[a])

[a] N.O.e. contacts were also observed for the carboxyl-reduced material.

TABLE III

^{1}H-N.m.r. data of carboxyl-reduced and Smith-degraded polysaccharide from *A. viridans*

	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
→3)-β-D-Qui*p*NAc(1→	4.76	3.90	3.73	3.25	3.57	1.35	
→3)-α-L-Alt*p*(1→	4.91	3.94	4.09	4.03	4.19	3.79	3.79
→3)-β-D-Glc*p*(1→	4.63	3.44	3.67	3.68	3.49	3.73	3.90

This structure was further established by subjecting the carboxyl-reduced PS to a Smith degradation, by which the terminal 4-*O*-[(*S*)-2-(1-hydroxy)propyl]-β-D-glucopyranosyl group should be removed. The signals in the ^{1}H-n.m.r. spectrum were assigned by H,H-COSY (Table III). Comparison of this spectrum with the ^{1}H-n.m.r. spectrum of the carboxyl-reduced PS (Table I) shows that the signals given by the β-D-Qui*p*NAc (except for H-1) and the α-L-Alt*p* residues are essentially unchanged, but that those given by H-3 and H-4 of the β-D-Glc*p* residue have shifted by 0.3 and 0.2 p.p.m., respectively, on removal of the terminal group.

As discussed before, α-L-Alt*p* in the carboxyl-reduced PS occurs in a ring conformation close to $^1C_4$. In the spectrum of the native PS, however, the H-1 signal of α-LAlt*p*A at δ 4.89 has a $^3J_{H,H}$ value of 7.5 Hz, indicating a conformation in which H-1 and H-2 are antiparallel. A similar conformational change was observed on carboxyl-reduction of the 2-acetamido-2-deoxy-L-altruronic acid residue in the *Shigella sonnei* O-antigen polysaccharide[8].

The structure of the *A. viridans* var. *homari* capsular polysaccharide contains some unusual features. Its tetrasaccharide repeating-unit has three different acidic sugars and two of these, namely, 4-*O*-[(*S*)-1-carboxyethyl]-D-glucose and L-altruronic acid, have not been found hitherto as components of any other naturally occurring material. Most hexuronic and aminodeoxyhexuronic acids are pairs of 5-epimers, *e.g.*, D-gluronic and L-iduronic acid or D-mannuronic and L-guluronic acid, indicating that epimerisation at C-5 takes place during biosynthesis. The finding of L-altruronic acid, which is the 5-epimer of D-galacturonic acid, is therefore not unexpected.

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 of alditol acetates were performed on an HP-54 fused-silica capillary column, using a temperature program from 210° (3 min) to 250° at 3°.min^{-1}. Partially methylated alditol acetates were analysed on the same column, using a temperature program from 190° (3 min) to 250° at 3°.min^{-1}, and on a DB 225 fused-silica capillary column, using a temperature program from 170° (2 min) to 200° at 2°.min^{-1}. G.l.c.–m.s. was performed on a Hewlett–Packard 5970 MSD instrument, using the columns described above. Absolute configurations were determined according to the procedure of Gerwig *et al.*[6].

Carboxyl-reduction of the native polysaccharide, by the method of Taylor *et al.*[9], was performed twice in order to effect complete reduction.

Methylation analysis was performed as described[10], except that the mixture containing the base was stirred for 5 min before the methyl iodide was added. The methylated products were purified by reversed phase chromatography on Sep-Pak C_{18}-cartridges[11].

N.m.r. spectroscopy. — N.m.r. spectra of solutions in deuterium oxide were recorded at 70°, using a JEOL GSX-270 or JEOL GX-400 instrument. Chemical shifts are reported in p.p.m., using sodium 3-trimethylsilylpropanoate-d_4 (δ_H 0.00) and 1,4-dioxane (δ_C 67.40) as internal references. COSY, relayed COSY, NOESY, phase-sensitive COSY, and C,H-COSY experiments were performed according to standard pulse sequences. In these correlation experiments, a 90° mixing pulse was used and, in the NOESY experiment, a mixing time of 250 ms.

Purification of the native polysaccharide. — Following enzymic treatment to remove contaminating nucleic acid and protein, a solution of the crude polysaccharide in 0.02M acetate buffer (pH 6.5) was chromatographed on a column of Sephacel-DEAE. The column was irrigated with the same buffer and then with a gradient (0→1M) of sodium chloride. Further purification was achieved by chromatography on DEAE Trisacryl. The polysaccharide (147 mg) in 0.01M phosphate buffer pH 6.0 (1 mL) was added to the top of a column (1 × 20 cm) of DEAE Trisacryl. Irrigation with the same buffer gave a small amount of neutral material, and elution with M sodium chloride gave the PS which was recovered by dialysis and freeze-drying (114 mg).

Smith degradation. — Sodium metaperiodate (215 mg) was added to a solution of carboxyl-reduced polysaccharide (45 mg) in 0.1M sodium acetate buffer (pH 3.9, 25 mL). The solution was kept for 5 days at 6°, after which ethylene glycol was added and the polymeric product was recovered by dialysis and freeze-drying (37 mg). The polyalcohol (23 mg) was obtained by reduction with sodium borohydride and conventional work-up. It was dissolved in 0.5M trifluoroacetic acid and the solution kept at room temperature (~22°). The hydrolysis of the modified residues was followed by ^{1}H-n.m.r. spectroscopy and had proceeded to ~90% after 18 h. The solution was then diluted with water and lyophilised. The product was fractionated by chromatography

on a column of Bio-Gel P-2, to yield two fractions, one polymeric (17 mg) and one of low molecular weight (5 mg).

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REFERENCES

1 J. E. Stewart, *Helgol. Wiss. Meeresunters.*, 37 (1984) 243–254.
2 L. Kenne, B. Lindberg, B. Lindqvist, J. Lönngren, B. Arie, R. G. Brown, and J. E. Stewart, *Carbohydr. Res.*, 51 (1976) 287–290.
3 B. A. Dmitriev, V. L. Lvov, and N. K. Kochetkov, *Carbohydr. Res.*, 56 (1977) 207–209.
4 P.-E. Jansson, B. Lindberg, J. Lönngren, C. Ortega, and W. Nimmich, *Carbohydr. Res.*, 132 (1984) 297–305.
5 S. J. Angyal and K. Dawes, *Aust. J. Chem.*, 21 (1968) 2747–2760.
6 G. J. Gerwig, J. P. Kamerling, and J. F. G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.
7 K. Bock and C. Pedersen, *Adv. Carbohydr. Chem. Biochem.*, 41 (1983) 27–66.
8 L. Kenne, B. Lindberg, K. Petersson, E. Katzenellenbogen, and E. Romanowska, *Carbohydr. Res.*, 78 (1980) 119–126.
9 R. L. Taylor, J. E. Shively, and H. E. Conrad, *Methods Carbohydr. Chem.*, 7 (1976) 149–151.
10 P.-E. Jansson, L. Kenne, H. Liedgren, B. Lindberg, and J. Lönngren, *Chem. Commun., Univ. Stockholm*, 8 (1976) 1–75.
11 T. J. Waeghe, A. G. Darvill, M. McNeil, and P. Albersheim, *Carbohydr. Res.*, 123 (1983) 281–304.