Carbohydrate Research, 210 (1991) 255–261
Elsevier Science Publishers B.V., Amsterdam

Structural studies of the extracellular polysaccharide elaborated by *Azotobacter vinelandii* strain 1484

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

and Wolfgang Nimmich
Institut für Med. Mikrobiologie und Epidemiologie, Wilhelm-Pieck-Universität Rostock, DDR 25 Rostock (G.D.R.)

(Received June 18th, 1990; accepted for publication, July 20th, 1990)

ABSTRACT

The structure of the extracellular polysaccharide from *Azotobacter vinelandii* strain 1484 has been investigated, specific degradations and n.m.r. spectroscopy being the main methods used. It is concluded that the polysaccharide is composed of tetrasaccharide repeating-units having the following structure,

→4)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-L-Rha*p*-(1→
2
↑
2
β-Sug*p*

in which Sug is 3-deoxy-D-*threo*-hexulosonic acid. The polysaccharide also contains a non-stoichiometric amount of *O*-acetyl groups, distributed over at least two positions.

INTRODUCTION

The extracellular polysaccharide elaborated by *Azotobacter vinelandii* strain 1484 was investigated by Claus[1], who showed that it was composed of L-rhamnose and a 3-deoxyhexulosonic acid, tentatively assigned the *threo* configuration. At that time, 3-deoxy-D-*manno*-octulosonic acid (Kdo) and neuraminic acid were the only 3-deoxy-ulosonic acids known as components of bacterial polysaccharides. We now report further studies of this *A. vinelandii* polysaccharide.

RESULTS AND DISCUSSION

The polysaccharide, which was purified by precipitation with Cetavlon, gave viscous solutions. After complete hydrolysis, L-rhamnose was the only sugar detected as the alditol acetate. On hydrolysis with acid under mild conditions, essentially only the acidic sugar was released, as observed by Claus.

* On leave from: Faculty of Chemistry, Montevideo University, Uruguay.

0008-6215/91/$03.50

The ^{1}H- and ^{13}C-n.m.r. spectra of the polysaccharide indicated heterogeneity and the presence of a non-stoichiometrical amount of *O*-acetyl groups, 0.17 mol per rhamnose residue, distributed over at least two positions. On *O*-deacetylation, a polysaccharide (PS) was obtained, which gave simpler n.m.r. spectra.

Methylation analysis of the PS gave 2,3-di-, 2,4-di-, and 4-*O*-methyl-L-rhamnose in the proportions 1:1:1. Methylation analysis of the polysaccharide (rhamnan) obtained after treatment of the PS with acid under mild conditions gave 2,3- and 2,4-di-*O*-methyl-L-rhamnose in the ratio 1:2. These results indicate that the PS is composed of chains of L-rhamnosyl residues, two-thirds of which are linked through O-3 and one-third through O-4, and that the acidic sugar is linked to O-2 of every second of the former residues.

From the ^{1}H- and ^{13}C-n.m.r. spectra of the PS and the rhamnan (Fig. 1), it was evident that the PS was composed of tetrasaccharide repeating-units, containing three L-rhamnopyranosyl residues and one residue of the acidic sugar. Most of the signals could be assigned using COSY, relayed COSY, and C,H-COSY spectra (Tables I and II). From the $J_{C-1,H-1}$ coupling constants[2], namely, 174, 172, and 163 Hz, it was further evident that two of the L-rhamnopyranosyl residues were α-linked and one residue was β-linked.

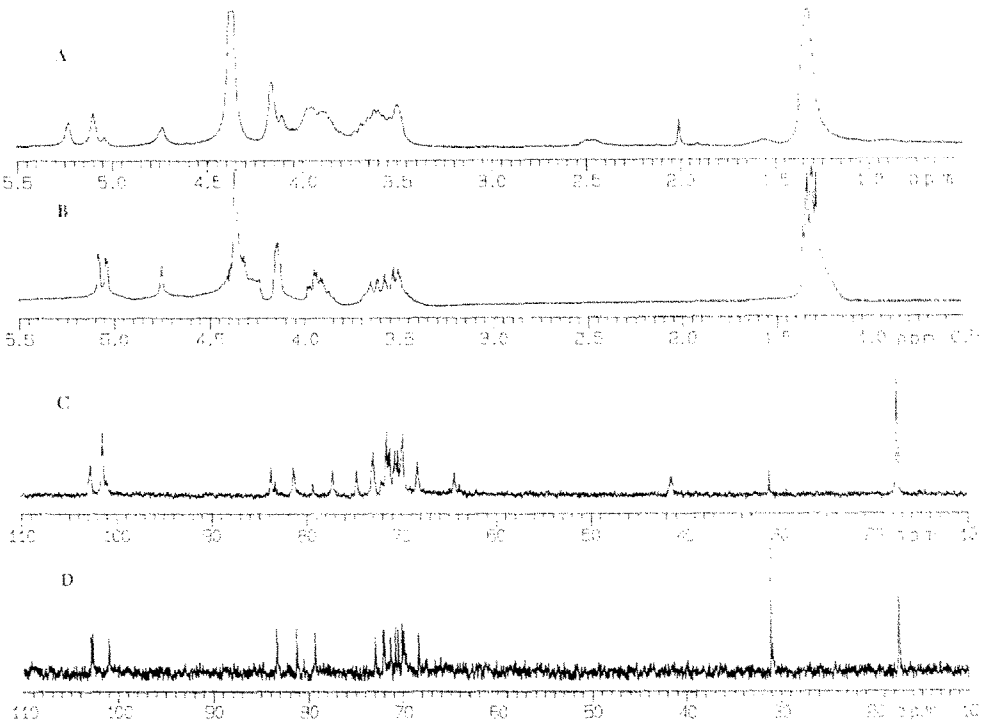


Fig. 1. ^{1}H-N.m.r. and ^{13}C-n.m.r. spectra of the *O*-deacetylated polysaccharide (A and C) and of the partially hydrolysed polysaccharide (B and D). The signal at δ 31.00 in the ^{13}C-n.m.r. spectra is for acetone used as the reference.

TABLE I

^{1}H-N.m.r. chemical shifts (δ)[a] for *O*-deacetylated and partially hydrolysed polysaccharides from *Azotobacter vinelandii*

	→4)-α-L-*Rha*p-(1→						→3)-α-L-*Rha*p-(1→					
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*
O-Deac PS	5.10	4.15	3.96	3.61	3.86	1.32	5.22	4.10	3.98	3.66	4.03	1.32
Part hydrolysed PS	5.08	4.16	3.96	3.62	3.95	1.32	5.04	4.16	3.92	3.58	3.90	1.30

	→3)-β-L-*Rha*p-(1→						β-D-threo-*Hex*p-(2→					
	H-1	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-3*a	*H-3*e	*H-4*	*H-5*	*H-6*a	*H-6*e
O-Deac PS	4.71	4.16	3.68	3.63	3.48	1.35	1.56	2.47	3.91	3.64	3.57	3.86
Part hydrolysed PS	4.75	4.13	3.67	3.51	3.50	1.38						
Methyl ester[b]							1.72 (−13.2, 11.0)	2.34 (4.9)	3.88 (8.9)	3.61 (10.3, 5.5)	3.38 (−10.6)	3.91

[a] Coupling constants (Hz) are given in parentheses. [b] Methyl (methyl 3-deoxy-α-D-*threo*-hexulopyranosid)onate. Signals from the OCH_3 and $COOCH_3$ groups were observed at δ 3.24 and 3.86, respectively.

TABLE II

^{13}C-N.m.r. chemical shifts (δ) for *O*-deacetylated and partially hydrolysed polysaccharides from *Azotobacter vinelandii*

	→4)-α-L-*Rha*p-(1→						→3)-α-L-*Rha*p-(1→					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
O-Deac PS	102.74	70.66	69.80	83.75	68.29	17.48	101.42	74.71	77.27	71.50	69.80	17.48
Part hydrolysed PS	102.71	70.50	69.98	83.32	68.34	17.43	102.88	70.80	79.29	72.07	70.04	17.48

	→3)-β-L-*Rha*p-(1→						β-D-threo-*Hex*p-(2→					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
O-Deac PS	100.99	71.23	81.37	71.93	72.93	17.48	174.48	n.d.	41.44	69.80	74.71	64.40
Part hydrolysed PS	100.99	71.36	81.24	71.96	72.93	17.38						

The structure of the rhamnan was determined from the ^{13}C-n.m.r. chemical shifts, the $J_{C,H}$ values, and the results of methylation analysis, using the CASPER program[3,4]. Of the three alternative structures shown below, **1** showed the best agreement between the observed and the computed spectrum, with a total deviation of 4.8 p.p.m. compared to 9.9 and 13.1 p.p.m. for structures **2** and **3**.

$$\underset{\text{I}}{\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)}\text{-}\underset{\text{II}}{\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)}\text{-}\underset{\text{III}}{\beta\text{-L-Rha}p\text{-}(1\rightarrow} \quad (1)$$

$$\rightarrow 4)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow \quad (2)$$

$$\rightarrow 4)\text{-}\beta\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow 3)\text{-}\alpha\text{-L-Rha}p\text{-}(1\rightarrow \quad (3)$$

The structure of the trisaccharide repeating-unit (**1**) thus obtained was confirmed by running a NOESY spectrum which showed, *inter alia*, inter-residue contacts between H-1 of RhaI and H-3 of RhaII, between H-1 of RhaII and H-3 of RhaIII, and between H-1 of RhaIII and H-4 of RhaI.

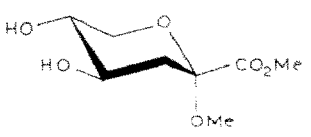
Inspection of the ^{13}C-n.m.r. spectra of the rhamnan and the PS (Table II) shows that significant differences are only observed for the C-2 signals of RhaII and, to a smaller extent, for the C-1 and C-3 signals of this residue. This demonstrates that the acidic sugar is linked to O-2 in RhaII.

In order to identify the acidic sugar, the PS was treated with methanolic hydrogen chloride, and the methyl ester methyl glycosides were isolated by chromatography, first on Bio-Gel P-2 and then on silica gel. The ^{1}H-n.m.r. spectrum showed that the product was a mixture of anomeric forms, but the signals of the major component were assigned, using 2D-techniques (Table I). From the magnitude of the coupling constants of the ring protons, it was evident that this was a glucoside of a 3-deoxy-*threo*-hexulopyranosonic acid.

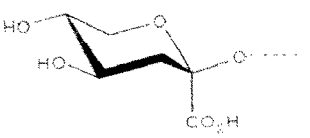
In order to determine the absolute configuration of the acid, the 3-deoxy-D- and -L-*threo*-hexulosonic acids were prepared from D- and L-glyceraldehyde, respectively, and oxalacetate[5]. Comparison of the gas chromatograms of their acetylated glycoside esters of (*S*)-butan-2-ol and that of the natural acid demonstrated that the latter is 3-deoxy-D-*threo*-hexulosonic acid.

It has been observed for neuraminic acid derivatives that the signals in the ^{1}H-n.m.r. spectrum for H-3*e* and H-3*a* differ by 0.4–0.6 p.p.m. when O-2 is axial, but by 0.9–1.3 p.p.m. when it is equatorial[6]. The corresponding differences for the major methyl ester methyl glycopyranoside of 3-deoxy-D-*threo*-hexulosonic acid and the corresponding group in the PS are 0.62 and 0.91 p.p.m., respectively, indicating that the former (**4**) is α-linked and the latter (**5**) β-linked.

From the combined evidence presented above, it is concluded that the extracellular polysaccharide from *Azotobacter vinelandii* strain 1484 is composed of tetrasaccha-

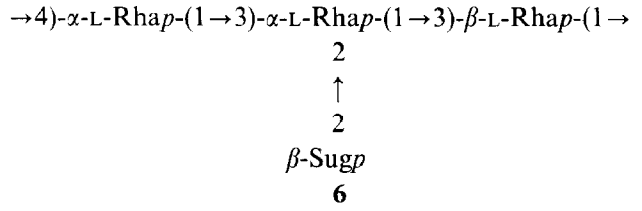


4



5

ride repeating-units having the structure **6**. In this structure, β-Sug*p* is the 3-deoxy-β-D-*threo*-hexulopyranosonic acid group (**3**). The polysaccharide also contains a low percentage of *O*-acetyl groups, which have not been located.



Several 3-deoxyulosonic acids have been detected as components of bacterial polysaccharides. This class of substances could previously have been overlooked because they are decomposed under the conditions of a conventional sugar analysis, but are now readily detected by n.m.r. spectroscopy. Of particular interest in connection with this work is the identification of 3-deoxy-D-*threo*-hexulosonic acid as a component of the LPS from *Vibrio parahaemolyticus* serotypes O7 and O12 (ref. 7).

EXPERIMENTAL

General methods. — Concentrations were performed under reduced pressure at bath temperatures below 40°. G.l.c. was performed on an HP-5890 chromatograph, equipped with fused-silica capillary columns HP-54 and DB-225. G.l.c.–m.s. was performed on an HP-5890 gas chromatograph linked to an HP-5970B mass detector, using an HP-54 fused-silica capillary column.

N.m.r. spectra for solutions in D_2O were recorded at 70° with a Jeol GX 270 spectrometer, using internal sodium 3-(trimethylsilyl)propanoate-d_4 (TSP, δ 0.00, ^1H) and acetone (δ 31.00, ^{13}C) as references. 2D-N.m.r. spectroscopy was performed with standard COSY, relayed COSY, C,H-COSY, and NOESY pulse sequences. In the NOESY experiment, a mixing time of 300 ms was used. Version 2 of CASPER[4] was used for the computerised structural analysis.

Isolation of the capsular polysaccharide. — Bacteria were cultivated on a nitrogen-free solid medium (40 plates). After 4 days at 28°, the bacterial growth was scraped off, suspended in water, and extracted for 15 min at 70° during stirring. The highly viscous suspension was centrifuged at 25 000 r.p.m. to get a clear supernatant. The polysaccharide was precipitated with Cetavlon and redissolved in M sodium chloride (200 mL), and the solution was diluted with water (800 mL). Precipitated RNA was removed by centrifugation and the polysaccharide (5 g) precipitated with ethanol.

O-*Deacetylation of the capsular polysaccharide.* — The polysaccharide (140 mg) was treated with 50% aqueous ammonia (8 mL) for 3 h at room temperature, and the solution was then freeze-dried. Gel filtration of the product on a column (80 × 2.6 cm) of Sephadex G-50 irrigated with water gave the *O*-deacetylated polysaccharide (84 mg).

Partial acid hydrolysis of the O-*deacetylated polysaccharide.* — *O*-Deacetylated

polysaccharide (65 mg) was hydrolysed with 0.1M trifluoroacetic acid for 30 min at 80°, and the solution was then diluted with water and freeze-dried. Gel filtration of the residue on a column of Sephadex G-25 eluted with water gave the rhamnan (42 mg).

Isolation of methyl (methyl 3-deoxyhexulosid)onates. — The *O*-deacetylated polysaccharide (30 mg) was treated with 0.5M hydrogen chloride in methanol for 1 h at 80°. The solvent was evaporated, and the residue applied to a Bio-Gel P-2 column, which was then irrigated with water. The fractions were assayed by t.l.c. ($CHCl_3$–MeOH, 19:1), and by ^1H-n.m.r. spectroscopy. Three fractions showed the presence of methyl glycosides of the hexulosonic acid, together with various amounts of methyl rhamnosides. The first of these fractions was further fractionated by column chromatography on silica gel, using the same solvent, to afford the product (1.5 mg).

Isolation of the 3-deoxyhexulosonic acid. — The *O*-deacetylated polysaccharide (15 mg) was treated with aqueous 2% acetic acid (2 mL) for 1 h at 100°, and then dialysed against water (10 mL). The water outside the bag was freeze-dried, affording the acid (1.1 mg). The acid, and the corresponding synthetic D- and L-acids, were transformed into the (*S*)-but-2-yl ester glycoside mixtures and analysed by g.l.c. as devised by Kondo *et. al.*[7].

*Synthesis of methyl (methyl 3-deoxy-*L- *and* -D-threo-*hexulopyranosid)onate.* — Oxalacetic acid (2.64 g) was dissolved in water (50 mL) at 4°, and the pH brought to 11 with 10M NaOH. L(or D)-Glyceraldehyde (260 mg) dissolved in water (1 mL) was added to this solution, and the pH adjusted to 11 as above. The mixture was kept at room temperature with good stirring for 2.5 h. The pH was then brought to 7 with Dowex 50 (H^+), and the resin filtered-off. The decarboxylation was performed for 10 min at 80°, the pH adjusted to 7 as before, and the solution kept for another 10 min at 80°.

The solution was freeze-dried, giving 3.1 g of solid material. Part of this residue (2.5 g) was dissolved in methanolic 1.1M HCl (45 mL) and sealed in a vial with a magnetic stirring bar. The methanolysis was performed at 85° for 2 h. The solvent was then evaporated, and the residue (1.1 g) was distributed between water and toluene (1:1, 6 mL). The aqueous layer was extracted with toluene (3 mL) and then concentrated to dryness. The residue was fractionated on a column of silica gel irrigated with $CHCl_3$–MeOH (19:1), to give the pure title compound (186 mg, 39%).

ACKNOWLEDGMENTS

This work was supported by grants from the Swedish Medical Research Council (03X-02522) and the Swedish National Board for Technical Development. A grant (to F.F.) from the International Science Programs, Uppsala University, is gratefully acknowledged. We are obliged to Dr. Göran Widmalm for the CASPER calculations. The *A. vinelandii* strain 1484 was kindly supplied by Dr D. Claus, University of Göttingen, F.R.G.

REFERENCES

1 D. Claus, *Biochem. Biophys. Res. Commun.*, 20 (1965) 745–751.
2 K. Bock and C. Pedersen, *J. Chem. Soc., Perkin Trans. 2*, (1974) 293–297.
3 P.-E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.*, 168 (1987) 67–77.
4 P.-E. Jansson, L. Kenne, and G. Widmalm, *Carbohydr. Res.*, 188 (1989) 169–191.
5 D. Portsmouth, *Carbohydr. Res.*, 8 (1968) 193–204.
6 J. Haverkamp, H. van Halbeck, L. Dorland, J. F. G. Vliegenthart, R. Pfeil, and R. Schauer, *Eur. J. Biochem.*, 122 (1982) 305–311.
7 S. Kondo, U. Zähringer, E. T. Rietschel, and K. Hisatsune, *Carbohydr. Res.*, 188 (1989) 97–104.
8 G. J. Gerwig, J. P. Kamerling, and J. F. G. Vliegenthart, *Carbohydr. Res.*, 62 (1978) 349–357.