Carbohydrate Research, 117 (1983) 125–131
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

STRUCTURAL STUDIES OF THE ANTIGENIC POLYSACCHARIDE OF *Eubacterium saburreum*, STRAIN T27

WATARU KONDO, FUTOSHI NAKAZAWA, MICHIKO SATO,
Department of Oral Microbiology, School of Dentistry, Niigata University, Gakko-cho-dori, Niigata (Japan)

AND TEIICHIRO ITO
Research Laboratories of Meiji Seika Kaisha Ltd., Morooka, Kohoku-ku, Yokohama (Japan)

(Received July 13th, 1982; accepted for publication, November 22nd, 1982)

ABSTRACT

The antigenic polysaccharide produced by *Eubacterium saburreum*, strain T27, is a homoglycan composed of D-*glycero*-D-*galacto*-heptose (Hep) residues having a nonasaccharide repeating-unit with the structure $\rightarrow$6)-[α-Hep*f*-(1$\rightarrow$4)]-β-Hep*p*-(1$\rightarrow$$_3$6)-[$\alpha$-Hep*f*-(1$\rightarrow$2), α-Hep*f*-(1$\rightarrow$4)]-β-Hep*p*-(1$\rightarrow$. The polysaccharide contains acetyl groups linked to O-2 (except to the 2,4,6-linked heptopyranosyl residue), O-3 and O-7 of part of both heptopyranosyl and heptofuranosyl residues. The assignment of an acetyl group at O-3 of part of the terminal heptofuranosyl and 4,6-linked heptopyranosyl groups is tentative.

INTRODUCTION

The structure of a cell-wall antigen produced by *Eubacterium saburreum*, strain L44 (ref. 1) was reported to be that of a linear polysaccharide composed of (1$\rightarrow$6)-linked D-*glycero*-β-D-*galacto*-heptopyranosyl residues, part of which are acetylated at O-7. The polysaccharide has been known as the only example of a bacterial homoglycan composed of heptose residues. Recently, a new strain, T27, of the same organism was isolated in our laboratory. The antigenic polysaccharide from strain T27 contains the same components as that of strain L44, but differs from the latter strain in its serological specificity. We report herein the structure of the new antigenic polysaccharide.

RESULTS AND DISCUSSION

Acid hydrolysis of the antigenic polysaccharide gave two compounds, in the ratio of 47:3. The first, isolated by p.c., was identified as D-*glycero*-D-*galacto*-heptose. The mass spectrum of its per-*O*-trimethylsilylalditol derivative showed that it was derived from a heptose. The ^{1}H-n.m.r. spectrum of the sugar was indistinguishable from that of an authentic sample[2], and its optical rotation, $[\alpha]_D^{18}$ +56° (*c*

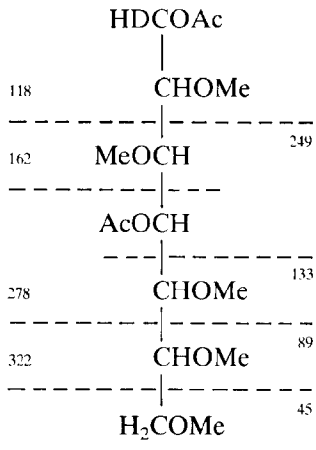
0008-6215/83/$ 03.00

1.3, water), was in good agreement with the published value[2]. The second compound, isolated by column chromatography on Dowex 1 (BO_4^{3-}), was identified as 1,6-anhydro-D-*glycero*-α-D-*galacto*-heptofuranose. It showed no reducing power and the optical rotation of the per-*O*-acetyl derivative, $[\alpha]_D^{28}$ +148° (*c* 0.6, chloroform), was in good agreement with the value reported for 2,3,5,7-tetra-*O*-acetyl-1,6-anhydro-D-*glycero*-α-D-*galacto*-heptofuranose[3]. The ^{1}H-n.m.r. spectrum of the per-*O*-acetyl derivative compound was identical with that of an authentic sample[3]. The mass spectrum of its per-*O*-trimethylsilyl derivative also gave results compatible with a 1,6-anhydroheptofuranose structure, showing a peak at *m/z* 465 (M^+ − 15) and intense peaks at *m/z* 319 and 217 (base peak). The intense peak at *m/z* 319 is characteristic for a hexofuranoside[4], and the peak at *m/z* 217 is either the base peak or the second, most intense peak in the mass spectra of furanosides. On the other hand, a peak at *m/z* 204, which was shown[4] to be much more prominent in the mass spectra of pyranosides than in those of furanosides, was obtained in low intensity. The result indicates that the sugar has a furanose ring structure. These data indicate that D-*glycero*-D-*galacto*-heptose is the sole sugar component of the antigenic polysaccharide, and suggest that 1,6-anhydro-D-*glycero*-α-D-*galacto*-heptofuranose was formed from the heptose residues during acid hydrolysis of the antigenic polysaccharide, under conditions similar to those reported by Angyal and Tran[3].

The ^{1}H-n.m.r. spectrum of the polysaccharide showed signals attributable to two anomeric protons at δ 5.20 ($J_{1,2}$ < 1 Hz) and 4.67 ($J_{1,2}$ 7 Hz), and for *O*-acetyl groups at δ 2.16 (s). This indicates that the heptose residues in the polysaccharide are present in two different anomeric or ring forms.

In the methylation analysis of the polysaccharide, the partially methylated sugars, identified as alditol acetates, were 2,3,5,6,7-penta-*O*-methylheptose (**1**), 2,3,7-tri-*O*-methylheptose (**2**), and 3,7-di-*O*-methylheptose (**3**) in the proportion of 5:3:1 (see Scheme 1). These results indicate that the antigenic polysaccharide contains a nonasaccharide repeating unit, and that, of the nine sugar residues, five occur as furanosyl end-groups, three as pyranosyl branching-residues *O*-glycosylated at O-4 and -6, and the remaining residue as a pyranosyl-branching residue *O*-glycosylated at O-2, -4, and -6.

The possibility that the branching residues are in furanosyl or septanosyl form was eliminated as follows. The polysaccharide was partially hydrolyzed (0.5M sulfuric acid, 100°, 2.5 h), fractionated by gel filtration, reduced with sodium borodeuteride, methylated, and hydrolyzed. Subsequently, the sugars in the hydrolyzate were reduced with sodium borohydride and acetylated, and the product was analyzed by g.l.c.–m.s. Three components were obtained that were identified as 6-*O*-acetyl-1,2,3,4,5,7-hexa-*O*-methyl-(1-^{2}H)heptitol (**4**), 1,5-di-*O*-acetyl-2,3,4,6,7-penta-*O*-methylheptitol (**5**), and 1,5,6-tri-*O*-acetyl-2,3,4,7-tetra-*O*-methylheptitol (**6**) (see Scheme 1). Compound **5** was obviously derived from heptopyranosyl residues located at the nonreducing ends of oligosaccharides, **6** from internal-chain heptopyranosyl residues, and **4** from reducing heptopyranose re-



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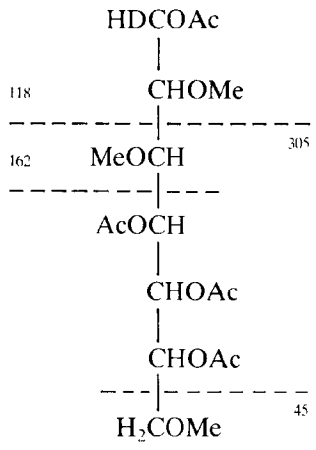

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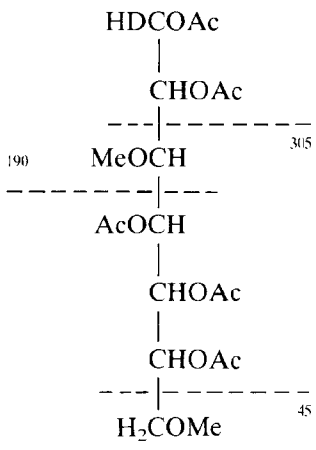

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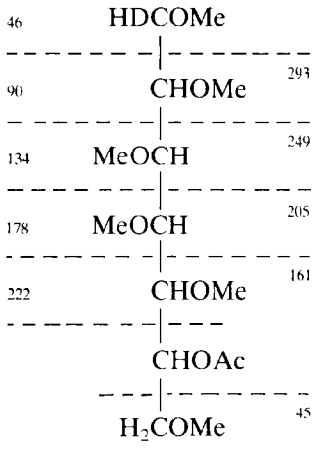

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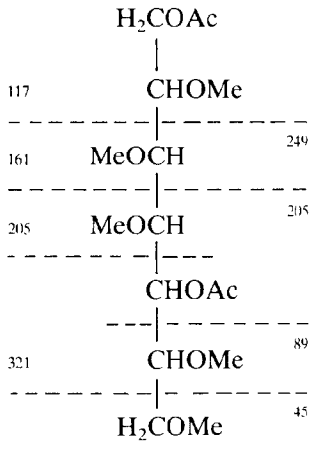

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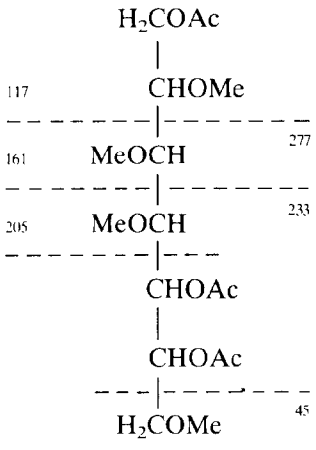

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Scheme 1 Fragmentation patterns of compounds **1–6**

sidues. Therefore, none of the heptosyl residues of the main polysaccharide chain could have been present in the furanose or septanose form.

Acid hydrolysis of the polysaccharide under mild conditions (50mM sulfuric acid, 80°, 3.5 h), followed by methylation analysis, gave, as alditol acetates, 2,3,5,6,7-penta-*O*-methylheptose, 2,3,4,7-tetra-*O*-methylheptose, and 2,3,7-tri-*O*-

methylheptose, in the proportion of 5:8:5. Assuming that none of the heptopyranosyl linkages of the original polysaccharide were cleaved during the mild acid hydrolysis, and considering that no 3,7-di-*O*-methylheptitol was observed, the sum of the last two alditols must account for four heptopyranosyl residues, and the 5:8:5 ratio of the three methylated compounds can, therefore, be expressed as 1.54:2.46:1.54 (Table I). As shown in Table I, the increase, after hydrolysis, of 2,3,4,7-tetra-*O*-methylheptose (+ 2.46) is proportional to the total decrease in 2,3,7-tri-*O*-methylheptose (− 1.46) and 3,7-di-*O*-methylheptose (− 1.00), and the decrease in 2,3,5,6,7-penta-*O*-methylheptose (− 3.46) is also proportional to the total decrease in 2,3,7-tri-*O*-methylheptose (− 1.46) and 3,7-di-*O*-methylheptose (2 × − 1.00) (Table I).

These results demonstrate that the terminal D-*glycero*-D-*galacto*-heptofuranosyl groups are attached to O-4 of the 4,6-*O*-diglycosylated D-*glycero*-D-*galacto*-heptopyranosyl residues, and to O-2 and O-4 of 2,4,6-*O*-triglycosylated D-*glycero*-D-*galacto*-pyranosyl residues. The absence of 3,7-di-*O*-methylheptose following mild-acid-catalyzed hydrolysis indicates that the *O*-D-*glycero*-D-*galacto*-heptofuranosyl-(1→2)-D-*glycero*-D-*galacto*-heptopyranosyl linkage is more readily hydrolyzable than the corresponding (1→4) linkage.

The *O*-acetyl groups were located by the method of de Belder and Norrman[5], *i.e.*, by protection of the free hydroxyl groups by treatment with methyl vinyl ether and a trace of *p*-toluenesulfonic acid, followed by methylation analysis. Four components were obtained that were identified as the alditol acetates of the heptose, 7-*O*-methylheptose, 2-*O*-methylheptose, and 3-*O*-methylheptose. The ratio of the four derivatives was found to be ~57:26:10:7. This result indicates that 26% of the heptose residues are acetylated at O-7, 10% at O-2, and 7% at O-3.

In order to establish the positions of the *O*-acetyl groups on the heptopyranosyl and -furanosyl residues, the polysaccharide was treated with methyl vinyl ether, methylated with ($^2H_3$)methyl iodide, hydrolyzed under mild conditions to remove the acetal groups, and remethylated with methyl iodide[6]. In this procedure, the substitution by *O*-acetyl groups in the original polysaccharide is reflected

TABLE I

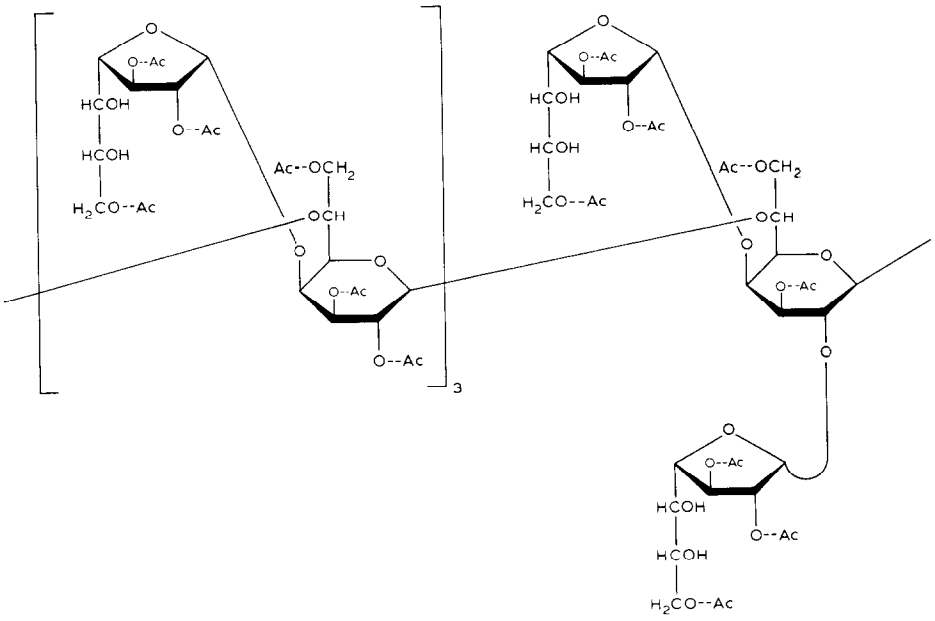
ALDITOL ACETATES OBTAINED BY METHYLATION ANALYSIS OF THE ORIGINAL AND MODIFIED ANTIGENIC POLYSACCHARIDES FROM *Eubacterium saburreum* STRAIN T27

O-Methyl-alditol acetate of D-glycero-D-galacto-*heptose*	*Polysaccharide*		*Decrease or increase after mild-acid hydrolysis*[a]
	Original[a]	*Mild-acid hydrolyzed*[a]	
2,3,5,6,7-Penta-	5.00	1.54	−3.46
2,3,4,7-Tetra-		2.46	+2.46
2,3,7-Tri-	3.00	1.54	−1.46
3,7-Di-	1.00		−1.00

[a]Molar ratios relative to original polysaccharide

in the substitution pattern of the ($^2H_3$)methyl groups. The hydrolyzate of the final product was reduced with sodium borodeuteride, acetylated, and analyzed by g.l.c.–m.s. Three components, 2,3,5,6,7-penta-*O*-methylheptose (**1**), 2,3,7-tri-*O*-methylheptose (**2**), and 3,7-di-*O*-methylheptose (**3**), were again obtained. M.s. (see Scheme 1) demonstrated that all the heptose derivatives contained (in part) ($^2H_3$)methyl groups at O-7 (CH_2OCD_3-7, *m/z* 48); except for **3**, at O-2 (CHDOAc-1–$CHOCD_3$-2, *m/z* 121); and at O-3 in **1** and **2** (CHDOAc-1–CHOMe-2–$CHOCD_3$-3, *m/z* 165) and in **3** (CHDOAc-1-CHOAc-2–$CHOCD_3$-3, *m/z* 193). The ion at *m/z* 165 in **1** and **2** is not unequivocal evidence for a ($^2H_3$)methyl group at O-3, because the same ion at *m/z* 165 is also given by the fragment that is not ($^2H_3$) methylated but at O-3 but at O-2 (CHDOAc-1–$CHOCD_3$-2–CHOMe-3). Accordingly, the identification of the position of the *O*-acetyl groups in **1** and **2** is tentative. These results demonstrate that *O*-acetyl groups are distributed between both heptopyranosyl and heptofuranosyl residues.

The optical rotation of the original polysaccharide, $[\alpha]_D^{23}$ +13.8° (*c* 1.0, water), decreased on mild acid hydrolysis, and a product, from which ~70% of the heptofuranosyl groups had been removed (Table I), showed $[\alpha]_D^{17}$ −6.7° (*c* 0.8, water). These results suggest a β form for the D-*glycero*-D-*galacto*-pyranosyl residues, and an α form for the D-*glycero*-D-*galacto*-heptofuranosyl groups. The ^{1}H-n.m.r. spectrum of the degraded polysaccharide obtained by mild-acid hydrolysis showed two signals in the anomeric region, one of which, at δ 4.67 ($J_{1,2}$ 7 Hz), was stronger than the other at δ 5.20 ($J_{1,2}$ low). These signals could be assigned to H-1 of the D-*glycero*-β-D-*galacto*-heptopyranosyl residues and to H-1 of the D-*glycero*-α-D-*galacto*-heptofuranosyl groups, respectively.



On the basis of the aforementioned evidence, structure **7** is proposed for the repeating unit of the polysaccharide of *E. saburreum*, strain T27. The assignment of an *O*-acetyl group at O-3 of part of the terminal heptofuranosyl groups and 4,6-linked heptopyranosyl residues is tentative.

EXPERIMENTAL

Culture conditions. — The same conditions were used as in the investigation[2] of the antigen from *E. saburreum*, strain O-2.

Antigen extraction and purification methods. — The antigenic polysaccharide was isolated as previously described[2]. The elution volume of the polysaccharide on a column (1.4 × 106 cm) of Sephadex G-100 was found to be 77 mL (Ve/Vo 1.38). The lyophilized cells (1 g) yielded 11.4 mg of antigenic polysaccharide.

Analytical methods. — These were the same as previously described[2]. G.l.c.–m.s. was performed with a Hitachi M-60 instrument. N.m.r. spectra were recorded with a JEOL FX-200 spectrometer, tetramethylsilane being the internal standard.

Identification of components. — The polysaccharide was hydrolyzed with M hydrochloric acid for 5 h at 100°. After cooling of the mixture, the excess of hydrochloric acid was removed by passage through a column of Dowex 1 (AcO^-) anion-exchange resin, and the effluent and water washings were evaporated to dryness. The hydrolyzate was reduced with sodium borodeuteride, per-*O*-(trimethylsilyl)ated, and analyzed by g.l.c.–m.s. G.l.c. (OV-17 column) of the product showed two components having retention times relative to per-*O*-(trimethylsilyl)inositol of 0.92 and 1.12, in the proportions 3:47. The first-eluted component was identified as 1,6-anhydro-D-*glycero*-α-D-*galacto*-heptofuranose, m.s.: *m/z* 465 (1, $M^+ - 15$), 375(1), 345(3), 344(4), 319(15), 245(3), 217(100), 191(8), 157(6), 147(21), 129(10), 117(5), 103(11), and 73(92). The sugar was isolated by applying the hydrolyzate to a column (0.75 × 46 cm) of Dowex 1 (BO_4^{3-}) and by eluting with 18mM potassium tetraborate. The elution volume of the sugar was found to be 40 mL. The sugar was per-*O*-acetylated to give a product having $[\alpha]_D^{28}$ +148° (*c* 0.6, chloroform); n.m.r. ($CDCl_3$): δ 5.58 (d, $J_{1,2}$ 4.5 Hz, H-1), 5.25 (d, $J_{2,3}$ 2.4, $J_{3,4}$ 0 Hz, H-3), 5.15 (m, H-2), 4.88 (dd, $J_{5,6}$ 10.0 Hz, H-5), 4.47 (d, $J_{4,5}$ 4.5 Hz, H-4), 4.35–4.0 (m, 3 H, H-6, 7, 7'), 2.15 (2 Ac), and 2.11 (2 Ac). The second-eluted component was identified as D-*glycero*-D-*galacto*-heptose[2].

Methylation analyses. — The polysaccharide was treated by the procedure previously described[2]. The retention times of the derived alditol acetates (relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol) on g.l.c. (OV-225 column, at 210°) were as follows: **1**, corresponding to 2,3,5,6,7-penta-*O*-methylheptose, *T* 1.76; **2**, corresponding to 2,3,7-tri-*O*-methylheptose, *T* 4.5; **3**, corresponding to 3,7-di-*O*-methylheptose, *T* 7.78; **4**, corresponding to 1,2,3,4,5,7-hexa-*O*-methylheptose, *T* 0.90; **5**, corresponding to 2,3,4,6,7-penta-*O*-methylheptose, *T* 2.01; and **6**, corresponding to 2,3,4,7-tetra-*O*-methylheptose, *T* 3.44.

Partial hydrolysis with acid. — The polysaccharide (8 mg) was hydrolyzed

with 0.5M sulfuric acid (2 mL) for 2.5 h at 100°, and the hydrolyzate was placed on a column (1 × 139 cm) of Sephadex G-25 which was eluted with water. The fractions other than those corresponding to monosaccharide and void-volume regions were pooled and reduced with sodium borodeuteride, and the product was methylated. The methylated product, which was isolated by partition between chloroform and water, was hydrolyzed, reduced with sodium borohydride, acetylated, and analyzed by g.l.c.–m.s. The proportions of **4** corresponding to 1,2,3,4,5,7-hexa-*O*-methylheptose, **5** corresponding to 2,3,4,6,7-penta-*O*-methylheptose, and **6** corresponding to 2,3,4,7-tetra-*O*-methylheptose were 27:40:33.

Another portion of the polysaccharide (8 mg) was hydrolyzed with 50mM sulfuric acid (2 mL) for 3.5 h at 80°. The degraded polysaccharide was recovered by dialysis, lyophilized, and subjected to methylation analysis.

Location of O-*acetyl groups.* — The polysaccharide (15 mg) and *p*-toluenesulfonic acid (5 mg) were dissolved in dimethyl sulfoxide (1.5 mL) in a stoppered flask. Methyl vinyl ether (2 mL) was added and the solution was kept for 4 h at 14–15°. The clear, yellow mixture was placed on a column (1.5 × 41 cm) of Sephadex LH 20, which was then eluted with anhydrous acetone. The separation was monitored by optical rotation, and the acetal derivative was eluted in the void volume, free of reagent. The acetalated polysaccharide was methylated, hydrolyzed, reduced with sodium borodeuteride, acetylated, and analyzed by g.l.c.–m.s. G.l.c. (OV-225 column, at 210°) showed the presence of four components having the retention times relative to D-glucitol hexaacetate of 1.35, 1.65, 2.11, and 2.41. They were identified as 1,2,3,4,5,6-hexa-*O*-acetyl-7-*O*-methyl-(1-^{2}H)heptitol (26%), 1,3,4,5,6,7-hexa-*O*-acetyl-2-*O*-methyl-(1-^{2}H)heptitol (10%), 1,2,4,5,6,7-hexa-*O*-acetyl-3-*O*-methyl-(1-^{2}H)heptitol (7%), and 1,2,3,4,5,6,7-hepta-*O*-acetyl-(1-^{2}H)heptitol (57%), respectively.

Another portion of the polysaccharide was treated with methyl vinyl ether and methylated with ($^2H_3$)methyl iodide. The product was hydrolyzed with 90% formic acid for 30 min at 40°, recovered by dialysis, and lyophilized. Remethylation with unlabelled methyl iodide was followed by hydrolysis, borodeuteride reduction, acetylation, and analysis of the product by g.l.c.–m.s.

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