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CHEMICAL STRUCTURE OF THE POLYSACCHARIDE ANTIGEN OF *Eubacterium saburreum*, STRAIN O2

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ABSTRACT

The polysaccharide antigen produced by *Eubacterium saburreum*, strain O2, is composed of (1→6)-linked β-D-*glycero*-D-*galacto*-heptopyranosyl residues, all of which are substituted with 6-deoxy-α-D-*altro*-heptofuranosyl groups at O-3.

INTRODUCTION

The structure of a cell-wall antigen produced by oral *Eubacterium saburreum* strain L44 was reported[1] to be a linear polysaccharide composed of (1→6)-linked β-D-*glycero*-D-*galacto*-heptosyl residues, part of which are acetylated at O-7. The antigen produced by strain L49 has also been investigated[2]. The polysaccharide antigen is composed of D-*glycero*-D-*galacto*-heptose and a new sugar, tentatively identified as 6-deoxy-D-*altro*-heptose. It contains chains of alternating (1→3)- and (1→6)-linked β-D-*glycero*-D-*galacto*-heptopyranosyl residues, the latter being substituted with 6-deoxy-α-D-*altro*-heptofuranosyl groups at O-3. The polysaccharide further contained *O*-acetyl groups, linked to O-7 of part of the heptosyl residues and to O-2 of part of the 6-deoxyheptosyl groups.

The serological activities of the polysaccharide antigens isolated from both L44 and L49 strains were reported to be destroyed by dilute alkali treatment[3,4], owing to the presence of *O*-acetyl groups in their antigenic structures. Recently, a new strain of *E. saburreum*, strain O2, was isolated in this laboratory. The polysaccharide antigen isolated from the new strain shows a different serological specificity from the aforementioned two strains (L44 and L49), and is alkali-stable. We now report studies on the structure of this antigen.

RESUTLS AND DISCUSSION

The antigen, which showed $[\alpha]_D^{22}$ +58° (*c* 1.8, water), on acid hydrolysis yielded two monosaccharides in the approximate ratio of 1 : 1, which were converted

into alditols by reduction with sodium borodeuteride, and trimethylsilylated. M.s. of these compounds showed that one monosaccharide derived from a heptose and the other from a 6-deoxyheptose. The monosaccharides were separated by p.c. on Whatman No. 1 paper with 6:4:3 (v/v) 1-butanol–pyridine–water as irrigant. The slower component, a heptose, was identified as D-*glycero*-D-*galacto*-heptose by optical rotation, $[\alpha]_D^{22}$ +58° (*c* 2.7, water), and by the n.m.r. spectrum, which was indistinguishable from that of an authentic sample[5] synthesized in this laboratory.

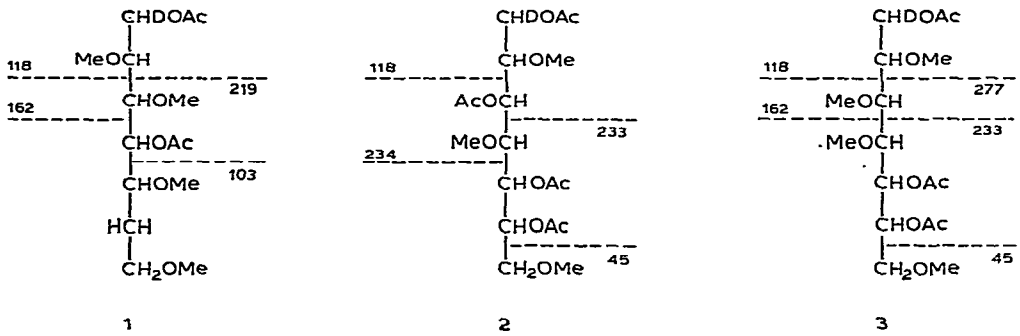
The faster component, a 6-deoxyheptose, was a syrupy substance showing $[\alpha]_D^{24}$ +39° (*c* 2.0, water). In p.c. and g.l.c., it was indistinguishable from a monosaccharide isolated from strain L49 (kindly supplied by Professor T. Hofstad, University of Bergen, Norway) and tentatively identified as 6-deoxy-D-*altro*-heptose. In the n.m.r. spectrum of the sugar, the signal of the methylene group was observed at τ 8.10 as a multiplet. At the lowest field were four doublets, two of which overlapped partially. These signals were assigned to the anomeric protons of two pyranose and two furanose forms[6]. Although the anomeric protons of furanoses are generally reported to resonate at fields lower than those of the anomeric protons of pyranoses, a few exceptions, such as α-D-talofuranose and α-D-talopyranose and the corresponding galactose derivatives, have been reported[6]. Therefore, it was difficult to assign each set of signals to the tautomeric forms. The observation that no doublet with a splitting of more than 7 Hz in the anomeric region of the spectrum was present suggests a *manno*, *altro*, *talo*, or *ido* configuration. D-Mannose has been reported to show only the signals of the two pyranose forms[6,7]. Therefore, it seemed unlikely that the sugar had the *manno* configuration. With respect to chemical shift and spacing of peak, the anomeric signals were quite similar to those of D-altrose[6], but were different from those of D-talose and D-idose[6]. Assuming that the anomeric signals of the sugar were corresponding to those of the tautomeric forms of D-altrose, the chemical shifts (τ) and the first order coupling constants (Hz) at 100 MHz are reported in Table I. The proportion of the forms of the sugar at equilibrium in deuterium oxide solution could also be estimated as α-pyranose, 28; β-pyranose, 52; α-furanose, 10; and β-furanose 10%. In the region below 210 nm, the optical rotatory dispersion increased sharply with decrease of the wavelength. High positive rotations in the region below 210 nm were observed for aldohexopyranoses having an equato-

TABLE I

CHEMICAL SHIFTS[a] AND FIRST-ORDER COUPLING CONSTANTS[b] (Hz) AT 100 MHz OF THE ANOMERIC PROTONS OF THE 6-DEOXYHEPTOSE COMPOUND, AND OF D-ALTROSE

Compound	*Form*			
	α-Pyranose	*β-Pyranose*	*α-Furanose*	*β-Furanose*
6-Deoxyheptose	5.10 (5.0)	4.88 (0.0)	4.73 (2.8)	4.69 (5.0)
D-Altrose[c]	5.03 (3.0)	4.91 (1.3)	4.77 (2.0)	4.73 (4.5)

[a](τ). [b]Values (Hz) of $J_{1,2}$ in parentheses. [c]Data from ref. 6.



Scheme 1. Mass spectral fragmentation of **1**, **2**, and **3**.

rial OH-4, such as D-altrose[8], whereas aldohexopyranoses having an axial OH-4, such as D-talose and D-idose, show[8] a sharp change toward the negative below 210 nm. These results confirm that the monosaccharide has the D-*altro* configuration.

The polysaccharide was methylated by the Hakomori procedure[9] and hydrolyzed, and the methylated derivatives were reduced with borodeuteride, acetylated, and analyzed by g.l.c.–m.s. Two main components (**1** and **2**) were obtained, which were derived, according to their mass spectra, from 6-deoxy-2,3,5,7-tetra-*O*-methylheptose and 2,4,7-tri-*O*-methylheptose, respectively. The primary fragments formed are shown in Scheme 1. The molar ratio of both derivatives was found to be about 1:1. These results demonstrate that the 6-deoxyheptose residues are terminal and in furanose form, and the galactoheptose residues are branch-points linked to O-3 and O-6.

Hydrolysis of the polysaccharide under mild conditions, followed by dialysis, gave a polymeric product that was mainly composed of galactoheptose. Methylation analysis of the polymer yielded, after reduction with borodeuteride and acetylation, only 1,5,6-tri-*O*-[1-^{2}H]acetyl-2,3,4,7-tetra-*O*-methylheptitol (**3**), demonstrating that the 6-deoxyheptofuranosyl groups are linked to O-3 of the branching heptopyranosyl residues.

Smith degradation of the original polysaccharide yielded a heptose polymer and a 2-deoxypentitol, the latter obviously deriving from the terminal 6-deoxyheptofuranosyl groups. On Smith degradation of the polymer obtained by acid hydrolysis under mild conditions, all heptose residues were degraded and erythritol was formed, in agreement with the postulated structure.

The optical rotation of the original polysaccharide, $[\alpha]_D^{22}$ +58°, decreased on mild acid hydrolysis, and a product, from which most of the 6-deoxyheptofuranosyl groups had been removed, showed $[\alpha]_D^{22}$ −27.5° (*c* 1.4, water). These results indicate that the D-*glycero*-D-*galacto*-heptopyranosyl residues are β-linked, and the 6-deoxy-D-*altro*-heptofuranosyl groups α-linked.

From the aforementioned evidence, it is concluded that *E. saburreum* strain O2 antigen is composed of (1→6)-linked β-D-*galacto*-heptopyranosyl residues, all of which are substituted at O-3 with 6-deoxy-α-D-*altro*-heptofuranosyl groups (**4**).

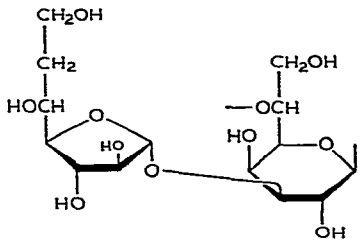


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Fig. 1 shows the serological reactions in gel between anti-O2 serum and untreated, and alkali-treated O2 antigen, and similarly treated L49 antigen. The alkali-treated O2 antigen exhibited complete identity with untreated O2 antigen against homologous anti-O2 serum. On the other hand, untreated L49 antigen reacted weakly with anti-O2 serum and produced a vague agar precipitation-band. However, when the antigen was treated with alkali, the reactivity of the antigen against anti-O2 serum markedly increased and produced a sharp precipitation line, which was spurred over by the line given by the O2 antigen. Thus, it was demonstrated that the deacetylated L49 antigen shows a reaction of partial identity with alkali-stable O2 antigen. This is to be expected, as both O2 and deacetylated L49 antigens have a common antigenic structure[2], namely, both contain (1→6)-linked *β*-D-*glycero*-D-*galacto*-heptopyranosyl residues that are substituted with 6-deoxy-*α*-D-*altro*-heptofuranosyl groups at O-3.

In addition, it was demonstrated that the precipitation reaction between O2 antigen and homologous anti-O2 serum was inhibited by a comparatively high concentration (0.5 mg/ml) of 6-deoxy-D-*altro*-heptose, but not with D-*glycero*-D-*galacto*-heptose, even at a concentration of 5 mg/ml, whereas the reaction between L49 antigen and homologous anti-L49 serum was inhibited neither with 6-deoxy-D-*altro*-heptose nor with D-*glycero*-D-*galacto*-heptose. These results suggest that the antigenic determinant group of L49 antigen may be a 2-*O*-acetyl-6-deoxy-D-*altro*-

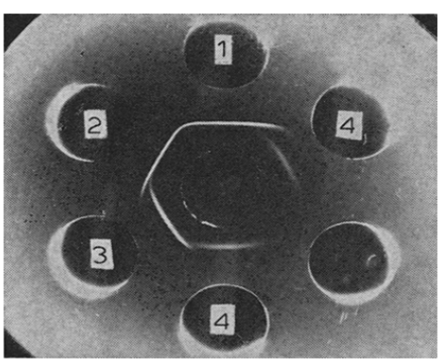

Fig. 1. Precipitation bands produced in agar by untreated and alkali-treated (0.01M NaOH, 30 min, 50°) O2 and L49 antigens (peripheral wells, 0.5 mg/ml) against anti-O2 serum (central well). Peripheral well: 1, O2 antigen; 2, alkali-treated O2 antigen; 3, alkali-treated L49 antigen; and 4, L49 antigen.

heptose, the acetyl group of which being readily eliminated by dilute alkali, which results in the reduction of reactivity with the homologous antiserum. This would explain the sensitivity of L49 antigen to alkali[4].

The comparatively high concentration of 6-deoxy-D-*altro*-heptose needed for the inhibition of precipitation reaction between O2 antigen and anti-O2 serum probably reflects the low proportion (10%) of α-furanose form of 6-deoxy-D-*altro*-heptose at equilibrium in deuterium oxide solution.

EXPERIMENTAL

Culture conditions. — The filamentous organism *E. saburreum* strain O2 and the other strains examined were grown anaerobically in the following medium: Proteose peptone No. 3 (Difco) (10 g), yeast extract (Difco) (5 g), sodium chloride (5 g), dipotassium hydrogenphosphate (2.5 g), D-glucose (10 g), and distilled water (1 litre) pH 7.0. Following incubation for 24 h at 37°, the cells were centrifuged, washed twice with saline solution, and kept as acetone-dried cells.

Extraction and purification methods. — The antigen was extracted essentially as described by Krause and McCarthy[10]. Formamide (20 ml) was added to acetone-dried cells (1 g), and the extraction was carried out with continuous stirring for 15 min at 150° (oil bath). After extraction, removal of the insoluble material by centrifugation was facilitated by the addition of 2 vol. of acid alcohol (95 vol. of ethanol and 5 vol. of M hydrochloric acid). The polysaccharide was collected from the alcoholic supernatant by the addition of acetone (5 vol.). The acetone-precipitable polysaccharide was dissolved in 0.02M Tris-hydrochloric acid buffer containing 15mM calcium chloride, pH 7.8 (10 ml). It was digested with Pronase for 24 h at 37°, with an enzyme concentration of 1 mg/ml. Gel filtration on a column (2.4 × 100 cm) of Sephadex G-100 was conducted with 0.02M phosphate buffer, pH 7.4, containing 0.02% of sodium azide. Serologically-active fractions (void-volume fraction) were pooled. The pooled fractions were then applied to a Sepharose 6B column (2.4 × 100 cm), and eluted with the same buffer. The elution volume of the serologically active polysaccharide was found to be 350 ml.

General analytical methods. — Optical rotations were determined with a Perkin–Elmer 241 photoelectric polarimeter. G.l.c. was performed with a Hitachi 063 instrument with flame-ionization detector, and g.l.c.–m.s. with a Hitachi RMU7M instrument. The tri-*O*-methylsilyl derivatives were analyzed by use of a glass column (0.3 × 300 cm) packed with 3% OV-17 on Shimalite W (80–100 mesh), the temperature being raised from 150 to 250° at a rate of 5°/min, and the *O*-methyl derivatives by use of a glass column (0.2 × 200 cm) containing 5% OV-225 on Gaschrom Q (100–120 mesh) at 210°. N.m.r. spectra were recorded at 100 MHz with a Varian XL-100 spectrometer equipped with an FT system, tetramethylsilane (τ 10.00) being the external standard. The optical rotatory dispersion curve was determined with a JASCO ORD/UV-5 spectrometer; measurements being made on aqueous solutions at a concentration of 0.1% at 24°.

Methylation analysis. — The polysaccharide (5 mg), in a small bottle sealed with a rubber cap, was dissolved in dry methyl sulfoxide (0.2 ml). The bottle was flushed with nitrogen, and 2M methylsulfinylsodium in methyl sulfoxide (0.2 ml) was added. The gelatinous solution was stirred with a magnetic stirrer for 4 h at room temperature. Methyl iodide (0.2 ml) was added dropwise with external cooling, and the solution was stirred for an additional 1 h. The clear solution was diluted with water (2 ml), dialyzed against distilled water, and evaporated to dryness. A solution of the methylated polysaccharide in 90% formic acid (3 ml) was kept for 2 h at 100°, concentrated to dryness, and then hydrolyzed with 0.25M sulfuric acid (3 ml) for 12 h at 100°. The hydrolyzate was neutralized with barium carbonate, and the sugars were reduced with sodium borodeuteride, acetylated, and analyzed by g.l.c.–m.s.

G.l.c. of this product showed two main components with a mobility (*t* value) relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol of 1.12 and 6.12 on an OV-225 column at 210°. One of the components (*t* 1.12) was identified as 1,4-di-*O*-acetyl-6-deoxy-2,3,5,7-tetra-*O*-methylheptitol. It gave the following ions on m.s. (relative intensities are in parentheses): 43(64), 45(83), 71(25), 73(21), 75(9), 87(8), 97(5), 101(27), 103(100), 113(9), 118(70), 127(6), 129(12), 143(4), 145(6), 159(8), 162(3), 187(18), and 219(8). The other compound (*t* 6.12) was identified as 1,3,5,6-tetra-*O*-acetyl-2,4,7-tri-*O*-methylheptitol, which gave the following ions on m.s. (relative intensities are in parentheses): 43(76), 45(13), 74(7), 87(16), 99(20), 101(15), 113(32), 117(8), 118(100), 128(11), 131(12), 141(7), 160(9), 173(6), 174(6), 202(5), 233(30), 234(19), and 349(2).

The polymeric product obtained after mild acid hydrolysis (0.05M HCl, 30 min, 100°) showed $[\alpha]_D^{22}$ −27.5° (*c* 1.4, water). The polymer was subjected to methylation analysis. G.l.c. (OV-225 column) of this product showed one main component (*t* 3.60, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol), which was identified as 1,5,6-tri-*O*-acetyl-2,3,4,7-tetra-*O*-methylheptitol. It gave the following ions on m.s. (relative intensities are in parentheses): 43(100), 45(21), 71(4), 75(9), 87(12), 99(14), 101(8), 102(40), 113(22), 117(7), 118(48), 129(7), 131(7), 143(21), 157(9), 162(7), 173(4), 203(4), 233(13), and 277(5).

Periodate-oxidation studies. — The untreated polysaccharide (5 mg) was dissolved in 0.1M sodium acetate buffer (pH 3.9, 5 ml), 0.2M sodium metaperiodate (1 ml) was added, and the mixture was kept in the dark for 10 days at 4°. Excess of periodate was decomposed with 1,2-ethanediol, and the oxidized material was purified by chromatography on a column (1.4 × 30 cm) of Sephadex G-15 and reduced with sodium borohydride. After removal of inorganic material by treatment with an ion-exchange resin and by addition and distillation of methanol, the product was hydrolyzed, methanolyzed, and per-*O*-trimethylsilylated. G.l.c. analysis of the product showed that the 6-deoxyheptose component had disappeared completely, and a degradation product with a mobility (*t* value) relative to trimethylsilylated inositol of 0.45 had been formed. The product was identified as a 2-deoxypentitol that gave the following ions on m.s. (relative intensities are in parentheses): 73(100), 103(70), 147(53), 205(49), 219(60), 307(47), 319(13), 321(20), 334(5), and 409(3).

The polymeric product obtained after mild acid hydrolysis was subjected to a Smith degradation, followed by hydrolysis, methanolysis, and trimethylsilylation. G.l.c. analysis of the product showed that all heptose residues had been degraded, and a degradation product with a mobility (t value) relative to trimethylsilylated inositol of 0.36 had been formed. This product was indistinguishable from erythritol on g.l.c.

Serological methods. — Antisera were produced in rabbits by intravenous injections of formalin-killed, whole cells. Difco special Agar Noble (1%) in saline solution was used for gel-diffusion tests. Deacetylation of the antigens was carried out by treatment with 0.01M sodium hydroxide for 30 min at 50°. Precipitation-inhibition test by sugars was carried out as follows: undiluted antiserum (25 µl) was mixed with an equal vol. of sugar solution. Following incubation at 37° for 30 min, the antigen (50 µl, 0.03 mg/ml) was added. The precipitation reaction was read after 2 h at 37°.

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