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STRUCTURAL STUDIES OF THE POLYSACCHARIDE ANTIGEN OF *Eubacterium saburreum*, STRAIN 49

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ABSTRACT

The polysaccharide antigen produced by *Eubacterium saburreum*, strain L 49, 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 contains *O*-acetyl groups, linked to O-7 of part of the heptosyl residues and to O-2 of part of the 6-deoxyheptosyl groups.

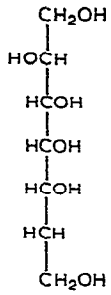
INTRODUCTION

Eubacterium saburreum is an anaerobic, Gram-positive, oral micro-organism. We recently reported[1] on the structure of a cell-wall antigen produced by *E. saburreum* strain 44. This antigen is 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 another strain, L 49, has also been isolated and investigated[2]. We now report on structural studies of this antigen.

RESULTS AND DISCUSSION

The antigen, which showed $[\alpha]_{578}$ +49°, on acid hydrolysis yielded D-*glycero*-D-*galacto*-heptose and another sugar in the approximate ratio 1.7:1. The D-*glycero*-D-*galacto*-heptose was identified by its optical rotation and by conversion into the crystalline diethyl dithioacetal, which was indistinguishable from an authentic sample. The other sugar was converted into its alditol by reduction with sodium borodeuteride, and acetylated. M.s.[3] of this product showed that it derived from a 6-deoxyheptose. 6-Deoxy-D-*manno*-heptose was recently found as a component of lipopolysaccharides from different strains of *Yersinia* (*Pasteurella*) *pestis*[4,5], and has

been synthesised[6]. The new sugar migrated slightly faster than 6-deoxy-D-*manno*-heptose on p.c., but the alditol acetates from the two sugars were not separated by g.l.c., using an ECNSS-M column. All hexitol acetates except the *manno* and *altro* derivatives are well-separated on this column[7]. By analogy, it therefore seemed likely that the new sugar had the *altro* or *talo* configuration. The derived alditol was oxidized with 0.1 molar equivalent of sodium metaperiodate, and the product was reduced, acetylated, and investigated by g.l.c.–m.s. The major reaction product was indistinguishable from 2-deoxy-D-*erythro*-pentitol tetra-acetate, prepared from 2-deoxy-D-*erythro*-pentose. This is the expected product from the *altro* derivative (**1**) as the *threo*-glycol grouping should be preferentially oxidized[8]. All the other isomers should give different products, and the *manno* isomer (**2**) gave the acetates of glycerol and 2-deoxytetritol as the main products using the same conditions of oxidation. The new sugar showed $[\alpha]_{578}$ +40°, which is not a very accurate value as only small amounts of amorphous material were available. D-Altrose and 6-deoxy-D-altrose show $[\alpha]_D$ +18° and +33°, respectively. The positive value for the new sugar therefore indicates that it has the D-*altro* configuration.



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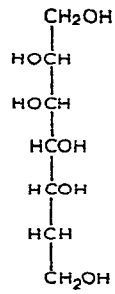

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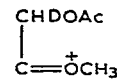

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The i.r. spectrum of the antigen showed absorption at 1735 cm^{-1}, thus indicating the presence of *O*-acyl groups, which were shown by their n.m.r. absorption at δ 2.05 (s) to be *O*-acetyl groups.

The molecular weight of the polysaccharide, $\bar{M}_w$ 40,000 and $\bar{M}_n$ 29,600, was determined by chromatography on a Sepharose 6B column, which had been calibrated with dextrans of known molecular weights[9].

Methylation analysis of the antigen and analysis of the methylated sugars by g.l.c.–m.s. of their alditol acetates[10] yielded 6-deoxy-2,3,5,7-tetra-*O*-methylheptose, 2,4,6,7-tetra-*O*-methylheptose, and 2,4,7-tri-*O*-methylheptose in the proportions 1.4:1:1.4. These figures, which do not indicate a simple repeating-unit, are in reasonably good agreement with the sugar analyses, which showed heptose and 6-deoxyheptose in the ratio 1.7:1. The results demonstrate that the 6-deoxyheptose is terminal and furanosidic, and that the heptose occurs as chain residues, linked to O-3, and as branching residues, linked to O-3 and O-6.

Hydrolysis of the polysaccharide under mild conditions, when mainly furanosidic linkages should be hydrolysed, followed by methylation analysis, yielded 2,3,4,7- and 2,4,6,7-tetra-*O*-methylheptose in the ratio 52:48. The result therefore demonstrates that the 6-deoxyheptofuranosyl groups are linked to O-3 of the branching heptopyranosyl residues.

The antigen was deacetylated and subjected to a modified Smith-degradation[11,12], that is periodate oxidation, reduction with borohydride, methylation, and hydrolysis under mild conditions. Part of the polyalcohol was subjected to sugar analysis (Table II). As expected, all 6-deoxyheptose had disappeared and D-galactose had been formed. The polymeric product obtained after mild hydrolysis was remethylated, using trideuteriomethyl iodide, and hydrolysed, and the resulting mixture of methylated sugars was analysed, as the alditol acetates, by g.l.c.–m.s. 2,4,6-Tri-*O*-methyl-D-galactose, carrying a trideuteriomethyl group at O-6, and 2,3,4,7-tetra-*O*-methyl-D-heptose, carrying a trideuteriomethyl group at O-3, were obtained in the ratio 49:51. These data further support the conclusion that the 6-deoxyheptofuranosyl groups are linked to O-3 of the branching heptopyranosyl residues.

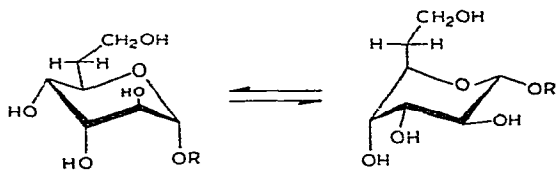
The *O*-acetyl groups were located by the method of de Belder and Norrman[13], that is by protection of the free hydroxyl groups by reaction with methyl vinyl ether and a trace of toluene-*p*-sulfonic acid, followed by methylation analysis. In addition to the alditol acetates of the heptose and 6-deoxyheptose, two other products were obtained. One was identified as deriving from the 7-*O*-methylheptose, which was also obtained from the *E. saburreum* L 44 antigen.

When the reduction was performed with sodium borodeuteride, the other component, which had a retention time of 0.95 relative to D-glucitol hexa-acetate, on m.s. gave a strong ion at *m/e* 118 (3), as given by the alditol acetates from sugars having a methoxyl group at C-2. Inasmuch as this ion, and that at *m/e* 43 ($CH_3-C\overset{+}{\equiv}O$), were the only significant ions in the spectrum, the identification of 6-deoxy-2-*O*-methylheptose in the sugar mixture is tentative only. The results, however, indicate that some 70% of the heptosyl residues are acetylated at O-7 and some 40% of the 6-deoxyheptosyl groups are acetylated at O-2.

In order to distinguish between *O*-acetylation on the chain and branching heptosyl residues, the *O*-acetyl groups were also located by treatment with methyl vinyl ether, methylation using trideuteriomethyl iodide, hydrolysis under mild conditions when essentially acetal groups should be hydrolysed off, and remethylation, using methyl iodide[14]. In this procedure, the substitution of *O*-acetyl groups in the native material is reflected in the substitution pattern of trideuteriomethyl groups. The hydrolysate of this product was reduced, acetylated, and analysed by g.l.c.–m.s. In addition to 2,4,6,7-tetra-*O*-methylheptose and 2,4,7-tri-*O*-methylheptose, 2,3,4,7-tetra-*O*-methylheptose was also obtained, demonstrating that some 6-deoxyheptofuranosyl groups had been removed during the mild hydrolysis. M.s. demonstrated that all the heptose derivatives contained, in part, trideuteriomethyl groups at O-7,

indicating that *O*-acetyl groups are distributed on both the chain and branching heptose residues.

The optical rotation of the polymer, $[\alpha]_D$ +49°, decreased on mild acid hydrolysis, and a product, in which most of the 6-deoxyheptofuranosyl groups had been removed, showed $[\alpha]_D$ +7°. These results indicate that the D-*glycero*-D-*galacto*-heptopyranosyl residues are β-linked and that the 6-deoxy-D-*altro*-heptofuranosyl groups are α-linked. Only few sugars occur as furanosides in Nature. In the α-D-*altro*-pyranose form (**4**), there are severe sterical interactions, which is probably the reason why the α-D-*altro*-furanose form (**5**), with all adjacent bulky substituents in *trans*-position, is preferred.



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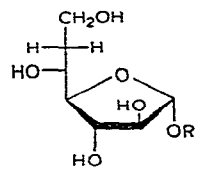

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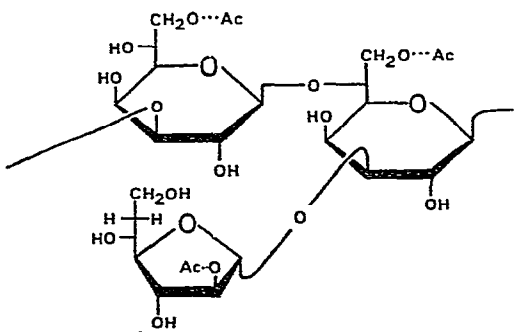
6-Deoxy-L-altrose has been isolated from a *Yersinia enterocolitica* lipopolysaccharide[15]. It seems possible that this sugar, too, occurs in the furanoid form in the polysaccharide.

From the combined evidence, it is evident that *E. saburreum* strain 49 antigen consists of chains of (1→3)- and (1→6)-linked β-D-*glycero*-D-*galacto*-heptopyranosyl residues, and that the latter are substituted at O-3 with 6-deoxy-α-D-*altro*-heptofuranosyl groups. *O*-Acetyl groups are present at O-7 in part of the heptosyl residues and, possibly, at O-2 in part of the 6-deoxyheptosyl groups. Bacterial polysaccharides are generally composed of oligosaccharide repeating-units. The simplest residue should be the trisaccharide **6**, but the analyses consistently indicate a higher percentage of branching than of chain residues. If the polysaccharide has a regular structure, it is therefore probably more-complicated.



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EXPERIMENTAL

General methods. — These were the same as in the investigation of the *E. saburreum* strain L 44 antigen[1].

Identification of sugar components. — The polysaccharide, isolated as previously described[2], showed $[\alpha]_{578}^{22}$ +49° (*c* 0.1, water). Acid hydrolysis gave two sugar components, identified by g.l.c.–m.s. of their alditol acetates (Table II) as a 6-deoxyheptose and a heptose, respectively. The sugars, from 10 mg of polysaccharide, were separated by p.c. on Whatman No. 1 paper, using 1-butanol–pyridine–water (6:4:3) as irrigant. The slower component (6 mg), $[\alpha]_{578}^{22}$ +60° (*c* 0.1, water), was transformed into its diethyl dithioacetal, m.p. 197–199°, which was indistinguishable (m.p., i.r.) from an authentic sample prepared from D-*glycero*-D-*galacto*-heptose[16].

The faster component, $[\alpha]_{578}^{22}$ +40° (*c* 0.1, water), was slightly faster than 6-deoxy-D-mannose on p.c. The R_{GLC} values were 1.3 and 1.2, respectively, in 1-butanol–pyridine–water (6:4:3).

The 6-deoxyheptose (2 mg) was reduced with sodium borohydride, and inorganic material was removed by ion-exchange, followed by distillation with methanol. The product in water (2 ml) was oxidized with 4.9mM sodium metaperiodate (0.5 ml) for 30 min at room temperature, reduced with sodium borodeuteride, acetylated, and investigated by g.l.c.–m.s. The main reaction product was indistinguishable from 2-deoxy-D-*erythro*-pentitol tetra-acetate on g.l.c., using different columns. The retention time for both components on ECNSS-M was 0.17, relative to D-glucitol hexa-acetate, and a mixture gave a single, symmetrical peak. M.s. of the two components were also identical, except for the shifts caused by the deuterium labelling.

Methylation analyses. — These were performed as previously described[1]. The identifications of the different methylated sugars from the m.s. of their alditol acetates were unambiguous and will not be discussed. The retention times of the different components are summarized in Table I.

TABLE I

G.L.C. OF ALDITOL ACETATES OBTAINED ON METHYLATION ANALYSIS OF ORIGINAL AND MODIFIED *Eubacterium saburreum* STRAIN L 49 ANTIGEN

Sugar	R_T[a]	*Original L 49* (*Mole %*)	*Partially hydrolysed L 49* (*Mole %*)	*L 49 after Smith degradation* (*Mole %*)	*L 49, O-acetyl determination* (*Mole %*)
2,3,5,7-6-Deoxy-Hep[b]	1.2	37.5	—	—	32
2,4,6-Gal[c]	2.0	—	—	49	—
2,4,6,7-Hep	4.1	26.5	48	—	22[d]
2,3,4,7-Hep	4.7	—	52	51	18[d]
2,4,7-Hep	7.3	36.0	—	—	27[d]

[a]Retention time on OV-225 column, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]6-Deoxy-2,3,5,7-tetra-*O*-methylheptose, *etc.* [c]Trideuteriomethylated on O-6. [d]Partly trideuteriomethylated on O-7.

Partial hydrolysis of the antigen. — The antigen (3 mg) in 0.05M sulfuric acid (2 ml) was kept at 80°, and the change in optical rotation was followed, using a 10-cm tube. After 3.5 h, when α_{578} had dropped from 0.163° to 0.040°, the solution was neutralized with barium carbonate and concentrated, and the monomer and polymer were separated by chromatography on a column (1.5 × 30 cm) of Sephadex G 15. The polymer, $[\alpha]_{578}^{22}$ +7° (*c* 0.1, water), was subjected to methylation analysis (Table I).

Periodate-oxidation studies. — The polysaccharide (6 mg), which had been deacetylated by treatment with 0.5M NaOH, 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 at 4° for 10 days. Excess of periodate was decomposed with ethylene glycol, and the oxidized material was purified by chromatography on a column (1.5 × 30 cm) of Sephadex G 15 and reduced with sodium borohydride. Part of the recovered material was used for sugar analysis (Table II).

TABLE II

G.L.C. OF ALDITOL ACETATES OF SUGARS AND MONO-*O*-METHYL SUGARS OBTAINED IN STUDIES OF THE *Eubacterium saburreum* STRAIN L 49 ANTIGEN

Sugar	R_T[a]	*Sugar analysis (Mole %)*	*Periodate–borohydride (Mole %)*	*O-Acetyl determination (Mole %)*
Galactose	0.90	—	40	—
2-*O*-Me-6-deoxy-D-*altro*-heptose	0.95	—	—	16
7-*O*-Me-D-*glycero*-D-*galacto*-heptose	1.10	—	—	43
6-Deoxy-D-*altro*-heptose	1.25	37	—	24
D-*glycero*-D-*galacto*-Heptose	2.22	63	60	17

[a]Retention time on an ECNSS-M column, relative to D-glucitol hexa-acetate

Another part was methylated, treated with 90% formic acid at 40° for 1 h, methylated with trideuteriomethyl iodide, converted into alditol acetates, and analysed by g.l.c.–m.s. (Table II).

Location of O-*acetyl groups.* — This was effected by treatment with methyl vinyl ether followed by methylation analysis (Table II), as previously described[1].

The polysaccharide (8 mg) was treated with methyl vinyl ether and methylated, using trideuteriomethyl iodide. The product was hydrolysed by treatment with 90% formic acid at 40° for 30 min, and recovered by dialysis and lyophilization. Re-methylation, using unlabelled methyl iodide, was followed by hydrolysis, borohydride reduction, and analysis of the product by g.l.c.-m.s.

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