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THE CELL-WALL ANTIGEN FROM *Eubacterium saburreum* STRAIN L 13, A NEW TYPE OF BIOPOLYMER

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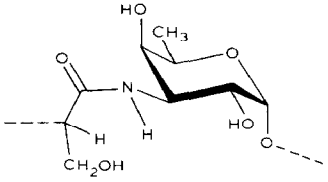
ABSTRACT

In the cell-wall antigen of *Eubacterium saburreum* strain L13, the main chain consists of alternating residues of 3-amino-3,6-dideoxy-α-D-galactopyranose and L-glyceric acid, joined *via* glycosidic and amidic linkages as depicted below. Every third 3-amino-3,6-dideoxy-α-D-galactopyranosyl residue has a β-D-fructofuranosyl group linked to its 2-position. The antigen also contains *O*-acetyl groups. According to the IUPAC–IUB recommendations, this new type of biopolymer is not a proper polysaccharide but it seems, nevertheless, eligible to be designated as a polysaccharide.



INTRODUCTION

The cell-wall antigens elaborated by different strains of *Eubacterium saburreum*, an anaerobic, Gram-positive oral organism, are polysaccharides with unusual structural features. For example, the L 44 antigen is a homopolysaccharide composed of (1→6)-linked β-D-*glycero*-D-*galacto*-heptopyranosyl residues[1]. Antigens from some other strains, *e.g.*, L 49[2], contain chains of this sugar substituted with 6-deoxy-α-D-*altro*-heptofuranosyl groups. The antigens from strains L 452[3] and L

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32[4] contain α-D-fucofuranosyl and 3,6-dideoxy-D-*arabino*-hexopyranosyl (tyvelosyl) groups, respectively. The antigen from strain V 5[5], finally, contains a 3-amino-3,6-dideoxyhexose, reported to have the D-*gluco* configuration, to which L-glyceric acid is amidically linked. The assignment of the D-*gluco* configuration was based on n.m.r. evidence; however, according to another group, the n.m.r. spectra indicate that the sugar has the D-*galacto* configuration[6]. We now report structural studies of the antigen from strain L 13. Preliminary studies of this antigen have been reported[7].

RESULTS AND DISCUSSION

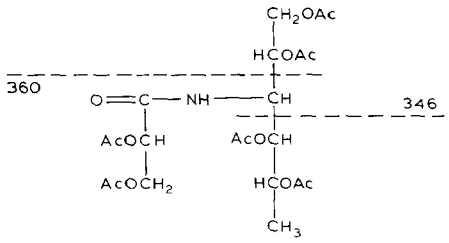
Previous studies indicated that the antigen from strain L 13 contained a hexulose component[7]. This was released on treatment with acid under mild conditions and, on borohydride reduction, yielded a mixture of mannitol and glucitol, identified by g.l.c.–m.s. of the acetates. This demonstrated that the sugar was fructose, which was shown to have the D configuration by the method of Gerwig *et al.*[8].

Acid hydrolysis of the polysaccharide under stronger conditions yielded another sugar, identified as a 3-amino-3,6-dideoxyhexose by g.l.c.–m.s. of its alditol acetate. This was indistinguishable from the 3-amino-3,6-dideoxy-D-galactose derivative, but well separated from the corresponding 3-amino-3,6-dideoxy-D-glucose derivative. N.m.r. evidence, given below, confirmed that the sugar has the *galacto* configuration. The hydrolysate also contained a hydroxycarboxylic acid, identified as L-glyceric acid by g.l.c.–m.s. of its acetylated (+)-2-butyl ester. This was indistinguishable from the corresponding derivative from L-glyceric acid, but different from the D-glyceric acid derivative.

The ^{1}H-n.m.r. spectrum of the antigen showed two signals for H-6 of 6-deoxyhexose residues at δ 1.17 and 1.21 (*J* 6.3 Hz, 9 H), a signal for *O*-acetyl groups (δ 2.10, 3 H), and a complex group of signals in the region for anomeric protons. After *O*-deacetylation, however, only two signals in this region at δ 5.11 (*J* 3.5 Hz, 2 H) and 5.33 (not well resolved, 1 H) were observed. The *O*-deacetylated polymer from which the fructosyl residues had been removed gave only one signal in the anomeric region, at δ 5.11 ($J_{1,2}$ 3.8 Hz), indicating that approximately every third amino sugar is substituted with fructose. The ^{13}C-n.m.r. spectrum of the antigen showed signals for C-6 of a 6-deoxyhexose (δ 17.1), for *O*-acetyl (δ 21.9), for carbon linked to nitrogen (several signals around δ 52), and for four anomeric carbons (δ 97.6, 99.9, 100.0, and 106.0).

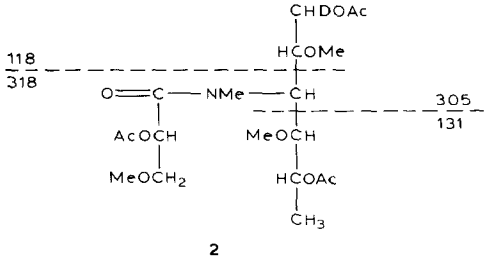
The antigen was solvolysed with liquid hydrogen fluoride[9], which should cleave glycosidic linkages but not amide linkages. G.l.c.–m.s. of the alditol acetate (**1**) of the sugar released on this treatment demonstrated that the amino group of the 3-amino-3,6-dideoxy-D-galactose is acylated with L-glyceric acid (the origins of two pertinent fragments are indicated in the formula).

The sugar analyses and the n.m.r. evidence indicated that the polysaccharide contained 3,6-dideoxy-3-(L-glyceroylamino)-D-galactose and D-fructose in the



relative proportions 3:1. The signal at δ 106.0 in the ^{13}C-n.m.r. spectrum could be assigned to the fructosyl group and indicated that it was β-furanosidic[10].

Methylation analysis of the antigen yielded a mixture of 3,6-dideoxy-2,4-di-*O*-methyl-3-methylamino-D-galactose and 3,6-dideoxy-4-*O*-methyl-3-methylamino-D-galactose in the ratio 2.4:1, analysed as the alditol acetates by g.l.c.–m.s. By performing the hydrolysis of the methylated polymer under mild conditions, 1,3,4,6-tetra-*O*-methyl-D-fructose was also obtained. This seemingly absurd result, indicating mainly terminal groups and no branching points, was explained when the methylated polymer was solvolysed with liquid hydrogen fluoride. The main component gave the alditol acetate **2**, demonstrating that one of the hydroxyl groups in the L-glyceric acid moiety is substituted in the polysaccharide.



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The origin of the main primary fragments in the mass spectrum are indicated in formula **2**. The secondary fragments were also in agreement with the postulated structure. That the primary and not the secondary hydroxyl group in the L-glyceric acid residue was methylated was not conclusively demonstrated at this stage, as the two alternatives should give similar mass spectra. However, n.m.r. evidence discussed below demonstrated that the secondary position is glycosylated.

On preparation of a sample of *O*-deacetylated antigen, the pH unintentionally became too low during the work-up and all β-D-fructofuranosyl groups were hydrolysed off. On methylation analysis of this product, alditol acetate **2** was the main component. An analogous component, in which the 2-position of the L-glyceric acid moiety was also methylated, was obtained in a low yield and was obviously derived from terminal groups. This polymeric material gave well resolved ^{1}H- and ^{13}C-n.m.r. spectra.

TABLE I

^{1}H-N M R DATA FOR THE *O*-DEACETYLATED FRUCTOSE-FREE POLYMER

Residue	*Hydrogen atom*	*δ*	J *(Hz)*
	H-1	5 11	$J_{1,2}$ 3 8
	H-2	3.94	$J_{2,3}$ ~10
α-D-Fuc*p*3N	H-3	4 31	$J_{3,4}$ 4
	H-4	3 80	$J_{4,5}$ 1
	H-5	4.15	$J_{5,6}$ 6
	H-6	1 17	
L-Glyceric acid	H-2′	4 31	$J_{2',3'} = J_{2',3''} = 5$ Hz
	H-3′, H-3″	~3 9	second-order spectrum

TABLE II

^{13}C-N M R DATA FOR THE LINEAR POLYMER OBTAINED AFTER *O*-DEACETYLATION AND MILD ACID HYDROLYSIS OF THE L13 ANTIGEN AND OF SOME MODEL SUBSTANCES

Substance	*C-1*	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*C-1′*	*C-2′*	*C-3′*
Polymer	99 5	67 0	52 0	71 2	68.8	16.1	173 2	79 8	62 4
Polymer, Δ(δH − δD)	−0 001	0.102	0.147	0.111	0.001	0 049	—	0.013	0 123
α-D-Fuc*p*3NAc[6]	98 1	67 2	52.2	71 7	67 5	16.9			
L-Glyceroyl residue[a]							175 0	73 6	64 7
D-Glyceric acid residue[b]							177 9	79 4	64 9

[a]Taken from ref. 5, but values increased by 0 5 p.p.m (*cf* ref 6) [b]From the ^{13}C-n m r spectrum of 2-*O*-α-D-mannopyranosyl-D-glyceric acid (Na salt)[12]

The signals in the ^{1}H-n.m.r. spectrum (Table I) were assigned by selective decoupling. The assignment of the signals for H-2′, H-3′, and H-3″ of the glyceric acid residue relies on the assumption that H-2′, being vicinal to a carboxyl group, should resonate at lower field than H-3′ and H-3″. The coupling constants of the ring protons confirmed that the 3-amino-3,6-dideoxyhexose has the *α-galacto* configuration. The specific rotation of the polymer, $[\alpha]_{578}^{20}$ +87°, although not very accurate because of the small amount of material available, indicates that this sugar has the D configuration.

The signals in the ^{13}C-n.m.r. spectrum (Table II) were assigned by selective proton decoupling and by comparison with model substances. It is evident from the spectrum that the polymer contains an unsubstituted primary hydroxyl group (*δ* 62.3), which must be O-3 of the L-glyceric acid residue. The differential isotope shifts, Δ (δH − δD)[11], further demonstrated free hydroxyl groups on the carbon atoms giving signals at *δ* 62.4, 67.0, and 71.2, assigned to C-3 of the acid and C-2 and C-4 of the amino sugar. The shift (16.2 p.p.m.) for C-6 is indicative of a 6-deoxyhexose having an axial substituent in the 4-position.

An estimation of the shifts for C-2, C-4, and C-2′, assuming different types

TABLE III

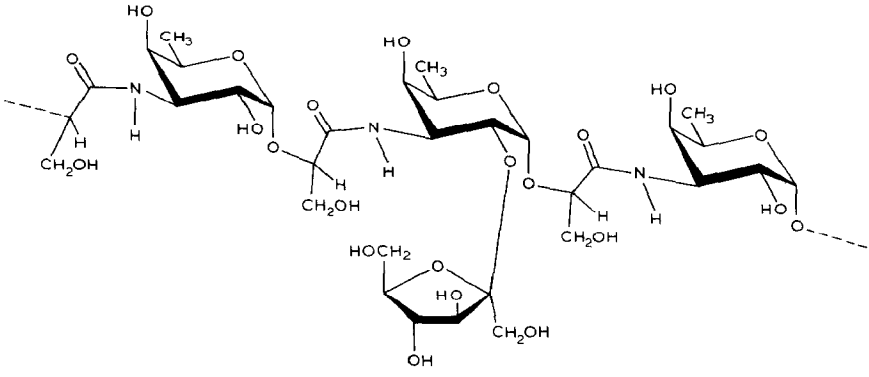
CALCULATED VALUES FOR THE ^{13}C-N M R SHIFTS OF C-2, C-4, AND C-2′ IN THE LINEAR POLYMER OBTAINED AFTER *O*-DEACETYLATION AND MILD HYDROLYSIS WITH ACID, UNDER ASSUMPTIONS GIVEN IN THE TEXT

Glycosylation at	*Shifts for C-2, C-4, and C-2′ (p.p.m., numerically descending order)*	*Σ Deviations from experimental values (p.p.m.)*
C-2	74.2, 73.6, 71.7	12.6
C-4	78.7, 73.6, 67.2	3.6
C-2′	80.6, 71.7, 67.2	1.4
Experimental values	79.8, 71.3, 67.0	—

of attachments, is given in Table III. It is assumed that glycosylation shifts the signal for the corresponding model substance downfield by 7 p.p.m. The signals are arranged in descending numerical order, and the sums of the deviations from the experimental values are given. As seen from Table III, there is good agreement for glycosylation at C-2′ but not for the other positions.

From the combined results, it is concluded that the *E. saburreum* L 13 antigen is composed of 3,6-dideoxy-3-(L-glyceroylamino)-α-D-galactopyranosyl residues, joined by glycosidic linkages to O-2 of the L-glyceric acid residue. Approximately every third of these residues has a β-D-fructofuranosyl group linked to O-2 of the amino sugar. Bacterial polysaccharides are often composed of oligosaccharide repeating-units and it seems likely, although not proved, that the antigen is composed of tetrasaccharide repeating-units having the structure **3**. The antigen further contains *O*-acetyl groups, approximately one group per "repeating unit", but these have not been located.

The *E. saburreum* strain L 13 antigen represents a new type of biopolymer, containing alternating amino sugar and hydroxycarboxylic acid residues, linked by glycosidic and amidic linkages. A polysaccharide is "a macromolecule consisting of a large number of monosaccharide residues joined to each other by glycosidic



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linkages"[13], and the antigen is consequently not a true polysaccharide. It seems, nevertheless, preferable to classify it as a polysaccharide rather than to invent a name for this new type of biopolymer.

EXPERIMENTAL

General methods. — Concentrations were performed at 40° in test tubes, by flushing with air. For g.l.c., an SE-30 glass-capillary column and an SE-54 fused-silica capillary column were used, generally with a temperature program (170→230°, 2°/min). G.l.c.–m.s. was performed on a Varian MAT 311 instrument, using glass columns packed with OV-17 and SE-30. Optical rotations were measured at 22°, using a Perkin–Elmer 241 polarimeter.

N.m.r. spectra, for solutions in deuterium oxide, were recorded at 70° (^{13}C) or 85° (^{1}H) with a JEOL FX 100 or GX 400 instrument. Chemical shifts are reported in p.p.m. downfield from external tetramethylsilane (^{13}C) and residual water as 4.17 p.p.m. downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS). The absolute configuration of the fructose was determined by the method devised by Gerwig *et al.*[8]. The absolute configuration of the glyceric acid was determined by g.l.c. [SE-54 column with a temperature program (100→190°, 2°/min)] of the acetylated (+)-2-butyl ester. The relative retention times for the D and L forms were 1.00 and 1.03, respectively.

Isolation of the antigen. — This was performed as previously described[14].

Sugar and methylation analyses. — For sugar analysis, the antigen was first hydrolysed with aqueous 10% acetic acid at 100° for 3 h and the product reduced with sodium borohydride. Part of the material was acetylated and analysed by g.l.c.–m.s. and showed the presence of approximately equal parts of fully acetylated mannitol and glucitol. The main part of the material was further hydrolysed with 2M trifluoroacetic acid at 100° for 16 h, and worked-up as described above. The ratio of fructose to 3-amino-3,6-dideoxy-D-galactose was 1:1.7 from the areas under pertinent peaks in the gas chromatogram. The alditol acetate from 3-amino-3,6-dideoxy-D-galactose had a somewhat longer retention time than the corresponding D-*gluco* derivative.

Solvolysis of the antigen with liquid hydrogen fluoride was performed at room temperature for 3 h. The 3,6-dideoxy-3-(L-glyceroylamino)-D-galactose formed was identified by g.l.c.–m.s. of its alditol acetate. The trimethylsilyl derivative of the alditol was also prepared and gave the expected mass spectrum[5].

Methylations[15,16] were performed with sodium methylsulfinylmethanide–methyl iodide in dimethyl sulfoxide. Methylated products were recovered by reversed-phase chromatography on Sep-Pak C_{18} cartridges[17]. The methylated polysaccharides were hydrolysed under mild conditions (50% aqueous acetic acid at 100° for 1 h) in order to identify the fructose derivative, with 2M trifluoroacetic acid at 100° for 16 h as in a conventional methylation analysis, and by solvolysis with hydrogen fluoride as described above, in order to obtain *N*-acylated sugar derivatives.

The two 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol derivatives obtained from the β-D-fructofuranosyl groups were separated on the SE-54 column (130°, isothermal) and had retention times of 0.77 and 0.82, relative to 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

O-*Deacetylation*. — This was performed by treatment of the antigen with 0.1M sodium hydroxide at room temperature for 16 h, and the product was isolated by chromatography on a small column of Sephadex G25 followed by freeze-drying.

In one experiment, when the solution after the deacetylation was treated with Dowex 50 (H^+) resin, the pH unintentionally became too low during the work-up and a fructose-free polymer was obtained.

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