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STRUCTURE OF THE LEVAN ELABORATED BY *Streptococcus salivarius* STRAIN 51: AN APPLICATION OF CHEMICAL-IONISATION MASS-SPECTROMETRY*

RICHARD A. HANCOCK, KEITH MARSHALL, AND HELMUT WEIGEL
The Bourne Laboratory, Royal Holloway College (University of London), Egham, Surrey TW20 0EX (Great Britain)

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ABSTRACT

The polysaccharide elaborated by *Streptococcus salivarius* strain 51 contains β-D-fructofuranose residues linked through positions 2 and 6, as well as 1, 2, and 6. The approximate numbers of terminal, non-reducing D-fructofuranose residues and those linked through positions 2 and 6, and through 1, 2, and 6 in the average repeating-unit are 1, 7, and 1, respectively. The branches through the β-(2→1)-linkage contain up to at least four D-fructofuranose residues. Chemical-ionisation mass-spectrometry aids the assignment of structures to *O*-acetyl-*O*-methylalditols obtained in methylation analysis.

INTRODUCTION

Strains of *Streptococcus salivarius* colonise in the human mouth and produce extracellular polysaccharides when grown on sucrose-containing media. These polysaccharides have previously been classed as levans, *i.e.*, D-fructans in which the principal glycosidic linkages are of the β-(2→6) type, the conclusions being based mainly on serological tests[1,2] and the infrared spectra[3] of the polysaccharides. As far as we are aware, the D-fructans have not been characterised chemically. Continuing our work on polysaccharides elaborated by organisms isolated from the human mouth[4], it seemed to us desirable to study in greater detail the chemical structure of these D-fructans. As a preliminary to detailed assignments of structures, we now report on the types and percentages of the glycosidic linkages, and the size of branches in the polysaccharide elaborated by *S. salivarius* strain 51.

RESULTS AND DISCUSSION

The extracellular polysaccharide was shown to be a D-fructan. Its negative rotatory power and the fact that invertase degraded the D-fructan almost completely to D-fructose indicated that it contained β-D-fructofuranose residues.

*Dedicated to the memory of Professor Edward J. Bourne.

TABLE I

PROPERTIES AND ANALYSIS OF HEXITOL-*2-d* DERIVATIVES OBTAINED FROM LEVAN

Component	*G.l.c.*		*C.i.–m.s.*		*E.i.–m.s.*		*Parent structural unit*
	T[a]	*Mole fraction*	$[M+1]^+$ (m/e)	*Hexitol*-2-d *derivative*	*Primary fragments* (m/e)	*Identity of hexitol derivative*	
A	0.79	0.14	324	Di-*O*-acetyl-tetra-*O*-methyl	45, 161, 162, 205, 206	3	11
B	1.70	0.75	352	Tri-*O*-acetyl-tri-*O*-methyl	45, 162, 189, 206, 233	5	12
C	3.95	0.11	380	Tetra-*O*-acetyl-di-*O*-methyl	189, 190, 233, 234	7	14

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol.

Since Lindberg and co-workers reported[5] that *O*-acetyl-*O*-methylalditols can be characterised by electron-impact (e.i.) mass-spectrometry, the procedures for the linkage analysis of polysaccharides have been greatly simplified[6]. The method involves methylation of the polysaccharide, followed by hydrolysis, reduction, acetylation, and g.l.c. of the alditol derivatives thus obtained. The results obtained with the levan of *S. salivarius* strain 51 when sodium borodeuteride[7] was used as the reducing agent are shown in Table I. It was expected that, on reduction, each *O*-methyl-D-fructose would give the corresponding D-glucitol and D-mannitol derivatives. However, g.l.c. of a sample of authentic 1,3,4,6-tetra-*O*-methyl-D-fructose, treated as described for the hydrolysate of the methylated levan, showed only a single peak, *i.e.*, 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol. Thus, the conditions used do not allow the separation of the corresponding D-glucitol and D-mannitol derivatives.

The assignment of structure to *O*-acetyl-*O*-methylalditols is based on the g.l.c. retention times (*T*-values) and the pattern of electron-impact (e.i.) induced fragmentations. Although it is possible by e.i. mass-spectrometry to determine the pattern of substitution in an *O*-acetyl-*O*-methylalditol, the assignment of its structure is aided by determining first the number of methoxyl groups; this number is often estimated from the *T*-values. The ambiguities inherent in this method can be avoided by determining the molecular weight of the *O*-acetyl-*O*-methylalditols. Molecular ions, $[M]^{+}$, of *O*-acetyl-*O*-methylalditols produced by electron-impact ionisation are unfortunately rarely stable enough to be discernible in the e.i. mass-spectra. However, determinations of molecular weight can easily be carried out by chemical-ionisation

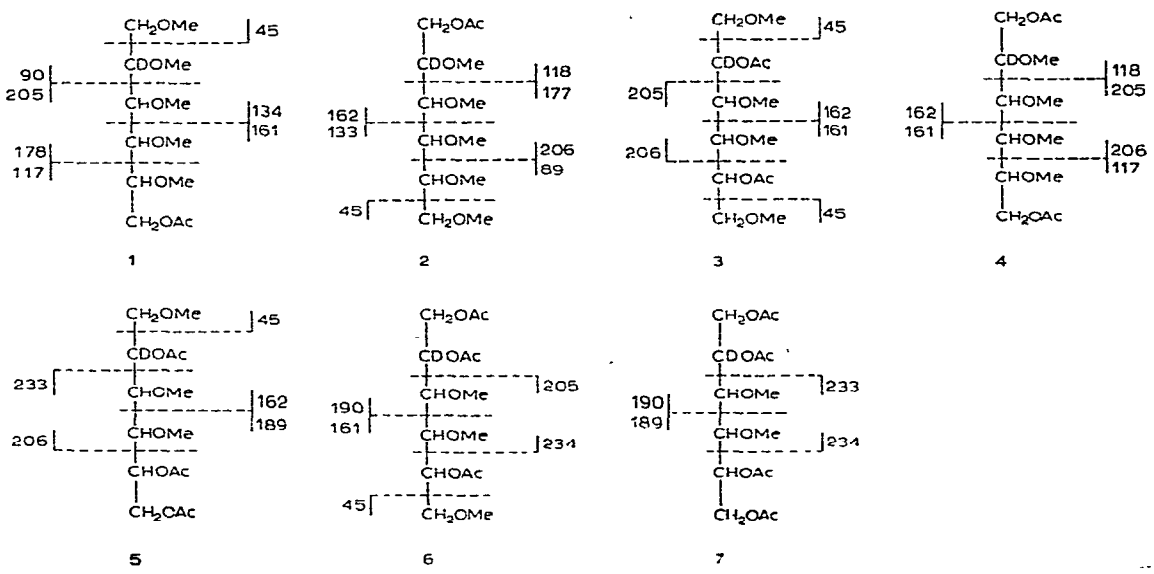

Fig. 1. E.i.-induced, primary fragmentation modes of *O*-acetyl-*O*-methylhexitol-*2-d* products (stereochemistry at asymmetric carbon atoms is not shown).

TABLE II

PROPERTIES AND ANALYSIS OF HEXITOL-2-*d* DERIVATIVES OBTAINED FROM DI- AND OLIGO-SACCHARIDES

Component	*G.l.c.* T[a]	*C.i.–m.s.*		*E.i.–m.s.*		*Parent structural unit*
		$[M+1]^+$ (*m/e*)	*Hexitol-2-*d *derivative*	*Primary fragments* (*m/e*)	*Identity of hexitol derivative*	
D[b–e]	0.50	296	*O*-Acetyl-penta-*O*-methyl	45, 89, 90, 117, 118, 133, 134, 161, 162, 177, 178, 205, 206.	1 and 2	8 and 9
E[b–e]	0.79	324	Di-*O*-acetyl-tetra-*O*-methyl	45, 161, 162, 205, 206.	3	11
F[d]	1.18	324	Di-*O*-acetyl-tetra-*O*-methyl	117, 118, 161, 162, 205, 206.	4	10
G[c–e]	1.70	352	Tri-*O*-acetyl-tri-*O*-methyl	45, 161, 162, 189, 190, 205, 206, 233, 234.	5 and 6	12 and 13
H[d,e]	3.95	380	Tetra-*O*-acetyl-di-*O*-methyl	189, 190, 233, 234	7	14

[a]Retention time relative to that of 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methyl-D-glucitol. [b]Obtained from disaccharide fraction. [c]Obtained from trisaccharide fraction. [d]Obtained from tetrasaccharide fraction. [e]Obtained from pentasaccharide fraction.

(c.i.) mass-spectrometry, as its commonly encountered feature is the abundance of $[M+1]^+$ ions. The molecular weights (M) of the alditol derivatives *A*, *B*, and *C* obtained from the levan (Table I), and determined from their $[M+1]^+$ ions in the c.i. mass-spectra, indicate them to be tetra-, tri-, and di-*O*-methylhexitol-*2-d* acetates, respectively. (C.i.-induced fragmentation modes of *O*-acetyl-*O*-methylalditols will be reported elsewhere). The e.i.-induced fragmentation patterns of the derivatives *A*, *B*, and *C* (Table I and Fig. 1) showed that they were, respectively, 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methyl- (3), 2,5,6-tri-*O*-acetyl-1,3,4-tri-*O*-methyl- (5), and 1,2,5,6-tetra-*O*-acetyl-3,4-di-*O*-methyl-hexitol-*2-d* (7).

The dubiety regarding the ring size of the D-fructose residues in the fructan was removed when the disaccharide fraction obtained by partial hydrolysis of the fructan (Table III) was reduced with sodium borodeuteride, followed by methylation, hydrolysis, reduction with sodium borodeuteride, and acetylation. The results (Table II and Fig. 1) of g.l.c.–m.s. (e.i. and c.i.) analysis show that the hexitol derivative *D* was a mixture of 6-*O*-acetyl-1,2,3,4,5-penta-*O*-methyl- (1) and 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methyl-hexitol-*2-d* (2), whereas the hexitol derivative *E* was 2,5-di-*O*-acetyl-1,3,4,6-tetra-*O*-methylhexitol-*2-d* (3). Hence, the disaccharide fraction contained *O*-β-D-fructofuranosyl-(2→6)-D-fructose (levanbiose) and *O*-β-D-fructofuranosyl-(2→1)-D-fructose (inulobiose). We therefore conclude that the fructan of *S. salivarius* strain 51 is composed of β-D-fructofuranose residues linked through positions 2 and 6, as well as 1, 2, and 6.

The chemical evidence for the suggestion that levans contain β-D-fructofuranose residues is the observation[8] that the tri-*O*-methyl-D-fructose obtained by methylation of a fructan, followed by hydrolysis, could be converted into a methyl 1,3,4,6-tetra-*O*-methyl-D-fructofuranoside by methylanosis at room temperature

TABLE III

DI- AND OLIGO-SACCHARIDES OBTAINED BY PARTIAL HYDROLYSIS OF LEVAN WITH ACID

Fraction	R_{Fru}[a]	*Yield (mg from 15 g of levan)*	*D.p.*[b]
Disaccharide	0.70	62	2.2
Trisaccharide *X*	0.47	213	3.3
Trisaccharide *Y*	0.42		
Tetrasaccharide *X*	0.31	146	3.6
Tetrasaccharide *Y*	0.27		
Pentasaccharide *X*	0.21	100	4.9
Pentasaccharide *Y*	0.18		
Hexasaccharide *X*	0.15	18	5.5
Hexasaccharide *Y*	0.12		
Heptasaccharide *X*	0.09		
Heptasaccharide *Y*	0.08		

[a]Paper-chromatographic migration rate relative to that of D-fructose. [b]Estimated by the method of Timell[14].

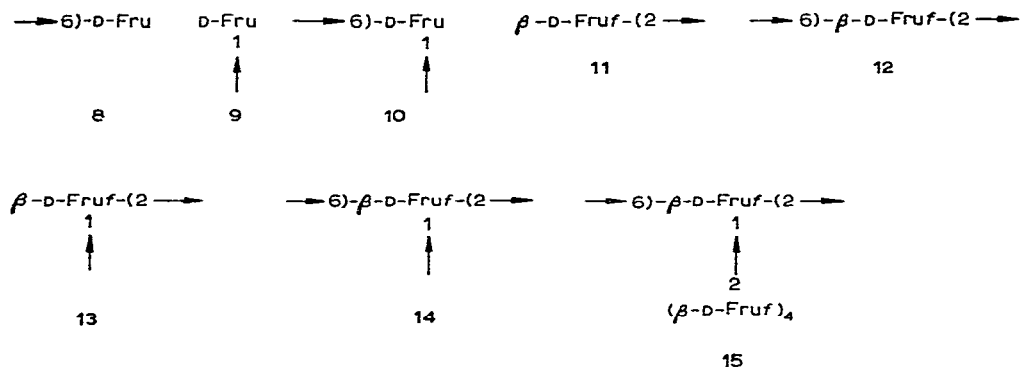

Fig. 2. Structural units of di- and oligo-saccharides, and levan.

and methylation. As 1,3,4-tri-*O*-methyl-D-fructose can give rise to furanosides and pyranosides, it is, however, likely that the intermediate, methyl 1,3,4-tri-*O*-methyl-D-fructofuranoside, was the kinetically controlled product of methanolysis. We believe that our results represent the first chemical evidence for levans to be composed of D-fructofuranose residues.

Complete methylation of polysaccharides is often assessed from the relative proportion of the terminal units and the branching residues. However, g.l.c. did not separate the tetra-*O*-methylhexitol acetate *A* (Table I) sufficiently from materials that arise from the reagents used. Nevertheless, when the methylated fructan was subjected to another methylation-analysis procedure, the molecular proportion of the tri-*O*-methyl- to di-*O*-methyl-hexitol derivatives remained constant. It was therefore assumed that the molecular ratio of the tetra-*O*-methyl- to di-*O*-methyl-hexitol acetates was unity. It could then be calculated that the approximate numbers of terminal, non-reducing D-fructofuranosyl units and those residues linked through positions 2 and 6, and through 1, 2, and 6 in the average repeating-unit of the levan are, respectively, 1, 7, and 1.

Partial, acid hydrolysis of the fructan yielded two series (*X* and *Y*) of oligosaccharides (Table III), for each of which the plot[9] R_M versus d.p. was a straight line. Although only 11% of all D-fructofuranose residues are branching residues, a considerable proportion of the disaccharide fraction was inulobiose. It is therefore likely that the susceptibilities to acid hydrolysis of the β-(2→1)- and β-(2→6)-D-fructosidic linkages are comparable, and that the oligosaccharides of one of the above series possessed also β-(2→1)-linkages.

Similar series of oligosaccharides, with d.p. up to 8, were obtained[10] by the action of an endo-hydrolase on the levan of *Aerobacter levanicum*. The members of one series reacted with triphenyltetrazolium chloride, and it was concluded that the reducing D-fructose residue in these was linked through position 1, and that the branches through the β-(2→1)-linkage in that levan contained up to seven D-fructose residues. Triphenyltetrazolium chloride can be used to distinguish between reducing

fructose residues linked through position 1 or 6. It fails, however, to distinguish between the former and those linked through positions 1 and 6.

It was conceived that the sequence of reduction with sodium borodeuteride, methylation, hydrolysis, reduction with sodium borodeuteride, acetylation, and analysis by g.l.c.–m.s. could also be used to ascertain the position of linkage to the reducing fructose residues in the oligosaccharides obtained by partial hydrolysis with acid. The results obtained with the tri-, tetra-, and penta-saccharide fractions (it was not practicable to separate the oligosaccharides of series *X* from those of series *Y*) are shown in Table II. The tri- and penta-saccharide fractions did not give all the hexitol derivatives which might be expected. It is significant, however, that each oligosaccharide fraction gave, *inter alia*, by the above sequence of reactions, 1-*O*-acetyl-2,3,4,5,6-penta-*O*-methylhexitol-2-*d* (**2**). Therefore, each oligosaccharide fraction contained a component in which the reducing D-fructose residue was linked only through position 1 (**9**). Methylation analysis of the fructan had already shown that those D-fructofuranose residues in the fructan that are linked through position 1 are also linked through positions 2 and 6, and hence constitute branching points (**14**). The di- and oligosaccharides (with d.p. $n+1$) which are terminated by D-fructose residues linked only through position 1 (**9**) thus contain n fructose residues of a branch of the original fructan. We now conclude that the branches through the β-(2→1)-linkage in the levan of *S. salivarius* strain 51 contain up to at least four D-fructofuranose residues (**15**).

The distribution of branching residues in the levan and the type of its branched structure will be reported elsewhere.

EXPERIMENTAL

Paper chromatography. — The solvent used was ethanol–butan-1-ol–water (40:11:19). Compounds were detected with silver nitrate in acetone–ethanolic sodium hydroxide[11]. Whatman No. 1 paper was used for the analysis of mixtures, and Whatman No. 17 paper for the fractionation of larger quantities of materials.

T.l.c. — Plates coated with silica gel (Polygram Sil G) were developed with butan-1-ol–acetic acid–water (4:1:5, upper layer). Compounds were detected by spraying with sulphuric acid (5% in ethanol) and heating at 120°.

G.l.c.–mass spectrometry. — (*a*) *C.i.–m.s.* A VG Micromass 16F mass spectrometer was used with 2-methylpropane, at a pressure of 0.5 torr, as the reactant gas. The gas chromatograph (Pye 104) contained a column (3.0 m × 5.0 mm) packed with 3% of OV 225 on Chromosorb Q (80–100 mesh), and was operated with temperature programming (170→225°) at $5°.min^{-1}$.

(*b*) *E.i.–m.s.* A Perkin–Elmer F11 gas chromatograph, operating at 190° and containing a glass column (4.0 m × 1.6 mm) packed with 3% of OV 225 on Chromosorb Q (80–100 mesh), was used. The carrier gas, helium, was removed from the effluent by passage through a Biemann separator. The effluent was then passed into a Hitachi RMS-4 mass spectrometer operating at 80 eV and 50-μamp target current.

Preparation of levan. — The lyophilysed *Streptococcus salivarius* strain 51 micro-organism was reactivated at 37° for 24 h in a medium containing D-glucose (0.5%), Brain Heart Infusion concentrate (Oxoid, 5 tablets/100 ml), Thioglucolate (Difco, 2.4%), and with a bottom layer of calcium carbonate (2 g/100 ml). A suspension (~5 ml) was then used to inoculate media (1.25 l) containing sucrose (8%), dipotassium hydrogen phosphate (0.2%), and Tryptone (Difco, 1%). After incubation at 37° for 40 h, the culture fluid was centrifuged at 1000 *g* for 20 min. The levan was precipitated from the clear, supernatant liquid by the addition of 2.3 volumes of ethanol, and sedimented by centrifugation at 1000 *g* for 20 min. The levan was dissolved in water and precipitated by the addition of ethanol as before. The procedure of redissolution and reprecipitation was repeated three times. The levan was freeze-dried to a white powder, and finally dried *in vacuo* over phosphoric oxide at 60°. The levan had $[\alpha]_D^{20}$ −59.3° (*c* 1.0, water), and the average yield (based on sucrose used) was 37% [Found: fructose (determined by the method of Wise *et al.*[12]), 93.8; ash, 4.7; N, 0.5%].

Degradation of levan by invertase. — Digests contained levan (0.5%, w/v) and *Candida utilis* invertase (0.4%, w/v; Sigma, grade X) in phosphate buffer (0.1M, pH 6.6), and were incubated under toluene at 37°. The reducing sugar content, as D-fructose, was determined by the method of Nelson[13], and indicated, after 350 h, that 90% of the levan had been degraded. Chromatographic analysis of the digest revealed the presence of only D-fructose.

Methylation of levan. — Levan (20 mg) was dissolved in methyl sulphoxide (4 ml) in a MacCartney flask containing a Teflon-covered magnetic follower, and fitted with a drilled cap with a rubber seal. Sodium methylsulphinylmethanide (~2M in dimethyl sulphoxide, 1 ml) was added with the aid of a syringe, and the mixture stirred for 6 h, with warming at 40° for the first hour. Methyl iodide (0.1 ml) was added and the mixture stirred for a further 18 h. Further portions of sodium methylsulphinylmethanide (as above, 1 ml) and methyl iodide (1.5 ml) were added in the manner described. The reaction mixture was then poured into water (20 ml), and the suspension was dialysed against running tap-water and then against deionised water. The methylated levan was extracted with chloroform (2 × 10 ml) and the extract evaporated to dryness. A portion of the methylated levan was subjected to another methylation procedure.

Characterisation and determination of O-*acetyl*-O-*methylhexitols obtained from methylated levan.* — A solution of the methylated levan (~10 mg) in a mixture of methanol (10 ml) and 2.5M oxalic acid (3 ml) was boiled under reflux for 18 h. The methanol was removed by distillation under reduced pressure, water (10 ml) was added, and the solution was boiled under reflux for 4 h. After neutralisation with calcium carbonate, sodium borodeuteride (10 mg; 98% D, Merck) was added, and the solution kept for 4 h. Deionisation was effected by treatment with Dowex 50W-X8 (H^+) resin, evaporation to dryness, and repeated distillation of methanol from the residue. The residue was acetylated by using acetic anhydride (1 ml) and pyridine (1 ml). After 20 min at 90°, the solution was evaporated and the residue analysed by

combined g.l.c.–m.s. Retention times (T) and peak areas were determined separately with a Pye 104 gas chromatograph, operating at 175°, containing a glass column (3.0 m × 5.0 mm) packed with 3% of OV 225 on Chromosorb Q (80–100 mesh), using nitrogen as carrier gas, and linked to a Hewlett Packard 3370B integrator. The results are listed in Table I.

Acid hydrolysis of levan. — (*a*) The levan (~10 mg) was hydrolysed with 5mM sulphuric acid (1 ml) at room temperature for 15 h. After neutralisation (with barium carbonate), paper chromatography revealed D-fructose as the sole reducing-sugar present.

(*b*) The levan (~10 mg) was hydrolysed with 5mM oxalic acid (1 ml) at 70° for 2 h. After neutralisation (with calcium carbonate), paper chromatography of the hydrolysate revealed the components shown in Table III. Chromatography of a deionised hydrolysate of a larger sample of levan (15 g) on a charcoal–Celite column (7 × 44 cm; elution with 1, 5, 10, and 20% ethanol), with purification of the fractions thus obtained by paper chromatography, gave samples of di-, tri-, tetra-, penta-, and hexa-saccharide fractions. The yields are shown in Table III.

Characterisation of oligosaccharides obtained from the levan. — The di-, tri-, tetra- and penta-saccharide fractions (see Table III, ~10 mg), separately dissolved in water (10 ml), were reduced with sodium borodeuteride (5 mg). After deionisation (as described above), each product was desiccated and then shaken with methyl iodide (0.2 ml), *N*,*N*-dimethylformamide (0.2 ml), and silver oxide (0.2 g) at room temperature for 18 h in the dark. Each mixture was filtered through glass-fibre paper, and the filtrate evaporated to dryness. Each methylated product was dissolved in a mixture of methanol (2 ml) and 0.5M oxalic acid (0.5 ml), and the solution was boiled under reflux for 18 h. Methanol was removed by evaporation under reduced pressure, water (2 ml) was added, and heating was continued for a further 3 h. Each reaction mixture was neutralised with calcium carbonate, and the products were reduced with sodium borodeuteride and acetylated as described above. The results of analysis by g.l.c.–m.s. of the *O*-acetyl-*O*-methylhexitol-*2-d* products are shown in Table II.

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