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STRUCTURAL STUDIES OF A TEICHOIC ACID FROM *Streptococcus agalactaie* TYPE III

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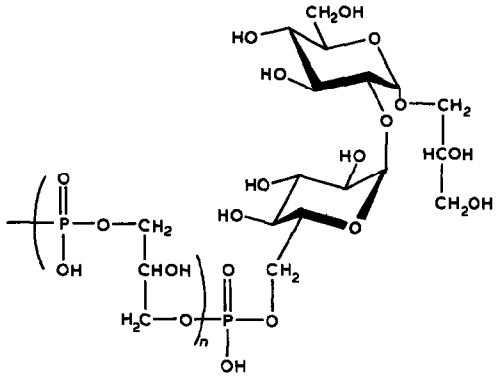
ABSTRACT

An unusual type of teichoic acid has been isolated from *Streptococcus agalactaie* type III. It has the same backbone as the lipoteichoic acid from *Streptococcus faecalis*, but is devoid of fatty acid residues, a phosphatidyl group, and substituents in the poly(glycerol phosphate) side-chain. The following structure, with $n \sim 20$, was determined mainly with the aid of n.m.r. spectroscopy.



INTRODUCTION

The lipoteichoic acids[1], also called membrane teichoic acids, of Gram-positive bacteria generally contain a chain of poly(glycerol phosphate) linked to a dihexosyl-diacylglycerol. Glycosyl residues and alanyl residues may be linked to the 2-positions in the poly(glycerol phosphate) chain and a phosphatidyl group to the disaccharide residue. The partial structure 1 has been suggested for the lipoteichoic

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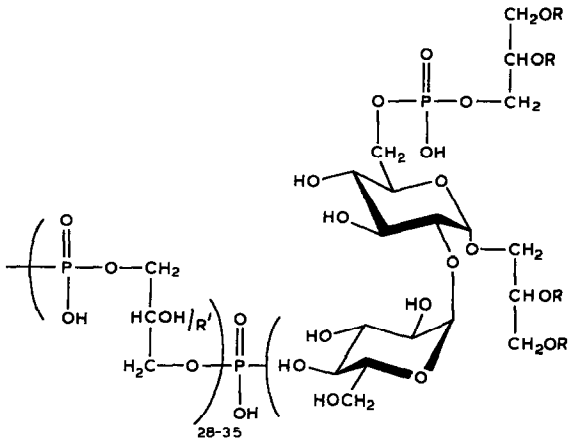
acid from *Streptococcus faecalis*[1,2]. In this structure, R stands for a fatty acid and R' for a kojibiosyl residue. It was not decided to which position the poly(glycerol phosphate) chain was linked.



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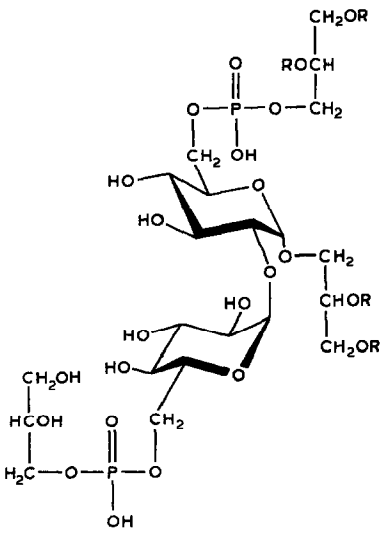
Gram-positive bacteria also contain glycolipids having the same general structure as the glycolipid part of the lipoteichoic acid. Thus, the glycolipid **2** has been isolated from *Streptococcus faecalis*[3]. Structure **2** has been fully determined and may indicate that the poly(glycerol phosphate) chain is linked to the 6'-position in the corresponding lipoteichoic acid. A similar structure is also found for the glycerophosphoglycolipids in *Streptococcus lactis*, in which the poly(glycosyl-



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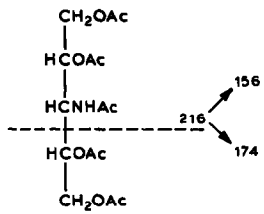
glycerol phosphate) chain is linked to the 6′-position and a fatty acid to the 6-position of the 1-*O*-α-kojibiosylglycerol residue[4].

We now report on the isolation and structural studies of a teichoic acid from *Streptococcus agalactaie* type III. Whereas *S. faecalis* belongs to the enterococcus type, with group D specificity, *S. agalactaie* belongs to group B of the pyogenic streptococci.

RESULTS AND DISCUSSION

S. agalactaie type III was fermented, the broth was concentrated and freeze-dried, and proteins were removed by partitioning between phenol and water. The aqueous phase was concentrated and fractionated on a column of DEAE-Sephacel. The column was eluted with a gradient of aqueous potassium chloride containing 0.01M TRIS acetate buffer of pH 7.8. After the group-specific and type-specific antigens, a polymer was eluted with 0.3→0.5M potassium chloride and was recovered by dialysis and freeze-drying.

The product contained approximately 30% of phosphate. An acid hydrolysate contained glycerol, D-glucose, and a component first assumed to be a 3-amino-3-deoxypentitol from the electron-impact mass spectrum of its acetate (*e.q.*, **3**). The spectrum showed a strong primary fragment at *m/z* 216 and the expected secondary fragments. It was soon found, however, that this component was the acetate (**4**) of tri(hydroxymethyl)aminomethane (TRIS) which gives the same predominating fragments and was used during the isolation of the polymer. This artefact was removed by filtering a solution of the polymer through a column of Dowex 50 (Na^+) resin. A similar observation, that contaminating TRIS may be mistaken for a sugar component, has recently been described by Robertson and Cain[5].



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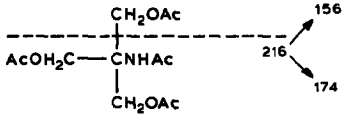

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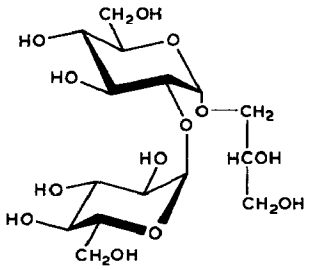
The ^{13}C-n.m.r. spectrum of the polymer demonstrated that it was a glycerol teichoic acid. The main signals given by C-1,C-3 and by C-2 of the glycerol residues were observed at δ 67.16 ($^2J_{C,P}$ 5.5 Hz) and 70.42 ($^3J_{C,P}$ 7.3 Hz), respectively. Several minor signals were also observed (see below). However, no signals for fatty acid residues were observed.

An oligosaccharide part of the teichoic acid was released by treatment with

aqueous 48% hydrogen fluoride and purified by gel-permeation chromatography. The acetylated product, available in small amounts only, was investigated by ^{1}H-n.m.r. spectroscopy at 400 MHz, with the help of two-dimensional techniques COSY and JRES. The chemical shifts and coupling constants of the protons (Table I) identified the substance as acetylated 1-*O*-α-kojibiosylglycerol (**5**). The only structural feature not determined was the absolute configuration of the glycerol residue. Pieringer[6], however, has demonstrated that enzyme preparations from *S. faecalis* catalyse the synthesis of 1,2-di-*O*-acyl-3-*O*-α-kojibiosyl-*sn*-glycerol[7], and it seems probable that the glycerol residue in **5** has the same configuration.



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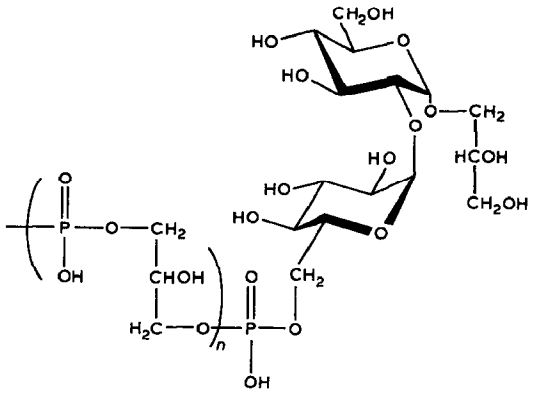

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The signals in the ^{13}C-n.m.r. spectrum of methyl α-kojibioside have been fully assigned[8] and the signals in the spectrum of the 1-*O*-α-kojibiosylglycerol could be assigned by analogy (Table II). Several signals in the spectrum of the teichoic acid could also be assigned. Because of the small shift differences, it was not possible, by comparison with model substances, to decide if the signals given by the phosphorylated moiety, at δ 65.24 ($^2J_{C,P}$ 4.3 Hz) and 71.85 ($^3J_{C,P}$ 7.9 Hz), derive from C-6,C-5 or from C-6′,C-5′ of the kojibiosyl residue.

In order to obtain further evidence, the ^{1}H-n.m.r. spectra of the teichoic

TABLE I

^{1}H-N.M.R. DATA[a] ($CDCl_3$) OF THE ACETYLATED OLIGOSACCHARIDE OBTAINED FROM THE TEICHOIC ACID BY DEPHOSPHORYLATION WITH AQUEOUS 48% HYDROGEN FLUORIDE AND ACETYLATION

Sugar residue	*H-1*	*H-1'*	*H-2*	*H-3*	*H-3'*	*H-4*	*H-5*	*H-6*	*H-6'*
α-D-Glc*p*-(1→	5.190		4.817	5.356		5.068	4.039	4.200	4.145
	(3.7)		(10.2)	(9.5)		(10.2)	(3.5;2.6)	(−12.5)	
→2)-α-D-Glc*p*-(1→	4.966		3.764	5.423		4.978	4.020	4.286	4.059
	(3.9)		(9.9)	(9.5)		(10.0)	(4.2;2.3)	(−12.2)	
→1)-glycerol	3.701	3.860	5.225	4.417	4.239				
	(−10.7;5.1)	(5.1)		(−12.4;3.8)	(5.9)				

[a]Chemical shifts are given relative to internal tetramethylsilane. Coupling constants in parentheses, within ±0.2 Hz.

TABLE II

SIGNALS IN THE ^{13}C-N.M.R. SPECTRA OF THE TEICHOIC ACID FROM *S. agalactaie* AND RELEVANT REFERENCE SUBSTANCES[a]

	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*
Teichoic acid (pD 7)						
Poly(glycerol phosphate)	67.16	70.42	67.16			
	(5.5)	(7.3)				
Glycerol 1-phosphate (term.)	67.40	71.64	63.21			
	(5.5)	(7.3)				
Phosphate-6-α-D-Glc*p*-(1→	97.29	72.18	73.70	~70.5[b]	71.85	65.24
					(7.9)	(4.3)
→2)-α-D-Glc*p*→	97.03	76.84	72.69	70.71	72.44	61.69
→1)-glycerol	63.58	71.49	69.96			
α-D-Glc*p*-(1→	97.15	72.25	73.84	70.68	72.42	61.64
→2)-α-D-Glc*p*-(1→	97.01	76.65	72.68	70.56	72.87	61.55
→1)-glycerol	69.97	71.50	63.58			
α-D-Glc*p*-(1→	97.66	72.33	73.86	70.80	72.41	61.74
→2)-α-D-Glc*p*-1-OMe[c]	97.51	77.04	72.55	70.68	72.91	61.65
α-D-Glc*p* 6-phosphate (pD 4)	93.04	72.42	73.55	70.37	71.47	65.04
					(7.7)	(5.1)
α-D-Glc*p*	92.99	72.47	73.78	70.71	72.37	61.84
Glycerol 1-phosphate (pD 4)	66.92	71.76	63.27			
	(4.9)	(6.8)				
Glycerol 1-phosphate (pD 7)	65.86	72.34	63.55			
	(4.4)	(5.9)				
Glycerol	63.36	72.91	63.36			

[a]The spectra were obtained at 70° for solutions in D_2O and the values expressed in p.p.m. downfield from tetramethylsilane, referenced indirectly to internal 1,4-dioxane (67.40). Coupling constants are given in parentheses. [b]Overlapping signals. [c]Ref. 8. The methoxyl carbon gives a signal at δ 55.77.

acid, 1-*O*-α-kojibiosylglycerol, α-D-glucopyranose 6-phosphate, and α-D-glucopyranose were compared (Table III). In the spectrum of the teichoic acid, only chemical shifts of signals given by the ring protons of the D-glucopyranosyl residues could be obtained, due to overlapping signals given by the poly(glycerol phosphate) chain. Introduction of a phosphate group in the 6-position of an α-D-glucopyranose influences the chemical shifts of H-4 and H-5 by +0.07 and +0.10 p.p.m., respectively. Corresponding downfield shifts (+0.12 and +0.14 p.p.m.) are observed for the terminal glucopyranosyl group, whereas the chemical shifts for H-4 and H-5 of the 2-linked glucopyranosyl residue are almost unaffected. Comparison of the chemical shifts of unsubstituted and phosphorylated residues thus shows that the poly(glycerol phosphate) chain is linked to O-6′ of the kojibiosyl residue, as in structure **6**. This is the same position as in the glycolipid **2**, isolated from *S. faecalis*[3]. Integration of signals in the ^{1}H-, ^{13}C-, and ^{31}P-n.m.r. spectra of the teichoic acid indicates that *n* in structure **6** is ~20. Information on the structure of the terminal glycerol group of the poly(glycerol phosphate) chain could be obtained from the ^{13}C- and ^{31}P-n.m.r. spectra. Comparison of the values found for the signals at δ 63.21, 67.40 ($J_{C,P}$ 5.5 Hz), and 71.64 ($J_{C,P}$ 7.3 Hz) with those for the monosodium

TABLE III

^{1}H-N.M.R. CHEMICAL SHIFTS[a] OF SOME PERTINENT SIGNALS GIVEN BY THE TEICHOIC ACID FROM *S. agalactaie* AND SOME RELEVANT MODEL SUBSTANCES

Sugar residue	*H-1*	*H-2*	*H-3*	*H-4*	*H-5*	*H-6*	*H-6'*
Teichoic acid							
α-D-Glc*p*-(1→	5.11	3.63	3.81	3.57	4.05	—	—
→2)-α-D-Glc*p*-(1→	5.17	3.72	3.85	3.49	3.70	—	—
1-*O*-α-Kojibiosylglycerol[b]							
α-D-Glc*p*-(1→	5.08	3.58	3.78	3.45	3.91	3.78	3.86
→2)-α-D-Glc*p*-(1→	5.15	3.69	3.85	3.47	3.70	3.77	3.88
α-D-Glucopyranosyl 6-phosphate[c]	5.23	3.56	3.73	3.50	3.94	4.08	4.08
α-D-Glc*p*	5.23	3.54	3.72	3.43	3.84	3.76	3.84

[a]Spectra were obtained for solutions in D_2O at 60°, and assignments were based on JRES, COSY, and relayed COSY spectra. [b]Signals for the glycerol part were obtained at δ 3.54, 3.83 (CH_2); 3.96 (CH); and 3.61, 3.69 (CH_2). [c]Monosodium salt.

salt of glycerol 1-phosphate (pD 4) at δ 63.27, 66.92 ($J_{C,P}$ 4.9 Hz), and 71.76 ($J_{C,P}$ 6.8 Hz) indicates that the poly(glycerol phosphate) chain is terminated by a monosubstituted, and not by a disubstituted, glycerol residue. This conclusion is shown by the occurrence of the uncoupled signal at δ 63.21, originating from the unphosphorylated hydroxymethyl group of the terminal glycerol group. In the ^{31}P-n.m.r. spectrum, a minor signal at 17.8 p.p.m. downfield of the main diphosphoryl signal shows that only a small part of the chains terminates with a cyclic phosphate group. This group had probably been formed by cleavage of the chain during the isolation of the teichoic acid. Similar cyclic phosphates were observed during depolymerisation of capsular antigens from *Haemophilus influenzae*[9].

The isolation of a teichoic acid from *S. agalactaie* with the same backbone as lipoteichoic acids from other *Streptococcus* species, but without fatty acid residues, a phosphatidyl group, and substituents in the poly(glycerol phosphate) chain, is an unexpected result. It is possible that this teichoic acid is an intermediate in the biosynthesis. It may, however, be an enzymic degradation product of a complete lipoteichoic acid, although this alternative seems less likely. The lack of lipophilic groups could explain its ability to be excluded from the cell wall into the surrounding medium.

EXPERIMENTAL

General methods. — Concentrations were performed under reduced pressure at bath temperatures below 40°. G.l.c. was performed on SE-54 fused-silica capillary columns. G.l.c.–m.s. was performed with a Varian MAT-311A instrument, using an SE-54 fused-silica capillary column. The n.m.r. spectra were recorded at 400 (^{1}H) and 100 MHz (^{13}C) with a JEOL GX-400 instrument, using internal tetramethylsilane (^{1}H, $CDCl_3$), sodium 3-trimethylsilylpropanoate-d_4 (^{1}H,

D_2O), or 1,4-dioxane (^{13}C; δ 67.40; D_2O) as references. Two-dimensional spectra, JRES, COSY, and relayed COSY, were obtained using a data matrix of (1K × 256) and (1K × 512) datapoints, respectively.

Isolation of S. agalactaie *teichoic acid.* — Cultures (70 L) of *S. agalactiae* type III, strain 878, were inactivated with aqueous 0.5% formaldehyde, and the supernatant solution was separated by continuous flow centrifugation, filtered through a 0.22-μm membrane, and concentrated twenty-fold by ultrafiltration. Four volumes of ethanol were added, and the precipitate was allowed to settle overnight and then collected by centrifugation using a Sorvall GSA rotor at 7000 r.p.m. for 20 min. The crude material was washed with ethanol followed by acetone, dried in a desiccator, and then extracted with cold phenol as described by Gotschlich *et al.*[10]. The material was suspended at 40 mg/mL in 0.4M acetate buffer (pH 7.0) and vigorously stirred with an equal volume of a solution prepared from phenol (100 g) and 0.4M acetate buffer (pH 7.0, 40 mL). The phase separation was achieved by centrifugation at 4° as described above. The aqueous phase was carefully removed and re-extracted twice with cold phenol, each time with a centrifugation step to separate the two phases. Finally, the phenol in the aqueous phase was removed by exhaustive dialysis against water, 2M $CaCl_2$ (0.05 vol.) was added, and the polysaccharides were precipitated by addition of 4 volumes of ethanol. Again, the precipitate was washed with ethanol followed by acetone and subsequently dried over P_2O_5.

Final purification was achieved by ion-exchange chromatography. A solution of the polysaccharide fraction (691 mg) in 0.01M TRIS acetate buffer (pH 7.8, 10 mL) was subjected to a brief sonication and then filtered through a 0.22-μm membrane. The solution was then applied to a K16/32 column (bed volume, 64 mL) of DEAE-Sephacel (Pharmacia) which was eluted with a gradient prepared from TRIS acetate buffer (250 mL) with the second vessel containing 0.5M KCl (250 mL). Fractions (5 mL) were collected and analysed for hexose by the phenol–sulfuric acid procedure[11]. The teichoic acid was eluted at a KCl concentration of >0.3M, well separated from group- or type-specific polysaccharides of *S. agalactaie* which were eluted at lower concentrations of KCl. No further material emerged from the column after raising the KCl concentration to 1M. The material was recovered by dialysis and freeze-drying. The yield of teichoic acid was 437 mg, or 63% of the amount applied. Part of the material (40 mg) was further purified by passing it through a column (5 × 0.5 cm) of Dowex 50 (Na^+) resin, using water (25 mL) as solvent. Pure teichoic acid (30 mg) was obtained after freeze-drying and this material was used in the n.m.r. analysis.

Analyses. — Column effluents were analysed for sugars by the phenol–sulfuric acid procedure. Sugar composition was determined by g.l.c. after hydrolysis (4M HCl, 2 h, 100°) of the polysaccharide and preparation of the alditol acetates. The main identifiable sugar component was glucose. In addition, a component with a large peak area was also present in all chromatograms and was later identified as TRIS acetate.

Phosphate was determined according to Ames[12].

Isolation of acetylated α-kojibiosylglycerol. — Teichoic acid (30 mg) was treated with aqueous 48% hydrofluoric acid (2 mL) for 4 days at 4°. The aqueous acid was removed *in vacuo*, the product was acetylated by treatment with acetic anhydride–pyridine (2 mL; 1:1) for 30 min at 100°, the mixture was concentrated to dryness, and the residue was purified by chromatography on a column (10 × 1 cm) of silica gel irrigated with ethyl acetate–toluene (3:2). The fraction containing the acetylated oligosaccharide was concentrated to dryness and the product (1 mg) was analysed by ^{1}H-n.m.r. spectroscopy.

Isolation of α-kojibiosylglycerol. — Teichoic acid (78 mg) was dephosphorylated as described above and the oligosaccharide obtained by gel-permeation chromatography, using a column (2.5 × 80 cm) of Bio-Gel P-2 irrigated with water. The elution was monitored by diffraction refractometry. The fraction containing α-kojibiosylglycerol (3 mg) was freeze-dried.

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