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^{13}C-N.M.R. SPECTRA OF SOME RIBITOL TEICHOIC ACIDS

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ABSTRACT

Proton-decoupled ^{13}C-n.m.r. spectra were determined for D_2O solutions of several wall teichoic acids containing glycosylated ribitol 1,5-diphosphate residues and for their dephosphorylated repeating-units. Assignments were made by correlating the chemical shift values observed with those reported for isolated constituents, allowing for perturbations of the latter resonances because of the presence of O–glycosyl or phosphodiester bonds. Anomeric configurations of hexopyranosyl residues and their position of substitution on ribitol were indicated from the distinctive chemical shifts of the carbons concerned. Three-bond $^{13}C–^{31}P$ couplings (6–8 Hz) were observed, and two-bond $^{13}C–^{31}P$ couplings were indicated by broadened signals. The lack of resolution for the latter resonances is probably due to the heterogeneous nature of the polymers.

INTRODUCTION

Teichoic acids[1] are natural polymers that occur in the walls and membranes of many Gram-positive bacteria. The wall teichoic acids consist of glycerol or ribitol (and occasionally glycosyl) residues joined through phosphodiesters, with some of the remaining, free hydroxyl groups glycosylated and/or esterified by D-alanine. Native, wall teichoic acids exist in combination with peptidoglycan, and this complex is a major cell metabolite (contributing up to 60% of cell dry-weight). Water-soluble teichoic acids may be extracted from cell-wall preparations by selective hydrolysis with dilute acid or alkali, and the rationale for this specific degradation lies in the nature of the linkage region of the copolymer, which has been shown[2] to be a tris-(glycerol phosphate)–2-acetamido-2-deoxyglucosyl phosphate oligomer.

Teichoic acids are antigenic, and have important functions such as selective ion transport[3] between the environment and the interior of the cell. They also act as receptor sites[4] during attack by phage. Elucidation of their structure is therefore of fundamental importance and has been the object of considerable effort for many

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years, with such studies relying heavily on chemical and enzymic degradations, binding to lectins, *etc.* The advent of Fourier-transform ^{13}C-n.m.r. spectroscopy has greatly facilitated structural studies of intact macromolecules, often providing such valuable information as the configurations at asymmetric centres which would be difficult to obtain by other means. Thus, the structure of the teichoic acid isolated from *Bacillus subtilis* var. *niger* was shown[5] by ^{13}C-n.m.r. spectroscopy to possess the repeating-unit 1-*O*-β-D-glucopyranosylglycerol joined through phosphodiesters at the remaining positions of glycerol. Similar studies of several other bacterial antigens have also utilised[6,7] this technique, thereby providing a relatively rapid, non-destructive method of structural analysis. We now describe results obtained from some teichoic acids containing ribitol 1,5-diphosphate* repeating-structures, substituted by glycosyl residues. Results from this investigation have proved useful in our concurrent studies of such complex systems as that[8] concerning the C-polysaccharide of *Streptococcus pneumoniae*.

EXPERIMENTAL

The teichoic acids from the following organisms were prepared (as described below): I, *Bacillus subtilis* W23 (ØR M12)[9]; II, *Bacillus subtilis* W23; III, *Staphylococcus aureus* 3528; IV, *Staphylococcus aureus* H; and V, *Staphylococcus aureus* H mutant (1AA)[10].

The bacteria were grown in 15-litre batches in tryptic soy broth, with the exception of *S. aureus* H (1AA) which was grown[10] in nutrient broth/succinate. Cells were harvested after growth for 18 h and disrupted by shaking for 2.5 min with No. 11 Ballotini beads in a Braun disintegrator. Cell walls were recovered by centrifugation at 10,000*g* for 30 min at 4°. The walls were freed of membrane and other contaminants by pouring a 30% (w/v) suspension of walls into an equal volume of boiling, 4% aqueous sodium dodecyl sulphate (SDS). The purified cell-walls were washed free of SDS with 6 changes of distilled water.

The teichoic acids from *B. subtilis* W23 and *S. aureus* H were extracted by hydrolysis of the walls in 0.5M NaOH at 20° for 1 h, followed by adjustment of the pH to 7.0 with hydrochloric acid. Salt was removed by dialysis against running water. The teichoic acids from the remaining bacteria were extracted by treatment of the walls with 10% (w/v) aqueous trichloroacetic acid (TCA) for 48 h at 4°. TCA was removed by extensive washing with ethyl ether. Alanine ester residues were removed by treatment with 0.1M $(NH_4)_2CO_3$ for several hours at room temperature. Nucleic acids were removed from both the acid and alkali extracts by fractionation on a "stacked column"[11] of Sephadex G-25 and G-75, by elution with 0.2M NaCl. The phosphorus-containing fractions which did not show any absorbance at 260 nm were

*Although the rules of carbohydrate nomenclature might prefer 2-*O*-substitution of L-ribitol, D-ribitol 5-phosphate (L-ribitol 1-phosphate) is derived biosynthetically from D-ribose 5-phosphate, and it is more convenient to consider ribitol teichoic acids as polymers of D-ribitol 5-phosphate.

combined, reduced in volume, and desalted on a column of Sephadex G-25. The resulting solution of teichoic acid was then lyophilised. The dephosphorylated repeating-units were obtained by hydrolysis of the teichoic acid in M NaOH at 100° for 3 h, followed by chromatography on Dowex-50(NH_4^+) resin. The eluate was treated at pH 9 with alkaline phosphomonoesterase (Boehringer) in the presence of toluene until >90% of the phosphate was present as inorganic phosphate. Products were re-*N*-acetylated, if required, by using 5% acetic anhydride–$NaHCO_3$. Finally, the glycosylated ribitols were purified by preparative paper chromatography on acid-washed Whatman 3MM paper. ^{13}C-N.m.r. spectra (external Me_4Si) were determined at 22.63 MHz with proton decoupling for solutions in D_2O at 32° and concentrations of 80–120 mg.ml^{-1}.

DISCUSSION

Table II lists the observed and calculated shift-values (relative to external Me_4Si) of the teichoic acids and the dephosphorylated repeating-units. The calculated shifts are obtained from the reported values (Table I) of individual constituents after allowing increments for the presence of glycosidic and phosphodiester bonds.

The repeating units prepared from *B. subtilis* W23 (II) and *S. aureus* H teichoic acids (IV and V) gave well-resolved spectra (each consisting of 11 signals) having chemical shifts in close agreement with the values calculated for the 4-*O*-glycosylated D-ribitols **4** and **5**, respectively. All three spectra possessed an isolated resonance in the region 80–82 p.p.m. downfield from Me_4Si, which may be assigned to C-4 of the D-ribitol moiety, *i.e.*, the glycosylated carbon. In addition, a characteristic anomeric-carbon signal was present in each spectrum with a chemical shift in accord[12] with the β configuration for **4** and[13] the α configuration for **5**. The spectrum of **4** was identical to that previously reported[7] for 4-*O*-β-D-glucopyranosyl-D-ribitol. There was a clear indication from the spectra of the nature of substitution, *i.e.*, the anomeric configuration of the glycoside and the involvement of C-4 of D-ribitol.

As expected, the teichoic acid of *B. subtilis* W23 (ØR M12) (I), *i.e.*, poly(ribitol 1,5-diphosphate), gave three signals in its ^{13}C-n.m.r. spectrum, with the resonances due to the primary carbon atoms perturbed by 4.5 p.p.m. to lower field relative to

TABLE I

^{13}C-N.M.R. SHIFTS OF CONSTITUENTS OF RIBITOL TEICHOIC ACIDS

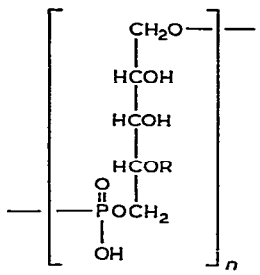
Constituent	*Chemical shifts (p.p.m. from Me_4Si)*							
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-6*	*CH_3*	*C=O*
Ribitol[7]	63.2	72.9	72.9	72.9	63.2	—	—	—
β-D-Glucopyranose[12]	97.1	75.6	77.3	71.2	77.3	62.4	—	—
2-Acetamido-2-deoxy-α-D-glucopyranose[13]	92.1	55.3	72.0	71.4	72.8	61.9	23.3	175.7

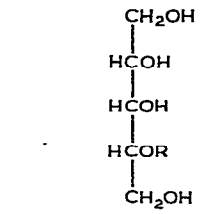
TABLE II

CALCULATED[a] AND OBSERVED ^{13}C-N.M.R. SHIFTS OF RIBITOL TEICHOIC ACIDS AND DEPHOSPHORYLATED REPEATING-UNITS

Teichoic acid		*Chemical shifts (p.p.m. from Me_4Si)*										
		C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*
I *B. subtilis* W23 (ØR M12)	Calc. for **1**	67.8	71.3	72.9	71.3	67.8						
	Obs.	67.7[b]	71.9[b]	72.4	71.9[b]	67.7[b]						
II *B. subtilis* W23	Calc. for **2**	67.8	71.3	71.9	80.3	66.8	104.1	74.6	77.3	71.2	77.3	62.4
	Obs.	67.8[b]	71.5	72.0	80.3[c]	65.8[b]	103.5	74.5	76.9	70.8	76.9	61.9
III *Staph. aureus* 3528	Calc. for **3**	67.8	71.3	71.9	80.3	66.8	99.1	54.3	72.0	71.4	72.8	61.9
	Obs.[e]	67.9[b]	71.4	72.3	78.8[d]	65.6[b]	97.6	55.1	72.3	71.4	73.2	61.9
IV *Staph. aureus* H	Obs.[e]	67.8[b]	71.4	72.1	79.2[d]	65.8[b]	97.7	55.0	72.1	71.4	73.2	61.9
V *Staph. aureus* H mutant (1AA)	Obs.[e]	67.8[b]	71.3	72.1	79.0[d]	65.6[b]	97.7	55.0	72.1	71.3	73.2	61.7
Dephosphorylated repeating-unit												
From II	Calc. for **4**	63.2	72.9	71.9	81.9	62.2	104.1	74.6	77.3	71.2	77.3	62.4
	Obs.	64.0	73.2	72.9	82.1	62.0	103.6	74.6	76.7	70.9	76.9	61.7
From IV	Obs.[e]	64.2	73.7	72.3	80.9	62.0	98.1	55.3	72.3	71.5	72.8	62.0
From V	Calc. for **5**	63.2	72.9	71.9	81.9	62.2	99.1	54.3	72.0	71.4	72.8	61.9
	Obs.[e]	63.8	73.4	71.9	80.6	61.7	97.9	55.1	71.9	71.1	72.5	61.7

[a]Chemical shifts are calculated from the reported values of individual units (see Table I) by allowing the following increments: +7 p.p.m. for the formation of a glycosidic bond at the anomeric carbon, +9 p.p.m. for the formation of a glycosidic bond at other carbons, −1 p.p.m. for the introduction of an adjacent bond[16], +4.6 p.p.m. for the formation of a phosphodiester, −1.6 p.p.m. for adjacent phosphodiester in ribitol[7]. [b]Coupled signal, coupling constant not determined. [c]Doublet, $J^{31}P-^{13}C = 5.9$ Hz. [d]Doublet, $J^{31}P-^{13}C = 7.9$ Hz. [e]Additional signals observed at ~175 (>C=O) and 23 p.p.m. ($H_3C-C\leqslant_O$).





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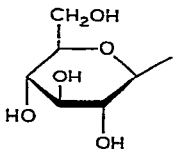

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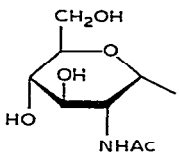

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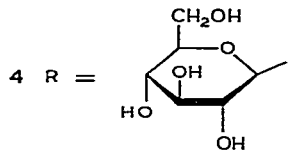

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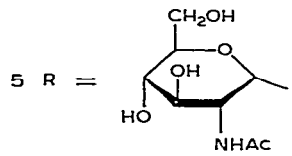

5 R =

their chemical shift in ribitol. Although coupling to phosphorus is undoubtedly present for two of the resonances, the signals were not fully resolved and therefore coupling constants were not determined. The effect of glycosylation of ribitol was seen in case II, which possesses a β-D-glucopyranosyl substituent at position 4 of the D-ribitol moiety. Relative to the unsubstituted polymer (I), the signals for C-3 and C-5 experienced small shifts to higher field, whilst that for C-4 was observed at almost 9 p.p.m. to lower field. In addition, the C-4 resonance appeared as a well-resolved doublet (J 5.9 Hz) due to three-bond coupling to phosphorus, as observed[5,7] in related systems. The β configuration of the glycoside was clearly demonstrated by the characteristic[12] signal observed for the anomeric carbon at 103.6 p.p.m. Overall, the spectrum was a composite of those observed for β-D-glucopyranose and poly-(ribitol 1,5-diphosphate), allowing for chemical-shift perturbations for those carbon atoms situated α and β to the glycosidic linkage. Similarly, the spectrum of II may be correlated with that of its repeating unit (**4**) by allowing for the change in chemical shifts of carbon atoms situated α and β to phosphodiesters.

Teichoic acid III, isolated from *S. aureus* 3528, possesses[14] the repeating-unit 4-*O*-(2-acetamido-2-deoxy-α-D-glucopyranosyl)-D-ribitol 1,5-diphosphate (**3**), and its spectrum was similar to that obtained from II inasmuch as the observed chemical shifts could be correlated with those of the constituent parts, allowing for the presence of glycosidic and phosphodiester bonds. Of particular note are the signal (Table II) for the anomeric carbon at 97.6 p.p.m., in accord[13] with the α configuration of the

glycoside, and the doublet (J 7.9 Hz) at 78.8 p.p.m. assigned to C-4 (the glycosylated carbon) of the D-ribitol moiety.

The ^{13}C-n.m.r. spectrum of teichoic acid IV from *S. aureus* H was almost identical (Table II) with that of III described above. This was somewhat surprising, since a mixture of α and β anomers of 2-acetamido-2-deoxyglucose has, on several occasions, been shown[15] to be present in specimens of IV, although the proportion of anomeric forms varied from preparation to preparation. A similar spectrum was obtained from V, which was extracted from a mutant of *S. aureus* H that had been phage-typed to ensure its authenticity. Neither for IV nor V was there any indication (in their spectra) of the presence of β-linked hexosamine residues, which, if present, would have been expected to give rise to characteristic resonances. For example, signals having chemical shifts of $\sim$103 (C-1), 58 (C-2), and 77 p.p.m. (C-5) should be present[13] from 2-acetamido-2-deoxy-β-D-glucosyl residues; however, these regions of the spectra of IV and V were devoid of any absorption. It therefore appears that our preparations contained almost exclusively α-linked hexosamine residues. This fact is borne out by the spectra obtained from the dephosphorylated repeating-units of IV and V (described earlier), which also showed the presence of only α-linked 2-amino-2-deoxyglucosyl residues.

The interpretation of spectra obtained from the glycosylated teichoic acids (II–V) has assumed complete substitution at C-4 of D-ribitol, which is not necessarily the case. *B. subtilis* W23 teichoic acid, for example, contained only 65–70% of the glucose required for complete substitution. However, the additional presence of ribitol 1,5-diphosphate units should have relatively small effects on the spectra described here; additional resonances would be accommodated within those already described, whilst heterogeneity due to incomplete glycosidation, together with variable chain-length of the polymers, may account for the observed broadening of some signals.

Finally, it should be noted that 2- and 4-*O*-glycosylation of D-ribitol would result in exactly the same spectra. It is therefore not possible to differentiate between these positional isomers by ^{13}C-n.m.r. spectroscopy.

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