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Note

Synthesis of 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-ribitol, antigenic determinant of *Staphylococcus aureus*

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Teichoic acids, the main constituents of the cell wall of Gram-positive bacteria, are responsible, among other properties, for the serological specificities of these organisms. The group-antigenic determinant of *Staphylococcus aureus* is a polymer composed of 4-*O*-(2-acetamido-2-deoxy-D-glucopyranosyl)-D-ribitol phosphate, in which repeating units are linked by phosphoric acid diester bonds. Both α- and β-D-glycosidic bonds are found in the disaccharide with predominance of β-D linkages[1,2]. Although these antigens have been exhaustively studied, no synthesis of the 4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-ribitol (**9**) antigenic determinant has been reported to date, except by Baddiley *et al.*[3], in order to confirm the occurrence of this disaccharide repeating unit in the natural polymer. Nevertheless, the structural proof was established only by comparison with the disaccharide obtained by hydrolysis of the natural teichoic acid, and the yields obtained were limited and did not allow the synthesis of large amounts of **9**. The unambiguous determination of structure of this product and the need of artificial antigens and immunoadsorbents having the same specificity, in order to study the group and type antigens of *S. aureus*, prompted us to realize an efficient synthesis of **9**, and to observe its inhibitory properties against the immune complex: poly-[4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-ribitol phosphate] ("poly Aβ")–anti-"poly Aβ".

The key step of the synthesis was the modified oxazoline method recently described by Kiso and Anderson[4,5] In our hands, this method was the most efficient (51% yield), as compared with the Koenigs–Knorr reaction (or Helferich modification) between 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[6] with **3** (best yield, 14%), or as compared with the "oxazoline method" described by Zurabyan and Khorlin[7] between **2** (ref. 8) and **3** (best yield, 29% for a five-fold excess of **2**). The method of Kiso and Anderson[4,5] was preferred to the phthalimido

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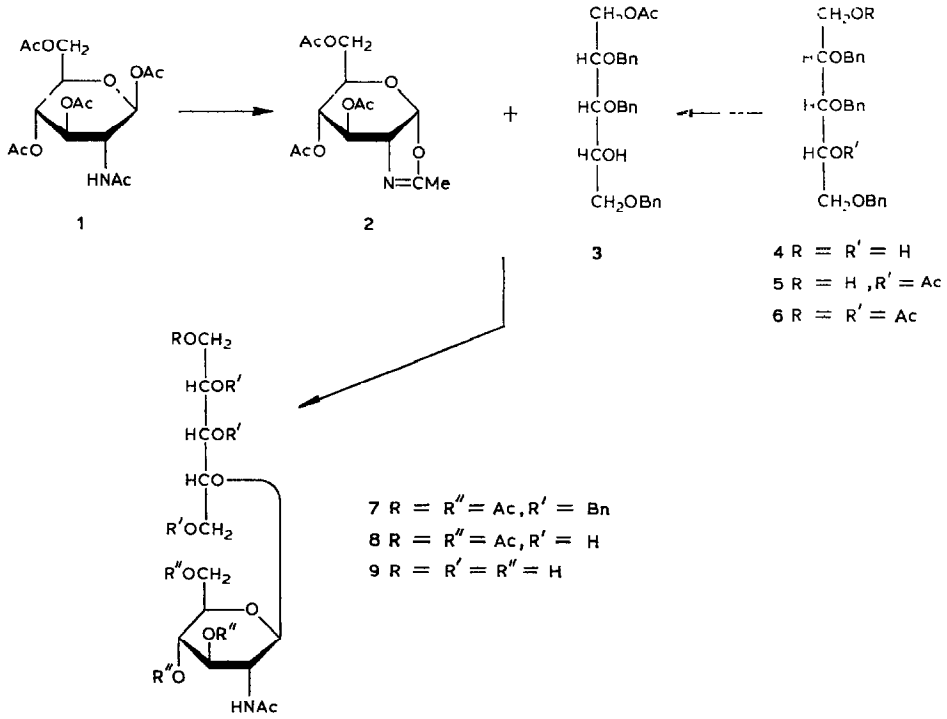

procedure[9] for the synthesis of **7** because of the fewer steps required in this reaction pathway. An improvement on the procedure of Kiso and Anderson[4,5] was obtained, in our case, by heating at reflux a dichloroethane solution (no reaction occurred after one week in dichloromethane at room temperature) and using a lower amount of iron trichloride as catalyst.

TABLE I

^{13}C-N.M.R. CHEMICAL SHIFTS (δ) OF RIBITOL INTERMEDIATES AND DISACCHARIDES[a]

Compounds	*Ribitol residue*					*2-Acetamido-2-deoxy-D-glucopyranosyl group*					
	C-1	*C-2*	*C-3*	*C-4*	*C-5*	*C-1'*	*C-2'*	*C-3'*	*C-4'*	*C-5'*	*C-6'*
Ribitol[b,c]	63.2	72.9	72.9	72.9	63.2						
3	64.5	78.6	80.4	70.9	72.6						
4	61.3	80.1[d]	80.5[d]	70.7	72.0						
5	60.8	79.8	78.4	72.8	68.9						
6	63.1	77.2[d]	78.1[d]	72.3	68.9						
7	62.9[d]	77.8[e]	78.5[e]	79.0[e]	70.5	100.8	55.1	73.2[f]	69.8	72.1[f]	63.6[d]
8[g]	65.7	68.7	68.7	80.5	60.6[d]	100.2	53.7	72.7[e]	68.7	72.1[e]	62.1[d]
9[b]	63.8	72.7[d]	72.6[d]	82.2	61.8	102.4	56.9	74.8	71.0	76.8	61.6
"Poly Aβ"[b]	68.4	71.1	72.6	80.6	66.4	102.4	56.9	74.8	71.0	76.8	61.6

[a]Spectra recorded for solution in acetone-d_6, unless otherwise stated, containing Me_4Si as internal standard. [b]For a solution in D_2O. [c]See Tarelli and Coley[12]. [d,e,f]Assignments could be inverted. [g]For a solution in $(CD_3)_2SO$.

Compound **1** is easily obtained as a crystalline material from 2-acetamido-2-deoxy-D-glucose by a two-step procedure[10] with a 72% overall yield. Compound **4** was prepared from D-ribose, by a slight modification of the procedure described by Austin *et al.*[11], in four steps (overall yield, 70%). The selective acetylation of **4** at low temperature gave **3** almost quantitatively. Its structure was ascertained by the formation of **5** and **6**, the latter compound being obtained by complete acetylation of **4**. The 4-*O*-acetyl-D-ribitol derivative **5** was obtained by tritylation of **4**, acetylation of the intermediate, and detritylation. The ^{13}C-n.m.r. assignments of **3–6** were in accordance with the expected structures (see Table I).

The disaccharide **7**, obtained in a crystalline form, was first debenzylated in acetic acid with 5% palladium-on-charcoal to give, in 90% yield, crystalline **8**, which was *O*-deacetylated with ammonia in methanol to yield **9** (90% yield). The ^{13}C-n.m.r. of **9** was in accordance with the results of Tarelli and Coley[12], and with the ^{13}C-n.m.r. spectrum of the natural teichoic acid from *S. aureus*.

Thus, the synthesis of the *S. aureus* antigenic determinant **9** was obtained with a 42% overall yield from the readily available starting materials **1** and **3**. This synthetic route constitutes a real improvement on the procedure described in the literature (~3% overall yield)[3], and afforded the amount of material necessary for the synthesis of artificial antigens related to *S. aureus*.

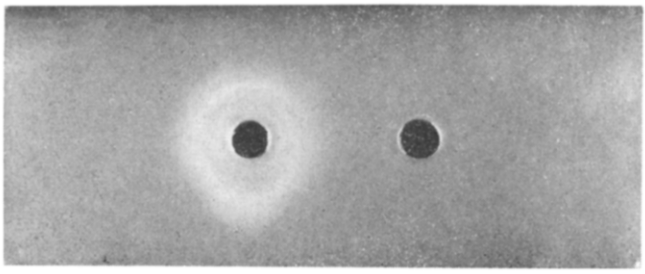

Fig. 1. One-dimensional gel immunodiffusion of ribitol teichoic acid against antiribitol teichoic serum inhibited by synthetic disaccharide **9**. The agar gel contained a 1:10 dilution of antiribitol teichoic acid serum: Left well, ribitol teichoic acid (12 μL, 0.125 g/L); right well, disaccharide **9** (12 μL, 0.5 mol/L).

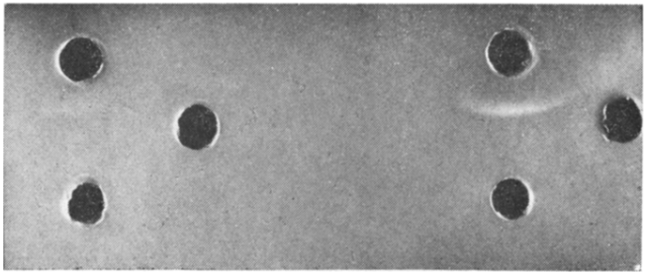

Fig. 2. Two-dimensional gel immunodiffusion of ribitol teichoic acid against antiribitol teichoic acid serum inhibited by synthetic disaccharide **9**: Top well, antiribitol teichoic acid serum diluted twice (12 μL); bottom well, ribitol teichoic acid (12 μL, 0.125 g/L); middle well, disaccharide **9** (12 μL), left (0.5 mol/L), right (0.03 mol/L).

The synthetic disaccharide **9** was tested by one- and two-dimensional immunodiffusion, as an inhibitor against the immunological system constituted by natural ribitol teichoic acid from *S. aureus* ("poly Aβ") and its specific antibodies raised in rabbits (see Figs. 1 and 2). As expected, immunoprecipitation occurred only between the serum and the ribitol teichoic acid, and not between the serum and synthetic **9**. A high concentration of **9** (0.5M) modified the appearance of the precipitation line in both gel-immunodiffusion experiments. Nevertheless, if almost no inhibition of precipitation was observed with a low concentration of **9** (30mM, Fig. 2), no inhibition at all was observed with 2-acetamido-2-deoxy-D-glucose at any concentration. This result ascertains that **9** is the antigenic immunodominant group of *S. aureus*.

EXPERIMENTAL

Chemical methods. — Melting points of compounds in capillary tubes were determined with a Büchi apparatus and are uncorrected. Optical rotations were measured for solutions in 1-dm tubes with a Perkin–Elmer 141 polarimeter. ^{13}C-N.m.r. spectra were recorded with a Varian XL-100 spectrometer operating at 25.2 MHz (internal Me_4Si). Column chromatographies were performed on silica gel Merck (230–400 mesh).

1-O-Acetyl-2,3,5-tri-O-benzyl-D-ribitol (**3**). — A solution of 2,3,5-tri-*O*-benzyl-D-ribitol[11] (**4**) (13.4 g, 31.7 mmol) in dry, alcohol-free dichloromethane (200 mL) containing dry pyridine (50 mL) was cooled to −78°. A stoichiometric amount of acetyl chloride (2.25 mL, 31.7 mmol) was added through a syringe, dropwise, over a 2-h period. After the mixture had been stirred for 3 h at −78°, methanol (1 mL) was added to stop the reaction. The mixture was allowed to reach room temperature, and then poured onto crushed ice (200 g). Pyridine was neutralized with M hydrochloric acid, and the organic extract washed once with water (100 mL) before being dried (sodium sulfate) and evaporated. The resulting oily material was chromatographed on silica gel (eluent: 1:2, v/v, ethyl acetate–hexane) to remove the unreacted material (3%), and **3** was recovered in an almost quantitative yield as a clear syrup, $[\alpha]_D^{20}$ +1.4° (*c* 5.0, chloroform); ^{13}C-n.m.r., see Table I.

Anal. Calc. for $C_{28}H_{32}O_6$: C, 72.39; H, 6.94. Found: C, 72.22; H, 6.83.

4-O-Acetyl-2,3,5-tri-O-benzyl-D-ribitol (**5**). — A solution of **4** (ref. 11, 18.0 g, 42.6 mmol) in pyridine (60 mL) was treated with chlorotriphenylmethane (12.0 g, 43.0 mmol) overnight at room temperature. Acetic anhydride (20 mL) was added and the mixture extracted after 12 h at room temperature. The unstable product obtained after washing and drying was immediately redissolved in dichloromethane (100 mL). Detritylation was performed at low temperature (−78°) by addition of a saturated solution of hydrogen bromide in acetic acid (7.7 mL, 45.0 mmol). After 15 min, the solution was poured onto water (300 mL) and rapidly neutralized with sodium carbonate. When the solution was neutral, the product was stable at room temperature, and the solution could be dried and evaporated without acetyl group migration. The dark-red mixture thus obtained was chromatographed on a silica

gel column (eluent: 1:4, v/v, ethyl acetate–hexane) to give **5** as a clear syrup (13.2 g, 66%), $[\alpha]_D^{23}$ −5.0° (*c* 7.3, chloroform); ^{13}C-n.m.r., see Table I.

Anal. Calc. for $C_{28}H_{32}O_6$: C, 72.39; H, 6.94. Found: C, 72.05; H, 6.78.

1,4-Di-O-acetyl-2,3,5-tri-O-benzyl-D-ribitol (**6**). — Compound **4** (ref. 11, 495 mg, 1.17 mmol) was acetylated overnight at room temperature in a mixture of pyridine (4 mL) and acetic anhydride (3 mL). The mixture was extracted as usual, and **6** was obtained after column chromatography (eluent: 1:2, v/v, ethyl acetate–hexane) as a clear syrup (422 mg, 71%), $[\alpha]_D^{23}$ −16.0° (*c* 4.1, chloroform); ^{13}C-n.m.r., see Table I.

Anal. Calc. for $C_{30}H_{34}O_7$: C, 71.13; H, 6.77. Found: C, 70.82; H, 6.64.

4-O-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1-O-acetyl-2,3,5-tri-O-benzyl-D-ribitol (**7**). — A mixture of **3** (621 mg, 1.34 mmol), 2-acetamido-1,3,4,6-tetra-*O*-acetyl-2-deoxy-β-D-glucopyranose[10] (**1**) (833 mg, 2.14 mmol), iron trichloride (271 mg, 1.67 mmol), and Drierite (800 mg) was dried under vacuum for 1 h at 60°. Dry, ethanol-free dichloroethane (12 mL) was introduced through a syringe into the flask, and the mixture gently boiled under reflux while being stirred. After 24 h, compound **1** (833 mg, 2.14 mmol, in the minimum amount of dichloroethane) was added again into the flask, and boiling under reflux continued; the same operation was repeated after 24 h, and the reaction stopped 48 h after the last addition. The cooled mixture was filtered, and the filtrate washed twice with water (20 mL), dried (sodium sulfate), and evaporated. The brown residue was chromatographed on silica gel (eluent: 1:1, v/v, ethyl acetate–ether). The main fraction crystallized after a few days to give **7** (542 mg, 51%), m.p. 81° (petroleum ether), $[\alpha]_D^{20}$ −15.3° (*c* 2.6, chloroform); ^{13}C-n.m.r., see Table I.

Anal. Calc. for $C_{42}H_{51}O_{14}N$: C, 63.55; H, 6.48; N, 1.76. Found: C, 63.59; H, 6.42; N, 1.68.

4-O-(2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1-O-acetyl-D-ribitol (**8**). — Compound **7** (500 mg, 0.63 mmol), dissolved in acetic acid (15 mL), was hydrogenolyzed in the presence of 5% palladium-on-charcoal (50 mg) at atmospheric pressure for 48 h. The mixture was filtered, and the filtrate evaporated. Toluene (25 mL) was added and evaporated twice from the residue, which was purified on a small silica gel column (eluent: 6:1, v/v, ethyl acetate–methanol); it crystallized after a few days. Recrystallization from methanol afforded **8** (300 mg, 91%), m.p. 162–163°, $[\alpha]_D^{20}$ −24.3° (*c* 2.0, methanol); ^{13}C-n.m.r., see Table I.

Anal. Calc. for $C_{21}H_{33}O_4N$: C, 48.18; H, 6.35; N, 2.68. Found: C, 48.36; H, 6.21; N, 2.59.

4-O-(2-Acetamido-2-deoxy-β-D-glucopyranosyl)-D-ribitol (**9**). — A solution of **8** (300 mg, 0.57 mmol) in dry methanol (3 mL) was cooled to ~0° and saturated with dry ammonia for 1 h. After being kept with stirring overnight at 4°, the mixture was evaporated to dryness, and three times, methanol (5 mL) was added to and evaporated from the residue, which was chromatographed on silica gel (eluent: 1:1, v/v, ethyl acetate–methanol). The pure oily **9** thus recovered (180 mg, 89%),

was freeze-dried to leave a white powder, m.p. 85–87°; $[\alpha]_D^{20}$ −11.6° (*c* 2.1, water); ^{13}C-n.m.r., see Table I.

Anal. Calc. for $C_{13}H_{25}NO_{10} \cdot 0.5\ H_2O$: C, 42.85; H, 7.19; N, 3.84. Found: C, 42.88; H, 7.49; N, 3.76.

Immunological methods. — Two-dimensional gel immunodiffusion was performed according to a modified Ouchterlony procedure[13]: buffered agar (1% agar in Tris · HCl, pH 7.2) was poured on microscope slides and allowed to gelify. Three 3-mm diameter wells, separated by 10 mm from each other and thus forming an equilateral triangle, were punched into the gel and filled, respectively with: (a) anti-ribitol teichoic acid serum (12 μL), prepared according to Oeding[14] and diluted twice; (b) ribitol teichoic acid (12 μL, 0.125 g/L); and (c) disaccharide **9** (12 μL, from 0.03 to 0.5 mol/L (Fig. 2). Reading was done after 24 h at 4°. Immunoprecipitation occurred as a bow-shaped line, and inhibition of the precipitation as a shortening of the precipitation line near the inhibitor well (Fig. 2).

In one-dimensional immunodiffusion, the gel was prepared by adding anti-ribitol teichoic acid serum in buffered agar at a 1:10 concentration. The gel was poured on the glass and allowed to gelify. Two wells were cut 1 cm apart and filled, respectively, with: (a) ribitol teichoic acid (12 μL, 0.125 g/L), and (b) disaccharide **9** (12 μL, from 0.03 to 0.5 mol/L). Reading was done after 24 h at 4°. Immunoprecipitation occurred as a circular line surrounding the hole containing the antigen, and inhibition of precipitation as a fading of this precipitation line near the well containing the inhibitor (Fig. 1).

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