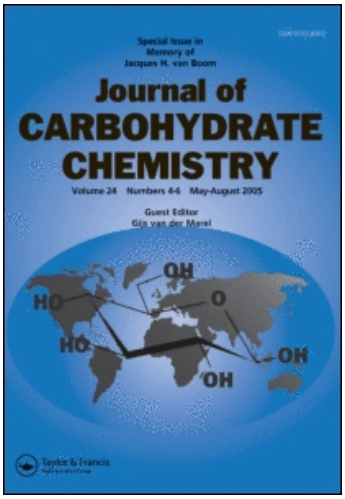

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STAPHYLOCOCCUS AUREUS ANTIGENS. PART III [1] : SYNTHESIS OF ARTIFICIAL DISACCHARIDIC ANTIGENS.

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ABSTRACT

Two artificial antigens related to S.aureus have been synthesized to elucidate the influence of the phosphodiester bond in the immunogenicity of the natural ribitol teichoic acid.

INTRODUCTION

Group antigens of S.aureus are teichoic acids composed of 4-O-(2-acetamido-2-deoxy-D-glucopyranosyl)-D-ribitol phosphate with disaccharide units linked by phosphoric diester bonds [2] (Scheme 1). Both anomeric configurations are encountered in the natural antigens with predominance of β-D-linkages.[2,3]

Before undertaking the synthesis of artificial antigens related to S.aureus, we first investigated the immunological properties of synthetic disaccharides 1 and 2 to confirm the pioneering results of J. Baddiley et al.[4] and to demonstrate that compounds 1 and 2 are the immunodominant components of the so called Aβ and Aα polysaccharides.[1,5]

The difficulties in the purification of the natural antigen and its low immunogenicity [6] were expected to be avoided with the synthesis of artificial antigens of similar specificities.

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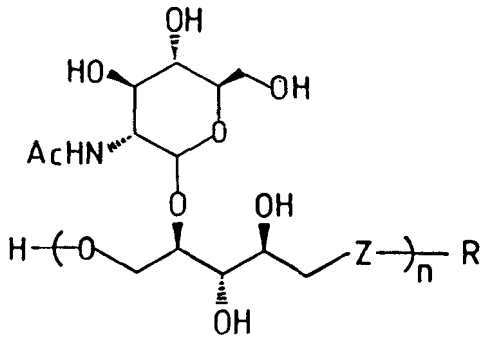


α-anomer : A-α polysaccharide Z = OPO_2^-, n = 20-50, R = OH

2 Z = O, n = 1, R = H

β-anomer : A-β polysaccharide Z = OPO_2^-, n = 20-50, R = OH

1 Z = O, n = 1, R = H

3 Z = $O(CH_2)_{10}CONH$, n = 30,40

R = BSA

4 Z = $OPO_2^-(CH_2)_8CONH$, n = 10-15

R = BSA

SCHEME 1

Since the role of the phosphate group in the immunogenicity of teichoic acids was not perfectly understood,[7] we undertook the synthesis of two artificial antigens 3 and 4 related to A β polysaccharide, but with 3 lacking the phosphodiester function.

RESULTS AND DISCUSSION

The coupling of disaccharide units to a carrier protein was realized with the 8-methoxycarbonyl group as a bridging arm [8] because of this group limited recognition[9] for immunization. This approach was used to obtain artificial conjugates from the disaccharide 1 corresponding to the major S.aureus antigen.

The reported strategy for the synthesis of **1**[5] had to be reinvestigated because it did not allow the coupling of the spacer on C-1 of the ribitol residue.

The expected haptens were obtained by two different routes (Scheme 2). In the first case, the bridging arm was introduced on C-1 of the ribitol residue before the glycosidation reaction, in the second case, after the glycosidation step.

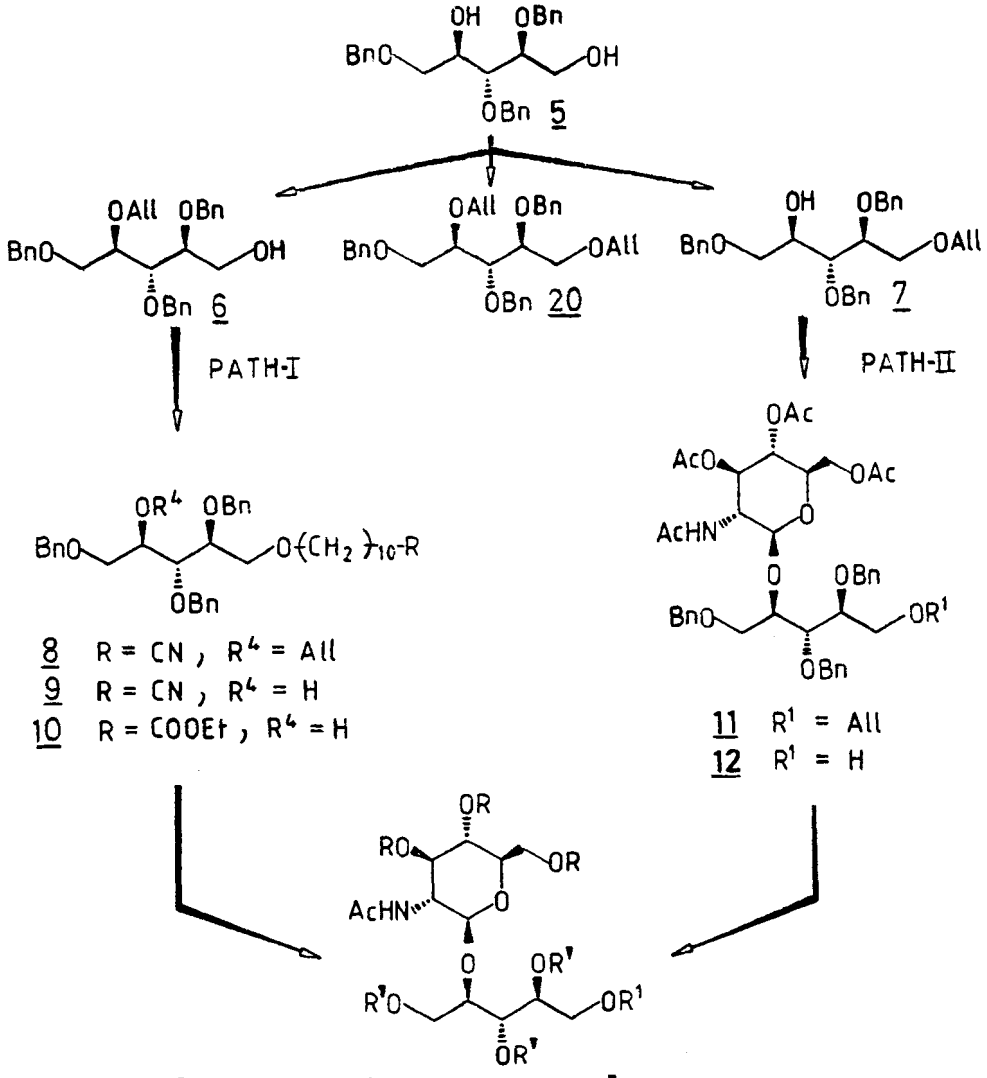
This strategy requires the occurence of a selectively - removable protecting group on C-4 (path I) or C-1 (path II) of the ribitol residue. Despite the poor regioselectivity of protection, the allyl group was chosen because of its good stability (no migration during the glycosidation as in the case of esters) , good selectivity of cleavage (in presence of other protective groups used in this work) and ease of separation of regioisomers **6** and **7**.

Treatment of 2,3,5-tri-O-benzyl-D-ribitol (**5**)[5] in DMF at room temperature with one equivalent of allyl chloride using sodium hydroxide as the base gave almost completely monoallylated compounds **6** and **7**, separated by high performance liquid chromatography, and recovered with 27% and 45% overall yields respectively.

The minor compound **6** was used in path I (Scheme 2). The direct etherification of **6** with 10-ethoxycarbonyl decanol in alkaline medium was unsuccessful, but compound **10** was obtained from **9** by the Pinner reaction with **6** and 11-bromoundecanitrile to give **8** (78% yield). The cleavage of the allyl protective group to **9** followed by treatment with ethanolic hydrogen chloride gave compound **10** , after hydrolysis (42% yield starting from **8**) . The structures of ribitol derivatives **5** to **10** were confirmed mainly by ^{13}C NMR (Table 1).

Glycosidation was then performed with the oxazoline prepared "in situ" from peracetylated glucosamine,[10] as already used for the synthesis of **1**,[5] to give the fully-protected hapten **13** (46% yield).

The major monoallylated compound **7** was used in path II (Scheme 2), with the same glycosidation procedure as previously mentionned, to give disaccharide **11** (71% yield). After deprotection of O-1 to give **12**, the condensation with 8-methoxycarbonyl octanol [8] was performed through a phosphite intermediate obtained with trichloroethylphosphorodi-



10 → 13 $R^1 = (CH_2)_{10}COOEt, R = Ac, R' = Bn$
15 $R^1 = (CH_2)_{10}COOEt, R = Ac, R' = H$
16 $R^1 = (CH_2)_{10}COOMe, R = H, R' = H$
12 → 14 $R^1 = PO(OCH_2CCl_3)O(CH_2)_8COOMe, R = Ac, R' = Bn$
17 $R^1 = PO(O^-)O(CH_2)_8COOMe, R = Ac, R' = Bn$
18 $R^1 = PO(O^-)O(CH_2)_8COOMe, R = Ac, R' = H$
19 $R^1 = PO(O^-)O(CH_2)_8COOMe, R = H, R' = H$

SCHEME 2

TABLE 1
^{13}C NMR Chemical Shifts (δ, ppm) of Ribitol Derivatives [a]

	C1	C2	C3	C4	C5	Allyl	
5	61.3	80.1[b]	80.5[b]	70.7	72.0	———	
6	61.5	80.4	79.1[b]	79.4[b]	70.8	71.5/115.7/135.9	
7	70.8	79.4[b]	80.4[b]	70.8	72.3[c]	72.5[c]/115.9/135.5	
20	70.7	79.4[b]	79.0[b]	78.9[b]	70.7	71.4/72.5/115.6/115.9/135.6/135.9	
8[d]	71.1[b]	79.5[c]	79.0[c]	78.9[c]	70.8[b]	71.4/115.6/135.9	(71.4)[b]
9[d]	71.1[b]	80.5[c]	79.4[c]	70.8	72.4	———	(71.4)[b]
10[d]	71.3[b]	80.6[c]	79.5[c]	70.9	72.5	———	(71.5)[b]

[a] Spectra recorded for solutions in acetone-d_6 containing Me_4Si as internal standard. All of the spectra contain additional signals (73.8 ± 0.1, 73.2 ± 0.1 , 72.2 ± 0.2) corresponding to benzylic methylene groups (plus aromatic signals).

[b,c] Assignments could be inverted ; in parentheses are values of chemical shifts of the first methylene group of the spacers.

[d] Additional signals 119.9 (CN) or 172.9/60.0/14.4 (COOEt) plus multiplets corresponding to the spacers.

TABLE 2
^{13}C NMR Chemical Shifts [δ ,ppm] of Disaccharidic Derivatives [a]

	Ribitol residue					Glucosamine moiety						
	C1	C2	C3	C4	C5	C1	C2	C3	C4	C5	C6	
11	72.1[c]	78.9[b]	78.9[b]	79.7[b]	70.6[c]	100.8	55.1	73.3	69.7	71.9	62.7	
12	61.5	80.4[b]	79.1[b]	79.7[b]	70.6	100.8	55.1	73.3	69.7	72.0	62.8	
13	71.6[c]	80.2[b]	79.2[b]	79.2[b]	71.0[c]	101.2	55.3	73.5	69.9	72.2	63.0	
14	67.6[c,e]	78.7[b]	78.4[b]	78.1[b]	70.4	100.7	55.0	73.2	69.7	72.1	62.8	(68.8)[c,e]
17	66.5[c,f]	79.9[b,f]	79.8[b]	77.2[b]	70.7	99.9	55.3	73.2	69.7	72.0	62.9	(67.0)[c,f]
18[g,i]	68.4[c,f]	69.9	69.9	80.2	62.0[b]	101.8	55.4	73.7	69.9	72.6	63.0[b]	(67.1)[c,f]
19[h,i,j]	68.3[f]	71.4[b]	72.0[b]	82.3	61.8[c]	102.4	57.0	74.8	71.0	76.8	61.6[c]	
16[h,i,j]	73.1[c]	73.3[b]	72.7[b]	82.4	61.8[d]	102.4	57.0	74.9	71.0	76.9	61.9[d]	(71.5)[c]

[a] Spectra recorded for solutions in acetone-d_6 unless otherwise stated (Internal Me_4Si). All of the spectra contain additional signals corresponding to protective groups and/or spacers.

[b,c,d] Assignments could be inverted ; in parentheses are values of chemical shifts for the first methylene group of the spacers.

[e] Doublet (corresponding to both diastereoisomers)

[f] Broad signals (long range coupling)

[g] Spectrum recorded in methanol -d_4

[h] Spectra recorded in D_2O (external Me_4Si)

[i] For assignment comparisons see reference 5.

[j] Spectra in perfect accordance with observed and calculated values described in reference 14.

chloridite followed by "in situ" oxidation.[11] The fully-protected phosphorylated hapten **14** was thus obtained with a 57% overall yield from **11**.

Compounds **13** and **14**, containing spacers of comparable length, were then deprotected before coupling to the carrier. Compound **13** was hydrogenolysed on palladium and treated, without purification, with methanol and sodium methylate to provide **16** (93% overall yield). Compound **14** was first treated with the Zn/Cu couple in DMF to give **17** before hydrogenolysis to **18** and methanolysis to **19**, as previously described (72% overall yield).

The structure of each disaccharide was carefully assigned, mainly by ^{13}C NMR (Table 2).

Haptens **16** and **19** were separately converted to the corresponding hydrazide[8] and used without purification for the coupling on bovine serum albumine, following Inman's procedure.[12] After dialysis against water and freeze-drying, the extent of coupling was estimated by the measurement of the N-acetylglucosamine content in each conjugate.[13] The percentage of incorporation of **16** to the protein to give **3**, was about three times higher than incorporation of **19** giving **4**. This limited coupling could possibly result from the cleavage of the phosphodiester bonds since the hydroxyl group on C-2 is well suited for the formation of a cyclic phosphate in alkaline or acidic medium.

Both conjugates obtained were used for immunization of rabbits and tested for specificity against staphylococcal antibodies. Immunochemical results will be published elsewhere.

EXPERIMENTAL

General procedures. Melting points in capillary tubes were determined with a Büchi apparatus and are uncorrected. Optical rotations were measured for solution in 1-dm tubes with a Perkin-Elmer 241 polarimeter. ^{13}C and ^{31}P NMR spectra were recorded with a Varian XL-100A spectrometer operating respectively at 25.3 MHz (internal Me_4Si) and 40.5 MHz (internal H_3PO_4). Column chromatography was performed on silica gel Merck (230-400 mesh) ; preparative H.P.L.C.'s were performed on a Waters Prep LC/System 500 with Prep PAK-500 silica gel

column working at 30 atm. pressure. Three solvent mixtures were used for thin layer chromatography: A (ethyl acetate, hexane 2 : 3); B (ethyl acetate, hexane 1 :4); C (ethyl acetate, ether 1 : 1).

Allylation of 2,3,5-tri-O-benzyl-D-ribitol **5**. Compound **5**[5] (27.92 g, 66.08 mmol) dissolved in DMF (200 mL) was treated for 30 min at room temperature with powdered sodium hydroxide (1.8 g, 45 mmol) before dropwise addition of allyl chloride (4 mL, 48.87 mmol). After 15 min, additional sodium hydroxide (0.6 g, 15 mmol) and allylchloride (1 mL, 12.22 mmol) were added. The reaction was completed by a third addition after 30 min (sodium hydroxide 15 mmol, allyl chloride 6.11 mmol). The TLC (solvent A) showed traces of unreacted material (R_F 0.24) together with three new compounds (R_F 0.85, 0.68 and 0.61). The mixture was then poured into acidic icy water, extracted with ether, dried and the ether evaporated. The syrup thus obtained (27.16 g) was then chromatographed on H.P.L.C. (eluent : ethyl acetate; hexane 1:4), and three products were successively eluted with a small amount of unreacted material **5**.

1,4-di-O-allyl-2,3,5-tri-O-benzyl-D-ribitol **20**. Syrup (0.99 g, 3%); $[\alpha]_D^{23}$ -3.0° (c 2.2, chloroform); ^{13}C NMR (table1); Anal. Calcd. for $C_{32}H_{38}O_5$: C, 76.46; H, 7.62. Found: C, 76.52; H, 7.67.

1-O-allyl-2,3,5-tri-O-benzyl-D-ribitol **7**. Syrup (13.76 g, 45%); $[\alpha]_D^{23}$ + 22.3° (c 4.7, chloroform); ^{13}C NMR (table 1); Anal. Calcd. for $C_{29}H_{34}O_5$: C, 75.30; H, 7.41. Found: C, 75.01; H, 7.42.

4-O-allyl-2,3,5-tri-O-benzyl-D-ribitol **6**. Syrup (8.25 g, 27%); $[\alpha]_D^{23}$ -6.9° (c 5.7, chloroform); ^{13}C NMR (table1); Anal. Calcd. for $C_{29}H_{34}O_5$: C, 75.30; H, 7.41. Found: C, 74.97; H, 7.66.

4-O-allyl-2,3,5-tri-O-benzyl-1-O-(11-cyanoundecanyl)-D-ribitol **8**. Compound **6** (8.02 g, 17.72 mmol) in dry DMF (3 mL) was introduced through a syringe into a mixture containing sodium hydride (4.5 g, 83.33 mmol) in dry freshly-distilled DMF (30 mL). After 30 min at room temperature under nitrogen, 1-bromo-11-cyanoundecane (8.72 g, 35.44 mmol) was added and the mixture stirred for 18h. The usual extraction procedure (icy water / ether) left an oily material (13 g, 84%) used without purification in the next step. A small sample of **8** only was purified for identification purposes (TLC: solvent B, R_F 0.40). $[\alpha]_D^{23}$ - 2.25° (c 4.8, chloro-

form): ^{13}C NMR (table 1); Anal. Calcd. for $C_{40}H_{53}O_5N$: C, 73.52; H, 8.51; N, 2.23. Found: C, 73.36; H, 8.60; N, 2.43.

2,3,5-tri-O-benzyl-1-O-(11-cyanoundecanyl)-D-ribitol **9**. A reaction mixture of crude compound **8** (5.1 g) in ethanol (200 mL) and water (20 mL) was refluxed for 7h in the presence of 1,4-diazabicyclo [2.2.2] octane (0.50 g) and Wilkinson's catalyst (0.50 g).[15] After 12h at room temperature, the mixture was poured into icy water and extracted with ether. After washing, drying and evaporation, the syrup thus obtained was redissolved in a 10: 1 acetone, water mixture (50 mL) and treated for 3h at room temperature with mercuric oxide (2.15 g, 9.94 mmol) and mercuric chloride (2.70 g, 9.94 mmol). After filtration and the usual extraction procedure,[16] the syrup recovered was purified by column chromatography to give **9**: (2.3 g, 56% overall yield from **6**; TLC: solvent B, R_F 0.21); $[\alpha]_D^{23}$ + 17.8° (c 4.7, chloroform); ^{13}C NMR (table 1); Anal. Calcd. for $C_{37}H_{54}O_5N$: C, 75.60; H, 8.40; N, 2.38. Found: C, 75.62; H, 8.53; N, 2.38.

2,3,5-tri-O-benzyl-1-O-(11-ethoxycarbonylundecanyl)-D-ribitol **10**. A solution of **9** (1.37 g, 2.83 mmol) in dry ethanol (20 mL) was saturated with hydrogen chloride. After 2h at room temperature, nitrogen was passed through the solution. After the solvent was evaporated and the residue stirred in water (30 mL) for 18h, the resulting mixture was extracted with ether, the ether solution washed with water, dried, and purified (TLC: solvent B, R_F 0.31); recovered oily material (**10**) (1.04 g, 72%); $[\alpha]_D^{20}$ +17.9° (c 4.6, chloroform); ^{13}C NMR (table 1); Anal. Calcd. for $C_{37}H_{54}O_7$: C, 73.78; H, 8.57. Found: C, 73.59; H, 8.56.

4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-1-O-allyl-2,3,5-tri-O-benzyl-D-ribitol **11**. The ribitol derivative **7** (9.09 g, 20.09 mmol), β-D-glucosamine pentaacetate (8.14 g, 20.91 mmol), anhydrous iron trichloride (2.0 g, 12.33 mmol) and Drierite (7.0 g) were dried *in vacuo* at 120° for 10 min in the reaction flask. After sealing, the vial was cooled to room temperature and flushed with dry nitrogen. Dry alcohol-free dichloroethane (110 mL) was introduced through a syringe and the mixture was then gently refluxed for 2h. The mixture was cooled to room temperature and additional reagents added to the reaction mixture (N,N,N',N'-tetramethylurea, 1 mL, 8.3 mmol; iron

trichloride 0.8 g, 4.9 mmol; β-D-glucosamine pentaacetate, 4.18 g, 10.73 mmol). After a 2-hour reflux the same operation was performed again, the mixture was refluxed for another 2 hours and then left at room temperature overnight. After filtration, the organic extract was evaporated and redissolved in ether (500 mL). The ethereal solution was washed once with 1% aqueous sodium bicarbonate (200 mL) and twice with water before evaporation. The brown syrup thus recovered was then chromatographed on silica gel using an elution gradient of ethyl acetate, hexane 1 : 1 to 2.5 : 1.

The purified material (11.76 g, 71%; TLC: solvent C, R_F 0.61); crystallized and was then recrystallized from ether/petroleum ether, mp 57-58 °C; $[\alpha]_D^{23}$ -7.6° (c 5.0, chloroform); ^{13}C (table 2); Anal. Calcd. for $C_{43}H_{53}O_{13}N$: C, 65.22; H, 6.74; N, 1.77. Found: C, 64.96; H, 7.06; N, 1.70.

4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy- β-D-glucopyranosyl)-2,3,5-tri-O-benzyl-D-ribitol **12**. The same experimental procedure was used as for the cleavage of **8** to **9** and a crystalline material was recovered after purification and recrystallization from ether (84% yield; TLC: solvent C, R_F 0.44), mp 65-66 °C; $[\alpha]_D^{23}$ -6.1° (c 2.7, chloroform); ^{13}C NMR (table 2); Anal. Calcd. for $C_{40}H_{49}O_{13}N$: C, 63.90; H, 6.57; N, 1.86. Found: C, 63.81; H, 6.56; N, 1.87.

4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β -D-glucopyranosyl)-2,3,5-tri-O-benzyl-1-O(11-ethoxycarbonylundecanyl)-D-ribitol **13**. The same glycosidation procedure was applied to compound **10** as for derivative **7**. An oily material was recovered after purification (48%; TLC: solvent C, R_F 0.58). $[\alpha]_D^{22}$ - 8.5° (c 3.5, chloroform); ^{13}C NMR (table 2); Anal. Calcd. for $C_{53}H_{73}O_{15}N$: C, 66.02; H, 7.63; N, 1.45. Found: C, 65.85; H, 7.86; N, 1.39.

4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β -D-glucopyranosyl)-2,3,5-tri-O-benzyl-D-ribitol-1-(2,2,2-trichloroethyl, 8-methoxycarbonyloctyl)-phosphate **14**. Disaccharide **12** (4.23 g, 5.63 mmol) dissolved in dry THF (12 mL) was added dropwise at -78 °C to a mixture of 2,4,6-trimethylpyridine (5 mL, 37.7 mmol), trichloroethylphosphorodichlorite (1.5 mL, 10.18 mmol) and THF (20 mL). After 5 min, 8-methoxycarbonyl octanol (3.7 g, 19.7 mmol) in THF (5 mL) was added and the mixture

was allowed to gradually reach -20 °C. Pyridine (8 mL) was then added, followed by the oxidative solution containing iodine (0.24 mmol) and 2,4,6-trimethylpyridine (0.48 mmol) per ml of solvent (THF, water 2:1). The latter solution was added until a deep-red color persisted. After evaporation of solvents, the mixture was redissolved in chloroform, washed with dilute aqueous sodium bisulfite, dilute hydrochloric acid, and water. Evaporation of solvents followed by column chromatography (eluent: ethyl acetate, ether 1:1) left a syrupy colorless material (4.34 g, 68%; TLC: solvent C, R_F 0.55). $[\alpha]^{23}$-12.6° (c 4.7, chloroform); ^{13}C NMR (table 2); ^{31}P NMR δ (ppm, $CDCl_3$) -2.45 and -2.30; Anal. Calcd. for $C_{52}H_{69}O_{18}NPCl_3$: C, 55.10; H, 6.14; N, 1.24; P, 2.73; Cl, 9.38. Found: C, 55.18; H, 6.35; N, 1.31; P, 2.66; Cl, 9.06.

4-O-(2-acetamido-2 deoxy-β-D-glucopyranosyl)-1-O-(11-methoxycarbonylundecanyl)-D-ribitol **16**. Compound **13** (1.36 g, 1.41 mmol) dissolved in acetic acid (30 mL) was hydrogenolyzed for 24 h in the presence of 5% palladium-on-charcoal (400 mg). After filtration and evaporation, the residue was dissolved without purification in methanol (40 mL) containing a trace of sodium. After overnight stirring and neutralization (acidic resin), the mixture was evaporated and purified by column chromatography (eluent: ethyl acetate, methanol 6:1) to leave a homogeneous material (93%) which was freeze-dried. $[\alpha]_D^{20}$ -8.0° (c 2.5, water); ^{13}C NMR (table 2); Anal. Calcd. for $C_{25}H_{47}O_{12}N$, H_2O: C, 52.53; H, 8.60; N, 2.45. Found: C, 52.60; H, 8.60; N, 2.50.

4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-2,3,5-tri-O-benzyl-D-ribitol-1-(8-methoxycarbonyloctyl)-phosphate **17**. Compound **12** (6.56 g, 5.79 mmol) dissolved in dry DMF (85 mL) was treated at 40 °C under nitrogen with zinc/ copper couple (7 g).[17] After filtration and evaporation, the recovered material was chromatographed on silica gel (ethyl acetate, methanol 2:1) to leave an amorphous material (4.55 g, 77%). ^{13}C NMR (table 2); ^{31}P NMR δ (ppm, $CDCl_3$): - 2.76.

4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranosyl)-D-ribitol-1-(8-methoxycarbonyloctyl)-phosphate **18**. The preceding material was hydrogenolyzed in DME for 30 h over 10% palladium-on-charcoal (700 mg). After filtration and evaporation, a solid material was recovered (93%). ^{13}C NMR (table 2); ^{31}P NMR δ (ppm, CD_3OD): -2.82.

4-O-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-ribitol-1-(8-methoxycarbonyloctyl)-phosphate (sodium salt) **19**. Compound **18** (2.64 g, 3.2 mmol) dissolved in dry methanol (100 mL) was treated with 1.05 eq. of sodium at 4 °C for 24h. After neutralization (H^+ resin) of the excess of sodium methylate, filtration and evaporation, compound **19** (97%) was recovered as a hygroscopic crystalline material. Pure **19** was obtained by treating the crude product with an excess cyclohexylamine. After evaporation and washing with ether, the purified compound was obtained by retreatment on a Na^+ resin and recrystallization from a mixture methanol, ethyl acetate: mp 59-61 °C: $[\alpha]_D^{22}$ -7.8° (c 0.4, water); ^{13}C NMR (table 2); ^{31}P NMR δ (ppm, D_2O) + 1.12; Anal. Calcd. for $C_{23}H_{43}O_{15}NPNa$, 2.5 H_2O: C, 41.07; H, 7.19; N, 2.08; P, 4.61. Found: C, 40.68; H, 6.71; N, 1.95; P, 4.53.

Preparation of artificial antigens **3** and **4**. Compounds **16** and **19** respectively were first activated at the terminal end of the spacer by hydrazinolysis according to Lemieux's procedure[8] (150 mg were stirred for 24 h in 3 mL of 85% aqueous hydrazine monohydrate). The hydrazides thus formed (40 mg) were separately dissolved in DMF (1 mL) and treated at -25 °C with a 4N hydrochloric acid solution in dioxane (70 μL) and then t-butyl nitrite (10 mg, 0.1 mmol) in DMF (0.1 mL). After 30 min at -25 °C, sulfamic acid (7 mg) in DMF (0.1 mL) was added and stirring was continued for 15 min. The acyl azides thus formed were used directly in solution. The above mixtures were added dropwise to BSA (65 mg) dissolved in aqueous 0.08 M $Na_2B_4O_7$ and 0.35 M $KHCO_3$ at 0°C. The solutions were dialyzed against water in a Diaflo ultrafiltration cell (equipped with a PM-10 membrane) and freeze-dried.

Incorporations of haptens were calculated by determination of the glucosamine content of "synthetic" glycoconjugates. The artificial antigens **3** and **4** were first hydrolyzed with 3N HCl at 95 °C for 4h and the N-acetyl glucosamine content determined, after reacetylation, by the modified Morgan-Elson reaction.[13] The numbers of moles of bound haptens per mole of BSA (Mw= 65000) are those given in Scheme 1.

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