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ω-AMINOALKYL β-GLYCOSIDES OF *N*-ACETYLMURAMYL-L-ALANYL-D-ISOGLUTAMINE, AND THEIR CONJUGATES WITH MENINGOCOCCAL GROUP C POLYSACCHARIDE

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ABSTRACT

The 6-aminohexyl β-glycoside of *N*-acetylmuramyl-L-alanyl-D-isoglutamine and its spacer-arm-linked analog (3.8 nm) were synthesized from 2-methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline, and coupled with meningococcal group C polysaccharide in attempts to enhance the immunogenicity of the polysaccharide antigen.

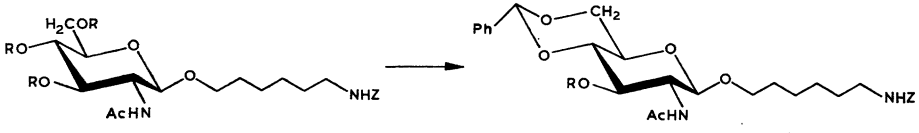
INTRODUCTION

In a preceding paper[1], we reported the synthesis of methyl β-glycosides of *N*-acetyl-6-*O*-(ω-aminoacyl)muramyl-L-alanyl-D-isoglutamines, and their conjugates with meningococcal group C polysaccharide, in attempts to enhance the immunogenicity of the polysaccharide antigen. Sela and his colleagues had predicted[2], and established[3,4], the importance of chemical attachment, to synthetic polypeptide antigens, of *N*-acetylmuramyl-L-alanyl-D-isoglutamine (MDP), the minimal adjuvant-active structure capable of replacing whole mycobacterial cells in complete Freund's adjuvant[5]. For example, the immunogenicity of the synthetic polypeptide poly(L-Tyr, L-Glu)-poly(DL-Ala)–poly(L-Lys) chemically linked to MDP was greatly enhanced when injected in aqueous solution into mice[4].

The methyl β-glycoside of *N*-acetylmuramyl-L-alanyl-D-isoglutamine has been reported to be more adjuvant-active than the corresponding methyl α-glycoside[6]. The size and orientation of the aglycon in MDP appear to have some influence on the biological activities; for instance, most of the responses of MDP are abolished when a *p*-aminophenyl group is introduced at the anomeric center. However, when the inactive *p*-aminophenyl β-glycoside was cross-linked with glutaraldehyde, several of the biological activities of MDP were recovered. Moreover, the cross-linked oligomer (molecular weight ~6000) was more active than MDP in protecting mice nonspecifically against bacterial challenge[7]. We now describe an alternative route[1] in the chemical attachment of MDP to meningococcal group C polysaccharide, namely, by the use of ω-aminoalkyl β-glycosides.

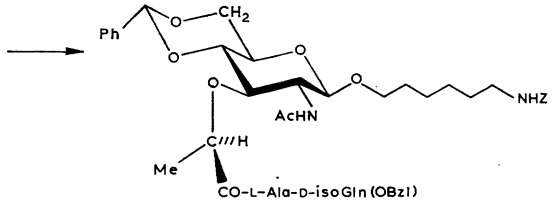
RESULTS AND DISCUSSION

2-Methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1-*d*]-2-oxazoline, prepared by the method of halide-ion participation in the presence of base[8], was treated with 6-(benzyloxycarbonylamino)-1-hexanol as reported in the literature[9]. The product, compound **1**, was deacetylated with sodium methoxide in methanol to



1 R = Ac
2 R = H
Z = $PhCH_2OCO$

3 R = H
4 R = CH_3CHCO_2H



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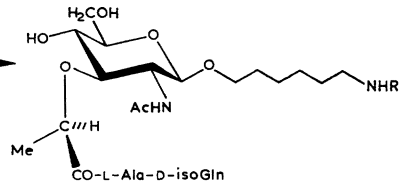
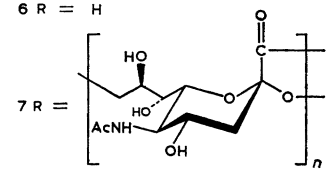


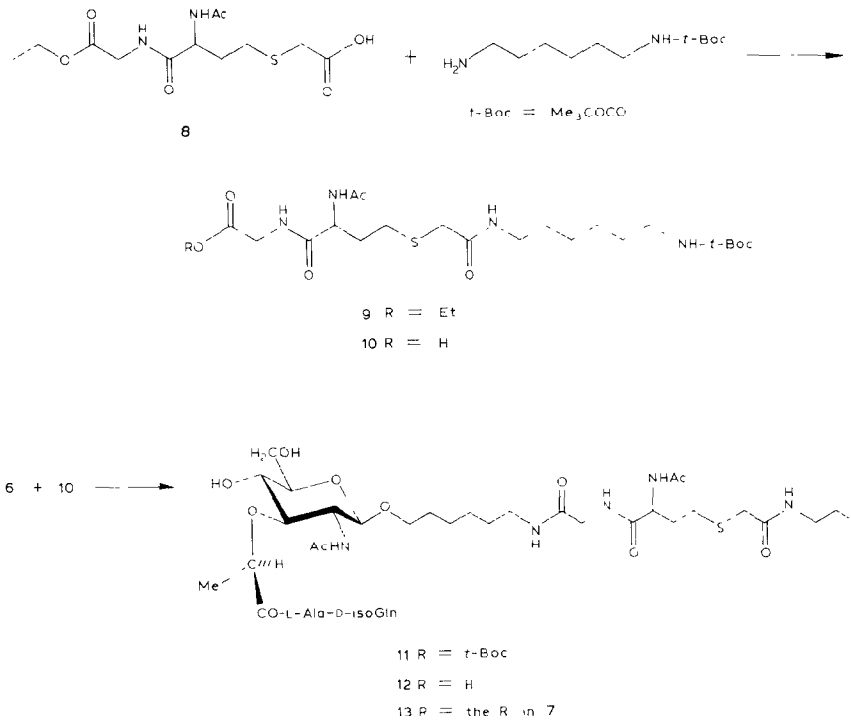
6 R = H



7 R =

give 6-(benzyloxycarbonylamino)-1-hexyl 2-acetamido-2-deoxy-β-D-glucopyranoside[9,10] (**2**). Acetalation of **2**, followed by alkylation with L-2-chloropropanoic acid[11] afforded **4** in good yield. The blocked glycopeptide **5** was prepared from **4** and L-alanyl-D-isoglutamine benzyl ester hydrochloride[12] by the mixed-anhydride method. Hydrogenolysis of **5** gave crystalline (6-aminohexyl) β-glycoside (**6**) of *N*-acetylmuramyl-L-alanyl-D-isoglutamine, which could be used as an intermediate for spacer-arm extension.

The 3.8-nm spacer-arm **9** was prepared from ethyl *N*-[*N*-acetyl-*S*-(carboxymethyl)-DL-homocysteinyl]glycinate[1] (**8**) and 1-amino-6-(*tert*-butoxycarbonylamino)-hexane[13]. Condensation of the carboxyl analog (**10**) of **9** with **6**, followed by acid treatment, gave the amine **12**. The two amine-containing ligands **6** and **12** were then coupled with meningococcal group C polysaccharide *via* the *N*-hydroxysuccini-



mide active ester, to give the conjugates **7** and **13**, respectively, as fluffy solids. Spinco analysis of **7** and **13** indicated the presence of 9.0 and 1.3% of MDP, respectively. Both conjugates exhibited enhanced antigenicity in the antigen–antibody, quantitative-precipitin assay. However, no enhancement of immunogenicity was observed *in vivo*.

EXPERIMENTAL

General methods. — Melting points were determined with a Thomas–Hoover Unimelt apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 241 polarimeter. Thin-layer chromatography (t.l.c.) was performed on silica gel GF_{254} (Analtech) plates, and the spots were detected by means of a ceric sulfate (1%)–sulfuric acid (10%) spray. Column chromatography was conducted on silica gel 60 (70–230 mesh, ASTM). N.m.r. spectra were recorded for solutions in chloroform-*d* (unless stated otherwise) at 300 MHz, with tetramethylsilane as the internal standard. Conventional processing consisted of drying organic solutions with anhydrous sodium sulfate, filtration, and evaporation of the filtrate under diminished pressure. Antigen–antibody, quantitative-precipitin assay was performed on immunodiffusion plates. Solvent *A* was 4:4:1 (v/v) $CHCl_3$–CH_3OH–H_2O.

*6-(Benzyloxycarbonylamino)hexyl 2-acetamido-4,6-O-benzylidene-2-deoxy-β-*D*-glucopyranoside* (**3**). — A mixture of **2** (17 g), anhydrous zinc chloride (20 g), and

benzaldehyde (90 mL) was stirred for 2 d at room temperature. The clear solution was diluted with ethyl ether; the resulting precipitate was collected by decantation, dissolved in chloroform, and the solution washed with water, dried, and evaporated to a syrup. Crystallization from ethanol afforded pure **3** (14.3 g, 70%): m.p. 208–210°, $[\alpha]_D^{27}$ −49.2° (*c* 1.5, DMF); n.m.r. ($CDCl_3$): δ 1.30–1.70 [m, $C(CH_2)_4C$], 2.03 (s, NHAc), 4.67 (d, $J_{1,2}$ 9.5 Hz, H-1), 5.12 (s, $CH_2C_6H_5$), and 5.56 (s, CHC_6H_5); *m/z* 542 (M^+).

Anal. Calc. for $C_{29}H_{38}N_2O_8$: C, 64.19; H, 7.06; N, 5.16. Found: C, 64.39; H, 7.36; N, 5.30.

*6-(Benzyloxycarbonylamino)hexyl 2-acetamido-4,6-O-benzylidene-3-O-(*D*-1-carboxyethyl)-2-deoxy-β-*D*-glucopyranoside* (**4**). — Sodium hydride (4.6 g) was added in portions to a solution of **3** (10.0 g) in dry oxolane (400 mL), and the mixture was boiled, with stirring, under reflux for 30 min, and allowed to cool. To this stirred mixture was added L-2-chloropropanoic acid[11] (10.0 g) dropwise, and the mixture was boiled under reflux for 4 h. The excess of sodium hydride was decomposed by addition of ethanol, and the solution was evaporated *in vacuo* to a residue which was dissolved in water. The solution was acidified to pH 2 with 2M HCl, and extracted with chloroform. The extracts were combined, dried, and evaporated to a residue which was placed on a column of silica gel, and eluted with 40:10:1 (v/v) chloroform–methanol–water. Compound **4** was isolated as an amorphous material (7.8 g, 69%): $[\alpha]_D^{27}$ −31.9° (*c* 1.0, DMF).

Anal. Calc. for $C_{32}H_{42}N_2O_{10} \cdot CH_3OH$: C, 61.29; H, 7.16; N, 4.33. Found: C, 61.31; H, 6.76; N, 4.14.

6-(Benzyloxycarbonylamino)hexyl β-glycoside (**5**) *of* N-*acetyl-4,6-O-benzylidenemuramyl-*L*-alanyl-*D*-isoglutamine benzyl ester*. — A mixture of **4** (2.0 g, 3.3 mmol), isobutyl chloroformate (0.52 mL, 3.9 mmol), and triethylamine (1.1 mL, 8.0 mmol) in dry DMF (10 mL) was stirred for 0.5 h at 0°, and to this mixture was added L-alanyl-D-isoglutamine benzyl ester hydrochloride[12] (1.57 g, 4.6 mmol). Stirring was continued for 1 h at room temperature, water (100 mL) and potassium carbonate (7.0 g) were added, and the mixture was stirred for 0.5 h at 0°, filtered, and the solid dissolved in DMF and precipitated with methanol, to give amorphous **5** (2.14 g, 72%); m.p. 209–210°, $[\alpha]_D^{27}$ −9.6° (*c* 1.3, DMF).

Anal. Calc. for $C_{47}H_{61}N_5O_{13} \cdot CH_3OH$: C, 61.59; H, 7.00; N, 7.48. Found: C, 61.49; H, 6.76; N, 7.37.

(6-Aminohexyl) β-glycoside (**6**) *of* N-*acetylmuramyl-*L*-alanyl-*D*-isoglutamine*. — A solution of **5** (1.28 g, 0.14 mmol) in 95% acetic acid (20 mL) containing palladium oxide (1.0 g) was hydrogenolyzed at 50 lb.in.$^{-2}$ for 2 d at room temperature. The catalyst was filtered off, and another batch (1.0 g) of palladium oxide was added. Hydrogenolysis was continued for 1 d, and the catalyst was filtered off, and washed with aqueous acetic acid. The filtrates were combined, and evaporated *in vacuo*, to give **6** (0.39 g, 47%); m.p. 224–227° (dec.), $[\alpha]_D^{27}$ −12° (*c* 1.3, methanol); R_f 0.11 (solvent *A*); n.m.r. (D_2O): δ 1.38, 1.45 (2 d, 2 $CHCH_3$), 1.97 (s, NHAc), 2.28 (t, CH_2COO^-), 3.0 (t, $CH_2N^+H_3$), and 4.47 (d, $J_{1,2}$ 9.0 Hz, H-1).

Anal. Calc. for $C_{25}H_{45}N_5O_{11}$: C, 50.75; H, 7.66; N, 11.83. Found: C, 50.34; H, 7.78; N, 11.50.

Ethyl N-[N-*acetyl*-S-{2-[6-(tert-*butoxycarbonylamino*)*hexylamino*]-2-*oxoethyl*}-DL-*homocysteinyl*]*glycinate* (**9**). — *p*-Nitrophenol (0.76 g, 5.5 mmol) was added to a solution of[1] **8** (1.52 g, 4.9 mmol) and DCC (1.25 g, 6.0 mmol) in dry DMF (6 mL). The mixture was stirred overnight at room temperature, and filtered, and the filtrate was added to a stirred solution of 1-amino-6-(*tert*-butoxycarbonylamino)hexane[13] (1.17 g, 5.4 mmol) in DMF (5 mL). After 3 h, the mixture was filtered, and the filtrate was evaporated *in vacuo* to a residue which was partitioned between chloroform and water. The organic layer was successively washed with aq. sodium hydrogencarbonate and water, dried, and evaporated to an oil, which was purified by column chromatography on silica gel with 19:1 (v/v) $CHCl_3$–MeOH as the eluant. Compound **9** was isolated as crystals (1.73 g, 68%); m.p. 86–87° (EtOAc).

Anal. Calc. for $C_{23}H_{42}N_4O_7S$: C, 53.26; H, 8.16; N, 10.80; S, 6.18. Found: C, 53.60; H, 8.50; N, 10.80; S, 6.05.

N-[N-*Acetyl*-S-{2-[6-(tert-*butoxycarbonylamino*)*hexylamino*]-2-*oxoethyl*}-DL-*homocysteinyl*]*glycine* (**10**). — Compound **9** (1.53 g, 2.9 mmol) was treated with 2.5M NaOH (1.2 mL) in 2:1 (v/v) ethanol–water (7 mL), and processed in the usual way, to give **10** (1.2 g, 84%); m.p. 114–116° (EtOAc–Et_2O).

Anal. Calc. for $C_{21}H_{38}N_4O_7S$: C, 51.41; H, 7.81; N, 11.42; S, 6.53. Found: C, 51.60; H, 7.96; N, 11.23; S, 6.58.

Compound **11**. — *p*-Nitrophenol (65 mg, 0.47 mmol) was added to a solution of **10** (127 mg, 0.26 mmol) and DCC (67 mg, 0.33 mmol) in DMF (5 mL), and the mixture was stirred for 3 h at room temperature. To this mixture was added a solution of **6** (135 mg, 0.22 mmol) in DMF (3 mL), and stirring was continued overnight. The mixture was filtered, and the filtrate was evaporated *in vacuo* to a residue which was placed on a column of silica gel and eluted with 6:4:1 (v/v) chloroform–methanol–water. The product was isolated as a foam (83 mg, 34%); $[\alpha]_D^{27}$ −1.1° (*c* 1.6, DMF); n.m.r. (CD_3OD): δ 1.43 [s, $C(CH_3)_3$], 1.94, 2.02 (2 s, 2 NHAc), 2.34 (t, CH_2COOH), and 2.68 (m, CCH_2S).

Anal. Calc. for $C_{46}H_{81}N_9O_{17}S \cdot 3\ H_2O$: C, 49.41; H, 7.84; N, 11.27. Found: C, 49.41; H, 7.69; N, 10.87.

Compound **12**. — A solution of **11** (102 mg, 96 μmol) in trifluoroacetic acid (1 mL) was kept for 5 min at 0°, and evaporated *in vacuo* to a residue which was placed on a column of silica gel, and eluted with solvent *A*. Compound **12** was isolated as a glass (44 mg, 48%); $[\alpha]_D^{27}$ +3.1° (*c* 0.12, methanol); R_F 0.11 (solvent *A*).

MDP–meningococcal group C polysaccharide conjugate (**7**). — A suspension of meningococcal group C polysaccharide (50 mg, containing 0.16 mmol of *N*-acetylneuraminic acid residues), *N*-hydroxysuccinimide (2.0 mg, 19 μmol) and 3-(3-dimethylaminopropyl)-1-ethylcarbodiimide hydrochloride (4.0 mg, 19 μmol) in dry DMF (1 mL) was stirred for 3 h at 0°. A solution of **6** (9.5 mg, 16 μmol) in DMF (1 mL) was added to the mixture, and the suspension was stirred for 3 h at 0°, diluted with water (4 mL), and dialyzed against distilled water, with three solvent

changes. The solution was then lyophilized, to give **7** as a fluffy solid that contained 9% of MDP (Spinco analysis).

Conjugate **13** was prepared from **12** and meningococcal group C polysaccharide similarly to **7**. Spinco analysis of the conjugate indicated the presence of only 1.3% of MDP.

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