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Glycopeptidolipids – a new class of artificial antigens with carbohydrate determinants. Synthesis of artificial antigen with type-specific oligosaccharide hapten from *Neisseria meningitidis* group B

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Synthetic lipopeptide *N*-palmitoyltyrosyl-seryl-seryl-asparaginyl-alanine, an analogue of B-mitogenic tripalmitoyl-pentapeptide from *Escherichia coli* lipoprotein, was coupled with an oligosaccharide hapten from *Neisseria meningitidis* lipooligosaccharide to give a glycopeptidolipid conjugate – the artificial antigen of a new type possessing the type-specific microbial determinant.

Keywords: Lipooligosaccharide, lipopeptide, glycopeptidolipid, artificial antigen.

Abbreviations: iBu, isobutyl; Bu^t, t-butyl; Boc, t-butoxycarbonyl; DCC, *N*,*N*′-dicyclohexylcarbodiimide; DMF, *N*,*N*-dimethylformamide; DMSO, dimethylsulfoxide; ONB, *N*-hydroxy-5-norbornen-2,3-dicarboximide ester; ONp, 4-nitrophenyl ester; Pal, palmitoyl; TEMED, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine; Z, benzyloxycarbonyl; KDO, 2-keto-3-deoxyoctonic acid; Hep, L-glycero-D-manno-heptose; TPP, *S*-[2,3-bis(palmitoyloxy)-(2*RS*)propyl]-*N*-palmitoylcysteinyl-seryl-seryl-asparaginyl-alanine.

Lipoproteins (LP) and lipopolysaccharides (LPS) are obligatory components of the Gram-negative bacteria envelope [1] and display high B-mitogenic and adjuvant activities [2, 3]. The immunoactive principles of the LP and LPS molecules have been shown to be the N-terminal tripalmitoylpentapeptide fragment [4] and the unique glycolipid, lipid A [5], respectively. In contrast to the nontoxic LP, LPS functions both as harmful endotoxins and as type-specific antigens [6]. The high toxicity of LPS preparations hampers their use as vaccine components despite anti-LPS antibodies being often bactericidal and protective [7]. The transfer of the type-specific carbohydrate chain of the LPS from highly toxic lipid A moiety to a deliberately nontoxic and B-mitogenic lipopeptide is expected to give an immunogenic glycopeptidolipid representing, to our knowledge, a new type of artificial antigen.

In this paper we describe the synthesis of such an immunogenic conjugate, prepared from synthetic *N*-palmitoyltyrosyl-seryl-seryl-asparaginyl-alanine and the oligosaccharide hapten of LPS from the *N. meningitidis* group B micro-organism.

Results and discussion

Choice of oligosaccharide hapten

Carbohydrate chains of microbial LPSs are composed of the O-specific polysaccharides attached to an oligosaccharide core [8], the latter being an inner part of the LPS molecule. However, LPSs of certain pathogenic bacteria (*Yersinia pestis*, *Bordetella pertussis*, *Neisseria meningitidis*, *Haemophilus influenzae*, etc.) possess no O-specific polysaccharides and, therefore, represent the lipooligosaccharides (abbreviated LOS) [9], and their serological specificities are determined by oligosaccharide haptens attached to lipid A components.

To synthesize the artificial glycopeptidolipid antigens, we decided to use the original microbial oligosaccharide haptens, and the oligosaccharide chain from *N. meningitidis* group B LOS was selected for this purpose. Two reasons

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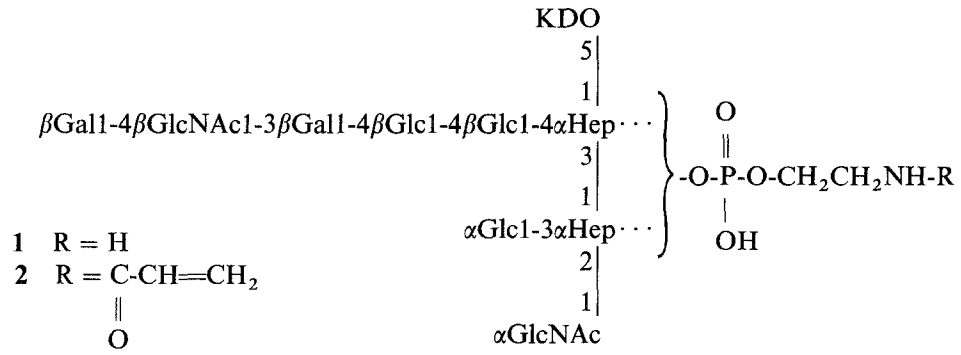


influenced our choice, namely that no efficient vaccine against *N. meningitidis* group B has been developed so far and that anti-LOS antibodies found in the blood of people ill with, and convalescing from meningitis were bactericidal ones [10].

Published results of haemagglutination [11], gel electrophoresis [12–15] and immunoblot [14, 15] studies have shown that LOS preparations from group B meningococci represent a mixture of related components, two or three of which being predominant in each sample. The components possessing long oligosaccharide chains harbour type-specific epitopes, whereas those with shorter chains express cross-reacting epitopes, thus causing a serological relationship among meningococci belonging to different serovars. Since the oligosaccharide chains differed from each other by molecular weight, sugar composition and serological specificity, it was concluded that the heterogeneity of the meningococcal LOS's was due to their carbohydrate moieties [16].

Chemical structures of different type-specific oligosaccharides obtained from the corresponding LOS preparations were established by Jennings *et al.* [17–20]. On the basis of literature data and our results of sugar composition and methylation analyses, the main oligosaccharide component of the LOS from the selected *N. meningitidis* strain B125 was deduced to be structure **1**. The exact attachment point of the phosphorylethanolamine residue in oligosaccharide **1** remained obscure; however, this was not of importance in this work.

The oligosaccharide fraction prepared by mild acetic acid hydrolysis of LOS from *N. meningitidis* B125 followed by purification on a Sephadex G-25 column was used for conjugation with the lipopeptide (see below) without preliminary separation into individual components. This was done with the aim of retaining all antigenic determinants which were present in the original LOS. By analogy, the oligosaccharide fractions from different *N. meningitidis* LOS preparations were successfully used for coupling with tetanus toxoid [21], and the artificial antigens thus obtained exhibited the original type-specificities and cross-reacting specificities. As oligosaccharide **1** contained a phosphorylethanolamine substituent with a free primary aminogroup, the latter was chosen as the attachment point with the lipopeptide. Moreover, the phosphoryl substituent was expected to play a role in the spacer-arm. However, the possibility existed [19] that phosphorylethanolamine residues could be involved in forming the type-specific epitopes. To investigate this possibility, two experiments were carried out. The original LOS was converted quantitatively into the corresponding 2,4-dinitrophenyl derivative [22] and the latter appeared to have an identical serological specificity when compared with the starting LOS. Additionally, the oligosaccharide material was *N*-acryloylated (oligosaccharide **2**) and subjected to copolymerization with acrylamide [23]. The water-soluble copolymer thus obtained contained one oligosaccharide chain on ten acrylamide residues and exhibited high serological specificity when reacted with anti-LOS antiserum, the optimal reacting dose being 20 times less than that of the original LOS sample. The results of these experiments indicated that phosphorylethanolamine substituents were not involved in the dominant epitopes in LOS from strain B125. Therefore, it was decided to use this substituent as a spacer group in the glycoconjugate synthesis.

Choice of lipopeptide

Tripalmitoylpentapeptide *S*-[2,3-bis(palmitoyloxy)-(2*RS*)-propyl] - *N* - palmitoylcysteinyl - seryl - seryl - asparaginyl - alanine (TPP) was shown to exhibit B-mitogenic activity as high as that of lipid A [24]. However, a chemical synthesis of TPP comprised fourteen steps [25], and material was not readily available. Soon afterwards, the synthesis of *N*-palmitoylseryl-seryl-asparaginyl-alanine was described, and the B-mitogenic activity of this simplified analogue was slightly lower in comparison to that of TPP [26]. The sequence of four aminoacids Ser-Ser-Asn-Ala was proved to be of importance for the expression of the immunostimulating activity either in TPP or *N*-palmitoyltetrapeptide [26]. Since TPP, expressing the highest B-mitogenic activity, possessed an additional aminoacid residue, we decided to synthesize an *N*-palmitoylpentapeptide derivative, *N*-palmitoyltyrosine being substituted for *N*-palmitoylcysteine. We selected *N*-palmitoyltyrosine because it was known that amphiphilic derivatives of this amino acid displayed the adjuvant activity

[27]. Moreover, the presence of tyrosine residues enhances the immunogenicity of poorly immunogenic antigens [28].

The synthesis of Pal-Tyr-Ser-Ser-Asn-Ala (**6**) was performed according to the scheme originally described for Pal-Ser-Ser-Asn-Ala [26], but with the essential difference that the activated esters method was used instead of the carbodiimide coupling procedure.

Z-Asn-OH
↓ 1. iBuOCOCl
↓ 2. H-Ala-OBu^t
Z-Asn-Ala-OBu^t 81% (**3**)
↓ 1. H_2/Pd
↓ 2. Z-Ser(Bu^t)-ONB
Z-Ser(Bu^t)-Asn-Ala-OBu^t 95% (**4**)
↓ 1. H_2/Pd
↓ 2. Z-Ser(Bu^t)-ONB
Z-Ser(Bu^t)-Ser(Bu^t)-Asn-Ala-OBu^t 90% (**5**)
↓ 1. H_2/Pd; 2. Boc_2Tyr-ONp
↓ 3. CF_3COOH; 4. Pal-ONB
Pal-Tyr-Ser-Ser-Asn-Ala 84% (**6**)

The use of the activated esters method appeared advantageous and led, for example, to the protected tetrapeptide derivative **5** with a total yield of 69%, the latter being twice as high as that described in the original synthesis [26]. Each elongation and deprotection step in the peptide synthesis was monitored by thin layer chromatography, reactions being continued until the starting materials disappeared. The structures of peptide derivatives **3**–**6** were proved by high resolution ^{1}H-NMR spectroscopy, and the spectra recorded contained the signals of all hydrogen atoms of the corresponding amino acid residues, those of amide group protons in peptide linkages being present in each spectrum and considered as a criterion for the enantiomeric purity of the synthesized peptide derivatives. In addition, the composition of lipopeptide **6** was confirmed by traditional amino acid analysis, and tyrosine, serine, aspartic acid and alanine were found in the hydrolysate in the molar ratio 1.0:2.0:1.0:1.0.

Synthesis of glycopeptidolipid conjugate

As mentioned above, the attachment of oligosaccharide **1** to lipopeptide **6** was planned to proceed via the free amino group of the phosphorylethanolamine residue. For this purpose, the carboxyl group of the alanine residue in lipopeptide **6** was activated by treatment with *N*-hydroxy-5-norbornen-2,3-dicarboximide. The active ester thus obtained was allowed to react with oligosaccharide **1** in DMF at room temperature until it gave a negative test with ninhydrin. The reaction mixture was processed using routine procedures, and the conjugate **7** was isolated by chromatography on a Sephadex G-25 column with 68% yield.

The hydrolysate of the conjugate **7** was subjected to amino acid analysis and tyrosine, serine, aspartic acid, alanine, glucosamine and ethanolamine were detected in the molar ratio 0.94:1.86:1.00:1.03:1.94:0.76, respectively, thus indicating the composition of the conjugate. Other ninhydrin-positive compounds, except small amounts of phosphorylethanolamine, were absent from the hydrolysate.

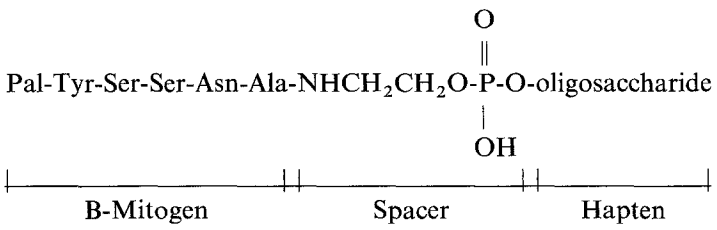
The conjugate was proved to be immunogenic in mice when a single 0.1–0.5 μg dose in buffered saline was administered intraperitoneally without any additional adjuvant. It was found that on day seven after immunization the antiserum possessed anti-LOS specificity, and the antibodies raised exhibited bactericidal and protective activities against homologous meningococci. These results will be published in detail elsewhere. As can be seen from these preliminary data, the immunogenic properties of synthesized glycoconjugate **7** resembled those of *N. meningitidis* LOS, which is known to stimulate formation of bactericidal antibodies which protect animals against infection with lethal doses of living meningococci [29].

Thus, the preparation of a semi-synthetic artificial antigen composed of the original microbial oligosaccharide hapten and synthetic lipopeptide possessing B-mitogenic activity is described. Since different oligosaccharide haptens could be prepared from numerous capsular and somatic microbial polysaccharides, the approach promises a wide application as a route to new chemical vaccines, and research in this direction is in progress.

Materials and methods

General methods

Monosaccharide analysis of the oligosaccharide and glycoconjugate hydrolysates (2 M HCl, 100 °C, 2.5 h) was performed with the use of (i) ion-exchange chromatography



(**7**)

on a column (18 cm × 0.37 cm) of DA × 8 resin (Durrum, USA) fitted in an LC-2000 instrument (Biotronic, Germany) and eluted with 0.5 M borax buffer, pH 8.0, at 65 °C, sodium bicinchoninate being used as a detecting reagent [30]; (ii) GLC of alditol acetates on an Ultra-1 capillary column (25 m) with chemically bound methylsilicone fitted in a Hewlett-Packard Model 5890 instrument, temperature range 200–290 °C with a gradient of 10 °C min^{-1}.

Analysis of amino acids, glucosamine and ethanolamine was carried out with the hydrolysates (4 M HCl, 100 °C, 16 h, under nitrogen) of peptides, oligosaccharides and glycoconjugates with the use of a LC-5001 chromatograph (Biotronic, Germany) supplied with a column (235 mm × 3.5 mm) packed with BTC 2710 resin (Biotronic) and eluted with 59.8 mM sodium citrate buffer, pH 4.92, according to the described programme [31].

TLC was performed on preformed Kieselgel 60 plates (Merck, Germany) in the following solvent systems (by vol): 1, ethyl acetate saturated with water; 2, chloroform:methanol:acetic acid (90:10:1); 3, ethyl acetate:chloroform:17% NH_4OH (98:6:1); 4, chloroform:methanol:32% acetic acid (60:45:20); 5, n-butanol:acetic acid:water (3:1:1); 6, n-butanol:pyridine:acetic acid:water (63:36:6:45). Ninhydrin and chlorine–benzidine reagents were used for detection.

^{1}H-NMR spectra were recorded at 37 °C for solutions in DMSO-d_6 using a Bruker WM500 instrument at 500 MHz, resonances being related to sodium 2,2-dimethyl-2-silanopentanesulphonate as an internal standard.

Solid-phase ELISA was carried out according to standard procedures [32] with the use of hyperimmune rabbit and mouse antisera raised against the cells of meningococci strain B125. Evaporations were performed *in vacuo* below 40 °C, and the solvents were distilled. Amino acid derivatives and corresponding reagents used for peptide synthesis were of chemical grade purity (Sigma, USA).

Isolation of lipooligosaccharide (LOS)

Cells of *N. meningitidis* (strain No. 125, group B, serotype 2b, immunotype 6) were cultivated in a fermenter Model ANKUM-2M (CIS) with the use of semisynthetic culture medium [33] containing rigorously hydrolysed casein, yeast dialysate and different salts at 37 °C and pH 7.4 in the exponential phase. The cells were harvested using a Pellicon Cassette System (Millipore, USA) with the molecular cut-off value at 10000 Da. The wet cells were extracted with hot aqueous phenol according to the modified Westphal method [34] as described in [35]. The isolated LOS was purified by sedimentation in an ultracentrifuge at 105000 × **g** for 4 h, the yield being 2.5%. A sample (10 mg) of LOS was treated with 2,4-dinitrofluorobenzene in the presence of triethylamine, as described in [22], to give the dinitriphenyl derivative (11 mg) as a yellow-coloured material.

Preparation of oligosaccharide **1**

A suspension of LOS (500 mg) in 1% acetic acid (150 ml) was heated at 100 °C for 2.5 h, cooled to room temperature and kept in a refrigerator overnight. The precipitate of lipid A was removed by centrifugation and the supernatant was concentrated and freeze-dried. The material (160 mg) was purified by gel filtration on a Sephadex G-25 (superfine) column (80 cm × 2 cm) in water, the elution curve being monitored at 205 nm by a Uvicord-2S (Pharmacia, Sweden). The oligosaccharide material eluted as a symmetrical peak and the corresponding fractions were freeze-dried to give oligosaccharide **1** (140 mg).

Preparation of N-acryloyl derivative **2**

To oligosaccharide material (10 mg) in water (0.5 ml), sodium carbonate (10 mg) and methanol (0.5 ml) were added. The mixture was cooled by ice and acryloyl chloride (10 µl) in ether (0.25 ml) was added dropwise with stirring, the funnel being rinsed with ether (0.25 ml) and washings added to the reaction mixture. This mixture was then stirred for 10 min at 0 °C, then kept for 16 h at room temperature, and diluted with 1 ml 0.1 M pyridine-acetate buffer, pH 5.5. The solution was concentrated to a small volume and chromatographed on a Sephadex G-15 column (50 cm × 1.5 cm) using 0.1 M pyridine-acetate buffer. The separation was monitored using a differential refractometer (Knauer, Germany), and the corresponding fractions were freeze-dried to give **2** (9 mg). The product had the same sugar composition as the original oligosaccharide **1** and did not react with ninhydrin.

Copolymerization of oligosaccharide **2** *with acrylamide*

To a solution of **2** (8 mg) and acrylamide (5 mg, ultra-grade) is deionized and deaerated water (0.5 ml) were added TEMED (2 µl) and ammonium persulphate (1 mg) under argon and with stirring. After 30 min, an additional portion of $(NH_4)_2S_2O_8$ (1 mg) was added, the mixture was kept for 16 h and diluted with 0.1 M pyridine-acetate buffer (1 ml). The reaction product was isolated by chromatography on a Sephadex G-25 column (60 cm × 2 cm) eluted with 0.1 M pyridine-acetate buffer. The material eluted at the void volume of the column was collected and freeze-dried to give the copolymer (10 mg, 82%). The sugar composition of the copolymer was analogous to that of oligosaccharide **2**.

N-Benzyloxycarbonylasparaginyl-alanine butyl ester (**3**)

A solution of Z-Asn (2.35 g) in DMF (10 ml) was cooled to −20 °C and treated in succession with *N*-methylmorpholine (1 ml) and chloroformic acid isobutyl ester (1.5 g). After 3 min, a solution of H-Ala-OBu^t·HCl (1.60 g) and *N*-methylmorpholine (1 ml) was added. The mixture was stirred for 3 h without cooling and then evaporated. The residue was dissolved in ethyl acetate (50 ml) and washed in sequence with 5% H_2SO_4 (2 × 5 ml), water (2 × 5 ml),

saturated solution of $NaHCO_3$ (2 × 5 ml) and water (2 × 5 ml) and then evaporated to dryness. The residue was ground to a powder under an ethyl acetate–hexane mixture (10 ml, 2:1 by vol), filtered and dried *in vacuo* to give dipeptide **3** (2.82 g, 81%). The material was homogeneous on TLC: R_F^1 0.30; R_F^2 0.41; R_F^3 0.12. ^{1}H-NMR Data: Ala,αH1 (4.09); βH3 (1.23); αNH (8.16); OBu^t (1.38). Asn,αH1 (4.38); βH2 (2.38 and 2.47); αNH (7.35); NH_2 (6.89 and 7.23); Z, CH_2 (5.01), aromatic ring (7.35).

N-Benzyloxycarbonyl(O-t-butyl)seryl-asparaginyl-alanine t-butyl ester (**4**)

A solution of dipeptide **3** (1.70 g) in ethanol (50 ml) was hydrogenated over 10% Pd/C catalyst for 5 h, then filtered and evaporated to dryness. The residue was dissolved in DMF (20 ml) and treated with Z-Ser(OBu^t)-ONB (1.92 g). After 15 h, the solution was evaporated, the residue was dissolved in ethyl acetate (50 ml) and washed with 5% H_2SO_4, water, saturated $NaHCO_3$ and water as described above. The solution was evaporated to dryness and the solid residue was ground to a powder under an ethyl acetate–hexane mixture (10 ml, 2:1 by vol), filtered and dried *in vacuo* to give tripeptide derivative **4** (2.20 g, 95%). The resulting material was homogeneous on TLC: R_F^1 0.28; R_F^2 0.44; R_F^3 0.10. ^{1}H-NMR Data: Ala,αH1 (4.08); βH3 (1.22); αNH (7.88); OBu^t (1.38). Asn,αH1 (4.56); βH2 (2.38 and 2.48); αNH (8.11); NH_2 (6.51 and 7.30). Ser,αH1 (4.08); βH2 (3.42 and 3.51); αNH (7.31); OBu^t (1.11); Z, CH_2 (5.33); aromatic ring (~7.35).

N-Benzyloxycarbonyl(O-t-butyl)seryl-(O-t-butyl)seryl-asparaginyl-alanine t-butyl ester (**5**)

A solution of the tripeptide derivative **4** (2.20 g) in ethanol (100 ml) was hydrogenated over 10% Pd/C catalyst for 5 h, then filtered and evaporated. The residue was dissolved in DMF (30 ml) and treated with Z-Ser(OBu^t)-ONB (1.82 g), and the reaction was carried out and processed as described in the previous synthesis, to give tetrapeptide derivative **5** (2.50 g), yield 89%. TLC data: R_F^1 0.27; R_F^2 0.43; R_F^3 0.09. ^{1}H-NMR Data: Ala,αH1 (4.08); βH_3 (1.23); αNH (7.83); OBu^t (1.38). Asn,αH1 (4.58); βH2 (2.43 and 2.52); αNH (8.04); NH_2 (6.88 and 7.30). Ser,αH1 (4.13); βH2 (3.47 and 3.52); αNH (7.33); OBu^t (1.12). Ser,αH1 (4.30); βH2 (3.46 and 3.52); αNH (7.31); OBu^t (1.11); Z, CH_2 (5.04); aromatic ring (~7.35).

N-Palmitoyltyrosyl-seryl-seryl-asparaginyl-alanine (**6**)

A solution of the tetrapeptide derivative **5** (1.20 g) in ethanol (50 ml) was hydrogenated as described above. The material obtained was dissolved in DMF (50 ml) and treated with *N,O*-(Boc)$_2$Tyr-ONp (504 mg), the mixture being stirred for 15 h at room temperature and then evaporated to dryness. The residue was dissolved in CF_3COOH (30 ml), kept for 1 h at 22 °C, concentrated to a small volume (5 ml) and diluted with ether (50 ml). The precipitated material was washed with ether (25 ml × 3) and dried. To a solution of the substance in DMF (20 ml) were added first triethylamine (0.25 ml) and then Pal-ONB (480 mg), and the reaction mixture was kept for 15 h at room temperature and diluted with ethylacetate (100 ml). After 2 h, a precipitate was filtered off, dissolved in DMF (10 ml), re-precipitated with ethyl acetate (50 ml) and dried *in vacuo* to give compound **6** (640 mg, 84%). TLC Data: R_F^4 0.53; R_F^5 0.37; R_F^6 0.76. ^{1}H-NMR Data (selected characteristic signals): Ala,αH1 (4.136); βH3 (1.256); αNH (7.856). Asn,αH1 (4.612); βH2 (2.417 and 2.565); αNH (8.088). Ser,αH1 (4.274); βH2 (3.549 and 3.665); αNH (7.964). Ser,αH1 (4.342); βH2 (3.567 and 3.653); αNH (8.039). Tyr,αH1 (4.474); βH2 (2.614 and 2.921); αNH (7.921); Pal,αH2 (2.002); ωH3 (1.345).

Preparation of glycopeptidolipid (**7**)

A solution of pentapeptide derivative **6** (200 mg) in DMF (5 ml) was cooled to −10 °C, and HONB (96 mg) and DCC (70 mg) were added in succession. After 1 h at −10 °C and 20 h at +4 °C, the mixture was filtered to remove the precipitate and evaporated, the residue was washed with ether (10 ml × 2) and dried *in vacuo* to give the activated ester of peptide **6** (260 mg), R_F^5 0.75. A solution of the material obtained (portion, 90 mg) in DMF (5 ml) was treated with oligosaccharide **1** (50 mg) and stirred for 48 h at room temperature. The reaction mixture was evaporated, the residue was dissolved in methanol (2 ml) and diluted with ethyl acetate (5 ml) to give a precipitate, which was washed with ethyl acetate (5 ml × 2) and dried. The residue was dissolved in a small volume of 5% acetic acid and purified by gel filtration on a Sephadex G-25 column (20 cm × 0.8 cm) and eluted with 5% acetic acid, the elution curve being monitored at 225 nm. The condensation product was eluted close to the void volume of the column as a symmetrical peak, and the corresponding fractions were pooled and freeze-dried to give conjugate **7** (42 mg, 68%) as a white cotton-like material.

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