Chemistry of Natural Compounds, Vol. 34, No. 1, 1998

IMMOBILIZATION OF SYNTHETIC GLYCOPEPTIDES ON POLYMERIC SUPPORTS

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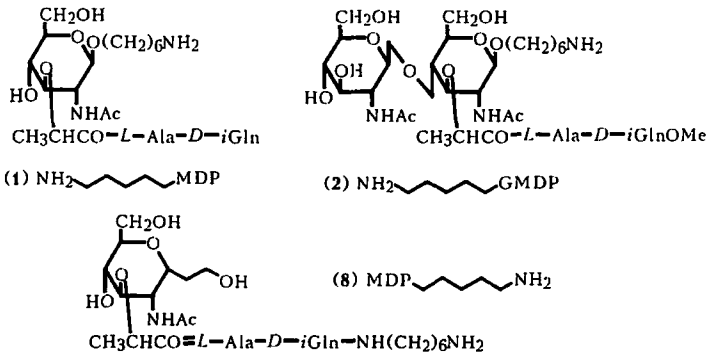
UDC 547.963.057

The synthesis of the 6-aminohexyl β-glycoside of O-(N-acetylglucosaminyl)-(β1→4)-MDP (GMDP) is described. Conjugates with polyacrylamide, including lipophilic conjugates (containing phosphatidylethanolamine residues) and biotinylated polymeric probes with MDP specificity have been obtained on the basis of ω-spacered glycopeptides. Conjugates of MDP with dextran have also been synthesized.

A report that macromolecular conjugates obtained by condensing N-acetylmuramoyl-*L*-alanyl-*D*-isoglutamine (MDP, muramoyldipeptide) with glutaraldehyde [1] or multi[poly-*L,D*-alanyl)-poly(*L*-lysine)] [2] possess considerably greater antiinfection activity than MDP itself has aroused great interest in conjugates of muramoyldipeptide. This is all the more the case since the production of synthetic macromolecular muramoyldipeptides has corresponded to Sela's idea [3] of the construction of completely synthetic vaccines consisting of synthetic antigens and adjuvants immobilized on natural or synthetic supports. Muramoyldipeptides having in their structure an active group (most frequently an amino group; more rarely a carboxy or thiol group) attached to a glycopeptide through a hydrocarbon, or other, spacer have been used for conjugation with polysaccharides [4], peptide hormones [5], components of lipid A [6], and compounds of different natures.

The preparation of macromolecular glycopeptides by conjugating MDP with oligo- and polypeptides containing lysine has been described [2, 7]. A conjugate of polyacrylamide (PAA) with MDP has been obtained by copolymerizing the 6-acryloylaminohexylamide of N-acetylmuramoyl-*L*-alanyl-*D*-isoglutamine with acrylamide [8]. The latter approach has been used for creating a conjugate of PAA with MDP and peptide antigens and a carbohydrate antigen of the Le^a blood group [9]. New possibilities in the production of polyacrylamide conjugates have been discovered by N. V. Bovin, who has proposed to achieve the immobilization of amino-containing ligands with poly(4-nitrophenyl acrylate) [10]. This method permits the quantitative introduction of up to 30 mole-% of amino components, including some of different natures, under mild conditions; the nitrophenyl groups that have not reacted are converted into amide groups by treatment with ammonia. The polymer is freed from low-molecular-mass products by gel filtration.

This method was taken as a basis for obtaining macromolecular glycopeptides in a polyacrylamide matrix. The 6-aminohexyl β-glycosides of MDP (**1**) [11] and of O-(N-acetylglucosaminyl)-(β1→4)-MDP (GMDP) (**2**) were used as haptens, which made it possible to obtain conjugates of PAA with MDP (**3**) and of PAA with GMDP (**4**) (ratio of acrylamide units and glycopeptide 5:1)



Simferopol' State University, 4 Yaltinskaya ul., Simferopol', Crimea, Ukraine. Translated from Khimiya Prirodnykh Soedinenii, No. 1, pp. 104-111, January-February, 1998. Original article submitted September 10, 1997.

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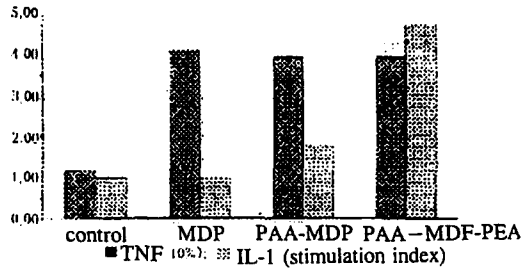
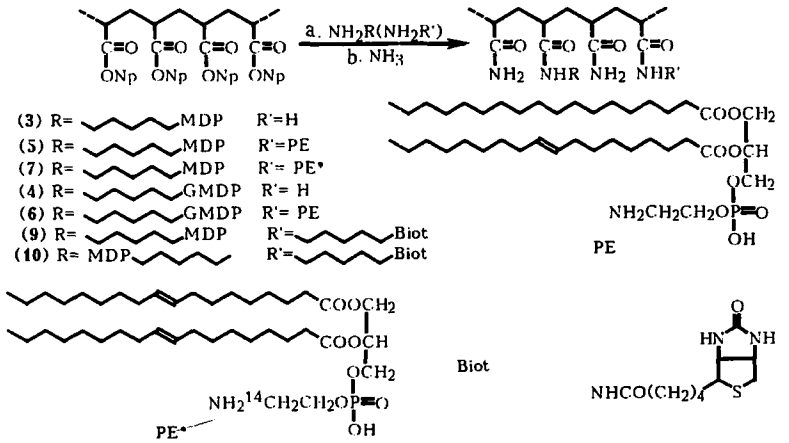


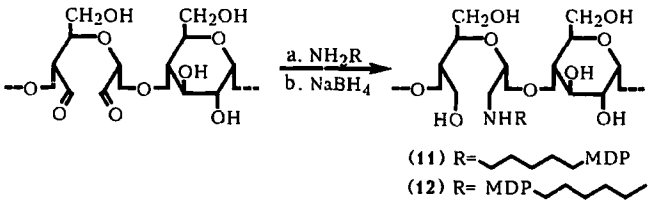
Fig. 1

To increase the lipophilicity of the macromolecular muramoyldipeptides, the glycopeptide (**1**) or (**2**) was co-immobilized on a polyacrylamide core with phosphatidylethanolamine (PEA) (ratio of acrylamide units to glycopeptide to PEA 5:1:1), giving conjugates (**5**) and (**6**). The use of ^{14}C-labeled PEA permitted the creation of the labeled conjugate (**7**), which is necessary for studying the mechanism of the action of polymeric forms of MDP.

Hitherto there has been no unambiguous opinion on the reception of muramoyldipeptide by immune cells. As tools for solving this question it is possible to use polymeric probes with MDP specificity consisting of conjugates of PAA with derivatives of muramoyldipeptide having a spacer at the glycosidic center [(6-aminohexyl glycoside of MDP (**1**)] or at the carboxy group of the isoglutamine residue (6-aminohexylamide of MDP (**8**) [12]) and with a fluorescent label (6-biotinylaminohexylamide). In conjugates (**9**) and (**10**) a ratio of acrylamide units to glycopeptide to label of 20:4:1 was used.

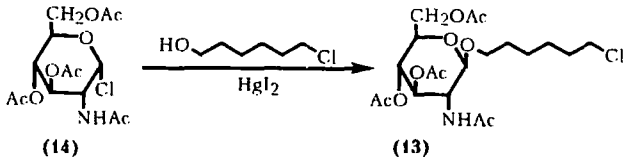


Dialdehydodextran (Dexal b) — a biodegradable polysaccharide having aldehyde groups — has been proposed as an alternative support. The condensation of Dexal b with the amino-spacered glycopeptides (**1**) and (**8**) was conducted in bicarbonate buffer. The unchanged carbonyl functions and the Schiff's base were reduced with an excess of sodium tetrahydroborate. After gel filtration, conjugates of dextran with MDP (**11**) and (**12**) having a ratio of haptens and initial aldehyde groups of 1:5 were isolated. Amino acid analysis confirmed the composition of the polymers.



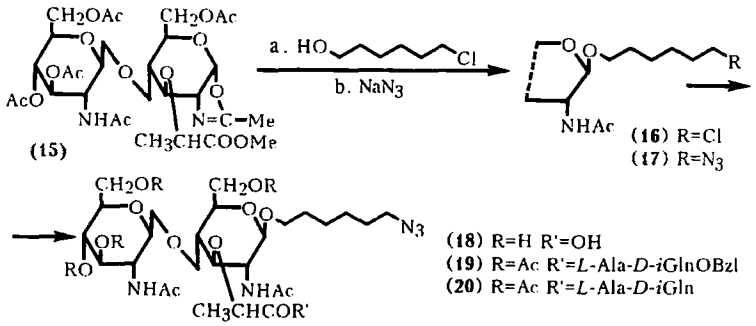
The synthesis of the 6-aminohexyl β-glucoside of MDP (**1**) was conducted by a modified method [11]. The initial fully acetylated 6-chlorohexyl β-glycoside of N-acetylglucosamine (**13**) was obtained by glycosylating 6-chlorohexan-1-ol with α-glucosaminyl chloride peracetate (**14**) in the presence of mercury(II) iodide [13]. In the PMR spectrum of compound (**13**), in addition to the signals of the protons of the glycoside residue, the signals of the protons of the aglycon were identified: a triplet from the terminal methylene group with CS 3.54 ppm, two multiplets of the nonequivalent protons of the α-methylene group

with the CSs 3.50 and 3.84 ppm, and multiplets of the protons of the other methylene groups with the CSs 1.40, 1.58, and 1.79 ppm. The β-configuration of the glycosidic center was shown by a doublet from the anomeric proton with the CS 4.67 ppm and the SSCC 8 Hz (see the Experimental part).



The synthesis of the 6-aminohexyl β-glucoside of GMDP (**2**) was based on the glycosylation of 6-chlorohexan-1-ol with the oxazoline (**15**) [14]. By treatment with sodium azide in a mixture of acetonitrile and DMFA in the presence of tetramethylammonium bromide, the 6-chlorohexyl β-glycoside (**16**) isolated by column chromatography was converted into the corresponding azide (**17**). The PMR spectrum of this compound included two signals of anomeric protons — doublets with the CSs 4.33 and 4.36 ppm and the SSCC 8 Hz, which confirmed the β-configuration of both glycosidic centers of the disaccharide (see the Experimental part). Its PMR spectrum contained the intense absorption at 2100 cm^{-1} that is characteristic for the azido group.

The ester protective groups in disaccharide (**17**) were eliminated. To improve solubility, the acid (**18**) was reacetylated and condensed with the methyl ester of *L*-alanyl-*D*-isoglutamine using dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (SuOH) as activating reagents. In the PMR spectrum of glycopeptide (**19**), in addition to the signals of the protons of the disaccharide fragment, characteristic signals of the protons of the dipeptide residue were identified — in particular, a triplet of the γ-methylene group of isoglutamine with the CS 2.30 ppm, a singlet of the methyl ester group with the CS 3.58 ppm, and two singlets of the nonequivalent protons of the primary amino group with the SCs 7.10 and 7.15 ppm. Final deacetylation gave the prespacered glycopeptide (**20**). Catalytic hydrogenation over palladium oxide or reduction of the azido groups with $NaBH_4$ led to the desired compound (**2**), which was used directly for condensation.



An investigation of conjugates of PAA with MDP (**3**) and of PAA with MDP-PEA (**5**) in tests of antitumoral activity (A. L. Rakhmilevich and B. B. Fuks, Institute of Human Morphology, Russian Academy of Medical Sciences) showed pronounced stimulation of the production of tumor necrosis factor (TNF) and of interleukin-1 (IL-1) by murine peritoneal macrophages under the conditions of desensitization to endotoxin.

EXPERIMENTAL

Melting points were determined on a PTP instrument, and optical rotations at 20-22°C on a Polamat-A polarimeter. PMR spectra were obtained on Varian VXR-300 (300 MHz) and Bruker WM-500 (500 MHz) instruments with tetramethylsilane as internal standard; chemical shifts (CSs) are given in ppm, δ-scale. IR spectra were recorded on a Specord 75-IR spectrophotometer (tablets with KBr). TLC was conducted on Kieselgel 60 F_{254} (Merck) and Silufol UV_{254} (Kavalier) plates. The substances were revealed by a 2% solution of sulfuric acid in ethanol, followed by heating at 150°C (Kieselgel) or by heating at 150-200°C (Silufol). The following solvent systems were used: 1) chloroform–benzene–ethanol (10:1:1); 2) chloroform–ethanol (5:1); 3) *n*-butanol–acetic acid–water (3:1:1); 4) ethanol–*n*-butanol–pyridine–water–acetic acid

(100:10:10:10:3). Column chromatography was conducted on the silica gels Kavalier L 100-160 μm and Aldrich 70-230 mesh. The elementary analyses of the compounds synthesized corresponded to the calculated values.

Reagents used were: sodium tetrahydroborate from Serva; *L*-1,2-dioleoylphosphatidyl-[2-^{14}C]ethanolamine from Amersham International Plc; Sephadex G-15 from Pharmacia; DCC from Aldrich; and HOSu from Merck.

DMFA was boiled with ninhydrin and distilled in vacuum.

Conjugate of PAA with MDP (3). A solution of 17 mg (88 μg-equiv.) of poly(4-nitrophenyl acrylate) in 1 ml of DMFA was treated with 11 mg (18 μmole) of the 6-aminohexyl β-glycoside of MDP (**1**) in 0.5 ml of DMFA and 2 drops of triethylamine. The reaction mixture was stirred for 12 h (TLC in systems 3 and 4 showed the absence of the amino-spacered glycopeptide). The solution was treated with 0.1 ml of 25% aqueous ammonia, and after 24 h it was diluted with 10 ml of water and then 16 mg (100%) of the polymer (**3**) was isolated by gel filtration.

The conjugate of PAA with GMDP (**4**) was obtained analogously.

Conjugate of PAA with MDP and PEA (5). A solution of 9 mg (47 μg-equiv) of poly(4-nitrophenyl acrylate) in 0.5 ml of DMFA was treated with 7 mg (11 μmole) of the 6-aminohexyl β-glycoside of MDP (**1**) in 0.5 ml DMFA, 0.2 ml of a solution of 9 mg of PEA in chloroform–methanol (1:1), and 3 drops of triethylamine. The synthesis was conducted as described above, giving 17 mg (94%) of polymer (**5**).

The conjugates of PAA with GMDP and PEA (**6**) and of PAA with MDP and [^{14}C]PEA (**7**) were obtained analogously.

Biotinylated Polymeric Probe (9). To a solution of 34 mg (175 μg-equiv.) of poly(4-nitrophenyl acrylate) in 0.5 ml of DMSO were added 21 mg (34 μmole) of the 6-aminohexyl β-glycoside of MDP (**1**) and 4 mg (8.5 μmole) of the 6-aminohexylamide of biotin in 0.5 ml [sic] and 3 drops of triethylamine. The synthesis was conducted as described above, giving 30 mg (84%) of the polymer (**9**).

The biotinylated polymeric probe (**10**) was synthesized analogously from the γ-(6-aminohexylamide) of MDP (**8**).

Conjugate of Dextran with MDP (11). To a solution of 75 mg of dialdehydodextran (containing 1.9 mmole of aldehyde groups per 1 g) in 3 ml of 2% $NaHCO_3$ was added 18 mg (29 μmole) of the 6-aminohexyl β-glycoside of MDP (**1**). The reaction mixture was kept at pH 8-8.5 for 24 h. After the absence of the amino component had been established (monitoring by TLC in system 3), 6.6 mg (174 mmole) of $NaBH_4$ was added and the mixture was stirred for 2 h, the pH of the medium being controlled (no greater than 9) by the addition of 5% acetic acid. The polymer (**11**) (75 mg; 88%) was isolated by gel filtration.

The dextran conjugate (**12**) was synthesized analogously from the γ-(6-aminohexylamide) of MDP (**8**).

6-Chlorohexyl 2-Acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-*D*-glucopyranoside (13). A solution of 500 mg (1.4 mmole) of 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-*D*-glucopyranosyl chloride (**14**) [15] in 10 ml of dry dichloroethane was treated with 1.16 equiv. of mercury(II) iodide and 187 mg (1.4 mmole) of 6-chlorohexan-1-ol. The reaction mixture was stirred in the presence of 3 Å molecular sieves until the glycosyl donor had disappeared (monitoring by TLC in system 1). The molecular sieves and the undissolved catalyst were filtered off, and the filtrate was diluted with chloroform and washed with potassium iodide solution and with water. The organic layer was separated off, dried with anhydrous Na_2SO_4, and evaporated. Purification by column chromatography (eluent: CCl_4 → CCl_4–propan-2-ol (10:1)) gave 326 mg (51%) of glycoside (**5b**), mp 112-114°C, $[\alpha]_{546}$ −21° (*c* 1.0; chloroform).

PMR (300 MHz, $CDCl_3$): 1.40, 1.58, 1.79 (10H, $(CH_2)_4$, m), 1.95, 2.02 (6H), 2.09 (12 H, NAc and 3 OAc, s), 3.50, 3.84 (2H, $-OCH_2-$, dt), 3.54 (2H, CH_2Cl, t), 3.70 (1H, H-5, ddd), 3.87 (1H, H-2, m), 4.14 and 4.27 (2H, H-6a, H-6b, $J_{5,6a}$ 2.5 Hz $J_{5,6b}$ 4.5 Hz, $J_{6a,6b}$ 12 Hz, dd), 4.68 (1H, H-1; $J_{1,2}$ 8.5 Hz, d), 5.07 (1H, H-4; $J_{4,5}$ 9.5 Hz, dd), 5.30 (1H, H-3; $J_{3,4}$ 9.5 Hz, $J_{2,3}$ 10 Hz, dd), 5.54 (1H, NH; J_{2NH} 8.5 Hz, d).

6-Chlorohexyl 2-Acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-*D*-glucopyranosyl)-6-O-acetyl-2-deoxy-3-O-[*D*-1-(methoxycarbonyl)ethyl]-β-*D*-glucopyranoside (16). A solution of 600 mg (0.91 mmole) of 2-methyl-({4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-*D*-glucopyranosyl)-6-O-acetyl-1,2-dideoxy-3-O-[*D*-1-(methoxycarbonyl)ethyl]-α-*D*-glucopyrano}-[2,1-*d*]-2-oxazoline) [14] in 12 ml of dry dichloroethane was treated with 122 mg (0.91 mmole) of 6-chlorohexan-1-ol and 15 mg of anhydrous TsOH. The reaction mixture was stirred at 80°C until the reaction was complete (monitoring by TLC in system 1). The solution so formed was neutralized with pyridine and evaporated. Column chromatography (eluent: chloroform → chloroform–ethanol (30:1)) led to the isolation of 420 mg (56%) of glycoside (**16**), mp 160-165°C (decomp.), $[\alpha]_{546}$ −41° (*c* 1.0; chloroform).

PMR (500 MHz, $CDCl_3$): 1.36, 1.58 ($[CH_2]n$, m), 1.95, 2.01 (9H), 2.06, 2.13 (18H, 2 NAc and 4 OAc, s), 1.35 (3H, $J_{Me,CH}$ 7 Hz, d), 3.25 (2H, $\underline{CH_2}Cl$, t), 3.50, 3.89 (2H, $-OCH_2-$, dt), 3.73 (3H, COOMe, s), 4.33 (1H, H-1; $J_{1,2}$ 8 Hz, d), 4.36 (1H, H-1′; $J_{1',2'}$ 8 Hz, d), 4.66 (1H, $\underline{CH}CH_3$, q), 6.08 and 7.01 (2H, 2NH, d).

6-Azidohexyl 2-Acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-*D*-glucopyranosyl)-6-O-acetyl-2-deoxy-3-O-[*D*-1-(methoxycarbonyl)ethyl]-β-*D*-glucopyranoside (17). A solution of 400 mg (0.49 mmole) of the chlorohexyl glycoside (**16**) in 5 ml of acetonitrile was treated with 160 mg (2.45 mmole) of sodium azide and 80 mg (0.49 mmole) of tetraethylammonium bromide, and the mixture was boiled for 24 h. Then it was evaporated, and column chromatography (eluent: chloroform → chloroform–ethanol (25:1)) yielded 340 mg (89%) of the azide (**17**); mp 176-178°C, $[\alpha]_{546}$ −42° (*c* 1.0; chloroform). IR spectrum (ν, cm^{-1}): 3280 (NH), 2930, 2860 (CH_2), 2100 (azide), 1780, 1230 (ester), 1670, 1550 (amide).

Methyl Ester of O-[6-Azidohexyl-2-acetamido-4-O-(2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-*D*-glucopyranosyl)-6-O-acetyl-2-deoxy-β-*D*-glucopyranosid-3-yl]-*D*-lactoyl-*L*-alanyl-*D*-isoglutamine (19). The neutrality of a solution of 300 mg (0.37 mmole) of the peracetate (**17**) in 5 ml of ethanol was established by the dropwise addition of 1 N KOH, and the course of the reaction was followed by TLC in system 2. After the end of saponification the solution was neutralized with KU-2 cation-exchanger (H^+), and the resin was filtered off and washed with 5 ml of ethanol. The filtrate was evaporated to dryness, and the residue was acetylated at room temperature with 2 ml of a 1:1 mixture of acetic anhydride and pyridine. The reaction mixture was evaporated, and the addition of ether precipitated 280 mg (95%) of the tetra-O-acetate of glucosaminylmuramic acid (**18**).

The acid (**18**) (270 mg, 0.34 mmole) was dissolved in 20 ml of dry THF and was activated with 78 mg (0.38 mmole) of DCC and 43 mg (0.38 mmole) of HOSu. After 12 h the precipitate of dicyclohexylurea was filtered off and washed with 5 ml of THF. The combined filtrate was treated with the trifluoroacetate of the methyl ester of *L*-alanyl-*D*-isoglutamine (obtained by the addition of trifluoroacetic acid to 135 mg (0.41 mmole) of the corresponding Boc derivative, followed by evaporation to dryness and neutralization with triethylamine). After completion of the reaction (monitoring by TLC in system 2), the precipitate of glycopeptide (**19**) was filtered off and purified by column chromatography (eluent: chloroform → chloroform–ethanol (20:1)). The yield of glycopeptide (**19**) was 260 mg (76%); amorphous white powder, $[\alpha]_{546}$ −44° (*c* 1.1; chloroform). PMR (500 MHz, DMSO-d_6): 1.28, 1.45, 1.51 ([CH_2]*n*, m), 1.77 s (6H), 1.91, 1.95, 1.96, 2.07 (18H, 2 NAc and 4 OAc, s), 1.24 and 1.27 (6H, J_{MeCH} 7 Hz, d), 2.30 (2H, γ-CH_2-Glu, t), 3.05 (2H, $\underline{CH_2}Cl$, t), 3.58 (3H, COOMe, s), 4.31 (1H, H-1; $J_{1,2}$ 8 Hz, d), 4.68 (1H, H-1; $J_{1,2}$ 8 Hz, d), 7.10 and 7.27 (2H, $CONH_2$, s), 7.48, 7.73, 8.03, and 8.13 (4H, 4 NH; d).

Methyl Ester of O-[6-Azidohexyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-6-O-acetyl-2-deoxy-β-*D*-glucopyranosid-3-yl]-*D*-lactoyl-*L*-alanyl-*D*-isoglutamine (20). A solution of 40 mg (0.04 mmole) of compound (**19**) in 2 ml of methanol was deacetylated by the addition of 2 drops of a 0.1 N solution of sodium methanolate in methanol. After 16 h, the solution was neutralized with KU-2 cation-exchanger (H^+), the resin was filtered off and washed with 3 ml of methanol, and the filtrate was evaporated. This gave 30 mg (91%) of the disaccharide-dipeptide (**20**).

Methyl Ester of O-[6-Aminohexyl 2-acetamido-4-O-(2-acetamido-2-deoxy-β-*D*-glucopyranosyl)-6-O-acetyl-2-deoxy-β-*D*-glucopyranosid-3-yl]-*D*-lactoyl-*L*-alanyl-*D*-isoglutamine (2). A. A solution of 15 mg (0.018 mmole) of the azide (**20**) in 2 ml of methanol was treated with 0.1 ml of acetic acid, and hydrogenation was carried out over 10 mg of palladium oxide at room temperature for 16 h. The catalyst was filtered off and washed with 2 ml of methanol, the filtrate was evaporated, and acetone precipitated 12 mg (83%) of the glycopeptide (**2**), which was used directly for conjugation.

B. To a solution of 15 mg (0.018 mmole) of the azide (**20**) in 1 ml of ethanol, 2 mg (0.054 mmole) of $NaBH_4$ was added. The reaction mixture was kept for 12 h and was then brought to pH 7 by the addition of 0.01 N HCl. According to TLC (systems 3 and 4), a substance analogous to that synthesized by method **A** was obtained.

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