Carbohydrate Research, 108 (1982) 139–147
Elsevier Scientific Publishing Company, Amsterdam – Printed in The Netherlands

Note

Synthesis and immunoadjuvant activity of 2-acetamido-1,5-anhydro-2-deoxy-3-*O*-[(*R*)-2-propanoyl-L-alanyl-D-isoglutamine]-D-glucitol ("1-deoxymuramoyl dipeptide") and its 6-(2-behenoyloxyisobutyrate)*

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(Received March 1st, 1982; accepted for publication, March 25th, 1982)

Our interest[1,2] in the immunostimulatory properties of *N*-acetylmuramoyl-L-alanyl-D-isoglutamine** ("muramoyl dipeptide; MDP") (**1**), the minimum structure of mycobacteria in Freund's complete adjuvant (FCA) necessary for immunoadjuvancy[3], also led us to investigate the effects, on biological activity, of modification of the 2-acetamido-2-deoxy-D-glucosyl moiety of the MDP structure. The objective of the study was to discover analogs not only having adjuvant activity enhanced over that of MDP, but also fewer and more tolerable side-effects, because, although MDP and known derivatives lack many of the toxic properties of FCA, immunotherapeutic applications remain restricted by the persistence of other undesirable side-effects, such as pyrogenicity[4,5], transitory leukopenia[4], and enhancement of endotoxic shock[6]. In a related approach toward obtaining glycopeptide adjuvants exhibiting lower toxicity, or pharmacodynamic advantages, or both, we reported[1] the synthesis and immunoadjuvant activities of several 2-acetamido-5-*O*-acetyl-6-*O*-acyl-2-deoxy-3-*O*-[(*R*)-2-propanoyl-L-alanyl-D-isoglutamine]-D-glucofuranoses, for which evidence was provided that they function as prodrug forms of 6-*O*-acyl derivatives of MDP.

In addition to synthesizing configurational[7] and positional[8] isomers of MDP, we also modified the 1-, 4-, and 6-hydroxyl groups in the 2-acetamido-2-deoxy-D-glucosyl residue. In most instances, replacement of the 4- or 6-hydroxyl group, or both, with other functional groups resulted in loss of humoral immunoadjuvant activity. Illustratively, 6-deoxy-MDP (**2**), 6-amino-6-deoxy-MDP (**3**), 6-acetamido-6-deoxy-MDP (**4**), and 6-deoxy-6-octadecanamido-MDP (**5**), when administered to

*Bacterial Cell-wall Constituents, Part VI. For Part V, see ref. 1.
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***N*-[2-*O*-(2-Acetamido-2,3-dideoxy-D-glucopyranose-3-yl)-D-lactoyl]-L-alanyl-D-isoglutamine.

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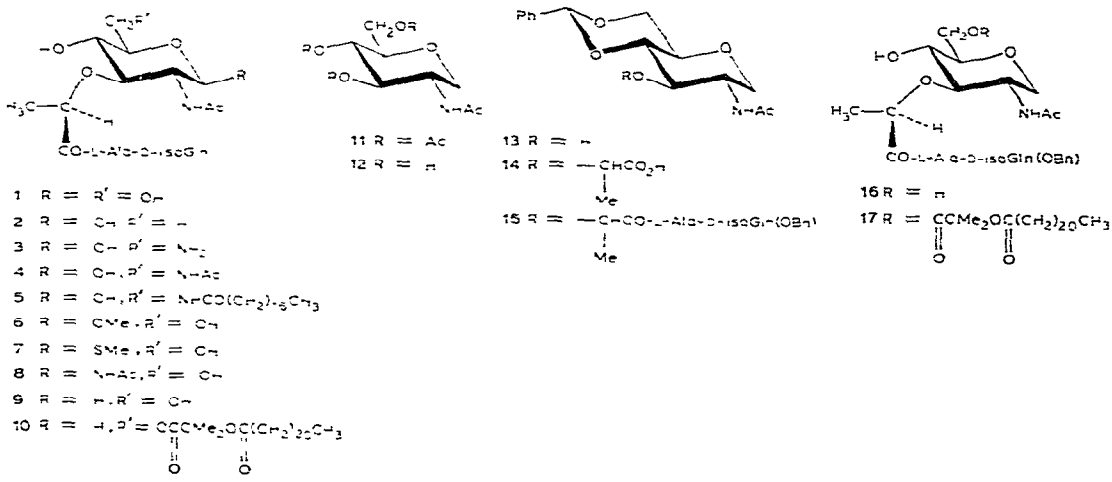

TABLE I

ANTIBODY RESPONSE OF MICE[a] INJECTED WITH BOVINE SERUM ALBUMIN (BSA) ALONE, OR IN COMBINATION WITH MDP OR MDP ANALOG

Experiment No.	*Injection*	*Geometric mean titer*[b]	
		50 μg/dose	*5 μg/dose*
1	BSA alone	3.89	
	BSA + MDP	342.10	[c]
	BSA + **6**	113.05	7.41
2	BSA alone	9.52	
	BSA + MDP	1536.00	[c]
	BSA + **7**	63.34	35.61
3	BSA alone	3	
	BSA + MDP	179.0	54.5[d]
	BSA + **8**	<3	<3
4	BSA alone	3.89	
	BSA + MDP	28.26	2.57
	BSA + **9**	241.90	7.78

[a]Groups of six ICR/Ha mice. [b]Results with 31-day postinjection sera, expressed as the reciprocal of the serum dilution. [c]Not tested at this dose. [d]10 μg/dose.

mice in an aqueous medium, did not elevate[9] antibody production against bovine serum albumin*** (BSA). However, unexpected results were obtained when chemical modifications were performed at the anomeric hydroxyl group. As the methyl β-pyranoside (**6**) of MDP had previously been found to have adjuvant activity (in-

***In contrast to these observations on the humoral component of the immune response, compounds **3**, **4**, and **5**, when formulated in Freund's incomplete adjuvant as a water-in-oil emulsion, were found by Hasegawa *et al.*[10] to be capable of inducing a delayed hypersensitivity (cellular immunity) reaction to ABA-*N*-acetyltyrosine in guinea pigs; compound **2** was reported to be inactive.

duction of experimental, allergic encephalomyelitis in guinea pigs) comparable to that of the parent reducing sugar[11], the corresponding methyl 1-thio-β-pyranoside (**7**) and *N*-acetyl-β-pyranosylamine derivative (**8**) were synthesized[8], in order that the effect, on adjuvant activity, of increasing steric bulk at C-1 might be examined. Adjuvant activity for these compounds was measured in terms of their ability (in saline) to enhance the antibody response in mice against BSA. Details of the biological assay are given in the Experimental section, and the results are presented in Table I. A Duncan statistical analysis[12] was performed on the log antibody titers. At 50 μg, the methyl β-pyranoside (**6**) of MDP had activity comparable to that of MDP at the same dose, whereas, at 5 μg, its activity was comparable to that of BSA alone. The methyl 1-thio-β-pyranoside (**7**) at 100 μg, and the 2-acetamido-*N*-acetyl-2-deoxy-glucosylamine analog **8** at 50 μg had lower activity than MDP at 50 μg.

Thus, although, in agreement with literature observations[11], the methyl β-pyranoside **6** was equipotent to MDP, a further increase in the size of the C-1 substituent (SCH_3, $NHCOCH_3$) resulted in loss of biological activity.

In the direction of decreasing the bulk at the anomeric center relative to MDP, the 1-hydroxyl group was replaced with a deoxy function, to afford 2-acetamido-1,5-anhydro-2-deoxy-3-*O*-[(*R*)-2-propanoyl-L-alanyl-D-isoglutamine]-D-glucitol (**9**). This nonreducing analog of MDP was synthesized from 2-acetamido-3,4,6-tri-*O*-acetyl-1,5-anhydro-D-glucitol (**11**), which was obtained by following the procedure described by Horton and Wolfrom[13] for the Raney nickel desulfurization of 2-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-2-thiopseudourea hydrochloride. Zemplén deacetylation of **11** afforded the known[14] 2-acetamido-1,5-anhydro-2-deoxy-D-glucitol (**12**). The 4- and 6-hydroxyl groups in **12** were blocked as the benzylidene acetal (**13**), and alkylation of the 3-hydroxyl group in **13** with (*S*)-2-chloropropanoic acid[15] gave the desired (*R*)-lactic acid ether **14**. The protected dipeptide was introduced by the mixed anhydride method (reaction of acid **14** with L-alanyl-D-isoglutamine benzyl ester hydrochloride[16] in *N*,*N*-dimethylformamide in the presence of 4-methylmorpholine and isobutyl chloroformate) to afford the fully blocked glycodipeptide **15**. The benzyl ester and benzylidene acetal protecting groups were removed by catalytic hydrogenolysis of **15** in glacial acetic acid.

Injection of 50 μg of 1-deoxy-MDP (**9**) in saline into mice significantly elevated antibody titers against BSA, with activity comparable to that of MDP at the same dose (see Table I). The surprising retention of adjuvant activity upon introduction of a deoxy function at the anomeric position of MDP is to be contrasted with the absence of such activity observed[9,10] with the positionally isomeric 6-deoxy-MDP (**2**). These results are indicative of a greater sensitivity of adjuvant activity to size, rather than polarity, of the C-1 substituent in the MDP molecule.

Although stimulation of antibody production in mice has even been observed with oral administration of MDP in an aqueous medium[17], enhancement of cell-mediated immunity in saline requires an increase in the lipophilic character of the molecule, such as can be obtained by acylation of the 6-hydroxyl group in the sugar moiety with a fatty carboxylic acid. Thus, delayed hypersensitivity reactions in the

TABLE II

ANTIBODY RESPONSE OF MICE INJECTED WITH BSA ALONE, OR IN COMBINATION WITH MDP OR COMPOUND **10**

Injection	*Anti-BSA antibody units/25 μL of serum*[a]
BSA alone (500 μg)	68 ±36
BSA + MDP (100 μg)	1569 ±412
BSA + **10** (50 μg)	4918 ±370

[a]Average of five Balb/cJ mice ± standard deviation. 100 Units equivalent to specific antibody contained in 25 μL of a 1:100 dilution of anti-BSA reference serum.

absence of a nonmetabolizable, oil component were recorded[18] with a number of 6-*O*-acyl derivatives of MDP. It was, therefore, of interest to prepare a 6-fatty acylate of 1-deoxy-MDP (**9**), in order to obtain a derivative capable of stimulating both the humoral and cellular components of the immune response.

Because the 6-(2-behenoyloxyisobutyrate) [6-(2-docosanoyloxy-2-methylpropanoate)] of MDP, a more lipophilic derivative than the corresponding 6-stearate (6-octadecanoate), was consistently found in our laboratories to have significantly greater adjuvant activity than MDP at both the 50- and 5-μg levels[1], we decided to introduce this fatty acyl group* at O-6 of 1-deoxy-MDP (**9**). 2-Acetamido-1,5-anhydro-6-*O*-(2-behenoyloxyisobutyryl)-2-deoxy-3-*O*-[(*R*)-2-propanoyl-L-alanyl-D-isoglutamine]-D-glucitol (**10**) was prepared by debenzylidenation of **15** to give diol **16**, regioselective acylation of the primary 6-hydroxyl group in **16** with 2-(behenoyloxy)-isobutyric acid[19] by the DMAP**-catalyzed DCC** method[20] to give **17**, and catalytic hydrogenolysis of the benzyl ester protecting group in **17**.

The antibody response measured against BSA with 6-*O*-(2-behenoyloxyisobutyryl)-1-deoxy-MDP (**10**) at 50 μg was significantly greater than that elicited by MDP at 100 μg (see Table II). Compound **10** at 5 μg per animal also significantly enhanced[21] the antibody response in mice to pig zona pellucida glycoprotein (5 μg), the response being comparable to that obtained with Freund's complete adjuvant. Immunization of female rabbits with pig zona preparations has been reported to inhibit pregnancy[22]. Moreover, when tested in rabbits at 50 μg/kg, compound **10** was found to be nonpyrogenic[23].

EXPERIMENTAL

Biological. — The immunoadjuvant activities of the compounds described herein, except **10**, were determined in the following way. Groups of six 5–6-week-old,

*Introduction of this particular acyl group at O-6 of the 5-acetylated dipeptidyl furanosyl system also gave rise to a significant enhancement of immunoadjuvant activity[1]. The medicinal-chemical rationale for selecting this carboxylic acid was presented in ref. 1.

**For explanation of the abbreviation, see the Experimental section.

female, ICR/Ha mice were subcutaneously injected between the shoulders with 0.25 mL of a mixture containing 100 μg of bovine serum albumin monomer (Miles Laboratories) and 50 or 5 μg of MDP analog dissolved, or dispersed by sonication, in phosphate-buffered saline (PBS). In all experiments, groups of mice were administered MDP (50 or 5 μg) with BSA (100 μg), as a positive control, or BSA (100 μg) with no analog, as a low response control. At 21 days post-immunization, all mice were boosted with 100 μg of BSA alone (no analog). Ten days layer (31 days after initial injection), all mice were bled, and the sera were assayed for anti-BSA, passive hemagglutination antibody titer. Each serum was adsorbed with two volumes of a 20% (v/v) suspension of washed, sheep red blood-cells in PBS, after which, serial two-fold dilutions were incubated with BSA-coated sheep cells. Settling patterns were determined, and group geometric mean passive hemagglutination antibody titers were calculated. The results are shown in Table I.

The activity of compound **10** was assayed in groups of five 8–10-week-old, female, Balb/cv mice obtained from Jackson Laboratories, Bar Harbor, Maine. Mice were immunized subcutaneously with 500 μg of bovine albumin (BSA, Pentex Fraction V from Miles Laboratories) in 0.2 mL of pyrogen-free saline, with, or without, 100 μg of MDP or 50 μg of **10**. The mice were challenged 21–25 days later with a subcutaneous injection of 100 μg of BSA, and bled, from the retroorbital plexus, 9–11 days later. The antibody titer was estimated in arbitrary units relative to a standard, high-titer serum, using a solid-phase radioimmunoassay, as previously described[24]. The results, given in Table II, are expressed as units of BSA-binding antibody. One hundred units was equivalent to the specific antibody contained in 25 μL of a 1 : 100 dilution of anti-BSA, reference serum. Units of unkown serum were calculated on the basis of a standard dilution curve of the reference serum.

Chemical. — General methods. Solutions were evaporated below 50° under diminished pressure. Melting points were determined with a Thomas–Hoover "Unimelt" apparatus and are uncorrected. Optical rotations were measured with a Perkin–Elmer Model 241 polarimeter. Mass spectra were recorded with an LKB Model 9000 spectrometer (electron impact). N.m.r. spectra were recorded at 300 MHz with a Varian SC-300 n.m.r. spectrometer. Chemical shifts are given on the δ scale. Spectra were measured at ambient temperature for solutions, as indicated, in dimethyl sulfoxide-d_6 or deuterium oxide, with tetramethylsilane (δ 0.00) as the internal standard. Spectra were analyzed on a first-order basis. T.l.c. was performed on plates (250 μm) of Silica Gel GF_{254} (Analtech), and indication was effected with ultraviolet light or a ceric sulfate (1%)–sulfuric acid (10%) spray. Column chromatography was conducted with silica gel No. 7734 (E. Merck; 70–230 mesh).

2-Acetamido-1,5-anhydro-4,6-O-benzylidene-2-deoxy-D-glucitol (**13**). — A mixture of 2-acetamido-1,5-anhydro-2-deoxy-D-glucitol[14] (**12**) (500 mg, 2.44 mmol) and anhydrous zinc chloride (700 mg) in benzaldehyde (10 mL) was stirred, with exclusion of moisture, for 3 h at room temperature. The product was precipitated by addition of water and hexane to the reaction mixture. The solid was filtered off, washed with copious amounts of water and then hexane, and dried *in vacuo* over phosphorus

pentaoxide; yield 530 mg (74%); n.m.r. (dimethyl sulfoxide-d_6): δ 7.90 (d, N*H*Ac), 5.62 (s, benzylic H), 5.27 (d, OH-3), 4.18 (dd, $J_{1e,2a}$ 5.0, $J_{1e,1a}$ 10.2 Hz, H-1*e*), and 1.83 (s, 3 H, NHAc).

*2-Acetamido-1,5-anhydro-4,6-O-benzylidene-3-O-[(R)-1-carboxyethyl]-2-deoxy-*D*-glucitol* (**14**). — To a stirred solution of **13** (530 mg, 1.81 mmol) in dry 1,4-dioxane (40 mL) was added sodium hydride (50% oil dispersion; 177 mg). The mixture was stirred, with exclusion of moisture, for 1 h at 95°. The temperature was then lowered to 65°, a solution of (*S*)-2-chloropropanoic acid[15] (353 mg, 3.25 mmol) in a small volume of 1,4-dioxane was added, and the mixture was stirred for 1 h at 65°. Additional sodium hydride (710 mg) and (*S*)-2-chloropropanoic acid (353 mg) were added, and the mixture was stirred overnight at 65°. The mixture was cooled, slowly poured into chilled (ice-bath) 50% acetic acid (25 mL), to decompose the excess of sodium hydride, evaporated, the residue partitioned between dichloromethane and water, and the organic layer separated, and evaporated. The resulting syrup was dissolved in a small volume of chloroform, and the solution was applied to a column of silica gel (packed as a slurry in chloroform) that was eluted with 175:5:1 chloroform–methanol–acetic acid. Evaporation of the appropriate fractions gave the desired lactic acid ether **14** as a white solid that was dried *in vacuo* over phosphorus pentaoxide: yield 210 mg (32%); m.p. 242–244° (dec.), $[\alpha]_D^{27}$ −26° (*c* 0.54, methanol); n.m.r. (dimethyl sulfoxide-d_6): δ 5.71 (s, benzylic H), 4.30 (q, $J_{CH_3,CH}$ 7.0 Hz, -OC*H*CO_2H), 4.21 (dd, $J_{1e,2a}$ 5.0, $J_{1e,1a}$ 10.3 Hz, H-1*e*), 1.85 (s, 3 H, NHAc), and 1.26 [d, CH_3 (lac)].

Anal. Calc. for $C_{18}H_{23}NO_7$ (365.39): C, 59.17; H, 6.34; N, 3.83. Found: C, 58.93; H, 6.41; N, 3.61.

*2-Acetamido-1,5-anhydro-4,6-O-benzylidene-2-deoxy-3-O-[(R)-2-propanoyl-*L*-alanyl-*D*-isoglutamine benzyl ester]-*D*-glucitol* (**15**). — To a solution of **14** (200 mg, 0.55 mmol) in dry *N,N*-dimethylformamide (2.3 mL) at −15° were successively added 4-methylmorpholine (60 µL, 0.55 mmol) and isobutyl chloroformate (71 µL, 0.55 mmol). After stirring for 3 min at −15°, a cooled solution of L-alanyl-D-isoglutamine benzyl ester hydrochloride[16] (219 mg, 0.64 mmol) and 4-methylmorpholine (70 µL, 0.64 mmol) in dry *N,N*-dimethylformamide (2.3 mL) was added. The mixture was stirred, with exclusion of moisture, for 4 h at −15°. The temperature was then allowed to rise to 0°, 2.5M aqueous potassium hydrogencarbonate (1 mL) was added, and the mixture was stirred for 30 min at 0°. The product was precipitated by addition of water (25 mL). The resulting solid was filtered off, washed with water, and dried by suction, and then *in vacuo* over phosphorus pentaoxide; yield 320 mg (89%); m.p. 249–252° (dec.); n.m.r. (dimethyl sulfoxide-d_6): δ 5.71 (s, benzylic H), 5.09 (s, -CO_2CH_2Ph), 4.21 (q, -OC*H*CO_2H), 2.36 (t, -CH_2CO_2Bzl), 2.02 and 1.78 (2 m, -$CH_2CH_2CO_2$Bzl), 1.81 (s, 3 H, NHAc), and 1.23 [d, CH_3(lac) and CH_3(ala)]; *m/z* 654 (M).

*2-Acetamido-1,5-anhydro-2-deoxy-3-O-[(R)-2-propanoyl-*L*-alanyl-*D*-isoglutamine)-*D*-glucitol* (**9**). — To a solution of **15** (100 mg, 0.15 mmol) in glacial acetic acid (8 mL) was added palladium oxide (200 mg). The mixture was stirred for 4 h at

room temperature under an atmosphere of hydrogen. The catalyst was removed by filtration through Celite, the filtrate was evaporated, and traces of solvent were co-evaporated several times with toluene and then methanol. The residue was dissolved in a small volume of methanol, and the product was precipitated by addition of diethyl ether. The solid was filtered off, washed with diethyl ether, and dried *in vacuo* over phosphorus pentaoxide; yield 69 mg (95%); $[\alpha]_D^{27}$ +15.7° (*c* 0.51, methanol); n.m.r. (deuterium oxide): δ 2.39 (t, -C*H*$_2$CO$_2$H), 2.18 and 1.98 (2 m, -C*H*$_2$C*H*$_2$CO$_2$H), 1.96 (s, 3 H, NHAc), and 1.24 and 1.18 [2 d, CH$_3$(ala) and CH$_3$(lac)].

Anal. Calc. for $C_{19}H_{32}N_4O_{10} \cdot 1.5\ H_2O$ (503.52): C, 45.32; H, 7.01. Found: C, 45.29; H, 7.23.

2-Acetamido-1,5-anhydro-2-deoxy-3-O-[(R)-*2-propanoyl*-L-*alanyl*-D-*isoglutamine benzyl ester*]-D-*glucitol* (**16**). — A mixture of **15** (190 mg, 0.29 mmol) in 60% acetic acid (8 mL) was stirred for several hours at 85°, until t.l.c. indicated complete conversion into a slower-moving material. The mixture was cooled, evaporated, and traces of solvent were coevaporated several times with toluene. Trituration of the resulting syrup with diethyl ether afforded 4,6-diol **16** as a white solid; yield 162 mg (99%); $[\alpha]_D^{27}$ +15.3° (*c* 0.72, methanol).

Anal. Calc. for $C_{26}H_{38}N_4O_{10} \cdot 1.5\ H_2O$ (593.65): C, 52.61; H, 6.96; N, 9.44. Found: C, 52.80; H, 6.92; N, 9.29.

2-Acetamido-1,5-anhydro-6-O-(*2-behenoyloxyisobutyryl*)-*2-deoxy-3*-O-[(R)-*2-propanoyl*-L-*alanyl*-D-*isoglutamine benzyl ester*]-D-*glucitol* (**17**). — To a solution of **16** (150 mg, 0.25 mmol) in dry *N*,*N*-dimethylformamide (3 mL) were successively added 4-(dimethylamino)pyridine (DMAP; 4 mg), 2-(behenoyloxy)isobutyric acid[19] (112 mg, 0.26 mmol), and dicyclohexylcarbodiimide (DCC) (55 mg, 0.27 mmol), and the mixture was stirred overnight at room temperature, at which time, sufficient dichloromethane was added to achieve dissolution. Additional acid (112 mg) and DCC (55 mg) were added, and the mixture was again stirred overnight at room temperature. This process was repeated a third time, with stirring continued for 3 d at room temperature. The mixture was then evaporated, the syrup taken up in dichloromethane, washed twice with 0.5M hydrochloric acid, once with saturated sodium hydrogencarbonate, and once with water, and evaporated. The residue was dissolved in a small volume of chloroform, and the solution was applied to a column of silica gel that was eluted with 20:1 chloroform–methanol. Evaporation of the appropriate fractions afforded the desired 6-(2-behenoyloxyisobutyrate) **17**; yield 144 mg (58%); n.m.r. (dimethyl sulfoxide-d_6): δ 5.58 (d, OH-4), 5.10 (s, -CO$_2$C*H*$_2$Ph), 2.36 (t, -C*H*$_2$CO$_2$Bzl), 2.26 [t, -OCOC*H*$_2$(CH$_2$)$_{19}$CH$_3$], 2.02 and 1.78 (2 m, -C*H*$_2$CH$_2$CO$_2$Bzl), 1.78 (s, 3 H, NHAc), 1.47 (s, 6 H, -CO-CMe$_2$-O-), and 0.86 [t, -(CH$_2$)$_{20}$C*H*$_3$].

2-Acetamido-1,5-anhydro-6-O-(*2-behenoyloxyisobutyryl*)-*2-deoxy-3*-O-[(R)-*2-propanoyl*-L-*alanyl*-D-*isoglutamine*]-D-*glucitol* (**10**). — To a solution of **17** (140 mg, 0.14 mmol) in glacial acetic acid (5 mL) was added palladium oxide (150 mg). The mixture was stirred under an atmosphere of hydrogen overnight at room temperature. The catalyst was removed by filtration through Celite, the filtrate evaporated, and

traces of solvent were coevaporated several times with toluene. The residue was dissolved in a small volume of chloroform, and the solution was applied to a column of silica gel that was eluted initially with 9:1 chloroform–methanol and then with 40:10:1 chloroform–methanol–water. Evaporation of the appropriate fractions gave the desired product as amorphous material from which diethyl ether was evaporated several times. The product was dried *in vacuo* over phosphorus pentaoxide; yield 105 mg (79%); $[\alpha]_D^{27}$ +16.4° (*c* 1.2, methanol); n.m.r. (dimethyl sulfoxide-d_6): δ 2.27 [t, -OCOCH_2(CH$_2$)$_{19}$CH$_3$], 2.19 (t, -CH_2CO$_2$H), 1.93 (m, -CHCH$_2$CO$_2$H), 1.79 (s, 3 H, NHAc), 1.46 (s, 6 H, -CO-CMe$_2$-O-), and 0.86 [t, -(CH$_2$)$_{20}$CH_3].

Anal. Calc. for $C_{45}H_{80}N_4O_{13} \cdot 2.5\ H_2O$ (930.21): C, 58.11; H, 9.21; N, 6.02. Found: C, 58.06; H, 9.05; N, 5.74.

ACKNOWLEDGMENTS

The authors thank Dr. David Wood and Mary Jo Staruch for determining the immunoadjuvant activity of compound **10**, Mr. Herman Flynn for 300-MHz, n.m.r.-spectral measurements, Mr. Jack Smith for mass-spectral measurements, Mr. Jack Gilbert and his associates for microanalyses, and Holly Matthews and Mr. Timothy Schofield for statistical analyses.

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