Synthesis and Immunoadjuvant Activities of 2-Acetamido-5-*O*-acetyl-6-*O*-acyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucofuranoses as Potential Prodrug Forms of 6-*O*-Acyl Derivatives of *N*-Acetylmuramyl Dipeptide[1]

Philippe L. Durette,*[†] Conrad P. Dorn, Jr.,[†] Arthur Friedman,[‡] and Abner Schlabach[‡]

Merck Sharp & Dohme Research Laboratories, Rahway, New Jersey 07065, and West Point, Pennsylvania 19486.
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2-Acetamido-5-*O*-acetyl-6-*O*-acyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucofuranoses, designed as prodrug forms of the corresponding immunoadjuvant-active 6-*O*-acyl derivatives of *N*-acetylmuramyl dipeptide (MDP), were synthesized from benzyl 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-β-D-glucofuranoside and found, when administered to mice in an aqueous medium, to elevate antibody production against bovine serum albumin. The 5,6-di-*O*-acetyl derivative **8** exhibited activity similar to that of MDP at 50 μg/dose. The antibody titer measured for the 5-*O*-acetyl-6-*O*-stearoyl compound **9** was comparable to that obtained with 6-*O*-stearoyl-MDP at 50 μg, and both compounds were more active than MDP at 5 μg. The more lipophilic 5-*O*-acetyl-6-*O*-[2-(behenoyloxy)isobutyryl] compound **10** was considerably more active than MDP at both 50 and 5 μg; moreover, its potent adjuvant activity was not diminished at the lower dose. The three 5-*O*-acetylated 6-*O*-acylated dipeptidyl furanose derivatives also significantly stimulated production of circulating antibodies against hepatitis B vaccine in mice; titers were comparable to those observed with the alum-adsorbed vaccine. The range of immunoadjuvant activities obtained with **8–10** and control compounds supports a prodrug mechanism for this class of furanoid MDP analogues.

Of the known immunoadjuvants, the most active is Freund's complete adjuvant (FCA), which is a water-in-oil emulsion containing the antigen in the aqueous phase and whole killed mycobacteria in paraffin oil.[2] The minimum structure necessary for the adjuvant activity of FCA has been identified[3] by chemical synthesis as a low molecular weight, hydrosoluble peptidoglycan fragment of the bacterial cell wall, viz., *N*-acetylmuramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, MDP). Addition of MDP to an emulsion of Freund's incomplete adjuvant with an antigen increases levels of antibodies against the antigen (humoral response) and induces delayed hypersensitivity (cellular immunity).[4,5] The synthetic immunoadjuvant has also been shown to enhance nonspecific immunity of adult,[6] as well as neonatal,[7] mice to infection by *Klebsiella pneumoniae*.

Stimulation of antibody production in mice has been observed even with oral administration of MDP in an aqueous medium;[8] however, 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 C-6 hydroxy in the carbohydrate moiety with a fatty carboxylic acid. Thus, delayed hypersensitivity reactions in the absence of a nonmetabolizable oil component were recorded with a number of 6-*O*-acyl derivatives of MDP.[5,9] Similar results were obtained when the fatty acid derivatives were incorporated into liposomes.[10] Their potential clinical utility in vaccines for the prevention of parasitic or bacterial infections was demonstrated by the effective immunization of owl monkeys against infection with a human malaria parasite, *Plasmodium falciparum*.[11] The successful replacement of FCA by liposome-incorporated 6-*O*-stearoyl-MDP was considered an important step toward the development of a safe and effective vaccine for human malaria.[11] An adjuvant capable of providing protection in the absence of liposomes would be even more clinically desirable.

Nevertheless, although MDP and its derivatives are devoid of many of the toxic properties of FCA (immunogenicity, arthritogenicity, sensitization to tuberculin, etc.), immunotherapeutic applications remain restricted by the persistence of other undesirable side effects, such as pyrogenicity,[12,13] transitory leukopenia,[12] and enhancement of endotoxic shock.[14] Consequently, the MDP structure has undergone extensive chemical modification in searches for adjuvant-active analogues having fewer and more tolerable side effects. Moreover, MDP appears to have pharmacokinetic limitations. In vivo administration of a radiolabeled form in mice results in extremely rapid excretion of intact material into the urine (90% after 2 h).[15]

[†] Rahway, NJ.
[‡] West Point, PA.

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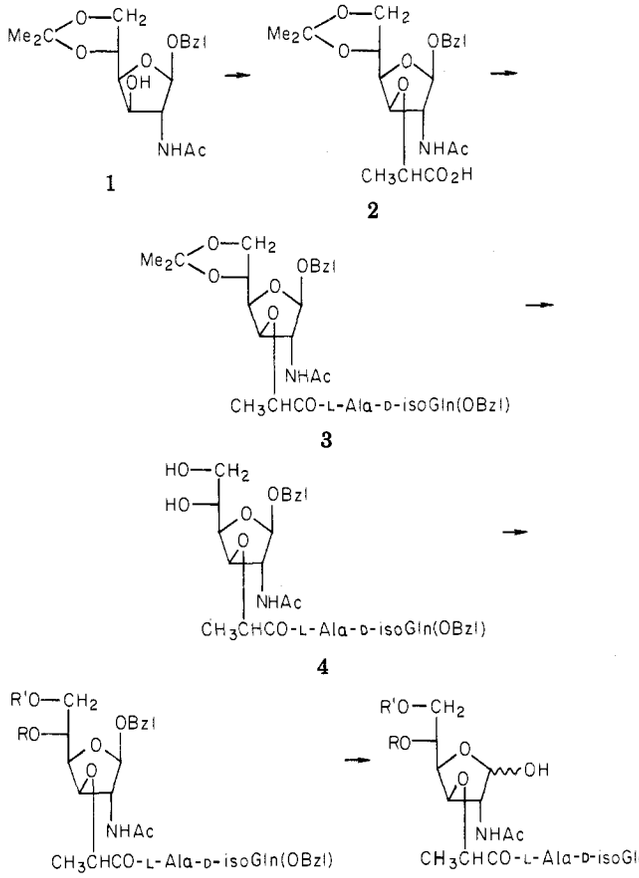
Scheme I[a]



[a] Ac = acetyl; Bzl = benzyl.

Immunoadjuvant activity could, therefore, in principle, be increased by modulation of the rapid clearance through slow-release forms. Control of in vivo distribution and presentation to target cells might also lead to improved biological properties.

As a novel approach toward obtaining glycopeptide adjuvants that exhibit lower toxicity and/or pharmacodynamic advantages, we report the synthesis and immunoadjuvant activities of several 2-acetamido-5-*O*-acetyl-6-*O*-acyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucofuranoses that were designed to function as prodrug forms of 6-*O*-acyl derivatives of *N*-acetylmuramyl dipeptide.

Chemistry. The starting material for the synthesis of the title compounds was the known benzyl 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-β-D-glucofuranoside (**1**),[16] which was converted into the (*R*)-lactic acid ether **2** by alkylation[17] with (*S*)-2-chloropropionic acid[18] in 1,4-dioxane in the presence of sodium hydride. The blocked dipeptide was next introduced by the mixed anhydride method (reaction of acid **2** with L-alanyl-D-isoglutamine benzyl ester hydrochloride[19] in DMF in the presence of *N*-methylmorpholine and isobutyl chloroformate) to afford the fully blocked dipeptidyl furanoside **3**. Deisopropylidenation was carried out with warm aqueous acetic acid. The resulting 5,6-diol **4** was treated with acetic anhydride in pyridine to give the protected 5,6-di-*O*-acetate **5**. The blocked 5-*O*-acetyl-6-*O*-acyl derivatives **6** and **7** were obtained by initial regioselective acylation of the primary C-6 hydroxy and subequent acetylation of the secondary C-5 hydroxy with acetic anhydride–pyridine. Selective stearoylation was achieved with stearoyl chloride in pyridine at room temperature, whereas acylation with 2-(behenoyloxy)isobutyric acid[20] was performed by the DMAP-catalyzed DCC method.[21] The benzyl ester and benzyl glycoside protecting groups in **5**–**7** were removed by catalytic hydrogenolysis in glacial acetic acid. In this fashion were obtained 2-acetamido-5,6-di-*O*-acetyl-2-deoxy-, 2-acetamido-5-*O*-acetyl-2-deoxy-6-*O*-stearoyl-, and 2-acetamido-5-*O*-acetyl-6-*O*-[2-(behenoyloxy)isobutyryl]-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucofuranose (**8**–**10**, respectively) (see Scheme I).

Table I. Antibody Response of Mice[a] Injected with Bovine Serum Albumin (BSA) Alone or in Combination with MDP or MDP Analogue

expt no.	injection	geometric mean titer[b] 50 μg/dose	5 μg/dose
1	BSA alone	28.54	
	BSA + MDP	592.21	28.12
	BSA + **12**	995.97	271.53
	BSA + **8**	271.53	31.12
	BSA + **9**	1408.52	28.12
2	BSA alone	<3	
	BSA + MDP	215.51	*c*
	BSA + **11**	384.00	4.36
3	BSA alone	<3	
	BSA + MDP	241.90	*c*
	BSA + **14**	<3	<3
	BSA + **13**	7.78	6.17
4	BSA alone	9.52	
	BSA + MDP	1536	*c*
	BSA + **17**	10.7	10.7
	BSA + **19**	768	431
5	BSA alone	3.89	
	BSA + MDP	28.26	<3 (2.57)
	BSA + **10**	215.51	543.06
	BSA + **9**	215.51	107.76
6	BSA alone	3.33	
	BSA + MDP	179.14	54.59[d]
	BSA + **19**	716.57	823.12
	BSA + **8**	113.49	13.08

[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.

Biological Results

The immunoadjuvant activities of the various peptidoglycans in saline were measured in terms of their ability to enhance the secondary antibody response of mice

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Table II. Antibody Response of Mice[a] Injected with Various Hepatitis B Vaccine Preparations

expt no.	preparation	no. of responders[b]	geometric mean titer[c]
1	aqueous	8	2.7
	alum	17	370.2
	8 (50 μg)	12	38.1
	9 (50 μg)	17	69.6
2	aqueous	6	2.5
	alum	17	286.5
	10 (50 μg)	20	303.6

[a] Groups of 20 female ICR mice. [b] The number of responders/total was analyzed by Fisher's exact test.[33] All preparations, except **8**, were significantly different from aqueous vaccine; only aqueous vaccine was significantly different from alum-adsorbed vaccine. [c] Results with 6-week postinjection sera expressed as the reciprocal of the serum dilution.

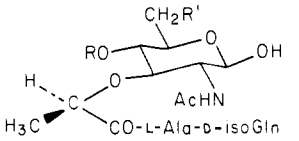
against bovine serum albumin (BSA) (humoral response). Details of the biological assay are given under Experimental Section. The results for six experiments are presented in Table I. A Duncan statistical analysis[22] was performed on the $\log_{10}$ antibody titers. In experiments 1 and 5, MDP at the 5 μg dose and BSA alone were not significantly different ($p > 0.05$), indicating that MDP is inactive at this dosage. The statistical evaluation of each compound is presented below. Comparable activity means there was no significant difference between the compounds ($p > 0.05$). Experiment 1: Compound **12** had activity comparable to MDP at 50 μg and greater than MDP at 5 μg. Compounds **8** and **9** had activity comparable to MDP at both 50 and 5 μg. Experiment 2: Compound **11** had activity comparable to MDP at 50 μg and BSA alone at 5 μg. Experiment 3: Compounds **13** and **14** had activity lower than MDP at 50 μg and comparable to BSA alone at 5 μg. Experiment 4: Compound **17** had activity lower than MDP at 50 μg and comparable to BSA alone at 5 μg. Compound **19** had activity comparable to MDP at 50 μg and greater than BSA alone at 5 μg. Experiment 5: Compounds **9** and **10** had activity greater than MDP at both 50 and 5 μg. Experiment 6: Compound **19** had activity comparable to MDP at 50 μg and greater than BSA alone at 5 μg. This compound at 5 μg had activity greater than MDP at 10 μg. Compound **8** had activity comparable to MDP at 50 μg and BSA alone at 5 μg. This compound at 5 μg had activity comparable to MDP at 10 μg.

The furanoid MDP analogues were tested for adjuvant activity by measuring their stimulatory effect on the production of circulating antibody following a primary immunization against hepatitis B vaccine in mice. Details of the vaccine preparations are given under Experimental Section. The results for two experiments were analyzed to compare hepatitis B vaccine with peptidoglycans as adjuvants to aqueous and alum-adsorbed vaccines. Data are presented in Table II. A Duncan analysis[22] of variance on the geometric mean titers showed all preparations to be significantly different ($p < 0.05$) from the aqueous vaccine. The following statistical evaluations were obtained. Experiment 1: Compound **9** at 50 μg had activity comparable to alum-adsorbed vaccine, and both were more active than aqueous vaccine. Compound **8** had activity comparable to **9** and greater than aqueous vaccine. Experiment 2: Compound **10** at 50 μg had activity comparable to alum-adsorbed vaccine, and both were more active than aqueous hepatitis B vaccine.

(22) SAS Institute Inc., Statistical Package Program, 1979.3.

Discussion

As part of a study of the effects of chemical modification of the carbohydrate moiety of the MDP molecule on immunoadjuvant activity, we were able to confirm published observations that derivatives in which the C-4 and/or C-6 hydroxys are acylated with short or long aliphatic carboxylic acids have activities comparable to that of MDP.[10,23,24] Thus, both 4,6-di-*O*-acetyl-MDP (**11**)[23] and



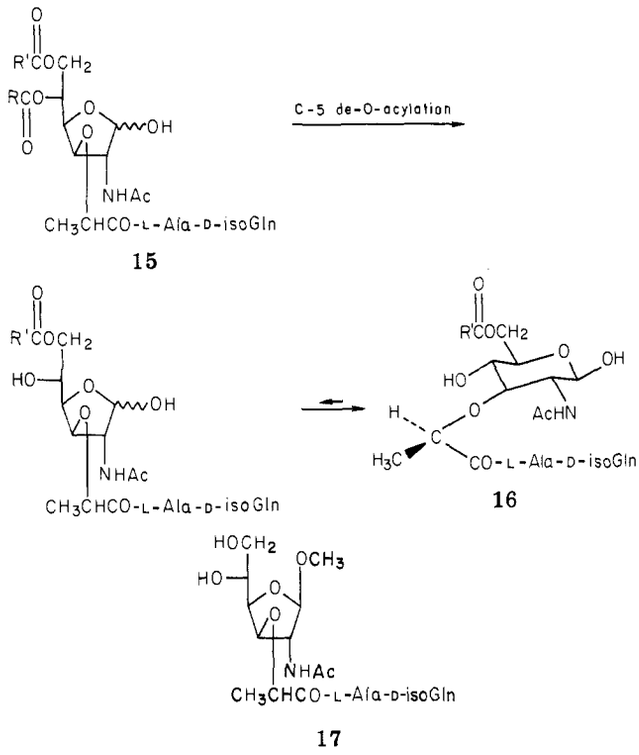
11, R = Ac; R' = OAc
12, R = H; R' = $OC(=O)(CH_2)_{16}CH_3$
13, R = H; R' = NHAc
14, R = H; R' = $NHC(=O)(CH_2)_{16}CH_3$
19, R = H; R' = $OC(=O)CMe_2OC(=O)(CH_2)_{20}CH_3$

6-*O*-stearoyl-MDP (**12**),[23] when injected into mice in saline, stimulated secondary antibody production against BSA (see Table I, experiments 1 and 2). However, surprisingly, both 2,6-diacetamido-2,6-dideoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucopyranose (6-acetamido-6-deoxy-MDP, **13**) and 2-acetamido-2,6-dideoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-6-(stearoylamino)-D-glucopyranose [6-deoxy-6-(stearoylamino)-MDP, **14**],[25,26] obtained by acylation of 6-amino-6-deoxy-MDP,[25,26] had no adjuvant activity at 5 and 50 μg/dose (see Table I, experiment 3). Since replacement of the acyloxy group with the hydrolytically more stable acylamino group abolished enhancement of the humoral response,[27] we speculated that the O-acylated derivatives were functioning as delivery systems for MDP.

If, indeed, ester hydrolysis were occurring in vivo (perhaps, from nonspecific esterase activity), we reasoned that 5-O-acylated furanoid analogues of MDP might serve as prodrug forms of MDP or its derivatives. Blocking of the sugar C-5 hydroxy as a carboxylic acid ester in an appropriately protected dipeptidyl furanoside prior to generation of the hemiacetal at the anomeric center (i.e., formation of a reducing sugar) would provide a structure having the carbohydrate moiety locked in the, presumably biologically inactive, five-membered ring form **15**. Only subsequent to C-5 de-O-acylation in vivo could tautomerization occur to afford the active pyranoid form **16**, the thermodynamically more stable sugar ring size assumed by MDP in aqueous solution[28] (see Scheme II). As a control, methyl 2-acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-β-D-glucofuranoside (**17**),[25] anticipated to be glycosidically stable in the furanoid form in vivo, was inactive at both 5 and 50 μg/dose (see Table I, experiment 4). The methyl β-pyranoside of MDP (**18**) had previously

(23) S. Kusumoto, S. Okada, K. Yamamoto, and T. Shiba, *Bull. Chem. Soc. Jpn.*, **51**, 2122 (1978).
(24) Y. Nagai, K. Akiyama, S. Kotani, Y. Watanabe, T. Shimono, T. Shiba, and S. Kusumoto, *Cell. Immunol.*, **35**, 168 (1978).
(25) P. L. Durette, unpublished results.
(26) A. Hasegawa, H. Okumura, M. Kiso, I. Azuma, and Y. Yamamura, *Carbohydr. Res.*, **79**, C20 (1980); *Agric. Biol. Chem.*, **44**, 1309 (1980).
(27) Although the 6-amido-6-deoxy-MDP derivatives **13** and **14** in saline did not enhance antibody titers against BSA in mice (see Table I, experiment 3), they have been reported,[26] when formulated in Freund's incomplete adjuvant as a water-in-oil emulsion, to induce in guinea pigs delayed hypersensitivity to *N*-acetyltyrosine-3-azobenzene-4'-arsonic acid.
(28) T. D. J. Halls, M. S. Raju, E. Wenkert, M. Zuber, P. Lefrancier, and E. Lederer, *Carbohydr. Res.*, 81, 173 (1980).

Scheme II



been found to have activity comparable to that of the parent reducing sugar.[24] That glycoside hydrolysis in **18** had not taken place in vivo antecedent to stimulation of the immune response at the cellular level was supported by the low activity observed with the corresponding methyl α-pyranoside.[24]

2-Acetamido-5,6-di-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucofuranose (**8**) was selected as a simple probe of the MDP-prodrug concept. It was synthesized from benzyl 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-β-D-glucofuranoside (**1**), which was chromatographically isolated in reasonable yield from the reaction of 2-acetamido-2-deoxy-D-glucose with 2,2-bis(benzyloxy)propane in DMF containing *p*-toluenesulfonic acid.[16] The blocked lactyl dipeptide fragment was incorporated at C-3, and the requisite 5,6-diol **4** was generated by hydrolysis of the isopropylidene acetal in **3**. Di-O-acetylation of **4** and subsequent removal of the protecting groups gave the desired furanose derivative **8**. Injection of 50 μg of this compound in saline into mice significantly elevated antibody titers against BSA, with activity comparable to that of MDP at the same dose. Activities for both **8** and MDP were comparably diminished at 5 μg/dose (see Table I, experiments 1 and 6). Compound **8** was also found to stimulate the production of circulating antibody against hepatitis B vaccine in mice. Antibody titers with **8** (50 μg) as an adjuvant were greater than those obtained with the aqueous vaccine alone (see Table II, experiment 1).

The observation of significant immunoadjuvant activity with compound **8** led us to the design and synthesis of 2-acetamido-5-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-6-*O*-stearoyl-D-glucofuranose (**9**) as a potential prodrug of 6-*O*-stearoyl-MDP (**12**). The latter MDP 6-acylate, when incorporated into liposomes with a protecting antigen, has shown promise as a safe and effective adjuvant suitable for use in humans.[11,29] At the 50 μg dose, **9** exhibited potent adjuvant activity with BSA, comparable to that obtained with 6-*O*-stearoyl-MDP (**12**) (see Table I, experiment 1). Statistically significant activity greater than that of MDP at the 5 μg level was seen in the experiment (no. 5) with a more typical, low GMT background for BSA alone. With the hepatitis B vaccine, compound **9** elicited an antibody response comparable to that of the alum-adsorbed vaccine; both preparations were more active than the aqueous vaccine alone. Moreover, as has been observed with lipophilic derivatives of MDP, compound **9**, as well as other 5-O-acetylated furanoid analogues of MDP having the C-6 hydroxy acylated with fatty carboxylic acids, should be capable, when administered in saline, of not only stimulating the humoral response but also inducing delayed hypersensitivity.

Since the 6-*O*-[2-(behenoyloxy)isobutyryl] derivative of MDP (**19**), a more lipophilic derivative than the corresponding 6-*O*-stearoyl derivative **12**, was found in our laboratories to have consistently, significantly greater adjuvant activity than MDP at both the 50 and 5 μg levels (see Table I, experiments 4 and 6), we also introduced this fatty acyl group at the C-6 position of our 5-acetylated dipeptidyl furanosyl system to further improve on biological activity. Compound **19** had been prepared[30] on the basis of reported observations with other drugs that such fatty acyl glycolate esters exhibit pharmacological advantages over stearoyl derivatives. Thus, prednisolone steaglate, the 21-stearoylglycolate of prednisolone, was found to be more active and less toxic than prednisolone itself and, in one study, provided for longer duration of antiinflammatory activity than prednisolone 21-stearate.[31] Behenate esters of bioactive alcohols have also been utilized for their prolonged activity and reduced side effects. As an illustration, the 3′-*O*-behenoyl derivative of 2,2′-anhydro-1-β-D-arabinofuranosylcytosine (cycloC) showed enhanced antiviral activity relative to that of cycloC itself.[20]

The antibody response measured against BSA with 2-acetamido-5-*O*-acetyl-6-*O*-[2-(behenoyloxy)isobutyryl]-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucofuranose (**10**) was significantly greater than that elicited by MDP at both 50 and 5 μg/dose (see Table I, experiment 5). Moreover, its potent adjuvant activity was not diminished at the lower 5 μg level. Compound **10** also significantly elevated antibody production against hepatitis B. Its activity at 50 μg was comparable to that obtained with the alum-adsorbed vaccine (see Table II, experiment 2).

The range of immunoadjuvant activities obtained with the three 5-O-acetylated and 6-O-acylated dipeptidyl furanose derivatives **8**–**10** and the control compounds, presented in Table I, supports the hypothesis that these furanoid analogues function as prodrug forms of the corresponding 6-*O*-acyl derivatives of MDP [and perhaps as prodrugs of MDP itself, since it is thought (vide supra) that 6-*O*-acyl-MDP derivatives serve as delivery systems for the free dipeptidyl saccharide]. The method devised for their synthesis should be conveniently applicable to the preparation of related structures containing any desired acyl group at the C-5 or C-6 position, as well as analogues chemically modified in the lactyl dipeptide moiety. The potent activities acquired render them attractive candidates for use as vaccine adjuvants with bacterial, viral, or parasitic antigens of therapeutic interest. Whether they, in addition, possess advantages of lower toxicity and/or

(29) A. C. Allison, *J. Reticuloendothel. Soc.*, **26**(Suppl), 619 (1979).

(30) C. P. Dorn, Jr., unpublished results.

(31) R. Tommasini, K. Bloch, J. Franceschini, A. Longoni, V. Mandelli, M. A. Parenti, N. Passerini, and G. Valzelli, *Arzneim.-Forsch.*, **16**, 164 (1966).

improved pharmacodynamical properties will require further study.

Experimental Section

Biology. (a) Bovine Serum Albumin. Groups of six, 5–6 week old female, ICR/Ha, mice were injected, subcutaneously 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 analogue 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 analogue, as a low response control. At 21 days postimmunization, all mice were boosted with 100 μg of BSA alone (no analogue). Ten days later (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 2 vol of a 20% (v/v) suspension of washed sheep red blood cells in PBS, after which serial twofold dilutions were incubated with BSA-coated sheep cells. Settling patterns were determined, and group geometric mean passive hemagglutination antibody titers were calculated. Results are shown in Table I.

(b) Hepatitis B Vaccine. Hepatitis B vaccine was prepared, as previously described,[32] from plasma collected from overtly healthy human donors who had hepatitis B antigenemia. An alum-adsorbed vaccine was used as a positive control. It was prepared in the following manner: 10% (w/v) $KAl(SO_4)_2 \cdot 12H_2O$ was added dropwise to aqueous vaccine with constant stirring in the ratio of 0.085 mL of $KAl(SO_4)_2$ per milliliter of vaccine containing 40 μg of antigen. The pH was then adjusted to 6.8 by the slow addition of 1 N NaOH with constant stirring, and the mixture was diluted to use level with phosphate-buffered saline, pH 7.5 (PBS). The MDP analogues were prepared by dissolving them in PBS at 200 μg/mL. Compound **10** was gently sonicated for 3 min by holding the tube in a sonicating water bath to facilitate dispersion. Equal volumes of test compound and aqueous vaccine at 5.0 μg/mL were mixed.

Five week old female ICR mice were obtained from the Merck Sharp & Dohme breeding colony. Groups of 20 mice were injected subcutaneously with 0.5 mL containing either 1.25 μg of aqueous vaccine, 1.25 μg of alum-adsorbed vaccine, or 1.25 μg of aqueous vaccine mixed with 50 μg of the MDP analogue. After 6 weeks, the mice were sacrificed and bled from the vena cava, and the sera were assayed for antibody by radioimmune assay (AUSAB, Abbott Laboratories, North Chicago, IL). Results are shown in Table II.

Chemistry. Solutions were evaporated below 50 °C 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. NMR spectra were recorded at 300 MHz with a Varian SC-300 NMR spectrometer. Chemical shifts are given on the δ scale. Spectra were measured at ambient temperature for solutions, as indicated, in chloroform-*d* or dimethyl sulfoxide-d_6, with tetramethylsilane (δ = 0.00) as the internal standard. Spectra were analyzed on a first-order basis. TLC 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).

Benzyl 2-Acetamido-3-*O*-[(*R*)-1-carboxyethyl]-2-deoxy-5,6-*O*-isopropylidene-β-D-glucofuranoside (2). To a stirred solution of benzyl 2-acetamido-2-deoxy-5,6-*O*-isopropylidene-β-D-glucofuranoside (1;[16] 1.88 g, 5.35 mmol) in dry 1,4-dioxane (75 mL) was added sodium hydride (50% oil disperison, 1.0 g). The mixture was stirred at 95 °C with exclusion of moisture, and additional 1,4-dioxane (70 mL) was added to disperse the gel that formed subsequent to the addition of sodium hydride. After 1 h, the temperature was lowered to 65 °C, and a solution of (*S*)-2-chloropropionic acid[18] (1.16 g, 10.7 mmol) in a small volume of 1,4-dioxane (3 mL) was added. The reaction mixture was stirred overnight at 65 °C and cooled, and excess sodium hydride was decomposed by the careful dropwise addition of water (100 mL). The resulting mixture was partially evaporated, and the aqueous residue was extracted with chloroform. The aqueous layer was cooled in an ice bath and acidified to pH 3 with 2.5 M hydrochloric acid. The mixture was immediately extracted with chloroform (3×), and the combined organic extracts were dried (sodium sulfate) and evaporated to afford the (*R*)-lactic acid ether **2** as a virtually chromatographically homogeneous gummy solid, which was employed without further purification in the condensation reaction with the blocked dipeptide: yield 1.92 g (85%); NMR ($CDCl_3$) δ 5.55 (d, NHAc), 5.04 (s, H-1), 4.78 (d, 1 H, OCHPh, J_{AB} = 11.8 Hz), 4.61 (d, 1 H, OCHPh), 4.43 (q, CH_3CHCO_2H, $J_{CH_3,CH}$ = 6.6 Hz), 1.98 (s, 3 H, NHAc), 1.44 (d, CH_3CHCO_2H), 1.42 and 1.34 (2 s, each 3 H, CMe_2).

Benzyl 2-Acetamido-2-deoxy-5,6-*O*-isopropylidene-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine benzyl ester]-β-D-glucofuranoside (3). To a solution of **2** (1.88 g, 4.44 mmol) in dry *N*,*N*-dimethylformamide (15 mL) at −15 °C were added successively *N*-methylmorpholine (0.49 mL, 4.45 mmol) and isobutyl chloroformate (0.58 mL, 4.47 mmol). After stirring for 5 min at −15 °C, a cooled solution of L-alanyl-D-isoglutamine benzyl ester hydrochloride[19] (1.53 g, 4.45 mmol) and *N*-methylmorpholine (0.49 mL, 4.45 mmol) in dry *N*,*N*-dimethylformamide (10 mL) was added. The mixture was stirred with exclusion of moisture for 4 h at −15 °C. The temperature was then allowed to rise to 0 °C, 2.5 M aqueous potassium hydrogencarbonate (8 mL) was added, and the mixture was stirred for 30 min at 0 °C. After addition of water (200 mL), the mixture was brought to pH 7 with 2.5 M hydrochloric acid and evaporated. The residue was partitioned between chloroform and water, and the organic layer was washed with water, dried (magnesium sulfate), and evaporated. The resulting syrup was dissolved in a small volume of chloroform, and the solution was applied to a column of silica gel that was eluted initially with 24:1 and subsequently 9:1 chloroform–methanol. Evaporation of the appropriate fractions gave **3** as a syrup, which was converted into an amorphous solid by trituration with diethyl ether: yield 2.0 g (63%); $[\alpha]^{27}_D$ −61° (*c* 1, chloroform); NMR (Me_2SO-d_6) δ 5.05 (s, CO_2CH_2Ph), 4.93 (s, H-1), 4.79 (d, 1 H, OCHPh), 4.56 (d, 1 H, OCHPh), 2.33 (t, CH_2CO_2Bzl), 2.00 and 1.79 (2 m, $CH_2CH_2CO_2Bzl$), 1.79 (s, 3 H, NHAc), 1.35 and 1.26 (2 s, each 3 H, CMe_2), 1.21 [d, CH_3(lac)], and 1.04 [d, CH_3(Ala)]. Anal. ($C_{36}H_{48}N_4O_{11} \cdot 0.5H_2O$) C, H, N.

Benzyl 2-Acetamido-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine benzyl ester]-β-D-glucofuranoside (4). A mixture of **3** (1.8 g, 2.5 mmol) in 65% acetic acid (100 mL) was stirred for 5 h at 40 °C, evaporated, and coevaporated several times with toluene to remove residual acetic acid. Trituration of the resulting syrup with diethyl ether afforded 5,6-diol **4** as an amorphous solid: yield 1.63 g (96%); $[\alpha]^{27}_D$ −73° (*c* 1, chloroform); NMR (Me_2SO-d_6) δ 5.04 (s, CO_2CH_2Ph), 4.86 (s, H-1), 4.75 (d, 1 H, OCHPh), 4.52 (d, 1 H, OCHPh), 2.31 (t, CH_2CO_2Bzl), 1.97 and 1.75 (2 m, $CH_2CH_2CO_2Bzl$), 1.76 (s, 3 H, NHAc), 1.21 [d, CH_3(lac)], 1.03 [d, CH_3(Ala)]. Anal. ($C_{33}H_{44}N_4O_{11} \cdot 0.5H_2O$) C, H, N.

Benzyl 2-Acetamido-5,6-di-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine benzyl ester]-β-D-glucofuranoside (5). A solution of **4** (500 mg, 0.74 mmol) in dry pyridine (6 mL) was treated with acetic anhydride (4 mL) overnight at room temperature. The mixture was then evaporated and coevaporated several times with toluene. The residue was dissolved in the minimal volume of chloroform, and the solution was passed through a short column of silica gel to remove minor impurities. The 5,6-diacetate **5** crystallized from ethanol–diethyl ether: yield 377 mg (67%); mp 164–166 °C, $[\alpha]^{27}_D$ −69° (*c* 1, chloroform); NMR (Me_2SO-d_6) δ 5.17 (o, H-5), 5.06 (s, CO_2CH_2Ph), 4.97 (s, H-1), 4.82 (d, 1 H, OCHPh), 4.64 (d, 1 H, OCHPh), 2.34 (t, CH_2CO_2Bzl), 2.04 and 2.00 (2 s, each 3 H, 2 OAc), 1.80 (s, 3 H, NHAc), 1.13 and 1.09 [2 d, CH_3(Ala) and CH_3(lac)]. Anal. ($C_{37}H_{48}N_4O_{13}$) C, H, N.

2-Acetamido-5,6-di-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucofuranose (8). To a solution of **5** (200 mg, 0.26 mmol) in glacial acetic acid (5 mL)

(32) E. B. Buynak, R. R. Roehm, A. A. Tytell, A. U. Bertland, G. P. Lampson, and M. R. Hilleman, *J. Am. Med. Assoc.*, **235**, 2832 (1976).

(33) R. A. Fisher, "Statistical Methods for Research Workers", 12th ed., Oliver and Boyd Ltd., Edinburgh, 1954, Sections 21.01 and 21.02.

was added palladium oxide (200 mg). The mixture was stirred for 18 h at room temperature under an atmosphere of hydrogen. The catalyst was removed by filtration through Celite, and the filtrate was evaporated and coevaporated several times with water and toluene. The residue was taken up in a small volume of methanol, and the solution was applied to a column of silica gel that was eluted initially with 9:1 chloroform–methanol, then 40:10:1 chloroform–methanol–water, and finally 6:4:1 chloroform–methanol–water. Evaporation of the appropriate fractions, followed by several coevaporations with methanol, gave the deblocked 5,6-diacetate **8** as an amorphous solid, which was dried in vacuo over phosphorus pentoxide: yield 130 mg (79%); $[\alpha]^{27}_D$ −10° (*c* 1.2, methanol). Anal. ($C_{23}H_{36}N_4O_{13}\cdot 2.5\ H_2O$) C, H, N.

Benzyl 2-Acetamido-5-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine benzyl ester]-6-*O*-stearoyl-β-D-glucofuranoside (6). To a solution of **4** (125 mg, 0.19 mmol) in dry pyridine (5 mL) was added stearoyl chloride (150 μL, 0.45 mmol). The mixture was stirred overnight at room temperature with exclusion of moisture. Additional stearoyl chloride (150 μL) was added, and the mixture was stirred a further 24 h. After evaporation and several coevaporations with toluene, the residue was dissolved in the minimal volume of chloroform, and the solution was applied to a column of silica gel that was eluted with 19:1 chloroform–methanol. Fractions containing pure 5-hydroxy-6-*O*-stearoyl compound were combined and evaporated to a syrup that was treated with acetic anhydride (0.6 mL) and pyridine (1 mL) overnight at room temperature. The mixture was then evaporated and coevaporated several times with toluene. The resulting material was dissolved in a small volume of chloroform, and the solution was applied to a column of silica gel that was eluted with 24:1 chloroform–methanol. Pure blocked 5-*O*-acetyl-6-*O*-stearoyl derivative (**6**) crystallized from diethyl ether: yield 109 mg (60%); mp 107–109 °C, $[\alpha]^{27}_D$ −54.1° (*c* 1.1, chloroform); NMR (Me_2SO-d_6) δ 5.15 (o, H-5), 5.05 (s, CO_2CH_2Ph), 4.96 (s, H-1), 4.81 (d, 1 H, OCHPh), 4.63 (d, 1 H, OCHPh), 2.33–2.27 [complex m, CH_2CO_2Bzl and $CH_3(CH_2)_{15}CH_2C(=O)$], 1.96 (s, 3 H, OAc), 1.79 (s, 3 H, NHAc), 1.12 and 1.10 [2 d, CH_3(Ala) and CH_3(lac)], 0.85 [t, $(CH_2)_{16}CH_3$]. Anal. ($C_{53}H_{80}N_4O_{13}$) C, H, N.

2-Acetamido-5-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-6-*O*-stearoyl-D-glucofuranose (9). A mixture of **6** (105 mg, 0.11 mmol) in glacial acetic acid (5 mL) containing 10% palladium on activated carbon (110 mg) was stirred under an atmosphere of hydrogen for 24 h at room temperature. The catalyst was removed by filtration through Celite, and the filtrate was evaporated and coevaporated several times with toluene. The residue was chromatographed on a column of silica gel that was eluted initially with 9:1 chloroform–methanol and subsequently with 40:10:1 chloroform–methanol–water. Fractions containing pure **9** were combined and evaporated, the resulting syrup was dissolved in a small volume of methanol, and the solution was filtered through glass-wool, evaporated, and lyophilized: yield 63 mg (71%); $[\alpha]^{27}_D$ −6.5° (*c* 1.1, methanol). Anal. ($C_{39}H_{68}N_4O_{13}\cdot 1.5H_2O$) C, H, N.

Benzyl 2-Acetamido-5-*O*-acetyl-6-*O*-[2-(behenoyloxy)-isobutyryl]-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine benzyl ester]-β-D-glucofuranoside (7). To a solution of **4** (150 mg, 0.22 mmol) in dichloromethane (5 mL) were added successively 4-(dimethylamino)pyridine (5 mg), 2-(behenoyloxy)isobutyric acid[20] (96 mg, 0.22 mmol), and *N*,*N*′-dicyclohexylcarbodiimide (46 mg, 0.22 mmol). After the solution was stirred for 24 h at room temperature, additional 2-(behenoyloxy)isobutyric acid (48 mg) and *N*,*N*′-dicyclohexylcarbodiimide (23 mg) were added, and the mixture was stirred a further 24 h at room temperature. The precipitated solid was removed by filtration, the filter was washed with dichloromethane, the combined filtrate and washings were evaporated to a syrup, which was dissolved in a small volume of chloroform and applied to a column of silica gel. Elution with 30:1 chloroform–methanol gave the 6-[2-(behenoyloxy)isobutyrate]-5-ol as a syrup, which was treated with acetic anhydride (0.6 mL) and pyridine (1 mL) overnight at room temperature. The mixture was then evaporated and coevaporated several times with toluene. The residue was chromatographed on a column of silica gel that was eluted with 24:1 chloroform–methanol. The syrup obtained by evaporation of fractions containing pure **7** crystallized from diethyl ether: yield 149 mg (60%); mp 92–94 °C; $[\alpha]^{27}_D$ −54.8° (*c* 0.9, chloroform); NMR (Me_2SO-d_6) δ 5.19 (o, H-5), 5.07 (s, CO_2CH_2Ph), 4.98 (s, H-1), 4.81 (d, 1 H, OCHPh), 4.63 (d, 1 H, OCHPh), 2.33 (t, CH_2CO_2Bzl), 2.28 [t, $CH_3(CH_2)_{19}CH_2C(=O)$], 1.97 (s, 3 H, OAc), 1.80 (s, 3 H, NHAc), 1.47 [s, 6 H, $C(=O)CMe_2O$], 1.13 and 1.10 [2 d, CH_3(Ala) and CH_3(lac)], 0.85 (t, $(CH_2)_{20}CH_3$). Anal. ($C_{61}H_{94}N_4O_{15}$) C, H, N.

2-Acetamido-5-*O*-acetyl-6-*O*-[2-(behenoyloxy)isobutyryl]-2-deoxy-3-*O*-[(*R*)-2-propionyl-L-alanyl-D-isoglutamine]-D-glucofuranose (10). To a solution of **7** (70 mg, 0.06 mmol) in glacial acetic acid (5 mL) was added palladium oxide (110 mg). The mixture was stirred under an atmosphere of hydrogen for 24 h at room temperature. The catalyst was removed by filtration through Celite, and the filtrate was evaporated and coevaporated several times with toluene. The resulting syrup was chromatographed on a column of silica gel that was eluted initially with 9:1 chloroform–methanol and subsequently 40:10:1 chloroform–methanol–water. Chromatographically homogeneous **10** was obtained upon evaporation of the appropriate fractions. Several coevaporations with diethyl ether and drying in vacuo over phosphorus pentoxide gave **10** as an amorphous solid: yield 32 mg (52%); $[\alpha]^{27}_D$ −8° (*c* 0.6, methanol). Anal. ($C_{47}H_{82}N_4O_{15}\cdot 2H_2O$) C, H, N.

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