Carbohydrate Research, 122 (1983) 87–98
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

CHEMICAL MODIFICATION OF THE C-6 SUBSTITUENT IN THE CARBOHYDRATE MOIETY OF *N*-ACETYLMURAMOYL-L-ALANYL-D-ISOGLUTAMINE (MDP), AND THE IMMUNOADJUVANT ACTIVITY*

HIROYUKI OKUMURA, YASUO TOKUSHIMA, IKUO SAIKI, ICHIRO AZUMA,
Institute of Immunological Science, Hokkaido University, Sapporo 060 (Japan)

MAKOTO KISO, AND AKIRA HASEGAWA
Department of Agricultural Chemistry, Gifu University, Yanagido, Gifu 501-11 (Japan)
(Received April 4th, 1983; accepted for publication, April 25th, 1983)

ABSTRACT

N-Acetyl-6-*O*-mesyl-, -6-*O*-methyl-, and -4,6-di-*O*-methyl-muramoyl-L-alanyl-D-isoglutamine and *N*-acetyl-6-chloro-, -6-bromo-, and -6-azido-6-deoxymuramoyl-L-alanyl-D-isoglutamine were synthesized from benzyl 2-acetamido-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside and its 6-*O*-mesyl derivative. The immunoadjuvant activity of the products was examined, in order to clarify the structural requirements for the activity of the carbohydrate moiety in *N*-acetylmuramoyl-L-alanyl-D-isoglutamine.

INTRODUCTION

In recent studies[1,2] on the structure–activity relationship of *N*-acetylmuramoyl-L-alanyl-D-isoglutamine** (MDP) (**15**), which is the minimal structure[3,4] required for the immunoadjuvant activity of whole mycobacterial cells in Freund's complete adjuvant, it was found that the 6-hydroxyl group of the carbohydrate moiety can be replaced by an amino or an acetamido or other acylamino group, with retention of the immunoadjuvant activity on the induction of delayed-type hypersensitivity in guineapigs. However, the substituents seem to be very critical for the activity, as replacement of the 6-hydroxyl group by a hydrogen atom almost abolishes the activity. The 2-acetamido group can be also replaced by a free amino, a methylamino, or an acylamino group[2,5,6] without decreasing the activity, whereas the 2-deoxy-D-*arabino*-hexose analog[5] is inactive. In order to examine the effect of functional groups on the manifestation of the activity, further chemical modification of the C-6 substituent of the carbohydrate moiety in MDP, and the immunoadjuvant activity of the products, are now described.

*Studies on Immunoadjuvant Active Compounds, Part XXII. For Part XXI, see ref. 1c.
**N-[2-O-(2-Acetamido-2,3-dideoxy-D-glucopyranose-3-yl)-D-lactoyl]-L-alanyl-D-isoglutamine.

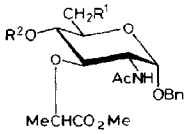
0008-6215/83/$ 03.00

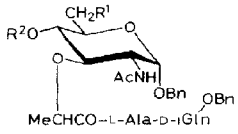
RESULTS AND DISCUSSION

We used benzyl 2-acetamido-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside[7] (**1**) and its 6-*O*-mesyl derivative[1a] (**2**) as starting materials for the synthesis of the muramoyl dipeptide analogs described herein.

Treatment of **1** with methyl iodide and silver oxide in dry 1,4-dioxane gave the 6-*O*-methyl derivative (**3**) of **1** in 71% yield, although the same treatment in *N*,*N*-dimethylformamide yielded the 4,6-di-*O*-methyl derivative **4** exclusively. Compound **2** was treated with two molar equivalents of tetrabutylammonium chloride in dry benzene, giving benzyl 2-acetamido-6-chloro-2,6-dideoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside (**5**) in 86% yield.

Saponification of the methyl ester group in **3** with 0.5M aqueous potassium hydroxide in methanol at 0° gave the free acid, which was used for the next reaction without purification. Coupling of the acid with L-alanyl-D-isoglutamine benzyl ester[8] was conducted with dicyclohexylcarbodiimide (DCC) and *N*-hydroxysuccinimide (HOSu) as the activating agents, to afford *N*-[2-*O*-(benzyl 2-acetamido-2,3-dideoxy-6-*O*-methyl-α-D-glucopyranoside-3-yl)-D-lactoyl]-L-alanyl-D-isoglutamine benzyl ester (**7**) in good yield. In the same way, coupling of the free acids derived from compounds **4** and **5** with the L-alanyl-D-isoglutamine derivative respectively yielded the corresponding dipeptides **8** and **9** in excellent yields.

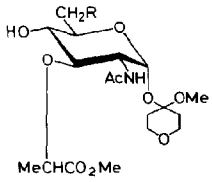




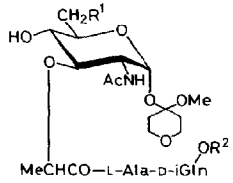
1 R^1 = OH, R^2 = H
2 R^1 = OMs, R^2 = H
3 R^1 = OMe, R^2 = H
4 R^1 = OMe, R^2 = Me
5 R^1 = Cl, R^2 = H
Bn = $PhCH_2$
Ms = $MeSO_2$

6 R^1 = OMe, R^2 = H
7 R^1 = OMe, R^2 = H
8 R^1 = OMe, R^2 = Me
9 R^1 = Cl, R^2 = H

Hydrogenolysis of the benzyl group in *N*-[2-*O*-(benzyl 2-acetamido-2,3-dideoxy-6-*O*-mesyl-α-D-glucopyranoside-3-yl)-D-lactoyl]-L-alanyl-D-isoglutamine benzyl ester[9] (**6**) with hydrogen in the presence of Pd-black catalyst, in methanol, gave *N*-acetyl-6-*O*-mesylmuramoyl-L-alanyl-D-isoglutamine (6-*O*-mesyl-MDP, **16**) in quantitative yield. By essentially the same procedure, compounds **7**–**9** yielded the corresponding carbohydrate analogs (**17**–**19**) of MDP, in almost quantitative yields.



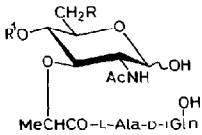
10 R = OMs
11 R = Br
12 R = N_3



13 R^1 = Br, R^2 = Bn
14 R^1 = N_3, R^2 = Me

We had shown[1a] that brief hydrogenolysis of *N*-[2-*O*-(benzyl 2-acetamido-6-bromo-2,3,6-trideoxy-α-D-glucopyranoside-3-yl)-D-lactoyl]-L-alanyl-D-isoglutamine benzyl ester gives the debenzyl ester derivative, but that prolonged hydrogenolysis yields *N*-acetyl-6-deoxymuramoyl-L-alanyl-D-isoglutamine (6-deoxy-MDP), instead of 6-bromo-MDP (**20**). Therefore, for the synthesis of 6-bromo-MDP (**20**) and 6-azido-MDP (**21**), which have hydrogenolytically sensitive groups, the benzyl group in **2** was first replaced by a different protecting group. Hydrogenolytic removal of the benzyl group in **2**, and treatment of the product with 5,6-dihydro-4-methoxy-2*H*-pyran in the presence of *p*-toluenesulfonic acid, in 1,2-dichloroethane and 1,4-dioxane at 0 to 10°, gave 2-acetamido-2-deoxy-6-*O*-mesyl-3-*O*-[D-1-(methoxycarbonyl)ethyl]-1-*O*-(tetrahydro-4-methoxypyran-4-yl)-α-D-glucopyranose (**10**) in 89% yield. The structure of compound **10** was based on n.m.r. spectroscopy; the n.m.r. spectrum of **10** showed the 4-OH signal as a doublet at δ 3.24 ($J_{4,OH}$ 5.4 Hz) and the H-1 as a doublet at δ 5.65 ($J_{1,2}$ 3.4 Hz), indicating the structure shown for the 4-hydroxy, α-D-pyranose form **10**.

Treatment of **10** with tetrabutylammonium bromide in dry benzene, or with sodium azide in *N*,*N*-dimethylformamide, respectively gave 2-acetamido-6-bromo- or -6-azido-2,6-dideoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-1-*O*-(tetrahydro-4-me-



15 R = OH, R^1 = H (MDP)
16 R = OMs, R^1 = H
17 R = OMe, R^1 = H
18 R = OMe, R^1 = Me
19 R = Cl, R^1 = H
20 R = Br, R^1 = H
21 R = N_3, R^1 = H

TABLE I

IMMUNOADJUVANT ACTIVITY OF SOME CARBOHYDRATE ANALOGS OF *N*-ACETYLMURAMOYL-L-ALANYL-D-ISOGLUTAMINE (MDP) ON THE INDUCTION OF DELAYED-TYPE HYPERSENSITIVITY TO ABA-TYROSINE IN GUINEAPIGS

Compound	*Compound No.*	*Dose (μg)*	*Skin reaction with ABA-BSA*[a] *(100 μg) at 24 h (diam. in mm ± SE*[b]*)*	
			Expt. I	*Expt. II*
6-*O*-Mesyl-MDP	**16**	100	19.3 ±0.6	22.0 ±0.5
		10		17.5 ±0.8
6-*O*-Methyl-MDP	**17**	100	13.8 ±0.7	
4,6-Di-*O*-methyl-MDP	**18**	100	11.9 ±1.3	
6-Deoxy-MDP[c]		100	(8.3 ±0.2)	
6-Chloro-MDP	**19**	100	13.3 ±1.1	15.1 ±0.7
6-Bromo-MDP	**20**	100	19.9 ±0.6	19.7 ±0.6
		10		18.5 ±1.2
6-Azido-MDP	**21**	100	12.9 ±0.9	15.0 ±0.7
6-Acetamido-MDP[c]		100	18.0 ±0.7	
MDP	**15**	100	20.8 ±0.4	21.1 ±0.8
		10		19.4 ±0.9
Control[d]		—	0	0

[a]Azobenzenearsonate–bovine serum albumin. [b]The data indicate the average diameter ± the standard error (SE) of the skin reaction of four guineapigs; the value in parentheses indicates the size of faint erythema. [c]See ref. 1a. [d]ABA-tyrosine in Freund's incomplete adjuvant.

thoxypyran-4-yl)-α-D-glucopyranose (**11** or **12**) in good yield. Saponification of the methyl ester group in **11** with 0.5M aqueous potassium hydroxide gave the acid derivative, which was coupled with L-alanyl-D-isoglutamine benzyl ester by using the DCC–HOSu method, to afford the dipeptide derivative **13** in good yield. In the same way, after saponification of **12**, coupling of the acid derivative thus obtained with L-alanyl-D-isoglutamine methyl ester yielded compound **14**.

Brief hydrogenolysis of **13** in 1,4-dioxane in the presence of 10% Pd–C catalyst, and hydrolysis of the product with 3:2 acetic acid–water, gave the desired *N*-acetyl-6-bromo-6-deoxymuramoyl-L-alanyl-D-isoglutamine (6-bromo-MDP, **20**). Compound **14** was treated with 0.5M aqueous potassium hydroxide to remove the methyl ester group, and the product hydrolyzed with 7:3 acetic acid–water, to afford the desired *N*-acetyl-6-azido-6-deoxymuramoyl-L-alanyl-D-isoglutamine (6-azido-MDP, **21**).

The immunoadjuvant activities[2] of compounds **16–21** on the induction of delayed-type hypersensitivity to *N*-acetyl-L-tyrosine-3-azobenzene-4′-arsonate (ABA-tyrosine) were examined in guineapigs (see Table I). 6-*O*-Mesyl-MDP (**16**) showed potent activity comparable to that of MDP at doses of both 100 and 10 μg, whereas 6-*O*-methyl-MDP (**17**) and 4,6-di-*O*-methyl-MDP (**18**) exhibited only weak activity. This suggests that introduction of a sulfonyl group, as well as of an acyl group[10], onto the 6-hydroxyl group in MDP does not lower the activity, but

that introduction of an ether substituent is unfavorable to the activity. 6-Chloro-MDP (**19**) showed weak activity compared to that of MDP, but this activity was stronger than that of *N*-acetyl-6-deoxymuramoyl-L-alanyl-D-isoglutamine[1a] (6-deoxy-MDP). 6-Bromo-MDP (**20**) had potent activity, even at a dose of 10 μg. These data indicate that the bulkiness of the C-6 substituent may be important for manifestation of the activity. As regards analogs in which the oxygen atom on C-6 is replaced by a nitrogen atom, 6-azido-MDP (**21**) showed distinct, but weak, immunoadjuvant activity compared to those of MDP and 6-acetamido-*N*-acetyl-6-deoxymuramoyl-L-alanyl-D-isoglutamine[1a] (6-acetamido-MDP), suggesting that a substituent capable of forming a hydrogen bond is also effective for the activity. As just described, the bulkiness of the substituent and an affinity of the functional group to immune competent cells may play an important role for manifestation of the immunoadjuvant activity.

EXPERIMENTAL

General methods. — Evaporations were conducted *in vacuo*. Preparative chromatography was performed on silica gel (Merck, 200 mesh), unless otherwise noted. Melting points were determined with a Yamato micro melting-point apparatus and are uncorrected. Specific rotations were determined with a Union PM-101 polarimeter, and i.r. spectra were recorded with a Shimadzu IR-27G spectrophotometer. N.m.r. spectra were recorded at 400 MHz with a JEOL FX-400 spectrometer for solutions in chloroform-*d* with tetramethylsilane as the internal standard, or in D_2O with sodium 4,4-dimethyl-4-silapentane-1-sulfonate (DSS), at a probe temperature of 23–25°. N.m.r. data were confirmed by use of decoupling techniques.

Benzyl 2-acetamido-2-deoxy-3-O-[D-1-(methoxycarbonyl)ethyl]-6-O-methyl-α-D-glucopyranoside (**3**). — To a solution of benzyl 2-acetamido-2-deoxy-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside[7] (**1**) (300 mg) in dry 1,4-dioxane (3 mL) were added methyl iodide (700 mg) and silver oxide (350 mg). The mixture was stirred in the dark overnight at room temperature, and then insoluble materials were removed by filtration. The filtrate was evaporated to a syrup which was chromatographed on a column of silica gel (30 g) with 100:1 and then 50:1 chloroform–methanol. The latter eluate gave compound **3** (220 mg, 71%); m.p. 140–141°, $[\alpha]_D^{20}$ +143° (*c* 0.4, chloroform); ν_{max}^{Nujol} 3320 (OH, NH), 1720 and 1225 (ester), 1650 and 1540 (amide), and 720 and 690 cm^{-1} (phenyl); n.m.r. data (in chloroform-*d*): δ 1.42 [d, 3 H, $J_{Me,CH}$ 7.3 Hz, Me (lac)], 2.02 (s, 3 H, AcN), 3.37 (s, 1 H, OH), 3.39 (s, 3 H, MeO), 3.75 (s, 3 H, CO_2Me), 4.51 and 4.66 (2 d, 2 H, J_{gem} 12.2 Hz, benzyl methylene), 4.69 [q, 1 H, CH (lac)], 5.32 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), 7.28–7.38 (m, 5 H, Ph), and 7.44 (d, 1 H, $J_{2,NH}$ 4.9 Hz, NH).

Anal. Calc. for $C_{20}H_{29}NO_8$: C, 58.38; H, 7.10; N, 3.40. Found: C, 58.21; H, 7.07; N, 3.59.

Benzyl 2-acetamido-2-deoxy-3-O-[D-1-(methoxycarbonyl)ethyl]-4,6-di-O-

*methyl-α-*D*-glucopyranoside* (**4**). — To a solution of **1** (300 mg) in dry *N*,*N*-dimethylformamide (3 mL) were added methyl iodide (700 mg) and silver oxide (350 mg), and the mixture was stirred in the dark overnight at room temperature. After removal of insoluble material by filtration, the filtrate was evaporated to a syrup which was chromatographed on a column of silica gel (30 g) with 100:1 chloroform–methanol, to afford compound **4** (260 mg, 81%): m.p. 100–101°, $[\alpha]_D^{25}$ +147.5° (*c* 0.4, chloroform); ν_{max}^{Nujol} 3300 (NH), 1725 and 1220 (ester), 1640 and 1545 (amide), and 720 and 690 cm^{-1} (phenyl).

Anal. Calc. for $C_{21}H_{31}NO_8$: C, 59.28; H, 7.34; N, 3.29. Found: C, 59.25; H, 7.35; N, 3.47.

*Benzyl 2-acetamido-6-chloro-2,6-dideoxy-3-*O*-[*D*-1-(methoxycarbonyl)ethyl]-α-*D*-glucopyranoside* (**5**). — To a solution of benzyl 2-acetamido-2-deoxy-6-*O*-mesyl-3-*O*-[D-1-(methoxycarbonyl)ethyl]-α-D-glucopyranoside[10] (**2**) (400 mg) in dry benzene (5 mL) was added tetrabutylammonium chloride (480 mg), and the mixture was heated, with stirring, overnight at 70°. After being cooled, the mixture was chromatographed on a column of silica gel (30 g), with 100:1 chloroform–methanol, to give a crystalline mass. Recrystallization from ethanol–ether afforded compound **5** (300 mg, 86%) as prisms: m.p. 165–165.5°, $[\alpha]_D^{20}$ +144° (*c* 0.5, chloroform); ν_{max}^{Nujol} 3320 (OH, NH), 1710 and 1230 (ester), 1650 and 1540 (amide), and 710 and 690 cm^{-1} (phenyl); n.m.r. data (in chloroform-*d*): δ 1.41 [d, 3 H, $J_{Me,CH}$ 6.8 Hz, Me (lac)], 2.01 (s, 3 H, AcN), 3.33 (d, 1 H, $J_{4,OH}$ 4.4 Hz, OH), 3.67 (m, 1 H, $J_{3,4} = J_{4,5}$ = 8.3 Hz, H-4), 3.72 (dd, 1 H, $J_{2,3}$ 10.3 Hz, H-3), 3.74 (2 H, H-6,6'), 3.76 (s, 3 H, COOMe), 3.83 (m, 1 H, $J_{5,6}$ 4.4, $J_{5,6'}$ 2.9 Hz, H-5), 3.95 (m, 1 H, H-2), 4.52 and 4.70 (2 d, 2 H, J_{gem} 11.7 Hz, benzyl methylene), 4.59 (q, 1 H, CH), 5.26 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), 7.19 (d, 1 H, $J_{2,NH}$ 6.4 Hz, NH), and 7.28–7.38 (5 H, Ph).

Anal. Calc. for $C_{19}H_{26}ClNO_7$: C, 54.87; H, 6.30; N, 3.37. Found: C, 54.66; H, 6.36; N, 3.13.

N-[*2-*O*-(Benzyl 2-acetamido-2,3-dideoxy-6-*O*-methyl-α-*D*-glucopyranoside-3-yl)-*D*-lactoyl]-*L*-alanyl-*D*-isoglutamine benzyl ester* (**7**). — To an ice-cooled solution of **3** (150 mg) in methanol (3 mL) was added 0.5M aqueous potassium hydroxide (1.5 mL), and the solution was stirred for 10 min at 0°. The solution was treated with Amberlite IR-120B (H^+) ion-exchange resin to remove the base, and the resin was filtered off and washed with methanol. The filtrate and washings were combined, and evaporated, to afford the free acid, which was used for the next reaction without purification.

To a cooled solution of the acid in dry oxolane (3 mL) were added *N*-hydroxysuccinimide (HOSu; 55 mg) and dicyclohexylcarbodiimide (DCC; 90 mg), and the mixture was stirred for 1 h at 0°. The 1,3-dicyclohexylurea formed was removed by filtration, L-alanyl-D-isoglutamine benzyl ester trifluoroacetate[9] (170 mg) and triethylamine (0.1 mL) were added to the filtrate, and the mixture was stirred for 3 h at room temperature. After evaporation of the solvent, the residue was chromatographed on a column of silica gel (30 g) with 50:1 and then with 20:1

chloroform–methanol. The latter eluate afforded 150 mg (60%) of **7** as crystals; m.p. 196–198° (dec.), $[\alpha]_D^{23}$ +105° (*c* 0.5, methanol); ν_{max}^{KBr} 3380 and 3280 (OH, NH), 1710 (C=O), 1640 and 1530 (amide), and 730 and 695 cm^{-1} (phenyl).

Anal. Calc. for $C_{34}H_{46}N_4O_{11}$: C, 59.46; H, 6.75; N, 8.16. Found: C, 59.61; H, 6.74; N, 8.27.

N-[2-O-(*Benzyl 2-acetamido-2,3-dideoxy-4,6-di-O-methyl-α-*D-*glucopyranoside-3-yl*)-D-*lactoyl*]-L-*alanyl*-D-*isoglutamine benzyl ester* (**8**). — Saponification of compound **4** (200 mg) in methanol (3 mL) with 0.5M aqueous potassium hydroxide (1.9 mL) gave an acid compound. Coupling of the acid with the protected L-alanyl-D-isoglutamine (218 mg) by using HOSu (70 mg), DCC (155 mg), and triethylamine (0.1 mL), according to the procedure already described, afforded compound **8** (297 mg, 90%) after purification by chromatography; m.p. 238–240° (dec.), $[\alpha]_D^{23}$ +108.7° (*c* 0.3, methanol); ν_{max}^{Nujol} 3270 (NH), 1735 (C=O), 1640 and 1540 (amide), and 740 and 690 cm^{-1} (phenyl).

Anal. Calc. for $C_{35}H_{48}N_4O_{11}$: C, 59.99; H, 6.90; N, 7.99. Found: C, 60.05; H, 6.92; N, 8.06.

N-[2-O-(*Benzyl 2-acetamido-6-chloro-2,3,6-trideoxy-α-*D-*glucopyranoside-3-yl*)-D-*lactoyl*]-L-*alanyl*-D-*isoglutamine benzyl ester* (**9**). — Saponification of compound **5** (300 mg) in methanol (5 mL) with 0.5M potassium hydroxide (2.9 mL), and coupling of the product with the L-alanyl-D-isoglutamine derivative (365 mg) by using HOSu (108 mg), DCC (163 mg), and triethylamine (0.12 mL), as described in the preparation of **7**, gave compound **9** (370 mg, 74%); m.p. 233–233.5° (dec.), $[\alpha]_D^{20}$ +92.0° (*c* 0.2, methanol); ν_{max}^{Nujol} 3280 (OH, NH), 1720 (C=O), 1650 and 1550 (amide), and 730 and 690 cm^{-1} (phenyl).

Anal. Calc. for $C_{33}H_{43}ClN_4O_{10}$: C, 57.34; H, 6.27; N, 8.11. Found: C, 57.14; H, 6.15; N, 8.25.

(N-*Acetyl-6-O-mesylmuramoyl*)-L-*alanyl*-D-*isoglutamine* (**16**). — *N*-[2-*O*-(Benzyl 2-acetamido-2,3-dideoxy-6-*O*-mesyl-α-D-glucopyranoside-3-yl)-D-lactoyl]-L-alanyl-D-isoglutamine benzyl ester[9] (**6**) (200 mg) was dissolved in methanol (7 mL), Pd-black catalyst was added, and the mixture was hydrogenolyzed, with stirring, overnight at room temperature. The catalyst was filtered off, and washed with methanol. The filtrate and washings were combined, and evaporated below 40°, to give amorphous **16** (157 mg, quantitative yield); $[\alpha]_D^{20}$ +58.0° (*c* 0.3, methanol; equil.); ν_{max}^{KBr} 3360 (OH, NH), 1710 (C=O), 1650 and 1530 (amide), and 1170 cm^{-1} (SO_2); n.m.r. data (in D_2O; α:β ratio at equilibrium was 71:29): δ 1.37 and 1.43 [4 d, 6 H, $J_{Me,CH}$ 6.8 and 7.3 Hz, 2 Me (lac and Ala)], 1.97 (s, 3 H, AcN), 1.97 and 2.19 [2 m, 2 H, β-CH_2 (isoGln)], 2.47 [t, 2 H, $J_{\beta,\gamma}$ 7.3 Hz, γ-CH_2 (isoGln)], 3.25 (2 s, 3 H, Ms), 3.52 (near t, $J_{3,4}$ 8.8 Hz, H-3β), 3.62 (near t, $J_{4,5}$ 9.8 Hz, H-4β), 3.64 (near t, $J_{3,4}$ 8.9, $J_{4,5}$ 9.8 Hz, H-4α), 3.72 (near t, H-3α), 3.75 (m, H-5β), 3.81 (near t, $J_{2,3}$ 9.8 Hz, H-2β), 3.98 (dd, $J_{2,3}$ 10.3 Hz, H-2α), 4.13 (m, H-5α), 4.18–4.30 [m, 2 H, 2 CH (lac and Ala)], 4.37 [dd, 1 H, $J_{\alpha,\beta}$ 9.8, $J_{\alpha,\beta'}$ 4.4 Hz, α-CH (isoGln), 4.54 (dd, 1 H, $J_{5,6'}$ 1.5, $J_{6,6'}$ 11.2 Hz, H-6'), 4.59 (dd, 1 H, $J_{5,6}$ 3.4 Hz, H-6), 4.72 (d, 1 H, $J_{1,2}$ 8.3 Hz, H-1β), and 5.17 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1α).

Anal. Calc. for $C_{20}H_{34}N_4O_{13}S \cdot H_2O$: C, 40.81; H, 6.16; N, 9.52. Found: C, 40.90; H, 6.05; N, 9.31.

(N-*Acetyl-6-O-methylmuramoyl*)-L-*alanyl*-D-*isoglutamine* (**17**). — Compound **7** (100 mg) in methanol (5 mL) and water (1 mL) was hydrogenolyzed in the presence of Pd-black catalyst, as described in the preparation of **16**, to give **17** (78 mg, quantitative yield) as an amorphous material; $[\alpha]_D^{23}$ +57.2° (*c* 0.5, methanol; equil.); ν_{max}^{KBr} 3300 (OH, NH), and 1650 and 1540 cm^{-1} (amide).

Anal. Calc. for $C_{20}H_{34}N_4O_{11} \cdot 1.5\ H_2O$: C, 45.02; H, 6.70; N, 10.50. Found: C, 45.02; H, 6.74; N, 10.38.

(N-*Acetyl-4,6-di-O-methylmuramoyl*)-L-*alanyl*-D-*isoglutamine* (**18**). — Hydrogenolysis of compound **8** (130 mg) with hydrogen in the presence of Pd-black catalyst, in methanol (9 mL)–water (1 mL), according to the procedure already described, gave **18** (102 mg, quantitative yield) as an amorphous material; $[\alpha]_D^{23}$ +69.6° (*c* 0.5, methanol; equil.); ν_{max}^{KBr} 3370 (OH, NH), and 1650 and 1530 cm^{-1} (amide).

Anal. Calc. for $C_{21}H_{36}N_4O_{11} \cdot 1.5\ H_2O$: C, 46.06; H, 7.18; N, 10.23. Found: C, 46.24; H, 6.93; N, 10.12.

(N-*Acetyl-6-chloro-6-deoxymuramoyl*)-L-*alanyl*-D-*isoglutamine* (**19**). — A mixture of **9** (100 mg) in methanol (10 mL) and water (2 mL) was hydrogenolyzed in the presence of Pd-black catalyst, with stirring, for 3 h at room temperature. After filtration of the catalyst and evaporation of the solvents, compound **19** (75 mg, 96%) was obtained as an amorphous solid; $[\alpha]_D^{20}$ +55.3° (*c* 0.3, methanol; equil.); ν_{max}^{KBr} 3300 (OH, NH), 1710 (C=O), and 1650 and 1530 cm^{-1} (amide); n.m.r. data (in D_2O; α:β ratio at equilibrium was 13:7): δ 1.37 and 1.42 [4 d, 6 H, $J_{Me,CH}$ 6.8 and 7.3 Hz, 2 Me (lac and Ala)], 1.97 (2 s, 3 H, AcN), 1.97 and 2.19 [2 m, 2 H, β-CH_2 (isoGln)], 2.47 [near t, 2 H, γ-CH_2 (isoGln)], 3.52 (near t, $J_{2,3}$ 10.3, $J_{3,4}$ 8.8 Hz, H-3β), 3.66 (near t, $J_{4,5}$ 9.8 Hz, H-4β), 3.67 (near t, $J_{3,4}$ 8.8, $J_{4,5}$ 9.8 Hz, H-4α), 3.71 (near t, H-3α), 3.71 (m, H-5β), 3.81 (near t, H-2β), 3.85 (dd, $J_{5,6}$ 4.3, $J_{6,6'}$ 12.0 Hz, H-6β), 3.89 (dd, $J_{5,6}$ 3.4, $J_{6,6'}$ 12.0 Hz, H-6α), 3.91 (dd, $J_{5,6'}$ 3.4 Hz, H-6'α), 3.93 (dd, $J_{5,6'}$ 2.0 Hz, H-6'β), 3.99 (dd, $J_{2,3}$ 10.3 Hz, H-2α), 4.10 (m, H-5α), 4.19–4.31 [m, 2 H, 2 CH (lac and Ala)], 4.37 [dd, 1 H, $J_{\alpha,\beta}$ 9.8, $J_{\alpha,\beta'}$ 4.4 Hz, α-CH (isoGln)], 4.72 (d, $J_{1,2}$ 8.3 Hz, H-1β), and 5.17 (d, $J_{1,2}$ 3.4 Hz, H-1α).

Anal. Calc. for $C_{19}H_{31}ClN_4O_{10} \cdot 1.5\ H_2O$: C, 42.42; H, 6.37; N, 10.41. Found: C, 42.61; H, 6.08; N, 10.11.

2-Acetamido-2-deoxy-6-O-mesyl-3-O-[D-*1-*(*methoxycarbonyl*)*ethyl*]-*1-O-*(*tetrahydro-4-methoxypyran-4-yl*)-α-D-*glucopyranose* (**10**). — To a solution of **2** (500 mg) in methanol (10 mL) was added Pd-black catalyst, and the mixture was hydrogenolyzed, with stirring, for 3 h at room temperature. After filtration of the catalyst and evaporation of the solvent, the 1-hydroxy compound was obtained. To an ice-cooled solution of this product in 1,2-dichloroethane (3 mL) and 1,4-dioxane (1 mL) were added 5,6-dihydro-4-methoxy-2*H*-pyran (0.3 mL) and *p*-toluenesulfonic acid monohydrate (4 mg), and the mixture was stirred for 4 h at 0 to 10°. After treatment with Amberlite IRA-410 (OH^-) ion-exchange resin, to remove

the acid, and filtration of the resin, the filtrate was evaporated. The residue was chromatographed on a column of silica gel (40 g) with 100:1, and then with 30:1, chloroform–methanol. The latter eluate afforded compound **10** (465 mg, 89%) as a syrup; $[\alpha]_D^{22}$ +83.1° (*c* 0.7, chloroform); ν_{max}^{film} 3300 (OH, NH), 1720 and 1255 (ester), 1650 and 1540 (amide), and 1170 cm^{-1} (SO_2); n.m.r. data (in chloroform-*d*): δ 1.45 [d, 3 H, $J_{Me,CH}$ 6.8 Hz, Me (lac)], 1.67–1.90 [m, 4 H, $(CH_2)_2C$ (pyran)], 2.02 (s, 3 H, AcN), 3.09 (s, 3 H, Ms), 3.21 [s, 3 H, MeO (pyran)], 3.24 (d, 1 H, $J_{4,OH}$ 5.4 Hz, OH-4), 3.78 (s, 3 H, COOMe), 3.53–3.81 [m, 7 H, H-3,4,5 and $(CH_2)_2O$ (pyran)], 3.89 (m, 1 H, $J_{2,3}$ 9.8 Hz, H-2), 4.28 (dd, 1 H, $J_{5,6'}$ 2.0, $J_{6,6'}$ 11.7 Hz, H-6'), 4.60 (dd, 1 H, $J_{5,6}$ 2.9 Hz, H-6), 4.72 [q, 1 H, CH (lac)], 5.65 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), and 7.79 (d, 1 H, $J_{2,NH}$ 4.4 Hz, NH).

Anal. Calc. for $C_{19}H_{33}NO_{12}S$: C, 45.68; H, 6.66; N, 2.80. Found: C, 45.36; H, 6.88; N, 2.67.

2-Acetamido-6-bromo-2,6-dideoxy-3-O-[D-*1*-(*methoxycarbonyl*)*ethyl*]-*1*-O-(*tetrahydro-4-methoxypyran-4-yl*)-α-D-*glucopyranose* (**11**). — To a solution of **10** (240 mg) in dry benzene (3 mL) was added tetrabutylammonium bromide (310 mg), and the mixture was stirred overnight at 70°. After the mixture had been cooled, it was chromatographed on a column of silica gel (40 g) with 50:1 chloroform-methanol, to afford compound **11** (171 mg, 73%) as crystals. Recrystallization of the product from ethanol–ether gave needles; m.p. 144–145°, $[\alpha]_D^{23}$ +116.8° (*c* 0.5, chloroform); ν_{max}^{Nujol} 3320–3280 (OH, NH), 1720 and 1230 (ester), and 1655 and 1540 cm^{-1} (amide); n.m.r. data (in chloroform-*d*): δ 1.44 [d, 3 H, $J_{Me,CH}$ 7.3 Hz, Me (lac)], 1.69–1.92 [m, 4 H, $(CH_2)_2C$ (pyran)], 2.02 (s, 3 H, AcN), 2.89 (d, 1 H, $J_{4,OH}$ 5.4 Hz, OH-4), 3.22 [s, 3 H, MeO (pyran)], 3.79 (s, 3 H, COOMe), 3.53–3.81 [m, 8 H, H-3,4,6,6' and $(CH_2)_2O$ (pyran)], 3.85 (m, 2 H, H-2,5), 4.66 [q, 1 H, CH (lac)], 5.62 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), and 7.61 (d, 1 H, $J_{2,NH}$ 4.9 Hz, NH).

Anal. Calc. for $C_{18}H_{30}BrNO_9$: C, 44.64; H, 6.24; N, 2.89. Found: C, 44.61; H, 6.30; N, 2.45.

2-Acetamido-6-azido-2,6-dideoxy-3-O-[D-*1*-(*methoxycarbonyl*)*ethyl*]-*1*-O-(*tetrahydro-4-methoxypyran-4-yl*)-α-D-*glucopyranose* (**12**). — To a solution of **10** (300 mg) in dry *N*,*N*-dimethylformamide (4 mL) was added sodium azide (300 mg), and the mixture was kept, with stirring, for 9 h at 70°, and then cooled. The insoluble materials were filtered off through Celite, and the filter cake was washed with chloroform. The filtrate and washings were combined, and extracted with chloroform. The extract was washed with H_2O, dried (sodium sulfate), and evaporated. The product, purified by chromatography on a column of silica gel (30 g) with 50:1 chloroform–methanol, was obtained as needles after crystallization from ether, wt. 190 mg (71%); m.p. 151.5°, $[\alpha]_D^{23}$ +115.2° (*c* 0.5, chloroform); ν_{max}^{Nujol} 3300–3280 (OH, NH), 2180 (N_3), 1720 and 1230 (ester), and 1660 and 1540 cm^{-1} (amide); n.m.r. data (in chloroform-*d*): δ 1.44 [d, 3 H, $J_{Me,CH}$ 6.8 Hz, Me (lac)], 1.68–1.95 [m, 4 H, $(CH_2)_2C$ (pyran)], 2.02 (s, 3 H, AcN), 2.95 (d, 1 H, $J_{4,OH}$ 4.9 Hz, OH-4), 3.23 [s, 3 H, MeO (pyran)], 3.45 (dd, 1 H, $J_{5,6}$ 4.9, $J_{6,6'}$ 12.7 Hz, H-6), 3.47 (dd,

1 H, $J_{5,6'}$ 3.4 Hz, H-6'), 3.79 (s, 3 H, COOMe), 3.54–3.81 [m, 6 H, H-3,4 and $(CH_2)_2O$ (pyran)], 3.85 (m, 2 H, H-2,5), 4.66 [q, 1 H, CH (lac)], 5.62 (d, 1 H, $J_{1,2}$ 3.4 Hz, H-1), and 7.61 (d, 1 H, $J_{2,NH}$ 4.9 Hz, NH).

Anal. Calc. for $C_{18}H_{30}N_4O_9$: C, 48.43; H, 6.77; N, 12.55. Found: C, 48.06; H, 6.76; N, 12.27.

N-[*2-O-{2-Acetamido-6-bromo-2,3,6-trideoxy-1-O-(tetrahydro-4-methoxypyran-4-yl)-α-D-glucopyranose-3-yl}-D-lactoyl*]-L-*alanyl*-D-*isoglutamine benzyl ester* (**13**). — Saponification of compound **11** (130 mg) in methanol (3 mL) with 0.5M aqueous potassium hydroxide (1.1 mL), and coupling of the product with the protected L-alanyl-D-isoglutamine (127 mg) by using HOSu (41 mg), DCC (68 mg), and triethylamine (0.1 mL) were performed as described for the preparation of **7**. The product was purified by chromatography on a column of silica gel (30 g) with 50:1, and then with 10:1, chloroform–methanol. From the latter eluate, compound **13** (195 mg, 93%) was obtained as a syrup; $[\alpha]_D^{22}$ +53.3° (*c* 1.2, chloroform); ν_{max}^{KBr} 3400–3250 (OH, NH), 1720 (ester), 1650 and 1530 (amide), and 695 cm^{-1} (phenyl).

Anal. Calc. for $C_{32}H_{47}BrN_4O_{12} \cdot H_2O$: C, 49.42; H, 6.35; N, 7.20. Found: C, 49.20; H, 6.61; N, 7.13.

N-[*2-O-{2-Acetamido-6-azido-2,3,6-trideoxy-1-O-(tetrahydro-4-methoxypyran-4-yl)-α-D-glucopyranose-3-yl}-D-lactoyl*]-L-*alanyl*-D-*isoglutamine methyl ester* (**14**). — Saponification of **12** (250 mg) with 0.5M potassium hydroxide (2.2 mL), as already described, gave the free acid; this was coupled with L-alanyl-D-isoglutamine methyl ester (210 mg), by using HOSu (84 mg) and DCC (138 mg), to afford the product. After purification by chromatography on a column of silica gel (40 g) with 10:1 chloroform–methanol, compound **14** was obtained as a syrup, wt. 255 mg (68%); $[\alpha]_D^{20}$ +74.4°; ν_{max}^{film} 3260 (OH, NH), 2070 (N_3), 1720 (ester), and 1660 and 1540 cm^{-1} (amide).

Anal. Calc. for $C_{26}H_{43}N_7O_{12} \cdot 1.5\ H_2O$: C, 46.42; H, 6.89; N, 14.57. Found: C, 46.78; H, 6.84; N, 14.26.

(N-*Acetyl-6-bromo-6-deoxymuramoyl*)-L-*alanyl*-D-*isoglutamine* (**20**). — To a solution of **13** (120 mg) in 1,4-dioxane (4 mL) was added 10% Pd–C catalyst (50 mg), and the mixture was hydrogenolyzed, with stirring, for 15 min at room temperature, the course of the reaction being monitored by t.l.c. The catalyst was removed by filtration, and the filtrate was evaporated, to give the acid compound. A solution of this product in 3:2 acetic acid–water was heated for 2.5 h at 45°, and then evaporated to an amorphous mass which was chromatographed on a column of silica gel (70 mesh, 30 g) with 75:25:1 chloroform–methanol–acetic acid, to give compound **20** (85 mg, 94%); $[\alpha]_D^{23}$ +39.0° (*c* 0.4, methanol; equil.); ν_{max}^{KBr} 3350–3280 (OH, NH), and 1650 and 1530 cm^{-1} (amide); n.m.r. data (in D_2O; α:β ratio at equilibrium was 67:33): δ 1.37 and 1.43 [4 d, 6 H, $J_{Mc,CH}$ 6.8 and 7.3 Hz, 2 Me (lac and Ala)], 1.95 and 2.16 [2 m, 2 H, β-CH_2 (isoGln)], 1.97 (2 s, 3 H, AcN), 2.38 [near t, 2 H, γ-CH_2 (isoGln)], 3.53 (m, H-5β), 3.63 (t, $J_{3,4}$ 9.3, $J_{4,5}$ 9.8 Hz, H-4α), 3.72 (near t, H-3α), 3.76 (2 d, $J_{5,6}$ 3.9, $J_{5,6'}$ 3.4 Hz, H-6,6'α), 3.80 (near t, $J_{2,3}$ 9.8, $J_{3,4}$ 8.8 Hz, H-2β), 3.98 (dd, $J_{2,3}$ 10.3, $J_{3,4}$ 9.3 Hz, H-2α), 4.02 (m, H-5α), 4.19–

4.32 [m, 2 H, 2 CH (lac and Ala)], 4.33 [dd, 1 H, $J_{\alpha,\beta}$ 9.8, $J_{\alpha,\beta'}$ 4.9 Hz, α-CH (isoGln)], 4.74 (d, $J_{1,2}$ 8.8 Hz, H-1β), and 5.17 (d, $J_{1,2}$ 3.4 Hz, H-1α).

Anal. Calc. for $C_{19}H_{31}BrN_4O_{10} \cdot 1.5\ H_2O$: C, 39.18; H, 5.88; N, 9.62. Found: C, 39.01; H, 5.40; N, 9.53.

(N-*Acetyl-6-azido-6-deoxymuramoyl*)-L-*alanyl*-D-*isoglutamine* (**21**). — To an ice-cooled solution of **14** (120 mg) was added 0.5M aqueous potassium hydroxide (1.1 mL), and the solution was kept for 30 min at room temperature. After treatment with IR-120B (H^+) ion-exchange resin, to remove the base, the resin was filtered off and washed with methanol. The filtrate and washings were combined and evaporated, to give the acid derivative. A solution of this product in 7:3 acetic acid–water was heated for 4 h at 45°; it was then evaporated below 40° to a syrup which was chromatographed on a column of silica gel (70 mesh, 15 g) with 75:25:1 chloroform–methanol–acetic acid. Compound **21** was obtained as an amorphous mass, wt. 64 mg (63%); $[\alpha]_D^{20}$ +49.3° (*c* 0.3, methanol; equil.); ν_{max}^{KBr} 3370–3280 (OH, NH), 2080 (N_3), 1710 (C=O), and 1650 and 1530 cm^{-1} (amide); n.m.r. data (in D_2O; α:β ratio at equilibrium was 13:7): δ 1.38 and 1.43 [4 d, 6 H, $J_{Me,CH}$ 6.8 and 7.3 Hz, 2 Me (lac and Ala)], 1.97 (s, 3 H, AcN), 1.99 and 2.20 [2 m, 2 H, β-CH_2 (isoGln)], 2.33 [m, 2 H, γ-CH_2 (isoGln)], 3.49 (near t, $J_{3,4}$ 8.8 Hz, H-3β), 3.56 (near t, $J_{4,5}$ 9.8 Hz, H-4β), 3.59 (near t, $J_{3,4}$ 8.8, $J_{4,5}$ 9.8 Hz, H-4α), 3.60 (H-6α), 3.62 (H-6'α), 3.69 (near t, H-3α), 3.80 (near t, $J_{2,3}$ 9.8 Hz, H-2β), 3.98 (dd, $J_{2,3}$ 10.3 Hz, H-2α), 3.99 (m, H-5α), 4.20–4.34 [m, 2 H, 2 CH (lac and Ala)], 4.37 [dd, $J_{\alpha,\beta}$ 8.8, $J_{\alpha,\beta'}$ 4.9 Hz, α-CH (isoGln)], 4.69 (d, $J_{1,2}$ 8.8 Hz, H-1β), and 5.17 (d, $J_{1,2}$ 3.4 Hz, H-1α).

Anal. Calc. for $C_{19}H_{31}N_7O_{10} \cdot 3\ H_2O$: C, 39.93; H, 6.52; N, 17.15. Found: C, 39.67; H, 6.12; N, 17.06.

Determination of immunoadjuvant activity. — Adjuvant activity was determined according to a reported method[2]. Briefly, four Hartley guineapigs in each group were immunized in their four footpads with *N*-acetyl-L-tyrosine-3-azobenzene-4'-arsonic acid (ABA-tyrosine) (50 μg) in Freund's incomplete adjuvant, with or without each compound. After 2 weeks, a skin test was performed with ABA-bovine serum albumin (100 μg), and the skin reaction (induration) was measured 24 h after intradermal injection of the test antigen.

ACKNOWLEDGMENT

We thank Miss Mutsuko Araki for typing the manuscript.

REFERENCES

1 (a) A. Hasegawa, H. Okumura, M. Kiso, I. Azuma, and Y. Yamamura, *Agric. Biol Chem*, 44 (1980) 1301–1308; (b) *ibid.*, 1309–1313; (c) H. Okumura, I. Azuma, M. Kiso, and A. Hasegawa, *Carbohydr Res*, 117 (1983) 298–303.

2 I. Azuma, H. Okumura, I. Saiki, M. Kiso, A. Hasegawa, Y. Tanio, and Y. Yamamura, *Infect. Immun*, 32 (1981) 1305–1308, 33 (1981) 834–839

3 F. ELLOUZ, A. ADAM, R. CIORBARU, AND E. LEDERER, *Biochem. Biophys. Res. Commun.*, 59 (1974) 1317–1325.
4 S. KOTANI, Y. WATANABE, F. KINOSHITA, T. SHIMONO, I. MORISAKI, T. SHIBA, S. KUSUMOTO, Y. TARUMI, AND K. IKENAKA, *Biken J.*, 18 (1975) 105–111.
5 A. HASEGAWA, H. OKUMURA, K. NISHIBORI, Y. KANEDA, M. KISO, AND I. AZUMA, *Carbohydr. Res.*, 97 (1981) 337–345.
6 H. OKUMURA, K. KAMISANGO, I. SAIKI, Y. TANIO, I. AZUMA, M. KISO, A. HASEGAWA, AND Y. YAMAMURA, *Agric. Biol. Chem.*, 46 (1982) 507–514; H. OKUMURA AND I. AZUMA, *ibid.*, 47 (1983) 847–854.
7 H. M. FLOWERS AND R. W. JEANLOZ, *J. Org. Chem.*, 28 (1963) 2983–2986.
8 S. KUSUMOTO, Y. TARUMI, K. IKENAKA, AND T. SHIBA, *Bull. Chem. Soc. Jpn.*, 49 (1976) 533–539.
9 P. L. DURETTE, A. ROSEGAY, M. A. R. WALSH, AND T. Y. SHEN, *Tetrahedron Lett.*, (1979) 291–294.
10 S. KOTANI, F. KINOSHITA, I. MORISAKI, T. SHIMONO, T. OKUNAGA, H. TAKADA, M. TSUJIMOTO, Y. WATANABE, K. KATO, T. SHIBA, S. KUSUMOTO, AND S. OKADA, *Biken J.*, 20 (1977) 95–103.