Synthesis and Immunoadjuvant Activity of Acylamino Analogs of *N*-Acetylmuramoyl-L-alanyl-D-isoglutamine

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A variety of long-chain fatty acylamino analogs of *N*-acetylmuramoyl-L-alanyl-D-isoglutamine (MDP), such as *N*-(acyl)muramoyl-dipeptides (**1**) and *N*-acetyl-6-(acylamino)-6-deoxymuramoyl-dipeptides (**2**), were synthesized to examine their immunological activities. All the *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**1**) were active as adjuvant on the induction of delayed-type hypersensitivity to azobenzenearsonate-*N*-acetyl-L-tyrosine in guinea pigs. *N*-(3-Hydroxy-2-tetradecyloctadecanoylamino)muramoyl-L-alanyl-D-isoglutamine (**1d**) and *N*-acetyl-6-deoxy-6-(3-hydroxy-2-docosylhexacosanoylamino)muramoyl-L-alanyl-, -L-valyl-, and -L-seryl-D-isoglutamines (**2**) showed antitumor activity by suppressing tumor (Meth-A fibrosarcoma) growth in syngeneic BALB/c mice.

The adjuvant-active minimal structure of bacterial cell wall peptidoglycans was established to be *N*-acetylmuramoyl-L-alanyl-D-isoglutamine^{2,3)} (MDP: **1a**), and the details of immunological properties were investigated. The relationship between the chemical structures and immunological activities of MDP derivatives and the analogs were examined by many groups.⁴⁾ It was found^{5~7)} that the L-alanine residue in MDP could be replaced by other suitable amino acids such as L-valine, L-serine, L-methionine or L-glutamine without decreasing the activity. We have demonstrated that the sugar skeleton in MDP is not restricted to 2-acetamido-2-deoxy-D-glucose. It can be replaced by 2-acetamido-2-deoxy-D-mannose and -D-galactose, D-glucose and 2-acetamido-6-amino- and -6-(acylamino)-2,6-dideoxy-D-glucose, while retaining the activity.^{8~13)}

On the other hand, some of the lipophilic MDP derivatives, *i.e.*, *N*-acetyl-6-*O*-

"mycoloyl"-muramoyl-dipeptide and *N*-acetyl-6-*O*-quinonyl-muramoyl-dipeptide, were shown to have not only adjuvant activity, but also antitumor activity.^{14~16)} In these compounds, the hydroxy group on C-6 of the carbohydrate moiety in MDP is esterified with "mycolic" acid or synthetic long-chain fatty acid.

Recently, we have found that the 2-amino group of the carbohydrate moiety in MDP can be acylated by myristoyl, stearoyl, 3-hydroxymyristoyl and 3-hydroxystearoyl groups¹⁾ instead of the acetyl group without decreasing the activity. In this paper, we describe the synthesis and immunological activities of *N*-(acyl)muramoyl-dipeptides (**1b~1f**) and *N*-acetyl-6-(acylamino)-6-deoxymuramoyl-dipeptides (**2A~2C**) bearing other long-chain fatty-acyl residues as the lipophilic group. We also discuss the effect of the alkyl-chain length of the *N*-acyl moiety for the adjuvant activity.

RESULTS AND DISCUSSION

Chemical synthesis

We used tetracosanoic, 2-tetradecylhexadecanoic, 3-hydroxy-2-tetradecyloctadecanoic, 3-hydroxy-2-docosylhexacosanoic, and mycolic acids,^{14,15} as the long-chain fatty acid.

Brief hydrogenolysis of benzyl 2-(benzyloxycarbonylamino)-2-deoxy-4,6-*O*-isopropylidene- α -D-glucopyranoside¹ (**3**) in the presence of 10% palladium-carbon catalyst, and subsequent treatment with *N*-(tetracosanoyloxy)succinimide or *N*-(2-tetradecylhexadecanoyloxy)succinimide afforded benzyl 2-deoxy-4,6-*O*-isopropylidene-2-(tetracosanoylamino)- and -2-(2-tetradecylhexadecanoylamino)- α -D-glucopyranosides (**4b**, **c**), respectively. The *N*-acyl derivatives **4b** and **4c** were condensed with L-2-chloropropanoic acid in the presence of sodium hydride, to give benzyl 3-*O*-(D-1-carboxyethyl)-2-deoxy-4,6-*O*-isopropylidene-2-(tetracosanoylamino)- and -2-(2-tetradecylhexadecanoylamino)- α -D-glucopyranosides (**6b**, **c**). In order to introduce α -branched β -hydroxylated fatty acids (mycoloyl type), compound **3** was first condensed with L-2-chloropropanoic acid to afford benzyl 2-(benzyloxycarbonylamino)-3-*O*-(D-1-carboxyethyl)-2-deoxy-4,6-*O*-isopropylidene- α -D-glucopyranoside (**5**), which was converted,

by selective hydrogenolysis of the benzyloxycarbonyl group and subsequent treatment with *N*-(3-hydroxy-2-tetradecyloctadecanoyl- or -2-docosylhexacosanonyl-oxy)succinimide or *N*-(mycoloyloxy)succinimide, into the corresponding *N*-fatty acyl, 3-*O*-(D-1-carboxyethyl) derivatives (**6d**~**f**). Compounds **6b**~**f** were coupled with L-alanyl-D-isoglutamine benzyl ester, using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide as the activating agents, to yield the protected *N*-(acyl)muramoyl-dipeptides (**7b**~**f**). Hydrolytic removal of the 4,6-*O*-isopropylidene group from com-

- a R¹ = COCH₃
- b R¹ = CO(CH₂)₂₂CH₃
- c R¹ = COCH(CH₂)₁₃CH₃
(CH₂)₁₃CH₃
- d R¹ = COCHCH(OH)(CH₂)₁₄CH₃
(CH₂)₁₃CH₃
- e R¹ = COCHCH(OH)(CH₂)₂₂CH₃
(CH₂)₂₁CH₃
- f R¹ = Mycoloyl

- R² = COCHCH(OH)(CH₂)₂₂CH₃
(CH₂)₂₁CH₃
- A R³ = L-Ala
- B R³ = L-Val
- C R³ = L-Ser

pounds **7b~f** with 80% acetic acid, followed by hydrogenolysis in the presence of palladium black catalyst, gave the desired *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**1b~f**).

For the synthesis of the 6-acylamino analogs, we employed benzyl 2-acetamido-6-azido-2,6-dideoxy-3-*O*-(*D*-2-propanoyl-L-alanyl-, -L-valyl-, and -*O*-benzyl-L-seryl-D-isoglutamine benzyl ester)- α -D-glucopyranosides¹¹ (**8A~8C**) as the synthetic intermediates. Selective reduction of the azide group in **8A~8C**, and subsequent condensation of the 6-amino compounds with *N*-(3-hydroxy-2-docosylhexacosanoyloxy)succinimide, gave the acylated derivatives (**9A~9C**). The protecting groups of **9A~9C** were removed by hydrogenolysis with palladium black catalyst to afford *N*-acetyl-6-deoxy-6-(3-hydroxy-2-docosylhexacosanoylamino)muramoyl-L-alanyl-, -L-valyl-, and -L-seryl-D-isoglutamines (**2A~2C**).

Adjuvant and antitumor activities

The adjuvant activity of the *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**1b~1f**)

was examined on the induction of delayed-type hypersensitivity to azobenzenearsonate-*N*-acetyl-L-tyrosine (ABA-tyrosine) in guinea pigs. As shown in Table I, all of the compounds tested were found to have adjuvant activity similar to or more potent than that of MDP. It was also found that the short-chain acylamino derivatives of MDP, *e.g.*, *N*-(myristoyl, stearoyl, 3-hydroxymyristoyl or 3-hydroxystearoyl)muramoyl-L-alanyl-D-isoglutamine, exhibit potent activity.¹¹ Recently, we reported the adjuvant activity of *N*-acetyl-6-(acylamino)-6-deoxymuramoyl-dipeptides.¹² In these compounds, *N*-acetyl-6-deoxy-6-(mycoloylamino)muramoyl-L-alanyl-D-isoglutamine had a distinct, but weak, adjuvant activity as compared to that of 6-acetamido or 6-(stearoylamino) analog of MDP in the same immune system. On the other hand, *N*-(mycoloyl)muramoyl-L-alanyl-D-isoglutamine (**1f**), which was mycoloylated at the C-2 amino group of the carbohydrate moiety in MDP, showed potent activity even at a dose of 33 μ g. The results suggest that attachment of the long-chain fatty acyl moiety to the C-2 amino group is

TABLE I. ADJUVANT ACTIVITY OF *N*-(ACYL)MURAMOYL-DIPEPTIDES ON THE INDUCTION OF DELAYED-TYPE HYPERSENSITIVITY IN GUINEA PIGS^a

Compound	Dose ^b (μ g)	Skin reaction ^c with ABA-BSA at 24 hr	Dose (μ g)	Skin reaction with ABA-BSA at 24 hr
	Expt. I		Expt. II	
1a (MDP)	100	24.6 $\pm$ 1.0	10	17.4 $\pm$ 0.7
1b	160	21.4 $\pm$ 1.4		ND ^d
1c	180	22.1 $\pm$ 0.8	18	22.9 $\pm$ 1.8
1d	200	22.4 $\pm$ 0.8	20	15.0 $\pm$ 0.7
1e	230	23.9 $\pm$ 0.5	23	20.4 $\pm$ 0.7
1f	330	23.6 $\pm$ 1.0	33	24.6 $\pm$ 1.3
Control:		0		(4.0 $\pm$ 0) ^e
ABA-tyrosine in FIA				

^a Four Hartley guinea pigs were immunized in four footpads with 50 μ g of azobenzenearsonate-*N*-acetyl-L-tyrosine (ABA-tyrosine) in Freund incomplete adjuvant (FIA) containing each compound. After 2 weeks, skin tests were performed with 100 μ g of ABA-bovine serum albumin (ABA-BSA), and the skin reactions were measured 24 and 48 hr after intradermal injection of the test antigen.

^b The doses of each compound are dependent on the molecular weights.

^c The data indicate the average diameter (mm) $\pm$ the standard error of the skin reaction (induration) of four guinea pigs at 24 hr.

^d ND, not done.

^e Only faint erythema was observed.

TABLE II. TUMOR SUPPRESSIVE ACTIVITY OF SYNTHETIC ACYLAMINO ANALOGS OF MDP^a

Compound	Dose (μ g)	No. of tumor-free mice/ No. of mice treated ^b	No. of tumor-free mice/ No. of mice reinoculated ^c
1c	100	0/10	
1d	100	6/10* ^d	6/6*
1e	100	3/10	
1f	100	0/10	
2A	100	9/11*	9/9*
2B	100	4/10**	
2C	100	5/10*	4/5*
Control		0/10	0/10

^a A mixture of tumor cells (Meth-A: 2×10^5) and each compound suspended in phosphate-buffered saline (PBS) was inoculated intradermally into the flank of BALB/c female mice. Four weeks later, tumor-free mice were reinoculated intradermally with the same tumor cells (2×10^5).

^b Results at 4 weeks after inoculation of the mixture.

^c Results at 2 weeks after reinoculation.

^d Statistical analysis by χ^2 -test of each group compared to control * $p < 0.01$, ** $p < 0.05$.

more favorable than that at C-6 substituent (amino group) for the development of the adjuvant activity.

We have demonstrated that the lipophilic derivatives of MDP, *e.g.*, 6-*O*-“mycoloyl”-MDP or 6-*O*-quinonyl-MDP, display anti-tumor activity on the suppression of tumor growth.^{14~16)} Therefore we examined the tumor (Meth-A fibrosarcoma) suppressive activity of *N*-(acyl)muramoyl-dipeptides (**1c**~**1f**) and *N*-acetyl-6-(acylamino)-6-deoxymuramoyl-dipeptides (**2A**~**2C**) in syngeneic BALB/c female mice. As summarized in Table II, compounds **1d**, **2A**, **2B** and **2C** were shown to be significantly active ($p < 0.05$ by χ^2 -test) on the suppression of tumor growth. Recently, it was also observed that *N*-acetyl-6-deoxy-6-(nocardomycoloylamino)muramoyl-L-alanyl-, and -L-seryl-D-isoglutamines showed potent tumor-suppressive activity in the same system. Moreover, the tumor-free mice rejected the same tumor cells after intradermal transplantation into the other site of the mice. These results indicate that compounds **1d**, **2A** and **2C** possess the antitumor activity based on a immune reaction to the transplantable tumor.

EXPERIMENTAL

Chemical synthesis. 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 IR spectra were recorded with a Shimadzu IR-27G spectrophotometer. NMR spectra were recorded at 60 MHz with a Hitachi R-20B spectrometer. Preparative chromatography was performed on silica gel (Merck Co.; 200 mesh) with the solvent systems specified. Evaporations were conducted *in vacuo*.

Benzyl 2-deoxy-4,6-O-isopropylidene-2-(tetracosanoylamino)- α -D-glucopyranoside (4b). To a solution of benzyl 2-(benzyloxycarbonylamino)-2-deoxy-4,6-*O*-isopropylidene- α -D-glucopyranoside¹⁾ (**3**) (2.0 g) in MeOH (15 ml) was added 10% Pd/C catalyst (0.5 g), and hydrogen was bubbled through, with stirring, for 1 hr at room temperature, the course of the reaction being monitored by TLC. The catalyst was filtered off, and the filtrate was evaporated below 40°C. To a solution of the residue in 1,4-dioxane (20 ml) was added *N*-(tetracosanoyloxy)succinimide (mp 101.5°C) (2.5 g) which was prepared from tetracosanoic acid by *N*-hydroxysuccinimide-dicyclohexylcarbodiimide method, and the mixture was stirred for 5 hr at room temperature, and then evaporated. The residue was chromatographed on a column of silica gel (100 g) with 100 : 1 CHCl₃-MeOH to give **4b** (2.21 g, 74%) as crystals, mp 94°C, $[\alpha]_D^{20} + 58.3^\circ$ ($c = 0.6$, CHCl₃); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3400 (OH), 3320 (NH), 2920 and 2850 (CH₂, CH₃), 1640 and 1530 (amide), 850 (Me₂C), and 720 and 690 (phenyl); NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 0.90 (3H, CH₃), 1.30 (42H, 21CH₂), 1.47 and 1.56 (6H, 2S, Me₂C), 3.27 (1H, bs, OH), 4.50 and 4.83 (2H, 2d, $J_{\text{gem}} = 12.0$ Hz, OCH₂Ph), 4.98 (1H, d, $J_{1,2} = 3.7$ Hz, H-1), 5.97 (1H, d, $J_{2,\text{NH}} = 8.0$ Hz, NH) and 7.45 (5H, s, Ph). *Anal.* Found: C, 72.66; H, 10.49; N, 2.22. Calcd. for C₄₀H₆₉NO₆: C, 72.79; H, 10.54; N, 2.12%.

Benzyl 2-deoxy-4,6-O-isopropylidene-2-(2-tetradecylhexadecanoylamino)- α -D-glucopyranoside (4c). Selective hydrogenolysis of **3** (500 mg) in MeOH (10 ml) in the presence of 10% Pd/C catalyst (200 mg) was carried out according to the procedure described above. To a solution of the amino derivative thus obtained, in 1,4-dioxane (5 ml), was added *N*-(2-tetradecylhexadecanoyloxy)succinimide (mp 50~51°C) (740 mg) which was prepared from 2-tetradecylhexadecanoic acid, and the mixture was stirred overnight at room temperature, and then evaporated. The residue was chromatographed on a column of silica gel (20 g) with 100 : 1 CHCl₃-MeOH to give **4c** (570 mg, 68%) as an amorphous solid, $[\alpha]_D^{23} + 52.8^\circ$ ($c = 0.8$, CHCl₃); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3450 and 3290 (OH, NH), 2920 and 2850 (CH₂, CH₃), 1635 and 1530 (amide), and 720 and 690 (phenyl); NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 0.88 (6H, 2CH₃), 1.27 (52H, 26CH₂), 1.45 and 1.53 (6H, 2s, Me₂C), 3.04 (1H, bs, OH), 4.48 and 4.71 (2H, 2d, $J_{\text{gem}} = 12.0$ Hz, OCH₂Ph), 4.95 (1H, d, $J_{1,2} = 4.0$ Hz, H-1), 5.92 (1H, d, $J_{2,\text{NH}} = 8.5$ Hz, NH), and 7.43 (5H, s, Ph). *Anal.* Found: C, 74.14; H, 10.93; N, 1.92. Calcd. for C₄₆H₈₁NO₆: C, 74.24; H, 10.97; N, 1.88%.

Benzyl 2-(benzyloxycarbonylamino)-3-O-(D-1-carboxyethyl)-2-deoxy-4,6-O-isopropylidene- α -D-glucopyranoside (5). To a stirred solution of **3** (1.3 g) in dry 1,4-dioxane (10 ml) was gradually added sodium hydride in oil suspension (560 mg; 50% of sodium hydride by weight), and the mixture was heated with stirring for 1 hr at 90°C. After being cooled to 60°C, L-2-chloropropanoic acid (410 mg) was gradually added to the mixture and the final mixture was stirred for 5 hr at 60°C. After being cooled, H₂O was added to the mixture to decompose the excess of sodium hydride, and then the pH of the mixture was adjusted to 3 with 2 M HCl. The mixture was extracted with CHCl₃ and the extract was washed with H₂O, dried (Na₂SO₄), and evaporated to a syrup which was purified on a column of silica gel (40 g) with 100 : 1 CHCl₃-MeOH. Compound **5** was obtained as a syrup, 890 mg (59%), $[\alpha]_D^{20} + 87.3^\circ$ ($c = 0.6$, CHCl₃); IR $\nu_{\text{max}}^{\text{film}}$ cm⁻¹: 3320 (NH), 1710 (COOH), 1690 and 1520 (amide), 850 (Me₂C), and 730 and 690 (phenyl); NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 1.43 (3H, d, $J_{\text{CH}_3,\text{CH}} = 7.0$ Hz, CH₃), 1.42 and 1.51 (6H, 2s, Me₂C), 5.13~5.20 (3H, CO₂CH₂Ph and H-1), 7.25~7.40 (2H, COOH and NH) and 7.41 (10H, s, 2Ph). *Anal.* Found: C, 62.64; H, 6.55; N, 2.85. Calcd. for C₂₇H₃₃NO₉: C, 62.90; H, 6.45; N, 2.72%.

Benzyl 3-O-(D-1-carboxyethyl)-2-deoxy-4,6-O-isopropylidene-2-(tetracosanoylamino)- α -D-glucopyranoside (6b). Condensation of **4b** (1.5 g) with L-2-chloropropanoic acid (300 mg) according to the procedure as described above was performed. The product was purified by chromatography on a column of silica gel (50 g) with 100 : 1 CHCl₃-MeOH. Compound **6b** was obtained as a syrup, 1.40 g (84%), $[\alpha]_D^{20} + 67.2^\circ$ ($c = 0.5$, CHCl₃); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3300 (NH), 2920 and 2850 (CH₂, CH₃),

1710 (COOH), 1640 and 1540 (amide), 850 (Me₂C), and 720 and 690 (phenyl); NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 0.90 (3H, CH₃), 1.28 (42H, 21CH₂), 1.44 (3H, d, $J_{\text{CH}_3,\text{CH}} = 7.0$ Hz, CH₃ of propanoyl), 1.42 and 1.53 (6H, 2s, Me₂C), 5.38 (1H, d, $J_{1,2} = 2.8$ Hz, H-1), 7.40 (5H, s, Ph) and 7.58 (1H, bs, NH). *Anal.* Found: C, 70.43; H, 10.19; N, 1.90. Calcd. for C₄₃H₇₃NO₈: C, 70.55; H, 10.05; N, 1.91%.

Benzyl 3-O-(D-1-carboxyethyl)-2-deoxy-4,6-O-isopropylidene-2-(2-tetradecylhexadecanoylamino)- α -D-glucopyranoside (6c). Condensation of **4c** (520 mg) with L-2-chloropropanoic acid (100 mg) according to the method as described in the preparation of **5** afforded **6c** (500 mg, 88%) as a syrup, $[\alpha]_D^{23} + 69.1^\circ$ ($c = 0.7$, CHCl₃). *Anal.* Found: C, 71.63; H, 10.41; N, 1.82. Calcd. for C₄₉H₈₅NO₈: C, 72.10; H, 10.50; N, 1.72%.

Benzyl 3-O-(D-1-carboxyethyl)-2-deoxy-2-(3-hydroxy-2-tetradecyloctadecanoylamino)-4,6-O-isopropylidene- α -D-glucopyranoside (6d). Compound **5** (130 mg) in MeOH (8 ml) was hydrogenated in the presence of 10% Pd/C catalyst for 1 hr at room temperature. The catalyst was removed off by filtration, and the filtrate was evaporated below 40°C. To a solution of the residue in 1,4-dioxane (6 ml), *N*-(3-hydroxy-2-tetradecyloctadecanoyloxy)succinimide (mp 73~74°C) (230 mg) and triethylamine (0.1 ml) were added. The mixture was stirred overnight at room temperature. After removal of the solvents, the residue was chromatographed on a column of silica gel (30 g) with 100 : 1 CHCl₃-MeOH to give **6d** (150 mg, 69%) as crystals, mp 149~150°C, $[\alpha]_D^{20} + 57.7^\circ$ ($c = 0.95$, CHCl₃); IR $\nu_{\text{max}}^{\text{KBr}}$ cm⁻¹: 3300 (OH, NH), 2920 and 2850 (CH₂, CH₃), 1710 (COOH), 1635 and 1550 (amide), 850 (Me₂C), and 725 and 690 (phenyl); NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 5.52 (1H, H-1), 7.40 (5H, s, Ph) and 8.60~8.90 (1H, COOH). *Anal.* Found: C, 71.41; H, 10.42; N, 1.71. Calcd. for C₅₁H₈₉NO₉: C, 71.20; H, 10.43; N, 1.63%.

Benzyl 3-O-(D-1-carboxyethyl)-2-deoxy-2-(3-hydroxy-2-docosylhexacosanoylamino)-4,6-O-isopropylidene- α -D-glucopyranoside (6e). Selective hydrogenolysis of the benzyloxycarbonyl group in **5** (400 mg) was carried out by the same way as described above. To a solution of the amino compound in 1,4-dioxane (4 ml) were added *N*-(3-hydroxy-2-docosylhexacosanoyloxy)succinimide (mp 93~94°C) (700 mg) and triethylamine (0.1 ml). The mixture was stirred for 20 hr at room temperature, and then evaporated. The residue was chromatographed on a column of silica gel (30 g) with 200 : 1 CHCl₃-MeOH to afford **6e** (558 mg, 66%) as a syrup, $[\alpha]_D^{22} + 55.0^\circ$ ($c = 0.8$, CHCl₃). *Anal.* Found: C, 73.71; H, 11.33; N, 1.20. Calcd. for C₆₇H₁₂₁NO₉: C, 74.19; H, 11.24; N, 1.29%.

Benzyl 3-O-(D-1-carboxyethyl)-2-deoxy-4,6-O-isopropylidene-2-(mycoloylamino)- α -D-glucopyranoside (6f). Selective hydrogenolysis of **5** (300 mg) was performed according to the procedure as described in the preparation

of **6d**. To a solution of the amino compound in 1,4-dioxane (3 ml) were added *N*-(mycoloyloxy)succinimide^{1(c)} (600 mg) and triethylamine (0.05 ml). The mixture was stirred for 48 hr at room temperature, and then evaporated. The residue was chromatographed on a column of silica gel (15 g) with 100 : 1 CHCl₃–MeOH to give **6f** (290 mg, 49%), mp 83~87°C, $[\alpha]_D^{23} + 34.8^\circ$ ($c = 1.0$, CHCl₃). *Anal.* Found: C, 76.75; H, 11.84; N, 1.13. Calcd. for C₉₉H₁₈₃N₉O_{9.5}: C, 77.23; H, 11.98; N, 0.91%.

Benzyl 2-deoxy-4,6-O-isopropylidene-3-O-(D-2-propanoyl-L-alanyl-D-isoglutamine benzyl ester)-2-tetracosanoyl-amino)-α-D-glucopyranoside (7b). To a solution of **6b** (300 mg) in 1,4-dioxane (5 ml) were added *N*-hydroxy-succinimide (HOSu) (70 mg) and dicyclohexylcarbodi-imide (DCC) (110 mg), and the mixture was stirred for 2 hr at 10°C. 1,3-Dicyclohexylurea formed was removed by filtration. L-Alanyl-D-isoglutamine benzyl ester trifluoroacetate¹⁷⁾ (210 mg) and triethylamine (0.1 ml) were added to the filtrate, and the mixture was stirred for 4 hr at room temperature, and then evaporated. The residue was chromatographed on a column of silica gel (30 g) with 100 : 1, and then 40 : 1 CHCl₃–MeOH. With the latter eluant, compound **7b** was obtained, wt. 350 mg (84%), mp 107~109°C, $[\alpha]_D^{18} + 61.6^\circ$ ($c = 0.63$, CHCl₃); IR $\nu_{\max}^{\text{KBr}}$ cm⁻¹: 3450 and 3380 (NH), 2920 and 2850 (CH₂, CH₃) 1730 (ester), 1650 and 1530 (amide), 850 (Me₂C), and 725 and 690 (phenyl); NMR $\delta_{\text{Me}_4\text{Si}}^{\text{CDCl}_3}$: 4.93 (1H, d, $J_{1,2} = 3.8$ Hz, H-1), 5.16 (2H, s, CO₂CH₂Ph), and 7.42 (10H, s, 2Ph). *Anal.* Found: C, 68.00; H, 9.00; N, 5.25. Calcd. for C₅₈H₉₂N₄O₁₁: C, 68.20; H, 9.08; N, 5.49%.

Other protected N-(acyl)muramoyl-L-alanyl-D-isoglutamines (7) were similarly prepared from the corresponding, protected *N*-(acyl)muramic acid (**6**) and L-alanyl-D-isoglutamine benzyl ester.

Benzyl 2-deoxy-4,6-O-isopropylidene-3-O-(D-2-propanoyl-L-alanyl-D-isoglutamine benzyl ester)-2-(2-tetradecyl-hexadecanoylamino)-α-D-glucopyranoside (7c). Yield 89%, mp 133~134°C, $[\alpha]_D^{22} + 61.0^\circ$ ($c = 0.8$, CHCl₃). *Anal.* Found: C, 69.47; H, 9.34; N, 5.05. Calcd. for C₆₄H₁₀₄N₄O₁₁: C, 69.53; H, 9.48; N, 5.07%.

Benzyl 2-deoxy-2-(3-hydroxy-2-tetradecylocta-decanoylamino)-4,6-O-isopropylidene-3-O-(D-2-propanoyl-L-alanyl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (7d). Yield 79%, mp 145~146°C, $[\alpha]_D^{20} + 54.2^\circ$ ($c = 0.9$, CHCl₃). *Anal.* Found: C, 68.72; H, 9.52; N, 4.87. Calcd. for C₆₆H₁₀₈N₄O₁₂: C, 68.95; H, 9.47; N, 4.87%.

Benzyl 2-deoxy-2-(3-hydroxy-2-docosylhexaco-sanoylamino)-4,6-O-isopropylidene-3-O-(D-2-propanoyl-L-alanyl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (7e). Yield 84%, mp 121~122°C, $[\alpha]_D^{22} + 46.3^\circ$ ($c = 0.8$, CHCl₃). *Anal.* Found: C, 71.59; H, 10.20; N, 3.99.

Calcd. for C₈₂H₁₄₀N₄O₁₂: C, 71.68; H, 10.27; N, 4.08%.

Benzyl 2-deoxy-4,6-O-isopropylidene-2-(mycoloyl-amino)-3-O-(D-2-propanoyl-L-alanyl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (7f). Yield 81%, mp 87°C, $[\alpha]_D^{23} + 33.6^\circ$ ($c = 1.0$, CHCl₃). *Anal.* Found: C, 74.86; H, 11.32; N, 2.80. Calcd. for C₁₁₄H₂₀₂N₄O_{12.5}: C, 74.87; H, 11.13; N, 3.06%.

N-(3-Hydroxy-2-tetradecyloctadecanoyl)muramoyl-L-alanyl-D-isoglutamine (1d). A solution of **7d** (90 mg) in 80% aqueous AcOH (5 ml) was heated for 2 hr at 45°C, and then evaporated. The residue was triturated with ethyl ether to give the diol derivative (86 mg, 99%), mp 169~172°C, $[\alpha]_D^{20} + 49.1^\circ$ ($c = 0.75$, MeOH). The product (75 mg) in a mixture of THF (5 ml) and AcOH (1 ml) was hydrogenated in the presence of palladium black catalyst for 24 hr at room temperature (25°C). After removal of the catalyst and evaporation of the solvents, the residue was chromatographed on a column of silica gel (15 g) with 50 : 1 CHCl₃–MeOH and 100 : 10 : 1 CHCl₃–MeOH–AcOH. The latter eluate afforded compound **1d** (59 mg, 83%), mp 162~165°C (dec.), $[\alpha]_D^{22} + 11.3^\circ$ (after 24 hr: $c = 0.32$, MeOH); IR $\nu_{\max}^{\text{KBr}}$ cm⁻¹: 3350 (OH, NH), 2920 and 2850 (CH₂, CH₃), and 1650 and 1550 (amide). *Anal.* Found: C, 61.89; H, 10.11; N, 5.73. Calcd. for C₄₉H₉₂N₄O₁₂·H₂O: C, 62.12; H, 10.00; N, 5.91%.

Other N-(acyl)muramoyl-L-alanyl-D-isoglutamines (1) were similarly prepared from the corresponding, protected muramoyl dipeptides (**7**).

N-(Tetracosanoyl)muramoyl-L-alanyl-D-isoglutamine (1b). mp 188~191°C (dec.), $[\alpha]_D^{22} + 16.1^\circ$ (after 24 hr: $c = 0.57$, MeOH). *Anal.* Found: C, 61.00; H, 9.40; N, 6.64. Calcd. for C₄₁H₇₆N₄O₁₁·1/2H₂O: C, 69.79; H, 9.58; N, 6.92%.

N-(2-Tetradecylhexadecanoyl)muramoyl-L-alanyl-D-isoglutamine (1c). mp 163~165°C (dec.), $[\alpha]_D^{22} + 7.7^\circ$ (after 24 hr: $c = 0.6$, 1 : 1 CHCl₃–MeOH). *Anal.* Found: C, 69.18; H, 11.15; N, 6.61. Calcd. for C₄₇H₈₈N₄O₁₁·H₂O: C, 69.47; H, 11.17; N, 6.90%.

N-(3-Hydroxy-2-docosylhexacosanoyl)muramoyl-L-alanyl-D-isoglutamine (1e). mp 170°C (dec.), $[\alpha]_D^{23} + 6.3^\circ$ (after 24 hr, $c = 0.38$, 1 : 1 CHCl₃–MeOH). *Anal.* Found: C, 66.88; H, 10.83; N, 4.45. Calcd. for C₆₅H₁₂₄N₄O₁₂·H₂O: C, 66.63; H, 10.84; N, 4.78%.

N-(Mycoloyl)muramoyl-L-alanyl-D-isoglutamine (1f). mp 169~170°C (dec.), $[\alpha]_D^{22} + 2.0^\circ$ (after 24 hr: $c = 1.0$, CHCl₃). *Anal.* Found: C, 69.62; H, 11.26; N, 2.98. Calcd. for C₉₇H₁₈₆N₄O_{12.5}·3H₂O: C, 70.07; H, 11.64; N, 3.37%.

Benzyl 2-acetamido-2,6-dideoxy-6-(3-hydroxy-2-

docosylhexacosanoylamino)-3-O-(D-2-propanoyl-L-alanyl-D-isoglutamine benzyl ester)- α -D-glucopyranoside (**9A**). To a solution of **8A**⁽¹¹⁾ (200 mg) in a mixture of MeOH (10 ml) and THF (5 ml) was added Raney-nickel catalyst (W-1), and the mixture was stirred overnight at room temperature. After removal of the catalyst and evaporation of the solvents, the residue was dissolved in THF (5 ml), and *N*-(3-hydroxy-2-docosylhexacosanoyloxy)succinimide (345 mg) was added. The mixture was stirred for 48 hr at room temperature, and then evaporated. The residue was chromatographed on a column of silica gel (50 g) with CHCl₃ and 50:1 CHCl₃-MeOH. With the latter eluant, compound **9A** (200 mg, 51%) was obtained, mp 216~218°C (dec.), $[\alpha]_D^{22} + 45.8^\circ$ ($c=0.8$, 1:1 CHCl₃-MeOH); IR $\nu_{\max}^{\text{KBr}}$: 3400 and 3270 (OH, NH), 2920 and 2850 (CH₂, CH₃), 1730 (ester), 1653 and 1540 (amide), and 720 and 690 (phenyl). *Anal.* Found: C, 70.27; H, 9.99; N, 5.06. Calcd. for C₈₁H₁₃₉N₅O₁₂: C, 70.75; H, 10.19; N, 5.09%.

Benzyl 2-acetamido-2,6-dideoxy-6-(3-hydroxy-2-docosylhexacosanoylamino)-3-O-(D-2-propanoyl-L-valyl-D-isoglutamine benzyl ester)- α -D-glucopyranoside (9B). Selective reduction of the azide group in **8B**⁽¹¹⁾ (200 mg), and subsequent coupling with the active ester (360 mg), as described for the preparation of **9A**, gave the product, which was purified by chromatography on a column of silica gel (30 g) with 100:1, and 20:1 CHCl₃-MeOH. The latter eluate gave compound **9B** (173 mg, 45%), mp 205~207°C (dec.), $[\alpha]_D^{22} + 29.7^\circ$ ($c=0.35$, 1:1 CHCl₃-MeOH). *Anal.* Found: C, 70.83; H, 10.06; N, 4.90. Calcd. for C₈₃H₁₄₃N₅O₁₂: C, 71.05; H, 10.27; N, 4.99%.

Benzyl 2-acetamido-2,6-dideoxy-6-(3-hydroxy-2-docosylhexacosanoylamino)-3-O-(D-2-propanoyl-O-benzyl-L-seryl-D-isoglutamine benzyl ester)- α -D-glucopyranoside (9C). Selective reduction of the azide group in **8C**⁽¹¹⁾ (150 mg), and subsequent coupling with the active ester (200 mg), according to the procedure described in the previous section, gave the product which was purified by chromatography on a column of silica gel (30 g) with 100:1 and 20:1 CHCl₃-MeOH. With the latter eluant, compound **9C** (85 mg, 31%) was obtained, mp 215~217°C (dec.), $[\alpha]_D^{22} + 41.3^\circ$ ($c=0.77$, 1:1 CHCl₃-MeOH). *Anal.* Found: C, 71.02; H, 9.75; N, 4.66. Calcd. for C₈₈H₁₄₅N₅O₁₃: C, 71.36; H, 9.87; N, 4.73%.

N-Acetyl-6-deoxy-6-(3-hydroxy-2-docosylhexacosanoylamino)muramoyl-L-alanyl-D-isoglutamine (2A). Compound **9A** (180 mg) was hydrogenated, in the presence of palladium black catalyst, in a mixture of THF (8 ml) and AcOH (2 ml), for 24 hr at 30°C. After removal of the catalyst and evaporation of the solvents, the product was purified by chromatography on a column of silica gel (20 g) with 50:1 CHCl₃-MeOH and 100:10:1 CHCl₃-MeOH-AcOH. The latter eluate gave compound **2A** (155 mg, 90%), mp 175~177°C (dec.), $[\alpha]_D^{22}$

+11.6° (after 24 hr: $c=0.5$, 1:1 CHCl₃-MeOH); IR $\nu_{\max}^{\text{KBr}}$ cm⁻¹: 3380 (OH, NH), 2920 and 2850 (CH₂, CH₃) and 1640 and 1540 (amide). *Anal.* Found: C, 65.76; H, 10.32; N, 5.39. Calcd. for C₆₇H₁₂₇N₅O₁₂·2H₂O: C, 65.38; H, 10.73; N, 5.69%.

N-Acetyl-6-deoxy-6-(3-hydroxy-2-docosylhexacosanoylamino)muramoyl-L-valyl-D-isoglutamine (2B). Hydrogenolysis of **9B** (35 mg) was performed according to the same way as described above, affording **2B** (20 mg, 66%), mp 172~174°C (dec.), $[\alpha]_D^{22} + 5.3^\circ$ (after 24 hr: $c=0.15$, 1:1 CHCl₃-MeOH). *Anal.* Found: C, 67.41; H, 10.98; N, 5.50. Calcd. for C₆₉H₁₃₁N₅O₁₂: C, 67.77; H, 10.80; N, 5.73%.

N-Acetyl-6-deoxy-6-(3-hydroxy-2-docosylhexacosanoylamino)muramoyl-L-seryl-D-isoglutamine (2C). Hydrogenolysis of **9C** (75 mg) was carried out according to the procedure described in the previous section, affording **2C** (59 mg, 96%), mp 177~180°C (dec.), $[\alpha]_D^{22} + 7.0^\circ$ (after 24 hr: $c=0.4$, 1:1 CHCl₃-MeOH). *Anal.* Found: C, 65.11; H, 10.33; N, 5.38. Calcd. for C₆₇H₁₂₇N₅O₁₃·H₂O: C, 65.49; H, 10.58; N, 5.70%.

Biological assay. Determination of adjuvant activity on the induction of delayed-type hypersensitivity and suppression test of transplantable tumor in syngeneic mice were carried out according to the methods reported previously.⁽¹⁶⁾

REFERENCES

- 1) M. Kiso, Y. Goh, E. Tanahashi, A. Hasegawa, H. Okumura and I. Azuma, *Carbohydr. Res.*, **90**, C8 (1981).
- 2) F. Ellouz, A. Adam, R. Ciorbaru and E. Lederer, *Biochem. Biophys. Res. Commun.*, **59**, 1317 (1974).
- 3) S. Kotani, I. Morisaki, Y. Watanabe, F. Kinoshita, T. Shimono, T. Shiba, S. Kusumoto, Y. Tarumi and K. Ikenaka, *Biken J.*, **18**, 105 (1975).
- 4) E. Lederer, *J. Med. Chem.*, **23**, 819 (1980).
- 5) A. Adam, M. Devys, V. Souvannavong, P. Lefrancier, C. Choay and E. Lederer, *Biochem. Biophys. Res. Commun.*, **72**, 339 (1976).
- 6) S. Kotani, Y. Watanabe, F. Kinoshita, I. Morisaki, K. Kato, T. Shiba, S. Kusumoto, T. Tarumi and K. Ikenaka, *Biken J.*, **20**, 39 (1977).
- 7) I. Azuma, K. Kamisango, I. Saiki, Y. Tanio, S. Kobayashi and Y. Yamamura, *Infect. Immun.*, **29**, 1193 (1980).
- 8) A. Hasegawa, K. Kaneda, M. Amano, M. Kiso and I. Azuma, *Agric. Biol. Chem.*, **42**, 2187 (1978).
- 9) M. Kiso, Y. Kaneda, Y. Goh, A. Hasegawa and I. Azuma, *Agric. Biol. Chem.*, **44**, 1971 (1980).
- 10) M. Kiso, Y. Kaneda, H. Okumura, A. Hasegawa, I. Azuma and Y. Yamamura, *Carbohydr. Res.*, **79**, C17 (1980).

- 11) (a) A. Hasegawa, H. Okumura, M. Kiso, I. Azuma and Y. Yamamura, *Carbohydr. Res.*, **79**, C20 (1980); (b) A. Hasegawa, H. Okumura, M. Kiso, I. Azuma and Y. Yamamura, *Agric. Biol. Chem.*, **44**, 1301 (1980); and (c) A. Hasegawa, H. Okumura, M. Kiso, I. Azuma and Y. Yamamura, *Agric. Biol. Chem.*, **44**, 1309 (1980).
 - 12) I. Azuma, H. Okumura, I. Saiki, Y. Tanio, M. Kiso, A. Hasegawa and Y. Yamamura, *Infect. Immun.*, **32**, 1305 (1981).
 - 13) I. Azuma, H. Okumura, I. Saiki, Y. Tanio, M. Kiso, A. Hasegawa and Y. Yamamura, *Infect. Immun.*, **33**, 834 (1981).
 - 14) T. Shiba, S. Okada, S. Kusumoto, I. Azuma and Y. Yamamura, *Bull. Chem. Soc. Jpn.*, **51**, 3307 (1978).
 - 15) S. Kusumoto, M. Inage, T. Shiba, I. Azuma and Y. Yamamura, *Tetrahedron Lett.*, 4899 (1979).
 - 16) I. Saiki, Y. Tanio, M. Yamawaki, M. Uemiya, S. Kobayashi, T. Fukuda, H. Yukimasa, Y. Yamamura and I. Azuma, *Infect. Immun.*, **31**, 114 (1981).
 - 17) S. Kobayashi, T. Fukuda, H. Yukimasa, M. Fujino, I. Azuma and Y. Yamamura, *Bull. Chem. Soc. Jpn.*, **53**, 2570 (1980).
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