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Synthesis and Structure-activity Relationship of *N*-(Acyl)muramoyl-dipeptides†

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A variety of *N*-(acyl)muramoyl-dipeptides were synthesized and their immunoadjuvant activities were examined on the induction of delayed-type hypersensitivity to *N*-acetyl-L-tyrosine-3-azobenzene-4′-arsonate in guinea pigs. It was found that introduction of acyl groups to the 2-amino group in muramoyl-L-valyl- or -L-seryl-D-isoglutamine reduced the activity with the increasing length of the acyl group, although all *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines were active as an adjuvant. The results suggest that development of the adjuvant activity of *N*-(acyl)muramoyl-dipeptides is closely related to both the length of the acyl group and the bulkiness of the peptide moiety.

N-Acetylmuramoyl-L-alanyl-D-isoglutamine (MDP; **21**), a synthetic low molecular weight compound, is the minimal structural requirement[1,2] for the adjuvant activity of bacterial peptidoglycan on antibody formation and cell-mediated immunity. On the basis of the findings, a great number of analogs have been synthesized by many groups and their immunological and antitumor activities have been investigated.[3]

In our studies on the structure-activity relationships of MDP, we have demonstrated that the sugar moiety as well as the peptide moiety plays an important role in developing the immunoadjuvant activity on the induction of delayed-type hypersensitivity to *N*-acetyl-L-tyrosine-3-azobenzene-4′-arsonate in guinea pigs. We have shown that replacement of the 2-acetamido-2-deoxy-D-glucose residue of the muramoyl moiety in MDP by some cyclitols or by 2-acetamido-2-deoxy-D-xylose, and deoxygenation at the C-4 or C-6 position decrease this activity drastically.[4,5] On the other hand, it has been shown that the 6-hydroxyl group can be replaced by an acetamido group, and that the 2-acetamido group can be also replaced by a free amino or a methylamino group without decreasing the activity.[5,6] Recently, we have found that the introduction of a long-chain fatty acid such as mycolic acid and α-branched-β-hydroxylated fatty acids to the 2-amino group, which is located near the lactoyl-dipeptide moiety in muramoyl-L-alanyl-D-isoglutamine by an amide linkage, does not decrease the adjuvant activity.[7,8] In this paper, we report the synthesis and immunoadjuvant activities of a variety of *N*-(acyl)muramoyl-dipeptides such as *N*-(acyl)muramoyl-L-alanyl-, -L-seryl- and -L-valyl-D-isoglutamines, and discuss the effect of the introduction of acyl groups to the 2-amino group of the muramoyl moiety in an MDP molecule on development of the activity.

RESULTS AND DISCUSSION

Benzyl 2-(decanoylamino)-2-deoxy-4,6-*O*-isopropylidene-α-D-glucopyranoside (**2**), benzyl 2-deoxy-4,6-*O*-isopropylidene-2-(tetradecanoylamino)-α-D-glucopyranoside (**3**) and benzyl 2-deoxy-4,6-*O*-isopropylidene-2-(octadecanoylamino)-α-D-glucopyranoside (**4**), which were prepared by the *N*-acylation of **1**[6] with *N*-(decanoyloxy)succinimide, *N*-(tetra-

† Dedicated to Dr. Edgar Lederer on the occasion of his 75th birthday.

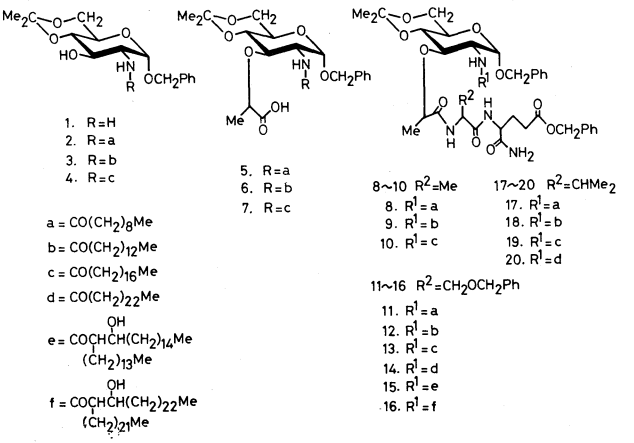

FIG. 1. Synthetic Route for *N*-(Acyl)muramoyl-dipeptides.

decanoyloxy)succinimide or *N*-(octadecanoyloxy)succinimide, were condensed with L-2-chloropropanoic acid in the presence of sodium hydride, to give the corresponding 3-*O*-(D-1-carboxyethyl) derivatives (**5**~**7**) in good yields. Compounds **5**~**7** were coupled with L-alanyl-, *O*-benzyl-L-seryl- or L-valyl-D-isoglutamine benzyl ester, using dicyclohexylcarbodiimide and *N*-hydroxysuccinimide as the activating agents, to yield the protected *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**8**~**10**), *N*-(acyl)muramoyl-L-seryl-D-isoglutamines (**11**~**13**) and *N*-(acyl)muramoyl-L-valyl-D-isoglutamines (**17**~**19**), respectively. The other protected *N*-(acyl)muramoyl-dipeptides (**14**~**16** and **20**) were prepared from the corresponding 3-*O*-(D-1-carboxyethyl) derivatives[8] described previously. Hydrolytic removal of the 4,6-*O*-isopropylidene group from compound **8**~**20** with 80% acetic acid, followed by hydrogenolysis in the presence of palladium black catalyst, gave the desired *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**22**~**24**), *N*-(acyl)muramoyl-L-seryl-D-isoglutamines (**30**~**35**) and *N*-(acyl)muramoyl-L-valyl-D-isoglutamines (**37**~**40**). The synthetic method for the other *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**25**~**28**) has been described previously.[8]

The immunoadjuvant activities[9] of these *N*-(acyl)muramoyl-dipeptides on the induction of delayed-type hypersensitivity to *N*-acetyl-L-tyrosine-3-azobenzene-4′-arsonate (ABA-tyrosine) in guinea pigs were examined (see Fig. 3). All the *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**22**~**28**) showed potent activities at a dose of 10 μg, comparable to that of MDP (**21**), as has been described previously.[7,8] In the case of the *N*-(acyl)muramoyl-L-valyl-D-isoglutamines (valine analogs of *N*-(acyl)muramoyl-dipeptide), the decanoyl and tetradecanoyl derivatives (**37**, **38**) showed clear activities at a dose of 100 μg, but were less active than *N*-acetylmuramoyl-L-valyl-D-isoglutamine (**36**). The octadecanoyl and tetracosanoyl derivatives (**39**, **40**) showed almost negligible activities because only faint erythemas were observed on the skin reaction. The same tendency was also ob-

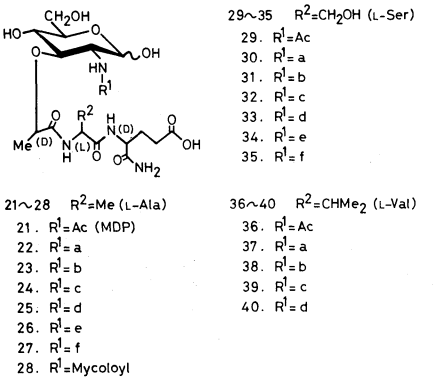

FIG. 2. Structures of *N*-(Acyl)muramoyl-dipeptides.
Structures of the acyl groups (**a**~**f**) are shown in Fig. 1.

served at a dose of 10 μg (data not shown). On the other hand, in the case of the *N*-(acyl)-muramoyl-L-seryl-D-isoglutamines (serine analogs of *N*-(acyl)muramoyl-dipeptide), although the decanoyl derivative (**30**) had an activity comparable to that of *N*-acetyl-muramoyl-L-seryl-D-isoglutamine (**29**), compounds **31**～**35**, bearing longer acyl group than tetradecanoyl, showed weak activities even at a dose of 100 μg, but were stronger than those of the corresponding valine analogs (**39**, **40**).

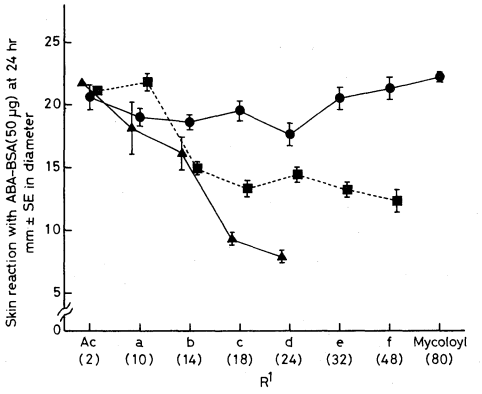

FIG. 3. Adjuvant Activity of *N*-(Acyl)muramoyl-dipeptides on the Induction of Delayed-type Hypersensitivity in Guinea Pigs.

The data indicate the average diameter (mm) ± the standard error (SE) of the skin reaction (erythema). The skin reaction of the control group (ABA-tyrosine in Freund incomplete adjuvant) was not observed. Structures of the acyl groups (R^1: **a**～**f**) are shown in Fig. 1. The numbers in parentheses indicate the total carbon numbers of the acyl groups.

●, R^2=Me (L-Ala), 10 μg; ■, R^2=CH_2OH (L-Ser), 100 μg; ▲, R^2=$CHMe_2$ (L-Val), 100 μg. ABA, *N*-acetyl-L-tyrosine-3-azobenzene-4′-arsonate; BSA, bovine serum albumin.

It is known that *N*-acetylmuramoyl-L-seryl-D-isoglutamine (**29**) and *N*-acetylmuramoyl-L-valyl-D-isoglutamine (**36**), the serine and valine analogs of MDP, are more active as adjuvants than MDP itself.[3,9] As described above, however, the introduction of acyl groups to the 2-amino group in muramoyl-L-seryl- or -L-valyl-D-isoglutamine decreased the activity with increasing length of the acyl group at a dose of 100 μg, although all *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**22**～**28**) showed an activity comparable to MDP (**21**), even at a lower dose (10 μg). Such a decrease of activity is more remarkable in the case of the valine analogs (**37**～**40**) than the serine analogs (**30**～**35**) of *N*-(acyl)muramoyl-dipeptide. The side chains of serine and valine are bulkier than that of alanine. This suggests that the development of the immunoadjuvant activity of *N*-(acyl)muramoyl-dipeptides is closely related to both the length of the acyl moiety (R^1) and the bulkiness of the side chain (R^2) in the peptide moiety, and that any increase in the bulkiness of R^2 reduces activity with the increasing length of R^1. Tables I and II show ^{1}H-NMR data for the *N*-(acyl)muramoyl-L-valyl-D-isoglutamines (**37**～**40**). These compounds exhibit similar chemical shifts in comparison with *N*-acetylmuramoyl-L-valyl-D-isoglutamine (**36**), and the coupling constants between NH and α-CH of the valine residues

TABLE I. ^{1}H-CHEMICAL SHIFTS (δ) FOR α-ANOMERS OF *N*-(ACYL)MURAMOYL-L-VALYL-D-ISOGLUTAMINES[a]

Compound	Sugar	Propanoyl		Valine		Isoglutamine
	H-1	α-CH	β-Me	α-CH	γ-Me[b]	α-CH
36	5.35	4.70	1.37	4.17	0.99	4.40
37	5.37	4.81	1.38	4.23	0.98	4.37
38	5.36	4.75	1.38	4.19	0.99	4.38
39	5.39	4.76	1.37	4.15	0.99	4.36

[a] Structures of the *N*-(acyl)muramoyl-L-valyl-D-isoglutamines (**36**～**39**) are shown in Fig. 3. ^{1}H-NMR spectra were measured at 400 MHz in acetone-d_6 containing 5% D_2O with TMS as an internal standard at 40°C.

[b] Average values of two methyl groups.

TABLE II. ^{1}H-COUPLING CONSTANTS (Hz) FOR α-ANOMERS OF *N*-(ACYL)MURAMOYL-L-VALYL-D-ISOGULTAMINES

Compound	Sugar	Propanoyl	Valine		Isoglutamine	
	$J_{1,2}$	$J_{\alpha,\beta}$	$J_{\alpha,\beta}$	$J_{\alpha,NH}$[a]	$J_{\alpha,\beta}$	$J_{\alpha,\beta'}$
36	3.4	6.4	7.8	8.2	9.3	4.9
37	3.4	6.4	6.8	8.2	9.3	4.9
38	3.4	6.8	7.3	8.0	9.3	4.9
39	3.1	6.6	7.3	7.6	9.3	4.9
40				7.6		

[a] Measured in DMSO-d_6.

TABLE III. PYROGENIC ACTIVITY OF *N*-(ACYL)MURAMOYL-DIPEPTIDES[a]

Compound	Dose (mg/kg of rabbit)	Elevation of body temp. (°C)[b]				
		Individuals			Total	Pyrogenicity
22	0.015	0.25	0.35	0.20	0.80	+
	0.075	**1.00**	**1.15**	**0.60**	**2.75**	
23	0.015	**0.95**	0.45	**0.60**	**2.00**	+
	0.075	**1.45**	**1.35**	**0.75**	**3.55**	
24	0.015	0.20	0.40	0.20	0.80	+
	0.075	**0.90**	**1.25**	0.45	**2.60**	
25	0.075	0.10	**0.85**	0.20	1.15	—[c]
	0.375	0.10	0.25	0.20	0.55	
26	0.075	0.20	0.15	0.50	0.85	—
	0.375	0.05	0.50	0.35	0.90	

[a] Structures of the *N*-(acyl)muramoyl-L-alanyl-D-isoglutamines (**22**～**26**) are shown in Fig. 3.
[b] Boldface numbers indicate definite pyrogenicity.
[c] Result of re-examination: **0.60**, 0.15, 0.00, 0.20, 0.15 (—).

were also similar to that of **36**. This result suggests that no marked change of conformation in the peptide moiety occurs. From the data, it may be concluded that the introduction of an acyl group to the 2-amino group of muramoyl-L-valyl-D-isoglutamine does not affect the conformation of the peptide moiety, but that it does interfere with the interaction of these compounds to immune competent cells.

It is known that MDP is adjuvant-active, but also pyrogenic.[10,11] A previous study has shown that 6-*O*-acylation of MDP with octadecanoic, corynomycolic (average carbon number is 31) and nocardomycolic (average carbon number is 51) acids did not diminish the pyrogenicity in rabbits, but that the 6-*O*-mycoloylated (mycolic acid: average carbon number is 80) derivative of MDP had no pyrogenic activity.[11] In this study, we examined the pyrogenic activity of the *N*-(acyl)-muramoyl-L-alanyl-D-isoglutamines in rabbits, using the method of "The Pharmacopeia of Japan," 10th Ed.[12] The decanoyl, tetradecanoyl and octadecanoyl derivatives (**22**～**24**) were definitely pyrogenic (Table III). However, pyrogenic activity in the *N*-(tetracosanoyl) and *N*-(3-hydroxy-2- tetradecyloctadecanoyl)muramoyl-L-alanyl-D-isoglutamines (**25**, **26**) was not observed, even at a dose of 375 μg per kg of rabbit. These compounds have a strong adjuvant activity as described previously, and it may be concluded that

there is no correlation between pyrogenic activity and adjuvant activity, at least for the induction of cell-mediated immunity.

It has been reported that MDP and its 6-*O*-octadecanoyl derivative augmented the non-specific resistance of mice to bacterial infections.[13,14] We examined the effect of compounds **22**~**26** on the resistance of mice to *Escherichia coli*, according to the method reported by Matsumoto *et al.*[14] These compounds were hardly effective in comparison with MDP and *N*-acetyl-6-*O*-(octadecanoyl)-muramoyl-L-alanyl-D-isoglutamine. The results indicate that replacement of the *N*-acetyl group with a long-chain fatty-acyl group reduces the protective activity.

EXPERIMENTAL

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 M Hz with a Hitachi R-20B spectrometer. Preparative chromatography was performed on silica gel (Merck Co., 200 mesh) with the solvent systems specified. All evaporation was conducted *in vacuo*.

Benzyl 2-(decanoylamino)-2-deoxy-4,6-O-isopropylidene-α-D-glucopyranoside (**2**). To a solution of benzyl 2-amino-2-deoxy-4,6-*O*-isopropylidene-α-D-glucopyranoside[7] (**1**; 1.5 g) in 1,4-dioxane (15 ml) was added *N*-(decanoyloxy)succinimide (2.5 g), which had been prepared from decanoic acid by the *N*-hydroxysuccinimide–dicyclohexylcarbodiimide method as described previously.[8] The mixture was stirred overnight at room temperature and then evaporated. The residue was purified by chromatography on a column of silica gel (50 g) with (a) $CHCl_3$ and (b) 100:1 $CHCl_3$–MeOH. Eluant (b) afforded **2** (2.13 g, 95%) as an amorphous solid, $[\alpha]_D^{20}$ +82.0° (c=0.6, $CHCl_3$); IR ν_{max}^{KBr} cm^{-1}: 3500–3300 (OH, NH), 2920 and 2850 (CH_2, Me), 1640 and 1540 (amide), 850 (Me_2C), and 720 and 690 (Ph); NMR $\delta_{TMS}^{CDCl_3}$: 0.90 (3H, Me), 1.30 (14H, $7CH_2$), 1.47 and 1.57 (6H, 2s, Me_2C), 2.22 (2H, CH_2CO), 4.50 and 4.81 (2H, 2d, J_{gem}= 12.0 Hz, OCH_2Ph), 4.98 (1H, d, $J_{1,2}$=4.0 Hz, H-1), 6.07 (1H, d, $J_{2,NH}$=8.0 Hz, NH) and 7.45 (5H, s, Ph). *Anal.* Found: C, 67.04; H, 8.91; N, 2.93. Calcd. for $C_{26}H_{41}NO_6$: C, 67.36; H, 8.91; N, 3.02%.

Benzyl 2-deoxy-4,6-O-isopropylidene-2-(tetradecanoylamino)-α-D-glucopyranoside (**3**). Coupling of **1** with *N*-(tetradecanoyloxy)succinimide was carried out according to a method similar to that described above. After purification, **3** was obtained as an amorphous solid, $[\alpha]_D^{18}$ +69.0° (c=0.4, $CHCl_3$). *Anal.* Found: C, 69.08; H, 9.48; N, 2.58. Calcd. for $C_{30}H_{49}NO_6$: C, 69.33; H, 9.50; N, 2.70%.

Benzyl 2-deoxy-4,6-O-isopropylidene-2-(octadecanoylamino)-α-D-glucopyranoside (**4**). Coupling of **1** with *N*-(octadecanoyloxy)succinimide was carried out according to a method similar to that described in the previous section. After purification, **4** was obtained as crystals, mp 90°C, $[\alpha]_D^{20}$ +66.7° (c=0.6, $CHCl_3$). *Anal.* Found: C, 70.47; H, 9.96; N, 2.25. Calcd. for $C_{34}H_{57}NO_6$: C, 70.92; H, 9.98; N, 2.43%.

Benzyl 3-O-(D-1-carboxyethyl)-2-(decanoylamino)-2-deoxy-4,6-O-isopropylidene-α-D-glucopyranoside (**5**). To a stirred solution of **2** (2.0 g) in dry 1,4-dioxane (15 ml) was gradually added sodium hydride (1.0 g), and the mixture was heated with stirring for 1.5 hr at 95°C. After being cooled to 60°C, L-2-chloropropanoic acid (0.8 g) was gradually added to the mixture, and the final mixture was stirred overnight at 70°C. After being cooled, H_2O was added to the mixture, and the pH of the mixture was then adjusted to 3 with 2 M HCl. The mixture was extracted with $CHCl_3$, and the extract was washed with H_2O, dried (Na_2SO_4) and evaporated. The residue was chromatographed on a column of silica gel (50 g) with 100:1 $CHCl_3$–MeOH to give **5** (1.84 g, 80%) as crystals, mp 113°C, $[\alpha]_D^{25}$ +103.3° (c=0.6, $CHCl_3$); IR ν_{max}^{KBr} cm^{-1}: 3300 (NH), 2850 and 2920 (CH_2, Me), 1710 (C=O), 1620 and 1580 (amide), 850 (Me_2C), and 730 and 690 (Ph); NMR $\delta_{TMS}^{CDCl_3}$: 1.25 (CH_2), 1.45 (3H, d, J=7.0 Hz, Me of propanoyl), 1.42 and 1.53 (6H, 2d, Me_2C), 5.43 (1H, H-1), 7.39 (5H, s, Ph), 7.85 (1H, NH) and 9.60 (1H, COOH). *Anal.* Found: C, 65.18; H, 8.57; N, 2.57. Calcd. for $C_{29}H_{45}NO_8$: C, 65.02; H, 8.47; N, 2.62%.

The other (D-1-carboxyethyl) derivatives (**6**, **7**) *were similarly prepared from the corresponding glycosides* (**3**, **4**).

Benzyl 3-O-(D-1-carboxyethyl)-2-deoxy-4,6-isopropylidene-2-(tetradecanoylamino)-α-D-glucopyranoside (**6**). $[\alpha]_D^{18}$ +80.6° (c=1.0, $CHCl_3$). *Anal.* Found: C, 66.69; H, 8.91; N, 2.20. Calcd. for $C_{33}H_{53}NO_8$: C, 66.97; H, 9.03; N, 2.37%.

Benzyl 3-O-(D-1-carboxyethyl)-2-deoxy-4,6-O-isopropylidene-2-(octadecanoylamino)-α-D-glucopyranoside (**7**). $[\alpha]_D^{25}$ +81.7° (c=0.6, $CHCl_3$). *Anal.* Found: C, 68.55; H, 9.54; N, 2.10. Calcd. for $C_{37}H_{61}NO_8$: C, 68.59; H, 9.48; N, 2.16%.

Benzyl 2-(decanoylamino)-2-deoxy-4,6-O-isopropylidene-3-O-(D-2-propanoyl-L-alanyl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (**8**). To a solution of **5** (300 mg) in 1,4-dioxane (3 ml) were added *N*-hydroxy-

succinimide (HOSu) (85 mg) and dicyclohexylcarbodiimide (DCC) (150 mg), and the mixture was stirred for 1.5 hr at 15°C. The 1,3-dicyclohexylurea which formed was removed by filtration. L-Alanyl-D-isoglutamine benzyl ester trifluoroacetate (300 mg) and triethylamine (0.1 ml) were added to the filtrate, and the mixture was stirred for 4 hr at room temperature. After evaporation of the solvent, the residue was chromatographed on a column of silica gel (30 g) with 100 : 1, and then 30 : 1 $CHCl_3$–MeOH. With the latter eluant, compound **8** (430 mg, 93%) was obtained as an amorphous solid, $[\alpha]_D^{25}$ +77.4° ($c=0.7$, $CHCl_3$); IR ν_{max}^{KBr} cm^{-1}: 3400 and 3300 (NH), 2920 and 2850 (CH_2, Me), 1730 (ester), 1650 and 1530 (amide), 850 (Me_2C), and 730 and 690 (Ph); NMR $\delta_{TMS}^{CDCl_3}$: 4.95 (1H, d, $J_{1,2}=4.0$ Hz, H-1), 5.17 (2H, s, $COOCH_2Ph$), and 7.43 (10H, s, 2Ph). *Anal.* Found: C, 63.80; H, 7.78; N, 6.70. Calcd. for $C_{44}H_{64}N_4O_{11}$: C, 64.05; H, 7.82; N, 6.79%.

*The other protected N-(acyl)muramoyl-L-alanyl-D-isoglutamines (***9**, **10***) were similarly prepared from the corresponding, protected N-(acyl)muramic acids (***6**, **7***) 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-(tetradecanoylamino)-α-D-glucopyranoside (***9***).* $[\alpha]_D^{20}$ +71.6° ($c=0.5$, $CHCl_3$). *Anal.* Found: C, 65.40; H, 8.24; N, 6.24. Calcd. for $C_{48}H_{72}N_4O_{11}$: C, 65.43; H, 8.24; N, 6.36%.

*Benzyl 2-deoxy-4,6-O-isopropylidene-2-(octadecanoylamino)-3-O-(D-2-propanoyl-L-alanyl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (***10***).* $[\alpha]_D^{25}$ +68.6° ($c=0.7$, $CHCl_3$). *Anal.* Found: C, 66.29; H, 8.68; N, 5.85. Calcd. for $C_{52}H_{80}N_4O_{11}$: C, 66.64; H, 8.60; N, 5.98%.

*Benzyl 2-(decanoylamino)-2-deoxy-4,6-O-isopropylidene-3-O-(D-2-propanoyl-O-benzyl-L-seryl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (***11***).* Treatment of **5** (200 mg) with HOSu (56 mg) and DCC (92 mg), and subsequent coupling with *O*-benzyl-L-seryl-D-isoglutamine benzyl ester (220 mg) according to the procedure described in the previous section, gave compound **11** (330 mg, 95%) as an amorphous solid, $[\alpha]_D^{22}$ +70.4° ($c=0.5$, $CHCl_3$). *Anal.* Found: C, 65.48; H, 7.49; N, 6.00. Calcd. for $C_{51}H_{70}N_4O_{12}$: C, 65.78; H, 7.58; N, 6.02%.

*The other protected N-(acyl)muramoyl-L-seryl-D-isoglutamines (***12**~**16***) were similarly prepared from the corresponding, protected N-(acyl)muramic acids and O-benzyl-L-seryl-D-isoglutamine benzyl ester.*

*Benzyl 2-deoxy-4,6-O-isopropylidene-3-O-(D-2-propanoyl-O-benzyl-L-seryl-D-isoglutamine benzyl ester)-2-(tetradecanoylamino)-α-D-glucopyranoside (***12***).* $[\alpha]_D^{20}$ +56.0° ($c=0.5$, $CHCl_3$). *Anal.* Found: C, 66.62; H, 7.89; N, 5.65. Calcd. for $C_{55}H_{78}N_4O_{12}$: C, 66.91; H, 7.96; N, 5.68%.

*Benzyl 2-deoxy-4,6-O-isopropylidene-2-(octadecanoylamino)-3-O-(D-2-propanoyl-O-benzyl-L-seryl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (***13***).* $[\alpha]_D^{18}$ +63.2° ($c=0.5$, $CHCl_3$). *Anal.* Found: C, 67.78; H, 8.26; N, 5.34. Calcd. for $C_{59}H_{86}N_4O_{12}$; C, 67.92; H, 8.31; N, 5.37%.

*Benzyl 2-deoxy-4,6-O-isopropylidene-3-O-(D-2-propanoyl-O-benzyl-L-seryl-D-isoglutamine benzyl ester)-2-(tetracosanoylamino)-α-D-glucopyranoside (***14***).* Benzyl 3-*O*-(D-1-carboxyethyl)- 2-deoxy-4,6-*O*-isopropylidene- 2-(tetracosanoylamino)- α-D-glucopyranoside[8] was coupled with protected L-seryl-D-isoglutamine. $[\alpha]_D^{18}$ +59.0° ($c=0.6$, $CHCl_3$). *Anal.* Found: C, 69.01; H, 8.67; N, 5.02. Calcd. for $C_{65}H_{98}N_4O_{12}$: C, 69.24; H, 8.76; N, 4.97%.

*Benzyl 2-deoxy-2-(3-hydroxy-2-tetradecyloctadecanoylamino)-4,6-O-isopropylidene-3-O-(D-2-propanoyl-O-benzyl-L-seryl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (***15***).* Benzyl 3-*O*-(D-1-carboxyethyl)-2-deoxy-2-(3-hydroxy-2-tetradecyloctadecanoylamino)-4,6-*O*-isopropylidene-α-D-glucopyranoside[8] was coupled with protected L-seryl-D-isoglutamine. $[\alpha]_D^{20}$ +55.6° ($c=0.5$, $CHCl_3$). *Anal.* Found: C, 69.45; H, 8.90; N, 4.81. Calcd. for $C_{73}H_{114}N_4O_{13}$: C, 69.82; H, 9.15; N, 4.46%.

*Benzyl 2-deoxy-2-(3-hydroxy-2-docosylhexacosanoylamino)-4,6-O-isopropylidene-3-O-(D-2-propanoyl-O-benzyl-L-seryl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (***16***).* Benzyl 3-*O*-(D-1-carboxyethyl)-2-deoxy-2-(3-hydroxy-2-docosylhexacosanoylamino)-4,6-*O*-isopropylidene-α-D-glucopyranoside[8] was coupled with protected L-seryl-D-isoglutamine. $[\alpha]_D^{22}$ +44.8° ($c=0.8$, $CHCl_3$). *Anal.* Found: C, 72.10; H, 9.86; N, 3.82. Calcd. for $C_{89}H_{146}N_4O_{13}$: C, 72.22; H, 9.94; N, 3.79%.

*The protected N-(acyl)muramoyl-L-valyl-D-isoglutamines (***17**~**20***) were similarly prepared from the corresponding, protected N-(acyl)muramic acids and L-valyl-D-isoglutamine benzyl ester.*

*Benzyl 2-(decanoylamino)-2-deoxy-4,6-O-isopropylidene-3-O-(D-2-propanoyl-L-valyl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (***17***).* $[\alpha]_D^{25}$ +71.4° ($c=0.7$, $CHCl_3$). *Anal.* Found: C, 64.74; H, 8.01; N, 6.68. Calcd. for $C_{46}H_{68}N_4O_{11}$: C, 64.76; H, 8.04; N, 6.57%.

*Benzyl 2-deoxy-4,6-O-isopropylidene-3-O-(D-2-propanoyl-L-valyl-D-isoglutamine benzyl ester)-2-(tetradecanoylamino)-α-D-glucopyranoside (***18***).* $[\alpha]_D^{20}$ +71.2° ($c=0.5$, $CHCl_3$). *Anal.* Found: C, 65.88; H, 8.45; N, 6.19. Calcd. for $C_{50}H_{76}N_4O_{11}$: C, 66.05; H, 8.42; N, 6.16%.

*Benzyl 2-deoxy-4,6-O-isopropylidene-2-(octadecanoylamino)-3-O-(D-2-propanoyl-L-valyl-D-isoglutamine benzyl ester)-α-D-glucopyranoside (***19***).* $[\alpha]_D^{25}$ +64.3° ($c=0.7$, $CHCl_3$). *Anal.* Found: C, 67.18; H, 8.79; N, 5.76. Calcd. for $C_{54}H_{84}N_4O_{11}$: C, 67.19; H, 8.77; N, 5.80%.

*Benzyl 2-deoxy-4,6-O-isopropylidene-3-O-(*D*-2-propanoyl-*L*-valyl-*D*-isoglutamine benzyl ester)-2-(tetracosanoylamino)-α-*D*-glucopyranoside* (**20**). $[\alpha]_D^{22}$ +60.0° (c=0.5, $CHCl_3$). *Anal.* Found: C, 68.71; H, 9.10; N, 5.38. Calcd. for $C_{60}H_{96}N_4O_{11}$: C, 68.67; H, 9.22; N, 5.34%.

*N-(Decanoyl)muramoyl-*L*-alanyl-*D*-isoglutamine* (**22**). A solution of **8** (420 mg) in 80% aqueous AcOH (5 ml) was heated for 2 hr at 45°C, and then evaporated. The residue was triturated with Et_2O to give the diol derivative (390 mg, 98%), $[\alpha]_D^{22}$ +83.6° (c=0.5, MeOH). The product (200 mg) in MeOH (10 ml) was hydrogenated in the presence of palladium black catalyst for 24 hr at room temperature. After removal of the catalyst and evaporation of the solvent, compound **22** (150 mg, 97%) was obtained as an amorphous solid, $[\alpha]_D^{20}$ +35.2° (c=0.5, MeOH; after 24 hr); IR ν_{max}^{KBr} cm^{-1}: 3400 (OH, NH), 2920 and 2845 (CH_2, Me), and 1650 and 1540 (amide); NMR (acetone-d_6–5% D_2O) δ_{TMS}: 0.88 (3H, Me of decanoyl), 1.29 (14H, CH_2), 1.36 and 1.39 (6H, 2d, J=7.0 Hz, Me of propanoyl and alanyl), 5.25 (d, $J_{1,2}$=2.9 Hz, H-1 of α-anomer). *Anal.* Found: C, 53.11; H, 8.06; N, 8.89. Calcd. for $C_{27}H_{48}N_4O_{11}\cdot 0.5H_2O$: C, 52.84; H, 8.05; N, 9.13%.

The other N-(acyl)muramoyl-dipeptides (**23**, **24**) *were similarly prepared from the corresponding, protected muramoyl dipeptides.*

*N-(Tetradecanoyl)muramoyl-*L*-alanyl-*D*-isoglutamine* (**23**). $[\alpha]_D^{20}$ +25.0° (c=0.4, MeOH; 24 hr). *Anal.* Found: C, 55.28; H, 8.66; N, 8.12. Calcd. for $C_{31}H_{56}N_4O_{11}\cdot 0.5H_2O$: C, 55.59; H, 8.58; N, 8.36%.

*N-(Octadecanoyl)muramoyl-*L*-alanyl-*D*-isoglutamine* (**24**). $[\alpha]_D^{20}$ +23.2° (c=0.5, MeOH). *Anal.* Found: C, 57.62; H, 9.13; N, 7.48. Calcd. for $C_{35}H_{64}N_4O_{11}\cdot 0.5H_2O$: C, 57.91; H, 9.02; N, 7.72%.

*N-(Decanoyl)muramoyl-*L*-seryl-*D*-isoglutamine* (**30**). $[\alpha]_D^{20}$ +39.6° (c=0.5, MeOH; 24 hr). *Anal.* Found: C, 50.01; H, 7.54; N, 8.39. Calcd. for $C_{27}H_{48}N_4O_{12}\cdot 1.5H_2O$: C, 50.07; H, 7.94; N, 8.65%.

*N-(Tetradecanoyl)muramoyl-*L*-seryl-*D*-isoglutamine* (**31**). $[\alpha]_D^{20}$ +37.6° (c=0.5, MeOH; 24 hr). *Anal.* Found: C, 53.98; H, 8.55; N, 7.96. Calcd. for $C_{31}H_{56}N_4O_{12}\cdot 0.5H_2O$: C, 54.29; H, 8.38; N, 8.17%.

*N-(Octadecanoyl)muramoyl-*L*-seryl-*D*-isoglutamine* (**32**). $[\alpha]_D^{20}$ +27.2° (c=0.6, MeOH; 24 hr). *Anal.* Found: C, 56.39; H, 8.94; N, 7.43. Calcd. for $C_{35}H_{64}N_4O_{12}\cdot 0.5H_2O$: C, 56.66; H, 8.83; N, 7.55%.

*N-(Tetracosanoyl)muramoyl-*L*-seryl-*D*-isoglutamine* (**33**). $[\alpha]_D^{20}$ +24.0° (c=0.5, MeOH; 24 hr). *Anal.* Found: C, 59.38; H, 9.45; N, 6.66. Calcd. for $C_{41}H_{76}N_4O_{12}\cdot 0.5H_2O$: C, 59.61; H, 9.39; N, 6.78%.

*N-(3-Hydroxy-2-tetradecyloctadecanoyl)muramoyl-*L*-seryl-*D*-isoglutamine* (**34**). $[\alpha]_D^{20}$ +5.0° (c=0.4, 1:1 $CHCl_3$–MeOH; 24 hr). *Anal.* Found: C, 61.35; H, 9.94; N, 5.66. Calcd. for $C_{49}H_{92}N_4O_{13}\cdot 0.5H_2O$: C, 61.67; H, 9.82; N, 5.87%.

*N-(3-Hydroxy-2-docosylhexacosanoyl)muramoyl-*L*-seryl-*D*-isoglutamine* (**35**). $[\alpha]_D^{22}$ +4.4° (c=0.5, 1:1 $CHCl_3$–MeOH; 24 hr). *Anal.* Found: C, 65.98; H, 10.78; N, 4.46. Calcd. for $C_{65}H_{124}N_4O_{13}\cdot 0.5H_2O$: C, 66.23; H, 10.69; N, 4.75%.

*N-(Decanoyl)muramoyl-*L*-valyl-*D*-isoglutamine* (**37**). $[\alpha]_D^{20}$ +34.4° (c=0.5, MeOH; 24 hr). *Anal.* Found: C, 54.16; H, 8.41; N, 8.65. Calcd. for $C_{29}H_{52}N_4O_{11}\cdot 0.5H_2O$: C, 54.28; H, 8.32; N, 8.73%.

*N-(Tetradecanoyl)muramoyl-*L*-valyl-*D*-isoglutamine* (**38**). $[\alpha]_D^{20}$ +33.2° (c=0.5, MeOH; 24 hr). *Anal.* Found: C, 56.60; H, 8.92; N, 7.88. Calcd. for $C_{33}H_{60}N_4O_{11}\cdot 0.5H_2O$: C, 56.80, H, 8.81; N, 8.03%.

*N-(Octadecanoyl)muramoyl-*L*-valyl-*D*-isoglutamine* (**39**). $[\alpha]_D^{20}$ +32.0° (c=0.5, MeOH; 24 hr). *Anal.* Found: C, 58.68; H, 9.35; N, 7.19. Calcd. for $C_{37}H_{68}N_4O_{11}\cdot 0.5H_2O$: C, 58.94; H, 9.22; N, 7.43%.

*N-(Tetracosanoyl)muramoyl-*L*-valyl-*D*-isoglutamine* (**40**). $[\alpha]_D^{23}$ +16.8° (c=0.5, 1:1 $CHCl_3$–MeOH; 24 hr). *Anal.* Found: C, 61.38; H, 9.80; N, 6.41. Calcd. for $C_{43}H_{80}N_4O_{11}\cdot 0.5H_2O$: C, 61.62; H, 9.74; N, 6.68%.

Determination of adjuvant activity. Four Hartley guinea pigs in each group were immunized in their four footpads with 50 μg of *N*-acetyl-L-tyrosine-3-azobenzene-4′-arsonate (ABA-tyrosine) in Freund incomplete adjuvant (FIA) with and without each compound. After 2 weeks, a skin test was performed with 50 μg of ABA-bovine serum albumin (ABA-BSA), and the sizes of the erythema and induration were measured 24 hr after intradermal injection of the test antigen.

Determination of pyrogenic activity. Pyrogenic activity was determined by the method of "The Pharmacopeia of Japan," 10th Ed.[12] Briefly, the test compound was dissolved or suspended in pyrogen-free physiological saline at an appropriate concentration. A 10-ml volume of the solution or suspension containing the required dose was injected intravenously into rabbits, and their rectal temperatures were measured continuously with an automatic body temperature-recording device. When at least two out of the three rabbits tested showed individual rises in temperature of 0.6°C or more above their control temperatures, the test compound was considered pyrogenic. When none of the three rabbits showed such a rise in temperature, the test material was regarded as nonpyrogenic.

Determination of protective activity. Protective activity was determined according to the method reported by Matsumoto *et al.*[14] Briefly, a solution of each compound (a dose equivalent to 100 μg of MDP) in phosphate-buffered saline (0.2 ml) was injected subcutaneously into STD-ddY mice. After 24 hr, *Escherichia coli* (6.4×10^6) was injected subcutaneously, and the survivors were recorded for 7 days after infection.

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