Chemical Synthesis of *N*-Acetylmuramyl Peptides with Partial Structures of Bacterial Cell Wall and Their Analogs in Relation to Immunoadjuvant Activities[1)]

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N-Acetylmuramyl peptides of successive length corresponding to the partial structures of cell wall in *Staphylococcus aureus* were synthesized in order to elucidate the minimum effective structure responsible for immunoadjuvant activity of bacterial cell walls. In view of the finding that *N*-acetylmuramyl-L-alanyl-D-isoglutamine was the least structure moiety for exhibition of the activity, nine analogs of either *N*-acetylmuramyl amino acid or *N*-acetylmuramyl dipeptide were also synthesized.

The fact that mycobacterium cell exhibits the immunoadjuvant activity had been first described by Freund in 1956.[2)] He observed the significant stimulation of both humoral and cellular immune responses to a protein antigen, when the antigen was administered together with killed cell of this bacterium. Successive investigations have revealed that this activity is not restricted to mycobacterium cells but manifests in many other bacterial cells commonly, and that water soluble fragments involving peptidoglycan are responsible for the activity.[3)] Meanwhile, the chemical structure of such bacterial peptidoglycan was clarified by many investigators.[4)] For example, the main chain of peptidoglycan in *Staphylococcus aureus* is composed of *N*-acetylglucosamines and *N*-acetylmuramic acids alternately, and tetrapeptide subunits of L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine are linked to the carboxyl groups of *N*-acetylmuramic acids. The carboxyl group of *C*-terminal D-alanine in one peptide subunit is connected to the ε-amino group of L-lysine in the another unit through a pentaglycine bridge, thus completing the network structure of cell wall. The peptidoglycans of many other bacteria have fundamentally similar structures, though the lysine residue is often replaced with α,ε-diaminopimelic acid and glycine residue in the bridge also with other amino acids.

Recently, Kotani *et al.*[1,5)] found the fact that *N*-acetylmuramyl peptides obtained by enzymatic digestion of cell wall, *i.e.*, N^{α}-(*N*-acetylmuramyl-L-alanyl-D-isoglutaminyl)-N^{ε}-(glycyl-glycyl)-L-lysyl-D-alanine from *S. aureus* and *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-diaminopimelyl-D-alanine and/or *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-*meso*-diaminopimelic acid from *Lactobacillus plantarum*, showed definite adjuvant activities. These muramyl peptides seemed to be the smallest chemical entities possessing adjuvant activity in the cell wall structure. Lederer *et al.*[6)] as well as Fleck *et al.*[7)] also reported that the monomeric subunit is enough essential for the adjuvant activities of peptidoglycan from their result of enzymatic degradations.

However, there seemed to remain still some ambiguity on possible disturbance by inevitable impurities which contaminated the enzymatic digests and caused the adjuvant activity. Furthermore, the method of enzymatic degradation has a limitation for the determination of the minimal structure for exhibition of the activity. In view of the above situation, the chemical synthesis would be expected to have obvious advantage particularly in this case, as one can prepare not only a satisfactory pure substance of natural structure but also tailor-made analogs to investigate a relation between the structure and the activity. We therefore synthesized several *N*-acetylmuramyl peptides corresponding to the partial structures of peptidoglycan in *S. aureus*, and could thus demonstrate clearly that *N*-acetylmuramyl-L-alanyl-D-isoglutamine is the least structure required for the adjuvant activity.[8)] The experimental results on the biological tests of these synthetic substances were already reported.[1,9)] In the present paper, we report the full details of the synthesis of the muramyl peptides.

As muramyl peptides of natural type structure involved in the *S. aureus* peptidoglycan, the following four compounds of successive length were prepared by total synthesis, *i.e.*, *N*-acetylmuramyl-L-alanine (**1d**), *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2d**), *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysine (**3d**) and *N*-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (**4d**).

These compounds were all preprared in principle by coupling of a protected *N*-acetylmuramic acid, *i.e.*, benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1′-carboxyethyl)-2-deoxy-α-D-glucopyranoside (**5**)[10)] with suitably protected peptide portions followed by deprotection. The all protecting groups were so selected as to be removed simultaneously by mild hydrogenolysis at the final synthetic step.

Each peptide portion was synthesized by conventional method using *t*-butoxycarbonyl (Boc) and benzyloxycarbonyl (Z) groups for α- and ε-amino protections respectively and benzyl ester for carboxyl protection. Although the synthesis of Boc-L-alanyl-D-isoglutamine benzyl ester (**2a**) and N^{α}-(Boc-L-alanyl-D-isoglutaminyl)-N^{ε}-Z-L-lysyl-D-alanine benzyl ester (**4a**) had been already described by Bricas *et al.*,[11)] synthetic procedure and yield of the products were eventually improved in this investigation. Thus, the dipeptide (**2a**) was prepared by coupling of Boc-L-alanine with *D*-isoglutamine benzyl ester using dicyclohexylcarbodiimide–*N*-hydroxysuccinimide. Hydrogenolysis of **2a** gave Boc-L-alanyl-D-isoglutamine, which was then coupled with benzyl N^{ε}-Z-L-lysinate or N^{ε}-Z-lysyl-L-D-alanine benzyl ester by means of mixed anhydride method to afford the protected tripeptide (**3a**) or

Table 1. List of *N*-acetylmuramyl amino acids and peptides synthesized (**1d**—**4d** and **6d**—**14d**) and their intermediates

1 R^1-L-Ala-OR^2

2 R^1-L-Ala-D-Glu(OR^2)-NH_2

3 R^1-L-Ala-D-Glu(L-Lys(R^3)-OR^2)-NH_2

4 R^1-L-Ala-D-Glu(L-Lys(R^3)-D-Ala-OR^2)-NH_2

6 R^1-D-Ala-OR^2

7 R^1-D-Glu(OR^2)-NH_2

8 R^1-L-Ala-L-Glu(OR^2)-NH_2

9 R^1-L-Ala-D-Glu(NH_2)-OR^2

10 R^1-L-Ala-L-Glu(NH_2)-OR^2

11 R^1-L-Ala-D-Glu(OR^2)-OR^2

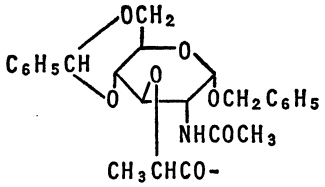
12 R^1-L-Ala-L-Glu(OR^2)-OR^2

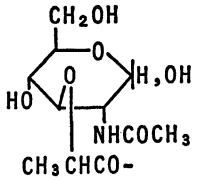
13 R^1-L-Ala-D-Asp(OR^2)-NH_2

14 R^1-L-Ala-D-Ala-NH_2

a : R^1=Boc, R^2=Bzl, R^3=Z

b : R^1=H, R^2=Bzl, R^3=Z

c : R^1=  =1-α-*O*-benzyl-4,6-*O*-benzylidene-*N*-acetylmuramyl, R^2=Bzl, R^3=Z

d : R^1=  =*N*-acetylmuramyl, R^2=R^3=H

e : R^1=R^2=R^3=H

Boc=*t*-butoxycarbonyl, Bzl=benzyl, Z=benzyloxycarbonyl

Table 2. Physical properties of protected dipeptides (**8a**—**14a**)

Compd	Yield (%)	Mp (°C)	$[\alpha]_D$[a]	Molecular formula	Anal. Found (Calcd) C%	H%	N%
8a	77	133.5—134.5	−21.8°[b]	$C_{20}H_{29}O_6N_3$	59.12 (58.95	7.28 7.17	10.34 10.31)
9a	71	143.5—145.5	−7.5°	$C_{20}H_{29}O_6N_3$	58.90 (58.95	7.14 7.17	10.33 10.31)
10a	74	136 —136.5	−1.9°	$C_{20}H_{29}O_6N_3$	59.06 (58.95	7.15 7.17	10.47 10.31)
11a	83	106.5—107.0	−8.2°	$C_{27}H_{34}O_7N_2$	64.82 (65.04	6.91 6.87	5.56 5.62)
12a	78	52.5— 53.5	−14.2°[c]	$C_{27}H_{34}O_7N_2$	65.01 (65.04	6.87 6.87	5.65 5.62)
13a	76	125.5—126.5	+12.2°[d]	$C_{19}H_{27}O_6N_3$	57.81 (58.00	6.89 6.92	10.69 10.68)
14a	70	128.0—128.5	+ 2.6°[e]	$C_{11}H_{21}O_4N_3$	50.93 (50.95	8.19 8.16	16.05 16.21)

a) *c* 2.0 in ethyl acetate at 20—22 °C unless otherwise noted. b) at 28 °C. c) *c* 2.2 at 23 °C. d) *c* 0.5 at 15 °C. e) *c* 1.0 at 17 °C.

tetrapeptide (**4a**). The Boc group in **2a** was removed with hydrogen chloride in acetic acid, while trifluoroacetic acid was employed for deprotection of those in **3a** and **4a**. The di-, tri- and tetrapeptides (**2b**, **3b**, and **4b**) with free amino terminals were thus obtained and then subjected to condensation with the muramic acid moiety (**5**).

Synthesis of muramyl peptides was first reported by Lanzilotti *et al.*[12] who prepared *N*-acetylmuramyl-L-alanyl-D-α- and γ-glutamyl-L-lysyl-D-alanyl-D-alanine by the same strategy mentioned above. They used Woodward's reagent K for coupling of **5** with peptides

TABLE 3. PHYSICAL PROPERTIES OF PROTECTED *N*-ACETYLMURAMYL AMINO ACIDS AND PEPTIDES (**6c—14c**)

Compd.	Method of coupling[a]	Yield (%)	Mp (°C)	$[\alpha]_D^{20}$ in DMF	Molecular formula	Anal. Found (Calcd) C%	H%	N%
6c	A	76	211—213	+105° (*c* 2.3)[c]	$C_{35}H_{40}O_9N_2$	66.55 (66.44	6.36 6.37	4.43 4.43)
7c	B	80	243—245[b]	+80.2° (*c* 1.2)	$C_{37}H_{43}O_{10}N_3$	64.14 (64.43	6.26 6.28	6.18 6.09)
8c	A	83	243—245[b]	+79.0° (*c* 1.9)	$C_{40}H_{48}O_{11}N_4 \cdot 1/2H_2O$	62.58 (62.40	6.26 6.42	7.28 7.28)
9c	A	87	246—248[b]	+88.2° (*c* 1.0)	$C_{40}H_{48}O_{11}N_4 \cdot 1/4H_2O$	62.70 (62.77	6.30 6.39	7.46 7.32)
10c	A	98	240[b]	+7.8° (*c* 1.0)	$C_{40}H_{48}O_{11}N_4 \cdot 1/2H_2O$	62.41 (62.40	6.37 6.42	7.42 7.28)
11c	A	70	211	+79.1° (*c* 1.0)	$C_{47}H_{53}O_{12}N_3 \cdot 1/2H_2O$	65.58 (65.57	6.28 6.32	4.91 4.88)
12c	A	75	208	+67.4° (*c* 0.9)	$C_{47}H_{53}O_{12}N_3$	66.00 (66.26	6.32 6.27	4.85 4.93)
13c	A	69	222—223[b]	+98.9° (*c* 1.0)[c]	$C_{39}H_{46}O_{11}N_4 \cdot 1/2H_2O$	62.00 (61.97	6.20 6.27	7.45 7.41)
14c	B	83	284.5[b]	+108° (*c* 0.55)[d]	$C_{31}H_{40}O_9N_4$	60.68 (60.77	6.52 6.58	8.84 9.15)

a) A : dicyclohexylcarbodiimide—*N*-hydroxysuccinimide, B : ethyl chloroformate. b) decomposition. c) at 25 °C. d) at 17 °C.

TABLE 4. *N*-ACETYLMURAMYL AMINO ACIDS AND PEPTIDES OF UNNATURAL STRUCTURES (**6a—14d**)

Compd[a]	Yield (%)	$[\alpha]_D$[c] after 3 min	after 24 hr	Molecular formula	Anal. Found (Calcd) C%	H%	N%
6d	82	+63.5°	+56.7°	$C_{14}H_{24}O_9N_2 \cdot 1/4H_2O$	45.75 (45.58	6.61 6.70	7.75 7.60)
7d	92	+52.7°	+47.4°	$C_{16}H_{27}O_{10}N_3$	45.44 (45.60	6.53 6.46	9.84 9.97)
8d	94	+16.5°	+12.2°	$C_{19}H_{32}O_{11}N_4 \cdot 1/2H_2O$	45.48 (45.50	6.58 6.63	11.35 11.17)
9d	85	+36.9°	+33.1°	$C_{19}H_{32}O_{11}N_4 \cdot 1/4H_2O$	45.96 (45.92	6.63 6.59	11.11 11.27)
10d	86	+42.4°	+38.5°	$C_{19}H_{32}O_{11}N_4$	46.05 (46.33	6.62 6.55	11.33 11.38)
11d	93	+35.5°	+32.4°	$C_{19}H_{31}O_{12}N_3 \cdot 1/2H_2O$	45.70 (45.41	6.35 6.42	8.26 8.36)
12d	95	+15.5°	+11.7°	$C_{19}H_{31}O_{12}N_3 \cdot 1/2H_2O$	45.58 (45.41	6.37 6.42	8.37 8.36)
13d[b]	81	+43.7°	+34.8°	$C_{18}H_{30}O_{11}N_4 \cdot 1/3H_2O$	44.73 (44.62	6.34 6.38	11.28 11.57)
14d	90	+50.0°	+43.3°	$C_{17}H_{30}O_9N_4 \cdot 1/2H_2O$	46.30 (46.04	6.94 7.05	12.76 12.64)

a) All these compounds except **13d** are colorless hygroscopic powder and do not show definite melting points. b) This compound was recrystallized from water—methanol—ether; mp 198 °C (decomp.). c) *c* 0.5 in H_2O at 15—17 °C.

without mention of the yield of the reaction. Thereafter, Chaturvedi *et al.*[13] reported the synthesis of *N*-acetylmuramyl-L-alanine, *N*-acetylmuramyl-L-alanyl-D-glutamic acid and several their analogs again by the same procedures and reagents. In our study, more convenient and simple methods generally applicable to the synthesis of muramyl peptides were investigated for this coupling.[14] Coupling of **5** with peptides by the active ester method was first examined using benzyl L-alaninate (**1b**). When the 1-succinimidyl ester of **5** was prepared using dicyclohexylcarbodiimide and allowed to react with **1b**, the fully protected *N*-acetylmuramyl-L-alanine (**1c**) was obtained in a fairly good yield. Its structure was confirmed by elemental analysis and NMR spectrum, in which two doublet signals of methyl groups attributed to the alanine and the muramic acid residues were clearly observed. The protected *N*-acetylmuramyl peptides **2c**, **3c** and **4c** were also obtained using the similar procedure by coupling of **5** with **2b**, **3b** and **4b**. In the latter two cases, 1-succinimidyl ester of **5** was isolated and recrystallized before the coupling.

Furthermore, it was found that this coupling proceeds also by the mixed anhydride method in a comparable yield.

The hydrogenolytic removal of the protecting groups in **1c** was carried out with palladium black in acetic acid. Although addition of hydrochloric acid increased the reaction rate considerably, it caused a formation of several by-products and removal of them required laborious process. Thus, **2c** was hydrogenolyzed in acetic acid without hydrochloric acid to afford pure **2d**. However, complete deprotections of **3c** and **4c** were only accomplished by addition of 0.5 equivalent of hydrochloric acid forming **3d** and **4d** in pure states, and use of 1 equivalent or more of the acid resulted in a formation of much amount of a by-product in each case. All the free *N*-acetylmuramyl peptides (**1d**—**4d**) precipitated as hygroscopic powder on addition of anhydrous ether to their ethanol or methanol solution. The powders thus obtained were found to contain considerable amount of the solvent alcohol from the results of elemental analyses and NMR spectra. The alcohol-free compounds adequate to biological tests and elemental analyses were prepared by lyophilization.

The *N*-acetylmuramyl amino acids and peptides of unnatural structures (**6d**—**14d**) were also synthesized by the similar procedures. The peptide portions were prepared by dicyclohexylcarbodiimide–*N*-hydroxysuccinimide method using Boc and benzyl groups for protections. After removal of Boc group, the products (**6b**—**14b**) were coupled with **5** by means of either the active ester or the mixed anhydride method, and the final hydrogenolysis afforded each *N*-acetylmuramyl peptide (**6d**—**14d**).

The free di-, tri- and tetrapeptides without sugar part, *i.e.*, L-alanyl-D-isoglutamine (**2e**), L-alanyl-D-isoglutaminyl-L-lysine (**3e**) and L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (**4e**) were also prepared in order to know the significance of *N*-acetylmuramyl moiety for the adjuvant activity. The dipeptide (**2e**) was obtained by hydrogenolysis of **2a** followed by removal of Boc group with trifluoroacetic acid. Dihydrochloride and bistrifluoroacetate of **3e** and bistrifluoroacetate of **4e**[11] were also prepared similarly from **3a** and **4a**, respectively.

Experimental

All melting points are uncorrected. Silica gel G according to Stahl, Merck, was used for thin-layer chromatography (tlc). Paper electrophoresis (PEP) was carried out using Toyo filter paper No. 51 in a pyridine–acetic acid–water (30 : 4 : 966 v/v/v) buffer solution.

1-α-O-Benzyl-4,6-O-benzylidene-N-acetylmuramyl-L-alanine Benzyl Ester (1c). Dicyclohexylcarbodiimide (0.82 g, 4.0 mmol) was added to an ice-cooled solution of benzyl 2-acetamido-4,6-*O*-benzylidene-3-*O*-(D-1'-carboxyethyl)-2-deoxy-α-D-glucopyranoside (**5**)[10] (1.88 g, 4.0 mmol) and *N*-hydroxysuccinimide (0.57 g, 5.0 mmol) in tetrahydrofuran (40 ml). The mixture was stirred in an ice bath for 3 hr and then at room temperature for 1 hr. Dicyclohexylurea formed was filtered off and washed with tetrahydrofuran. The combined filtrate and washings were again cooled in an ice bath and there were added benzyl L-alaninate *p*-toluenesulfonate (1.54 g, 4.4 mmol) and triethylamine (0.62 ml, 4.4 mmol). The mixture was stirred overnight at room temperature. After evaporation of the solvent, the residue was dissolved in chloroform, worked up as usual and the product was recrystallized from ethanol; yield, 1.89 g (75%); mp 211.5—216.5 °C. A sample for elemental analysis was recrystallized again from ethanol; mp 215.5—217 °C; $[\alpha]_D^{20}$ +85.6 ° (*c* 2.1, *N,N*-dimethylformamide). NMR (in $CDCl_3$): δ1.37 and 1.44 (each 3H, d, J=7 Hz), 1.90 (3H, s; CH_3CO) and 7.2—7.5 (15H; aromatic).

Found: C, 66.47; H, 6.42; N, 4.65%. Calcd for $C_{35}H_{40}O_9N_2$: C, 66.44; H, 6.37; N, 4.43%.

N-Acetylmuramyl-L-alanine (1d). A solution of **1c** (1.26 g, 2.0 mmol) in acetic acid (100 ml) containing 6M hydrochloric acid (1 ml) was hydrogenolyzed in the presence of palladium black at room temperature for 31 hr. The solvent was removed *in vacuo*, the syrupy residue was dissolved in water and subjected to column chromatography on a mixture of Darco G-60 active charcoal (30 g) and Hyflo Super-Cel (90 g). Elution with water–ethanol (9 : 1) afforded a fairly pure product, which was passed through a column of Dowex 50×8 (H^+ form) to remove the ninhydrin-positive impurity. The eluate was evaporated *in vacuo* and the residue was dissolved in absolute ethanol. Hygroscopic solid was formed on addition of absolute ether; yield, 0.490 g (67%). This product was dissolved in water and lyophilized to give colorless hygroscopic solid, which was subjected to biological test and elemental analysis. $[\alpha]_D^{17}$ +35.6 ° (after 3 min), +30.9 ° (after 19 hr) (*c* 0.51, H_2O).

Found: C, 46.08; H, 6.76; N, 7.54%. Calcd for $C_{14}H_{24}O_9N_2$: C, 46.15; H, 6.64; N, 7.69%.

t-Butoxycarbonyl-L-alanyl-D-isoglutamine Benzyl Ester (2a). *N*-Hydroxysuccinimide (2.25 g, 19.6 mmol) and dicyclohexylcarbodiimide (4.04 g, 19.6 mmol) were added to an ice-cooled solution of Boc-L-alanine (3.71 g, 19.6 mmol) in tetrahydrofuran (70 ml). After stirring for 10 min, there was added a cold mixture of benzyl D-isoglutaminate hydrochloride (5.33 g, 19.6 mmol) and triethylamine (2.70 ml, 19.6 mmol) in tetrahydrofuran (100 ml), and the mixture was stirred in an ice bath. Stirring was continued overnight, allowing the temperature of the mixture to reach to room temperature. Triethylamine hydrochloride and dicyclohexylurea formed were filtered off and the filtrate was worked up as usual. Recrystallization was effected from ethyl acetate–hexane; yield, 7.28 g (91%); mp 137.5—138.5 °C (lit,[11] 133—134 °C); $[\alpha]_D^{28}$ −8.1 ° (*c* 1.0, methanol).

Found: C, 58.64; H, 7.15; N, 10.41%. Calcd for $C_{20}H_{29}O_6N_3$: C, 58.95; H, 7.17; N, 10.31%.

L-Alanyl-D-isoglutamine Benzyl Ester (2b) Hydrochloride. Dry hydrogen chloride was passed through a solution of **2a** (3.70 g, 9.1 mmol) in acetic acid (50 ml) for 1 hr. After evaporation of the solvent *in vacuo*, the syrupy residue crystallized on trituration with ether. Recrystallization was effected from ethanol–ether; yield, 2.76 g (89%); mp 149.5—151 °C; $[\alpha]_D^{23}$ +10.8 ° (*c* 2.1, ethanol).

Found: C, 51.68; H, 6.41; N, 11.91; Cl, 9.97%. Calcd for $C_{15}H_{21}O_4N_3Cl \cdot 1/4H_2O$: C, 51.87; H, 6.24; N, 12.10; Cl, 10.21%.

1-α-O-Benzyl-4,6-O-benzylidene-N-acetylmuramyl-L-alanyl-D-isoglutamine Benzyl Ester (2c). i) *Dicyclohexylcarbodiimide–N-hydroxysuccinimide Method*: The procedure is similar to that described above for **1c**. Compound **5** (2.83 g, 6.0 mmol) was converted into its 1-succinimidyl ester with dicyclohexylcarbodiimide (1.24 g, 6.0 mmol) and *N*-hydroxysuccinimide (0.69 g, 6.0 mmol) in tetrahydrofuran (80 ml) and then treated with **2b** hydrochloride (2.06 g, 6.0 mmol) and triethylamine (0.85 ml, 6.0 mmol). The insoluble material

was collected by filtration, and dissolved in *N,N*-dimethylformamide. After filtration of insoluble triethylamine hydrochloride, the product was precipitated on addition of ether; yield, 3.43 g (75%). Recrystallization was effected from *N,N*-dimethylformamide-95% ethanol; mp 226.5—227.5 °C (decomp.); $[\alpha]_D^{25}$ +88.5 ° (*c* 2.1, *N,N*-dimethylformamide).

Found: C, 62.36; H, 6.39; N, 7.23%. Calcd for $C_{40}H_{48}O_{11}N_4 \cdot 1/2H_2O$: C, 62.40; H, 6.42; N, 7.28%.

ii) *Mixed Anhydride Method*: Compound **2a** (408 mg, 1.0 mmol) was dissolved in trifluoroacetic acid (2 ml). After standing at room temperature for 15 min, excess trifluoroacetic acid was removed *in vacuo*, and the remaining **2b** trifluoroacetate was dried over potassium hydroxide *in vacuo*. Ethyl chloroformate (0.10 ml, 1.0 mmol) and *N*-methylmorpholine (0.11 ml, 1.0 mmol) were added to a solution of **5** (472 mg, 1.0 mmol) in tetrahydrofuran (10 ml) at −19 °C. The mixture was stirred at this temperature for 15 min, then a cold solution of **2b** trifluoroacetate above obtained and *N*-methylmorpholine (0.11 ml, 1.0 mmol) in tetrahydrofuran (10 ml) was added. The mixture was stirred at room temperature for 22 hr. Then, water (100 ml) was added and the product was collected by filtration; yield, 600 mg (79%). It was reprecipitated from *N,N*-dimethylformamide-ether; mp 224—225 °C (decomp.); $[\alpha]_D^{19}$ + 83.4 ° (*c* 2.5, *N,N*-dimethylformamide). The IR spectrum of this compound was identical with that of the product in (i).

N-*Acetylmuramyl*-L-*alanyl*-D-*isogultamine* (***2d***). A solution of **2c** (1.52 g, 2.0 mmol) in acetic acid (50 ml) was hydrogenolyzed in the presence of palladium black at room temperature for 2 days. After removal of the catalyst by filtration, the filtrate was diluted with water (300 ml) and then evaporated *in vacuo* at 35 °C. After the residue was dissolved in methanol and the solvent was evaporated *in vacuo*, the final residue was dissolved in a small amount of absolute ethanol. Addition of absolute ether afforded colorless hygroscopic powder; yield, 918 mg (94%). This substance was further subjected to lyophilization. $[\alpha]_D^{15}$ +36.9° (after 3 min), +33.1° (after 25 hr) (*c* 0.51, H_2O).

Found: C, 45.73; H, 6.81; N, 11.36%. Calcd for $C_{19}H_{32}O_{11}N_4 \cdot 1/4H_2O$: C, 45.92; H, 6.59; N, 11.27%.

t-*Butoxycarbonyl*-L-*alanyl*-D-*isoglutamine*. This compound was prepared by hydrogenolysis from **2a** (7.34 g, 18.0 mmol) according to Bricas *et al.*[11]; yield, 6.87 g (94%); mp 99.5—101 °C (lit,[11] 94—98 °C); $[\alpha]_D^{28}$ −8.2° (*c* 1.0, methanol).

Found: C, 49.97; H, 7.70; N, 10.11%. Calcd for $C_{13}H_{23}O_6N_3 \cdot CH_3CO_2C_2H_5$: C, 50.36; H, 7.71; N, 10.37%.

N^{α}-(t-*Butoxycarbonyl*-L-*alanyl*-D-*isoglutaminyl*)-N^{ε}-*benzyloxycarbonyl*-L-*lysine Benzyl Ester* (***3a***). Triethylamine (0.82 ml, 5.8 mmol) and ethyl chloroformate (0.56 ml, 5.8 mmol) were added to a solution of Boc-L-alanyl-D-isoglutamine (2.35 g, 5.8 mmol) in *N,N*-dimethylformamide (15 ml) at −23 °C. After the mixture was stirred at this temperature for 15 min, a cooled solution of benzyl N^{ε}-Z-L-lysinate *p*-toluenesulfonate (3.15 g, 5.8 mmol) and triethylamine (0.82 ml, 5.8 mmol) in *N,N*-dimethylformamide (15 ml) was added and the mixture was stirred at room temperature for 15 hr. The precipitate of triethylamine hydrochloride was filtered off, the solvent was evaporated *in vacuo*. The residue was then dissolved in ethyl acetate and worked up as usual. The product was recrystallized from 99% ethanol–hexane; yield, 3.13 g (81%); mp 130—137 °C; $[\alpha]_D^{25}$ −13.8° (*c* 1.8, *N,N*-dimethylformamide).

Found: C, 60.53; H, 7.04; N, 10.06%. Calcd for $C_{34}H_{47}O_9N_5 \cdot 1/2C_2H_5OH$: C, 60.67; H, 7.27; N, 10.11%.

1-α-O-*Benzyl*-*4,6*-O-*benzylidene*-N-*acetylmuramic Acid 1*-*Succinimidyl Ester*. To an ice-cooled solution of **5** (4.72 g, 10 mmol) in tetrahydrofuran (60 ml) were added *N*-hydroxysuccinimide (1.38 g, 12 mmol) and dicyclohexylcarbodiimide (2.06 g, 10 mmol). After stirring in an ice bath for 3 hr, the mixture was worked up as usual and the product was recrystallized from ethyl acetate–hexane; yield, 4.52 g (79%); mp 178—179 °C.

N^{α}-(*1*-α-O-*Benzyl*-*4,6*-O-*benzylidene*-N-*acetylmuramyl*-L-*alanyl*-D-*isoglutaminyl*)-N^{ε}-*benzyloxycarbonyl*-L-*lysine Benzyl Ester* (***3c***). Trifluoroacetic acid (4 ml) was added to **3a** (1.34 g, 2.0 mmol) and the mixture was stirred at room temperature. After 10 min, absolute ether was added to the resulting solution and the precipitate of free amino tripeptide derivative (**3b**) trifluoroacetate was collected by filtration (1.24 g). 1-Succinimidyl ester of **5** (1.14 g, 2.0 mmol) was added to an ice-cooled solution of **3b** trifluoroacetate thus obtained and triethylamine (0.28 ml, 2.0 mmol) in a mixture of tetrahydrofuran (20 ml) and *N,N*-dimethylformamide (20 ml). The mixture was stirred at room temperature for 15 hr. The gelatinous solid was collected by filtration, washed with ethyl acetate and dried (1.00 g). By addition of ether to the mother liquor followed by recrystallization from *N,N*-dimethylformamide–water, further amount of the product was recovered (0.46 g); total yield, 1.46 g (79%); 221—224 °C (decomp.); $[\alpha]_D^{25}$ +53.9 ° (*c* 1.9, *N,N*-dimethylformamide).

Found: C, 62.00; H, 6.51; N, 8.36%. Calcd for $C_{54}H_{66}O_{14}N_6 \cdot H_2O$: C, 62.29; H, 6.58; N, 8.07%.

N-*Acetylmuramyl*-L-*alanyl*-D-*isogultaminyl*-L-*lysine* (***3d***). A solution of **3c** (256 mg, 0.25 mmol) in acetic acid (15 ml) was hydrogenolyzed in the presence of 1 M hydrochloric acid (0.12 ml, 0.12 mmol) and palladium black. After the catalyst was removed, the mixture was diluted with water (100 ml) and evaporated *in vacuo* at 35 °C. After this procedure had been repeated, the aqueous solution was passed through a column of Amberlite IR-45 (OH^- form) to remove hydrogen chloride. The eluate was evaporated *in vacuo* and the residue was dissolved in absolute methanol. Colorless hygroscopic powder was obtained on addition of absolute ether; yield, 134 mg (86%). It was reprecipitated from absolute methanol–absolute ethanol and then lyophilized. $[\alpha]_D^{28}$ +18.3 ° (after 3 min), +14.7° (after 19 hr) (*c* 1.0, H_2O).

Found: C, 47.23; H, 7.12; N, 13.27%. Calcd for $C_{25}H_{44}O_{12}N_6 \cdot H_2O$: C, 47.01; H, 7.26; N, 13.16%.

N^{α}-t-*Butoxycarbonyl*-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-D-*alanine Benzyl Ester*. To an ice-cooled solution of N^{α}-Boc-N^{ε}-Z-L-lysine (2.89 g, 7.6 mmol) in tetrahydrofuran (10 ml), there were added dicyclohexylcarbodiimide (1.56 g, 7.6 mmol) and then a solution of triethylamine (1.07 ml, 7.6 mmol) and benzyl D-alaninate *p*-toluenesulfonate (2.66 g, 7.6 mmol) in tetrahydrofuran (10 ml). The mixture was allowed to stand at room temperature for 20 hr. The product was recrystallized from ethyl acetate–hexane; yield, 2.91 g (71%); mp 103.5—104.5 °C (lit,[11] 96—100 °C); $[\alpha]_D^{28}$ +6.4 ° (*c* 1.1, methanol).

Found: C, 64.33; H, 7.26; N, 7.68%. Calcd for $C_{29}H_{39}O_7N_3$: C, 64.30; H, 7.26; N, 7.76%.

N^{ε}-*Benzyloxycarbonyl*-L-*lysyl*-D-*alanine Benzyl Ester* p-*Toluenesulfonate*. This compound was prepared from N^{α}-Boc-N^{ε}-Z-L-lysyl-D-alanine benzyl ester according to Bricas[11]; yield, 13.0 g (98%); mp 122—123 °C (lit,[11] 122—124 °C).

N^{α}-(t-*Butoxycarbonyl*-L-*alanyl*-D-*isoglutaminyl*)-N^{ε}-*benzyloxycarbonyl*-L-*lysyl*-D-*alanine Benzyl Ester* (***4a***). Ethyl chloroformate (0.77 ml, 8.0 mmol) and triethylamine (1.13 ml, 8.0 mmol) were added to a solution of Boc-L-alanyl-D-isoglutamine (3.24 g, 8.0 mmol) in *N,N*-dimethylformamide (20 ml) at −18 °C. After stirring at −18 °C for 15 min,

a cold solution of N^{ε}-Z-L-lysyl-D-alanine benzyl ester *p*-toluenesulfonate (4.91 g, 8.0 mmol) and triethylamine (1.13 ml, 8.0 mmol) in *N,N*-dimethylformamide (20 ml) was added and the mixture was stirred at room temperature for 15 hr. Triethylamine hydrochloride was filtered off and the solvent was evaporated *in vacuo*. The residue was dissolved in ethyl acetate saturated with water, worked up as usual, and the product was recrystallized from *N,N*-dimethylformamide–ether; yield, 4.65 g (78%), mp 191—194.5 °C (lit,[11] 196—198 °C); $[\alpha]_D^{26}$ −10.8° (*c* 2.2, acetic acid).

Found: C, 60.11; H, 7.11; N, 11.08%. Calcd for $C_{37}H_{52}O_{10}N_6$: C, 59.98; H, 7.08; N, 11.35%.

*N^{α}-(1-α-O-Benzyl-4,6-O-benzylidene-N-acetylmuramyl-L-alanyl-D-isogultaminyl)-N^{ε}-benzyloxycarbonyl-L-lysyl-D-alanine Benzyl Ester (**4c**).* This compound was prepared in a similar way to that described for **3c**. Trifluoroacetic acid salt of **4b**, obtained from **4a** (1.41 g, 1.9 mmol), was neutralized with triethylamine (0.26 ml, 1.9 mmol) and treated with 1-succinimidyl ester of **5** (1.02 g, 1.8 mmol) in a mixture of tetrahydrofuran (40 ml) and *N,N*-dimethylformamide (20 ml). The product was recrystallized from *N,N*-dimethylformamide–water, then from *N,N*-dimethylformamide–ether; yield, 1.24 g (63%); mp 221 °C (decomp.); $[\alpha]_D^{23}$ +50.4 ° (*c* 2.1, *N,N*-dimethylformamide).

Found: C, 61.64; H, 6.56; N, 8.74%. Calcd for $C_{57}H_{71}O_{15}N_7 \cdot H_2O$: C, 61.55; H, 6.62; N, 8.82%.

*N-Acetylmuramyl-L-alanyl-D-isoglutaminyl-L-lysyl-D-alanine (**4d**).* This compound was prepared from **4c** (547 mg, 0.50 mmol) by the same procedure described for **3d**. The final product was precipitated from absolute methanol–absolute ether; yield, 295 mg (85%). A sample after lyophilization showed the following data: $[\alpha]_D^{15}$ +14.9° (after 3 min), +15.4° (after 25 hr) (*c* 0.51, H_2O).

Found: C, 47.42; H, 7.13; N, 13.75%. Calcd for $C_{28}H_{49}O_{13}N_7 \cdot H_2O$: C, 47.38; H, 7.24; N, 13.82%.

*t-Butoxycarbonyl-L-alanyl-L-isoglutamine Benzyl Ester (**8a**) and the Analogous Protected Dipeptides (**9a—14a**).* These compounds as shown in Table 1 were all prepared in similar manners to that described for **2a**. The yields and physical constants are summarized in Table 2.

*Protected N-Acetylmuramyl Amino Acids (**6c** and **7c**) and N-Acetylmuramyl Dipeptides (**8c—14c**).* These compounds were prepared by coupling of **5** with the corresponding amino acid benzyl esters (**6b** or **7b**) or dipeptides having free amino groups (**8b—14b**) using either dicyclohexylcarbodiimide—*N*-hydroxysuccinimide or ethyl chloroformate as described for **2c**. While **8b** hydrochloride was prepared from **8a**[15] just as described for **2b**, compounds **9b—14b** were obtained as their trifluoroacetic acid salt by treating **9a—14a** with trifluoroacetic acid at room temperature. The yields and the physical constants of the products (**6c—14c**) are summarized in Table 3.

*N-Acetylmuramyl Amino Acids (**6d** and **7d**) and N-Acetylmuramyl Dipeptides (**8d—14d**).* Compounds **6c—14c** were hydrogenolyzed in acetic acid in the presence of palladium black at room temperature. The product was isolated as described for **2d** and lyophilized. The yields, $[\alpha]_D$ and the results of elemental analyses are summarized in Table 4.

*L-Alanyl-D-isoglutamine (**2e**).* Boc-L-alanyl-D-isoglutamine (300 mg, 0.74 mmol) was dissolved in trifluoroacetic acid (1.5 ml), and the solution was set aside at room temperature for 30 min. On addition of absolute ether to the solution, trifluoroacetate of **2e** precipitated as hygroscopic powder. Removal of trifluoroacetic acid with a column of Amberlite IR-45 (OH^- form) followed by lyophilization afforded hygroscopic solid; yield, 132 mg (81%).

Found: C, 43.40; H, 7.01; N, 19.14%. Calcd for $C_8H_{15}O_4N_3 \cdot 1/4H_2O$: C, 43.33; H, 7.05; N, 18.95%. It was recrystallized from water–ethanol; mp 83 °C (decomp); $[\alpha]_D^{28}$ +14.4° (*c* 0.96, H_2O).

*L-Alanyl-D-isoglutaminyl-L-lysine (**3e**).* i) *Dihydrochloride*: Compound **3a** (0.50 g, 0.75 mmol) was dissolved in ethanol (50 ml) and hydrogenolyzed in the presence of palladium black. After removal of the solvent *in vacuo*, the residue was dissolved in methanol. Addition of ether afforded Boc-L-alanyl-D-isoglutaminyl-L-lysine (0.33 g, 99%). To a solution of this compound (190 mg) in acetic acid (6 ml) was added acetic acid (10 ml) previously saturated with dry hydrogen chloride. After standing at room temperature for 15 min, absolute ether was added to the mixture and the precipitate was collected and dried over phosphorus pentaoxide and potassium hydroxide *in vacuo*. Reprecipitation from absolute methanol–absolute ether afforded very hygroscopic powder; yield, 136 mg (76% from **3a**). This was further purified by treatment with charcoal and lyophilized for elemental analysis. $[\alpha]_D^{28}$ +2.06° (*c* 4.5, H_2O).

Found: C, 39.90; H, 7.08; N, 16.34; Cl, 16.95%. Calcd for $C_{14}H_{29}O_5N_5Cl_2$: C, 40.19; H, 6.99; N, 16.74; Cl, 16.95%.

ii) *Bistrifluoroacetate*: Compound **3a** (67 mg, 0.1 mmol) was hydrogenolyzed in acetic acid (5 ml) in the presence of palladium black. After evaporation of the solvent *in vacuo*, the residue was treated with trifluoroacetic acid (2 ml) at room temperature for 60 min. Addition of absolute ether to the mixture afforded hygroscopic powder, yield, 45 mg (76%). This compound showed a single ninhydrin-positive spot identical with the product obtained in (i) on tlc (1-butanol–acetic acid–water, 4 : 1 : 2) and PEP.

*L-Alanyl-D-isoglutaminyl-L-lysyl-D-alanine (**4e**) Bistrifluoroacetate.* This compound was prepared from **4a** (185 mg, 0.25 mmol) according to Bricas *et al.*[11] The procedure is similar to that described for **3e** (ii). Yield, 155 mg (96%); $[\alpha]_D^{19}$ + 2.24° (*c* 0.45, H_2O) (lit,[11] $[\alpha]_D^{25}$ +1.6±0.5° (*c* 0.45, H_2O).

Found: C, 39.35; H, 5.60; N, 12.85%. Calcd for $C_{21}H_{34}O_{10}N_6F_6$: C, 39.13; H, 5.32; N, 13.04%.

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14) After we presented our preliminary communication,[1] Arendt *et al.* also reported the synthesis of **1c** by coupling of **5** with benzyl L-alaninate using *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline (EEDQ); A. Arendt, A. Kolodziejczyk, and T. Sokolowska, *Roczniki Chem.*, **48**, 1707 (1974). They removed the benzyl ester group in **1c** by alkaline hydrolysis with the intention of use of the resultant 1-*O*-benzyl-4,6-*O*-benzylidene-*N*-acetylmuramyl-L-alanine for their further synthetic program of more complex mucopeptides. Indeed, they reported more recently the synthesis of *N*-acetylmuramyl pentapeptide containing diaminopimelic acid; *ibid.*, **48**, 1921 (1974). On the contrary, our approach differs from theirs in the strategy that the peptide chains were stepwise elongated from *C*-terminal except at γ-glutamyl linkage, thus minimizing the possibility of racemization of each amino acid residue in the molecule.

15) **8b** hydrochloride: yield, 87% from **8a**; mp 112.5—114 °C; $[\alpha]_D^{20}$ +7.30 (*c* 2.0, ethanol). Found: C, 52.85; H, 6.74; N, 11.76; Cl, 9.99%. Calcd for $C_{15}H_{22}O_4N_3Cl \cdot 1/2\text{-}C_2H_5OH$: C, 52.38; H, 6.87; N, 11.46; Cl, 9.66%.