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Syntheses of Several Muramyl Peptides in Relation to Chemical Structures of Less Immunoadjuvant Bacterial Cell Walls

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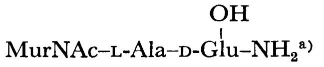
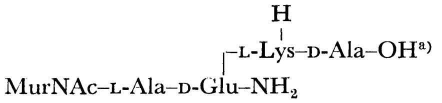
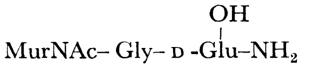
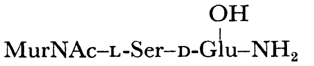
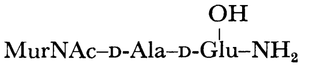
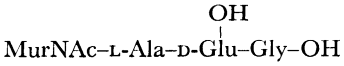
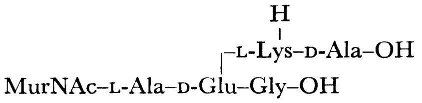
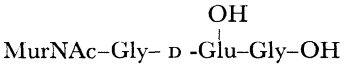
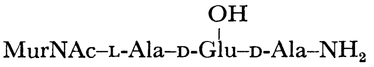
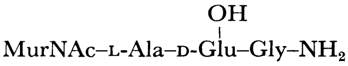
Various structural analogs of *N*-acetylmuramyl-L-alanyl-D-isoglutamine, which had been shown to be the minimum structure required for the immunoadjuvant activity of bacterial cell walls, were synthesized in order to investigate in more detail the relationship between the activity and chemical structures. Particularly, the compounds of the structures corresponding to the cell walls of special bacteria without immunoadjuvant activity were prepared in this study. Several novel procedures including the preparation of a branched glutamyl peptide were newly exploited to accomplish the syntheses of muramyl peptides with complicated structures. As a result of this synthetic study, new informations were obtained on the structural requirement for the immunoadjucant activity.

Since *N*-acetylmuramyl-L-alanyl-D-isoglutamine ($\text{MurNAc–L-Ala–D-Glu(OH)–NH}_2$)[1] (**1**) had been shown to be the minimum structure required for the immunoadjuvant activity of the bacterial cell walls,[2,3] investigations on the relationship between the structure and the adjuvant activity from the view point of synthetic approaches were much urged. In fact, we reconfirmed the more significance of the structure of $\text{MurNAc–L-Ala–D-Glu(OH)–NH}_2$ (**1**) by comparison with various synthetic analogs.[2]

Thereafter, we extended the study in the above line to the investigation on the structural relation of adjuvant inactive cell walls. According to the recent study of Kotani *et al.*,[4] there have been known quite a few bacteria whose cell walls do not show the immunoadjuvant activity, although usual bacteria possess the significant activities. It should be noted that most of inactive bacteria belong to plant pathogenic ones and are not familiar to mammals. Although $\text{MurNAc–L-Ala–D-Glu(OH)–NH}_2$ (**1**) mentioned above is the common structural unit in many bacterial peptidoglycan, a number of variations are often encountered especially in adjuvant inactive bacteria, for instance, replacement of L-Ala with Gly or L-Ser residue, or linkage of α-carboxyl group of D-Glu residue to the other amino acid.[5] In these respects, we synthesized now *N*-acetyl-

Table 1. Synthetic *N*-acetylmuramyl peptides

MurNAc peptides		Corresponding original bacterium[b)]
 $\text{MurNAc–L-Ala–D-}\overset{\text{OH}}{\overset{\vert}{\text{Glu}}}\text{–NH}_2{}^{\text{a)}}$	(**1**)	*Staphylococcus aureus*
 $\text{MurNAc–L-Ala–D-}\overset{\ulcorner\text{L-}\overset{\text{H}}{\overset{\vert}{\text{Lys}}}\text{–D-Ala–OH}^{\text{a)}}}{\text{Glu}}\text{–NH}_2$		*Staphylococcus aureus*
 $\text{MurNAc– Gly– D -}\overset{\text{OH}}{\overset{\vert}{\text{Glu}}}\text{–NH}_2$	(**2c**)	*Microbacterium lacticum*[c)]
 $\text{MurNAc–L-Ser–D-}\overset{\text{OH}}{\overset{\vert}{\text{Glu}}}\text{–NH}_2$	(**3c**)	*Eubacterium limosum*[c)]
 $\text{MurNAc–D-Ala–D-}\overset{\text{OH}}{\overset{\vert}{\text{Glu}}}\text{–NH}_2$	(**4c**)	
 $\text{MurNAc–L-Ala–D-}\overset{\text{OH}}{\overset{\vert}{\text{Glu}}}\text{–Gly–OH}$	(**5c**)	*Micrococcus lysodeikticus*
 $\text{MurNAc–L-Ala–D-}\overset{\ulcorner\text{L-}\overset{\text{H}}{\overset{\vert}{\text{Lys}}}\text{–D-Ala–OH}}{\text{Glu}}\text{–Gly–OH}$	(**6c**)	*Micrococcus lysodeikticus*
 $\text{MurNAc–Gly– D -}\overset{\text{OH}}{\overset{\vert}{\text{Glu}}}\text{–Gly–OH}$	(**7c**)	*Corynebacterium poinsettiae*
 $\text{MurNAc–L-Ala–D-}\overset{\text{OH}}{\overset{\vert}{\text{Glu}}}\text{–D-Ala–NH}_2$	(**8c**)	*Arthrobacter* sp. (NCIB 9423)
 $\text{MurNAc–L-Ala–D-}\overset{\text{OH}}{\overset{\vert}{\text{Glu}}}\text{–Gly–NH}_2$	(**9c**)	*Arthrobacter atrocyaneus*

a) These are prepared in our previous study. See Ref. 2b in the text. b) The names of the corresponding representative bacteria are given, that have the partial structure mentioned in each line. See Ref. 9. c) In the cell walls of these bacteria, the α-carboxyl group of glutamic acid does not exist actually as a simple amide but further links with a basic amino acid participating in a cross linkage.

muramyl peptides corresponding to the partial structures of inactive cell walls in order to investigate whether changes in rather small moieties are reflected in the inactivities of cell walls.

The *N*-acetylmuramyl peptides (**2c**—**9c**) prepared in this study are summarized in Table 1 together with the names of the representative bacteria in which the corresponding structures are involved. For the synthesis of these compounds, the same strategy was employed as described previously for the muramyl dipeptide (**1**).[2] Thus, peptide portions (**2a**—**9a**) were prepared in principle by conventional methods using Boc[1] and Bzl[1] groups for amino and carboxyl protections, respectively. After removal of the *N*-terminal Boc groups, the resulting peptide benzyl esters were coupled with protected muramic acid, *i.e.*, 1-α-*O*-benzyl-4,6-*O*-benzylidene-*N*-acetylmuramic acid (**10**), by means of the active ester method to give protected muramyl peptides (**2b**—**9b**), whose protecting groups were then removed simultaneously by catalytic hydrogenolysis. This procedure is illustrated for the preparation of **2c** in Fig. 1 for an example. In some syntheses, modifications were required as described below.

The first group of muramyl peptides (**2c**, **3c**, and **4c**) were synthesized in order to elucidate the role of the amino acid adjacent to the muramic acid moiety; the L-Ala residue in **1** was replaced with Gly or L-Ser as models of plant pathogenic and adjuvant-inactive cell walls and with D-Ala as non-natural peptidoglycan analog. Thus, the synthesis of MurNAc–L-Ser–D-Glu(OH)–NH_2 (**3c**) and MurNAc–D-Ala–D-Glu(OH)–NH_2 (**4c**) were performed in a similar manner to that illustrated for MurNAc–Gly–D-Glu(OH)–NH_2 (**2c**) (Fig. 1) except that the mixed anhydride method was used for the coupling of **10** with the peptide moieties.

In the another group of the analogs prepared so far, the α-carboxyl group of D-Glu is linked with an amino acid (**5c**, **6c**, and **7c**) or an amino acid amide (**8c** and **9c**) in place of ammonia in **1**. This type of variations has been mostly found also in adjuvant-inactive cell walls. For instance, the muramyl tri- and pentapeptides, *i.e.*, MurNAc–L-Ala–D-Glu(OH)–Gly–OH (**5c**) and MurNAc–L-Ala–D-Glu(L-Lys–D-Ala–OH)–Gly–OH (**6c**) are involved in the cell wall of *Micrococcus lysodeikticus* which shows exceptionally no adjuvant activity though this bacteria is familiar to human beings.

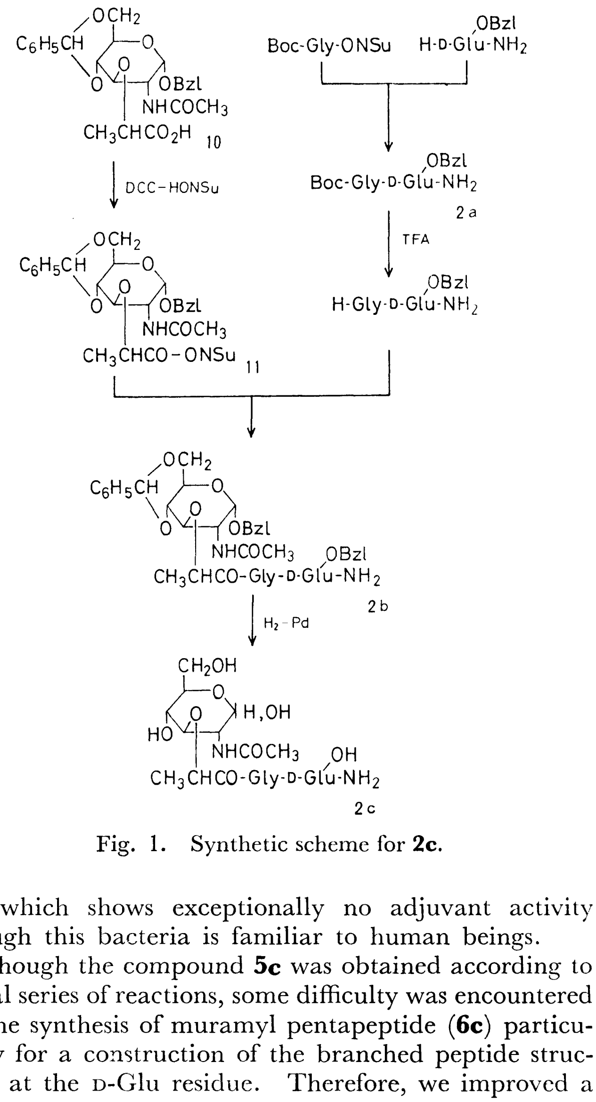


Fig. 1. Synthetic scheme for **2c**.

Though the compound **5c** was obtained according to usual series of reactions, some difficulty was encountered in the synthesis of muramyl pentapeptide (**6c**) particularly for a construction of the branched peptide structure at the D-Glu residue. Therefore, we improved a classical reaction of Boc-D-glutamic anhydride with H–Gly–OBzl to obtain the key intermediate Boc–D-Glu(OH)–Gly–OBzl (**13**) directly by a simple procedure. In this reaction, the isomeric γ-peptide, Boc–D-Glu(Gly–OBzl)–OH (**14**), formed simultaneously must be removed anyhow. LeQuesne and Young[6a] employed fractional extraction for this purpose, but their pro-

TABLE 2. PHYSICAL AND ANALYTICAL DATA OF SYNTHETIC *N*-ACETYLMURAMYL PEPTIDES

Compd[a]	Yield (%)	$[\alpha]_D$ [b]		(*c*)	Molecular formula	Found (Calcd)		
		after 5 min	after 24 h			C%	H%	N%
4c	97	+67°	+64°	(0.45)	$C_{19}H_{32}O_{11}N_4 \cdot 1/2H_2O$	45.58 (45.50	6.63 6.63	11.21 11.17)
5c	60	+42° [c]	+39° [d]	(0.48)	$C_{21}H_{34}O_{13}N_4 \cdot 3/4H_2O$	44.62 (44.72	6.30 6.34	10.51 9.93)
6c	90	+19°	+19°	(0.63)	$C_{30}H_{51}O_{15}N_7 \cdot 3/2H_2O$	46.54 (46.38	6.81 7.01	12.64 12.62)
7c	95	+45°	+42°	(0.54)	$C_{20}H_{31}O_{13}N_4 \cdot 3/4H_2O$	43.83 (43.75	5.95 5.97	10.27 10.21)
8c	98	+49°	+48°	(0.52)	$C_{22}H_{37}O_{12}N_4$	46.70 (46.88	6.60 6.62	12.36 12.43)
9c	94	+42°	+38°	(0.50)	$C_{21}H_{35}O_{12}N_5 \cdot H_2O$	44.31 (44.44	6.35 6.57	12.44 12.34)

a) All these compounds were obtained by precipitation from abs ethanol–abs ether followed by lyophilization. See Ref. 18 in the text. b) In H_2O at 25—28 °C. c) At 19 °C. d) At 15 °C.

cedure was too tedious to be applied in a preparative scale.[6b] We improved the procedure as follows. Boc-D-glutamic anhydride was added to a solution of H–Gly–OBzl in THF and the resulting mixture of α- and γ-peptides (**13** and **14**) was dissolved in ethyl acetate. On addition of 1 equivalent of DCHA[1] to this solution, the desired α-peptide (**13**) was obtained as pure DCHA salt in 60% yield, where the γ-peptide (**14**) remained in the mother liquor. The idea came from the method of a selective precipitation of α-esters of *N*-protected glutamic acid,[7] but no one has yet applied it in direct peptide formation. The structure of the α-peptide obtained was checked after deprotection by amino acid analyzer and TLC in comparison with the authentic free dipeptides, *i.e.*, H–D-Glu(OH)–Gly–OH (**15**) and H–D-Glu(Gly–OH)–OH (**16**), which were obtained *via* unequivocal ways (see experimental). Meanwhile, we recognized a fact that the amount of α-peptide (**13**) decreased slightly in the presence of DCHA as compared to that in its absence at the stage of anhydride ring opening. The ratio of α- and γ-peptides was measured for the reaction mixture of Boc–D-glutamic anhydride and H–Gly–OBzl after deprotection by amino acid analyzer. That in the presence of DCHA was actually 2.0: 1.0 and in its absence 2.6: 1.0. These ratios did not change in a range of the reaction temperature between −70 and 20 °C. Furthermore, the same technique in our method was also applicable to the reaction of Z-D-glutamic anhydride with H–Gly–OBzl as described in the experimental section. In this case, the pure γ-peptide, *i.e.*, Z–D-Glu(Gly–OBzl)–OH, could be also isolated from the mother liquor, besides the crystalline α-peptide DCHA salt.

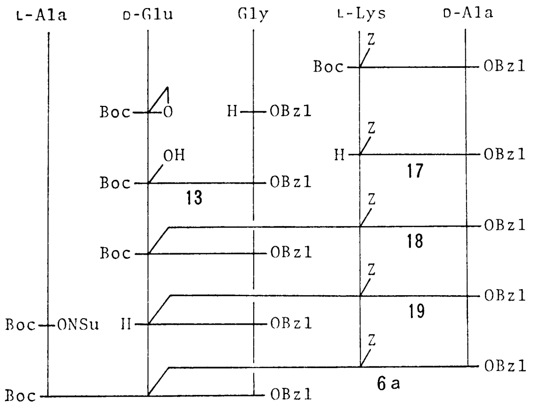
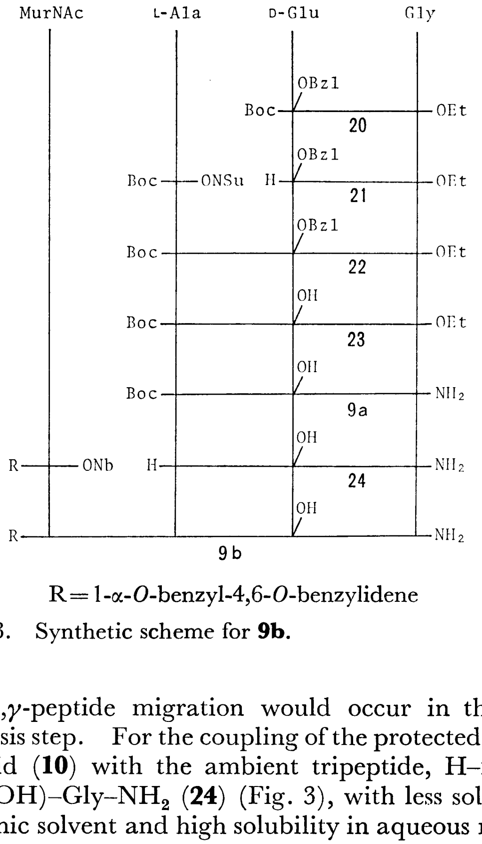


Fig. 2. Synthetic scheme for **6a**.

Concerning with the muramyl pentapeptide (**6c**), Boc–D-Glu(OH)–Gly–OBzl (**13**) was first condensed with H–L-Lys(Z)–D-Ala–OBzl (**17**) to give a branched tetrapeptide, *i.e.*, Boc–D-Glu(L-Lys(Z)–D-Ala–OBzl)–Gly–OBzl (**18**), whose Boc group was then selectively removed by treating with TFA (100 equivalents) at room temperature for 5 min.[8] The resulting H–D-Glu(L-Lys(Z)–D-Ala–OBzl)–Gly–OBzl (**19**) was successively coupled with Boc–L-Ala–OH. After removal of the Boc group, the product was condensed with the protected muramic acid 1-succinimidyl ester (**11**) to give the muramyl pentapeptide derivative (**6b**). Finally, hydrogenolysis of **6b** afforded MurNAc–L-Ala–D-Glu(L-Lys–D-Ala–OH)–Gly–OH (**6c**).

The muramyl tripeptide containing glycine, *i.e.*, MurNAc–Gly–D-Glu(OH)–Gly–OH (**7c**) was also prepared in a similar manner to that described above for **5c**.

Concerning with the syntheses of two muramyl tripeptides containing acid amide groups at their termini, *i.e.*, MurNAc–L-Ala–D-Glu(OH)–D-Ala–NH$_2$ (**8c**) and MurNAc–L-Ala–D-Glu(OH)–Gly–NH$_2$ (**9c**), the former (**8c**) could be prepared by conventional method starting from D-alanine amide, whereas a modified procedure should be used for the latter (**9c**) to avoid difficulties in experimental procedures due to high solubility of glycine amide derivatives in water. Thus, a tripeptide ester, *i.e.*, Boc–L-Ala–D-Glu(OBzl)–Gly–OEt (**22**), was first prepared by conventional method and its terminal ethyl ester was converted into amide function after selective removal of γ-benzyl ester group (Fig. 3). Otherwise, α,γ-peptide migration would occur in the ammonolysis step. For the coupling of the protected muramic acid (**10**) with the ambient tripeptide, H–L-Ala–D-Glu(OH)–Gly–NH$_2$ (**24**) (Fig. 3), with less solubility in organic solvent and high solubility in aqueous media, we adopted HONb[1] ester method, particularly since this active ester has been known to afford relatively good yields in condensation in aqueous media.[9] In fact, the desired 1-α-*O*-benzyl-4,6-*O*-benzylidene-MurNAc-L-Ala-D-Glu(OH)-Gly-NH$_2$ (**9b**) was obtained in a satisfactory yield by this method. The product (**9b**) was then hydrogenolyzed to give the free *N*-acetylmuramyl tripeptide (**9c**). This advantageous method for the coupling of the active ester of muramic acid with a free peptide moiety was further utilized in our another synthesis of MurNAc–L-$[\text{U-}^{14}C]$Ala–D-Glu(OH)–NH$_2$ successfully.[10]



R = 1-α-*O*-benzyl-4,6-*O*-benzylidene

Fig. 3. Synthetic scheme for **9b**.

The immunoadjuvant activities of the muramyl peptides (**2c**—**9c**) prepared above were measured by Kotani *et al.*[11,12] From these results, the relationship between the structure and the adjuvant activity had been already discussed.[11] For the reference, only the conclusion is now summarized as follows. i) The L-Ala residue in MurNAc–L-Ala–D-Glu(OH)–NH_2 (**1**) can be replaced with L-Ser without significant decrease in the activity. On the other hand, less activity is observed in the Gly analog and complete inactivity in the D-Ala analog. ii) Coupling of another amino acid residue at the α-carboxyl terminal of D-glutamic acid in **1** in place of the simple amide caused a remarkable decrease in the activity, while replacement with an amino acid amide kept the high activity. Therefore, the termination with an amide group in the molecule of muramyl peptide seems to be advisable in order to maintain the adjuvant activity in higher level.

Experimental

All melting points are uncorrected. Silica gel 60 (0.063—0.2 mm), Merck, was used for column chromatography and silica gel G, Merck, for thin layer chromatography. THF as solvent was purified by refluxing with $LiAlH_4$ followed by distillation and stored with Na-wire.

Boc-Gly–D-Glu(OBzl)–NH$_2$ (2a). Boc-Gly–ONSu (2.72 g, 10.0 mmol) was added to a mixture of H–D-Glu(OBzl)–NH_2·HCl (2.36 g, 8.7 mmol) and triethylamine (1.50 ml, 11.0 mmol) in THF (40 ml). After the usual work-up, the product was recrystallized from ethyl acetate–hexane; yield, 3.08 g (90%); mp 112—113 °C; $[\alpha]_D^{29}$ +4.95° (*c* 2.00, ethyl acetate).

Found: C, 57.76; H, 6.90; N, 10.64%. Calcd for $C_{19}H_{27}O_6N_3$: C, 58.00; H, 6.92; N, 10.68%.

1-α-O-Benzyl-4,6-O-benzylidene-MurNAc-Gly-D-Glu(OBzl)-NH$_2$ (2b). Compound **2a** (2.36 g, 6.0 mmol) was dissolved in TFA (6 ml) and allowed to stand at room temperature for 1 h. On addition of abs ether and hexane, an oily layer separated. The supernatant was removed by decantation, the remaining oil was dissolved in THF, and then the solvent was removed *in vacuo*. The residue solidified by drying over KOH *in vacuo*. It was triturated with abs ether and filtered to give H–Gly–D-Glu(OBzl)–NH_2 TFA salt; yield, 2.08 g (85%).

A solution of 1-α-*O*-benzyl-4,6-*O*-benzylidene-MurNAc-ONSu[2b] (**11**) (0.57 g, 1.0 mmol) in DMF (10 ml) was added to a solution of the TFA salt obtained above (0.41 g, 1.0 mmol) and triethylamine (0.14 ml, 1.0 mmol) in THF (15 ml) with stirring. After the mixture had been kept at room temperature overnight, THF was evaporated *in vacuo* and ethyl acetate was added to the remaining mixture. The resulting gelatinous solid was filtered and recrystallized from dioxane–water; yield, 0.57 g (75%); mp 234 °C (dec); $[\alpha]_D^{28}$ +82° (*c* 0.95, DMF).

Found: C, 61.50; H, 6.24; N, 7.40%. Calcd for $C_{39}H_{46}O_{11}N_4 \cdot H_2O$: C, 61.24; H, 6.33; N, 7.33%.

MurNAc-Gly–D-Glu(OH)–NH$_2$ (2c). A solution of **2b** (0.30 g, 0.39 mmol) in acetic acid (20 ml) was hydrogenolyzed in the presence of palladium black catalyst. After removal of the catalyst, the filtrate was diluted with water and then evaporated *in vacuo*. The residue was crystallized from methanol; yield, 0.19 g (quantitative); mp 234 °C (dec); $[\alpha]_D^{28}$ +35° (after 5 min), +33° (after 24 h) (*c* 0.46, H_2O).

Found: C, 44.08; H, 6.32; N, 11.55%. Calcd for $C_{18}H_{30}O_{11}N_4 \cdot 1/2H_2O$: C, 44.35; H, 6.41; N, 11.49%.

Boc-L-Ser–D-Glu(OBzl)–NH$_2$ (3a). A mixture of Boc–L-Ser–OH (1.54 g, 7.5 mmol), H–D-Glu(OBzl)–NH_2·HCl (2.04 g, 7.5 mmol), and *N*-methylmorpholine (0.82 ml, 7.5 mmol) in THF was treated with DCC (1.70 g, 8.2 mmol) and HOBt[1] (1.01 g, 7.5 mmol) under ice-cooling. After the usual work-up, the product was recrystallized form ethyl acetate–hexane; yield, 3.13 g (98%); mp 73—75 °C; $[\alpha]_D^{29}$ +0.70° (*c* 2.98, ethyl acetate).

Found: C, 56.61; H, 7.02; N, 9.90%. Calcd for $C_{20}H_{29}O_7N_3$: C, 56.72; H, 6.90; N, 9.92%.

1-α-O-Benzyl-4,6-O-benzylidene-MurNAc-L-Ser-D-Glu(OBzl)-NH$_2$ (3b). Compound **3a** (1.27 g, 3.0 mmol) was dissolved in TFA (3 ml) and set aside at room temperature for 35 min. Evaporation of TFA *in vacuo* afforded H–L-Ser–D-Glu(OBzl)–NH_2 TFA salt as a syrup, which was dried over KOH *in vacuo*. Ethyl chloroformate (0.29 ml, 3.0 mmol) was added to a solution of the protected *N*-acetylmuramic acid (**10**) (1.42 g, 3.0 mmol) and triethylamine (0.42 ml, 3.0 mmol) in THF (20 ml) at −19 °C. After being stirred at the same temperature for 15 min, a solution of the above TFA salt and triethylamine (0.42 ml, 3.0 mmol) in THF (15 ml) was added to the mixture, and stirring was continued overnight. Gelatinous solid obtained by addition of water was collected by filtration; yield, 1.95 g (82%). Recrystallization was effected from dioxane–water; mp 197 °C (dec); $[\alpha]_D^{28}$ +81.6° (*c* 1.12, DMF).

Found: C, 60.56; H, 6.19; N, 7.08%. Calcd for $C_{40}H_{48}O_{12}N_4 \cdot H_2O$: C, 60.44; H, 6.34; N, 7.05%.

MurNAc–L-Ser–D-Glu(OH)–NH$_2$ (3c). Compound **3b** (0.30 g, 0.38 mmol) was dissolved in acetic acid (35 ml) and hydrogenolyzed in the presence of palladium black catalyst. After addition of water, the solvent was evaporated *in vacuo* and the residue was precipitated from abs ethanol–abs ether to afford colorless hygroscopic solid. It was again dissolved in a small amount of water, and subjected to lyophilization;[13] yield, 0.11 g (55%); $[\alpha]_D^{28}$ +39° (after 5 min), $[\alpha]_D^{29}$ +35° (after 24 h) (*c* 0.49, H_2O).

Found: C, 44.29; H, 6.41; N, 10.91%. Calcd for $C_{19}H_{32}O_{12}N_4 \cdot 1/2H_2O$: C, 44.10; H, 6.43; H, 10.83%.

Boc–D-Ala–D-Glu(OBzl)–NH$_2$ (4a). This compound was prepared from Boc–D-Ala–OH (1.25 g, 6.6 mmol) and H–D-Glu(OBzl)–NH_2·HCl (1.79 g, 6.6 mmol) by means of the DCC–HONSu method in THF and recrystallized from ethyl acetate–hexane; yield, 2.33 g (87%); mp 130.5—131.5 °C; $[\alpha]_D^{29}$ +19.2° (*c* 1.88, ethyl acetate).

Found: C, 59.03; H, 7.20; N, 10.37%. Calcd for $C_{20}H_{29}O_6N_3$: C, 58.95; H, 7.17, N, 10.31%.

1-α-O-Benzyl-4,6-O-benzylidene-MurNAc–D-Ala-D-Glu(OBzl)-NH$_2$ (4b). Compound **4a** (1.22 g, 3.0 mmol) was treated with TFA (3 ml) and the resulting H–D-Ala–D-Glu(OBzl)–NH_2 was coupled with **10** (1.42 g, 3.0 mmol) by means of the mixed anhydride method as described for **3b**; yield, 1.69 g (73%). Recrystallization was effected from dioxane–water; mp 223 °C (dec); $[\alpha]_D^{28}$ +83.5° (*c* 1.09, DMF).

Found: C, 62.40; H, 6.39; N, 7.05%. Calcd for $C_{40}H_{48}O_{11}N_4 \cdot 1/2H_2O$: C, 62.40; H, 6.42; N, 7.28%.

MurNAc–D-Ala–D-Glu(OH)–NH$_2$ (4c) and Other MurNAc Peptides (5c—9c). These compounds were prepared by hydrogenolytic deprotection in acetic acid of **4b** and **5b**—**9b** whose syntheses are described below, followed by precipitation from abs ethanol–abs ether in a similar way to that for **3c**. The hygroscopic powders thus obtained were dissolved in water and lyophilized,[13] respectively. The yields, $[\alpha]_D$ values and the results of elemental analyses are summarized in Table 2.

Boc–D-Glu(OBzl)–Gly–OBzl (12). Boc–D-Glu(OBzl)–ONSu (8.69 g, 20.0 mmol) was added to a mixture of H–

Gly–OBzl·HCl (4.03 g, 20.0 mmol) and triethylamine (2.82 ml, 20.0 mmol) in THF (100 ml). After the usual work-up, the product was recrystallized from ethyl acetate–hexane; yield, 8.67 g (89%); mp 71—72 °C; $[\alpha]_D^{20}$ +6.99° (*c* 1.03, ethyl acetate).

Found: C, 64.07; H, 6.75; N, 5.83%. Calcd for $C_{26}H_{32}O_7N_2$: C, 64.45; H, 6.66; N, 5.78%.

*Boc–*L*-Ala–*D*-Glu(OBzl)–Gly–OBzl* (**5a**). The TFA salt of H–D-Glu(OBzl)–Gly–OBzl obtained from **12** (4.32 g, 8.9 mmol) was neutralized with triethylamine and then coupled with Boc–L-Ala–OH (1.70 g, 9.0 mmol) by means of DCC in a usual manner. The product was recrystallized from ethyl acetate–hexane; yield, 2.38 g (48%); mp 107 °C; $[\alpha]_D^{19}$ +6.5° (*c* 1.01, DMF).

Found: C, 62.68; H, 6.77; N, 7.63%. Calcd for $C_{29}H_{37}O_8N_3$: C, 62.69; H, 6.71; N, 7.56%.

*1-α-O-Benzyl-4,6-O-benzylidene-MurNAc–*L*-Ala–*D*-Glu(OBzl)–Gly–OBzl* (**5b**). Compound **5a** (0.28 g, 0.50 mmol) was treated with TFA as described in the synthesis of **3b**. The syrupy H–L-Ala–D-Glu(OBzl)–Gly–OBzl TFA salt solidified on trituration with abs ether. It was filtered and dried over KOH *in vacuo*. A mixture of **10** (0.24 g, 0.51 mmol), HONSu (0.063 g, 0.55 mmol) and DCC (0.10 g, 0.49 mmol) in THF was stirred under ice-cooling for 4 h. After *N,N'*-dicyclohexylurea had been filtered off, the filtrate was again cooled in an ice bath, and a solution of the TFA salt and triethylamine (0.70 ml, 0.50 mmol) in a small amount of THF was added. The mixture was stirred overnight, and gelatinous solid was collected by filtration and then recrystallized from dioxane–water; yield, 0.28 g (63%); mp 220 °C (dec); $[\alpha]_D^{19}$ +68° (*c* 0.49, DMF).

Found: C, 64.48; H, 6.23; N, 6.26%. Calcd for $C_{49}H_{56}O_{13}N_4$: C, 64.74; H, 6.21; N, 6.16%.

*Boc–*D*-Glu(OH)–Gly–OBzl* (**13**) *DCHA Salt*. A mixture of H–Gly–OBzl·HCl (8.87 g, 44.0 mmol) and triethylamine (6.20 ml, 44.0 mmol) in THF (100 ml) was stirred at room temperature for 3 h, and then triethylamine hydrochloride was filtered off. The filtrate was cooled in an ice bath and Boc-D-glutamic anhydride (9.17 g, 40.0 mmol) was added with stirring. After being stirred in an ice bath for 6 h, the solvent was evaporated *in vacuo*. The residue was dissolved in ethyl acetate, washed with 1 M citric acid and water, and dried over Na_2SO_4. After addition of DCHA (8.0 ml, 40 mmol), the mixture was left stand at room temperature to afford crude crystalline material, which was collected by filtration (20.1 g). This was recrystallized from methanol–ethyl acetate–hexane; yield, 12.40 g (53%); mp 164—167 °C. The mother liquor of the recrystallization was condensed *in vacuo*, and the residue was shaken with ethyl acetate and 1 M citric acid. The organic layer was washed with water, dried and again treated with DCHA (2.0 ml, 10 mmol) to give an additional amount of the product; 1.46 g (6%); mp 165.5—167.5 °C.

Found: C, 64.30; H, 8.67; N, 7.04%. Calcd for $C_{31}H_{49}O_7N_3 \cdot 1/4H_2O$: C, 64.17; H, 8.60; N, 7.24%.

Compound **13** was hydrogenolyzed in the presence of palladium black and then treated with TFA to give a free dipeptide, which was identified with the authentic specimen of H–D-Glu(OH)–Gly–OH (**15**) described below by means of TLC and amino acid analysis.

Determination of the Ratio of **13** *to* **14**. A solution of H–Gly–OBzl (0.11 g, 0.55 mmol) in THF (3 ml) was prepared as above and stirred at −70, 0, or 20 °C with or without addition of DCHA (0.10 ml, 0.50 mol). Boc-D-glutamic anhydride (0.11 g, 0.50 mmol) was added to the solution and the mixture was stirred overnight. After the solvent was removed *in vacuo*, the residue was dissolved in ethyl acetate and washed with 1 M citric acid and water. Hydrogenolysis with palladium black followed by treatment with TFA at room temperature afforded a mixture of free dipeptide **15** and **16**, whose ratio was determined by means of amino acid analyser. The standard color values for each substance were obtained using the pure authentic samples described below.

*DCHA Salts of Z–*D*-Glu(OH)–Gly–OBzl and Z–*D*-Glu(Gly–OBzl)–OH*. For the synthesis of these compounds, a similar procedure to that described for **13** was employed. A solution of Z-D-glutamic anhydride (0.53 g, 2.0 mmol) in THF (10 ml) was added to an ice-cooled solution of H–Gly–OBzl (2.2 mmol) in THF (8 ml). After being stirred overnight, the mixture was worked up as above and dissolved in ethyl acetate. After addition of DCHA (0.40 ml, 2.0 mmol), the mixture was left stand in a refrigerator for 4 days to afford Z–D-Glu(OH)–Gly–OBzl DCHA salt; yield, 0.57 g (48%); R_f on TLC, 0.60 ($CHCl_3$–acetone–acetic acid, 16:4:1). When the mother liquor was seeded with Z–D-Glu(Gly–OBzl)–OH DCHA salt and again left stand in a refrigerator for further 2 days, the γ-peptide DCHA salt was solidified; yield, 0.52 g (43%); R_f on TLC, 0.50.

*H–*D*-Glu(OH)–Gly–OH* (**15**). *i) TFA Salt as a Standard Sample*: Boc–D-Glu(OBzl)–Gly–OBzl (**12**) (0.70 g, 1.4 mmol) was deprotected by hydrogenolysis followed by action of TFA to afford white precipitate; yield, 0.43 g (94%); mp 139—140 °C (dec); R_f on TLC, 0.41 (1-butanol–acetic acid–water, 4:1:2).

*ii) From Z–*D*-Glu(OH)–Gly–OBzl DCHA Salt*: This salt (0.30 g, 0.50 mmol) was washed with aqueous citric acid and hydrogenolyzed in ethyl acetate with palladium black. The product was recrystallized from water–ethanol–ether; yield, 0.070 g (68%); mp 177.5—178.5 °C (dec).

Found: C, 40.74; H, 5.98; N, 13.66%. Calcd for $C_7H_{12}O_5N_2 \cdot 1/8H_2O$: C, 40.72; H, 5.98; N, 13.57%.

*H–*D*-Glu(Gly–OH)–OH* (**16**). *i) TFA Salt as a Standard Sample*: Coupling of Boc–D-Glu(OH)–OBzl derived from its DCHA salt (2.60 g, 5.0 mmol) with H–Gly–OBzl *p*-toluenesulfonate (1.69 g, 5.0 mmol) by means of ethyl chloroformate afforded Boc–D-Glu(Gly–OBzl)–OBzl, which was recrystallized from ethyl acetate–hexane; mp 98—99 °C.

Found: C, 64.42; H, 6.63; N, 5.81%. Calcd for $C_{26}H_{32}O_7N_2$: C, 64.45; H, 6.66; N, 5.78%.

This dipeptide (0.23 g, 0.47 mmol) was deprotected as described in the preparation of **15** to afford **16** TFA salt; yield, 0.14 g (94%); mp 42 °C (dec); R_f on TLC, 0.29 (1-butanol–acetic acid–water, 4:1:2).

*ii) From Z–*D*-Glu(Gly–OBzl)–OH DCHA Salt*: A similar procedure to that described above for **15** afforded a product, which was recrystallized from water–ethanol–ether; mp 196—197 °C (dec).

Found: C, 40.55; H, 5.90; N, 13.53%. Calcd for $C_7H_{12}O_5N_2 \cdot 1/8H_2O$: C, 40.72; H, 5.98; N, 13.57%.

*H–*L*-Lys(Z)–*D*-Ala–OBzl* (**17**) *HCl Salt*. A 2 M solution of dry HCl in ethyl acetate (60 ml, 120 mmol) was added to a solution of Boc–L-Lys(Z)–D-Ala–OBzl[2b)] (8.21 g, 15.2 mmol) in ethyl acetate (70 ml) under ice-cooling. After the mixture had been kept at room temperature overnight, the crystalline product was filtered; yield, 7.00 g (95%); mp 142—144 °C.

Found: C, 59.51; H, 6.70; N, 8.73; Cl, 7.42%. Calcd for $C_{24}H_{32}O_5N_3Cl \cdot 1/4H_2O$: C, 59.74; H, 6.79; N, 8.71; Cl, 7.35%.

*Boc–*D*-Glu(*L*-Lys(Z)–*D*-Ala–OBzl)–Gly–OBzl* (**18**). A solution of **13** DCHA salt (1.20 g, 2.1 mmol) in $CHCl_3$ (30 ml) was shaken twice with 0.3 M citric acid solution, washed with water, dried over $MgSO_4$, and then added to a mixture of **17** HCl salt (1.00 g, 2.1 mmol) and triethylamine (0.29

ml, 2.1 mmol) in $CHCl_3$ (30 ml). To the total mixture was added DCC (0.50 g, 2.4 mmol) with stirring under ice-cooling. Stirring was continued at room temperature for 67 h with further addition of DCC (0.80 g, 3.9 mmol) during the first 48 h. After the usual work-up, the product was purified by silica gel column chromatography ($CHCl_3$–acetone, 1: 1), and then recrystallized successively from methanol–ether and ethanol–water; yield, 0.92 g (54%); mp 175.5—176 °C; $[\alpha]_D^{22}$ +1.9° (*c* 2.16, DMF).

Found: C, 62.92; H, 6.92; N, 8.69%. Calcd for $C_{43}H_{55}O_{11}N_5$: C, 63.14; H, 6.78; N, 8.56%.

Boc–L-Ala–D-Glu(L-Lys(Z)–D-Ala–OBzl)–Gly–OBzl (**6a**). Compound **18** (0.87 g, 1.1 mmol) was dissolved in TFA (8 ml). After 2.5 min at room temperature, abs ether and hexane were added to the mixture. The resulting gelatinous precipitate was crystallized from abs ethanol–ethyl acetate–hexane to give **19** TFA salt; yield, 0.86 g (98%).

To a suspension of **19** TFA salt (0.40 g, 0.48 mmol) obtained above in THF (20 ml) was added 1 M triethylamine in THF (0.48 ml, 0.48 mmol). The mixture was then cooled in an ice bath and Boc–L-Ala–ONSu (0.29 g, 1.0 mmol) was added. After being stirred overnight, the insoluble gelatinous solid was filtered and washed successively with acetone, water, acetone and then ether; yield, 0.29 g (67%); mp 173—174 °C (with softening at 167 °C); $[\alpha]_D^{26}$ +0.50° (*c* 1.99, DMF).

Found: C, 61.58; H, 6.82; N, 9.27%. Calcd for $C_{46}H_{60}O_{12}N_6$: C, 62.14; H, 6.80; N, 9.45%.

1-α-O-Benzyl-4,6-O-benzylidene-MurNAc-L-Ala-D-Glu(L-Lys(Z)-D-Ala-OBzl)-Gly-OBzl (**6b**). Compound **6a** (0.30 g, 0.34 mmol) was dissolved in TFA(2.5 ml). After 2.5 min at room temperature, abs ether was added and the precipitate of the TFA salt was filtered. It was dissolved in DMF (30 ml), treated with 1 M triethylamine in THF (0.32 ml, 0.32 mmol), and then cooled in an ice bath. 1-*α*-*O*-Benzyl-4,6-*O*-benzylidene-MurNAc-ONSu (**11**) (0.28 g, 0.50 mmol) was added to the cooled solution, and the mixture was stirred overnight. The insoluble solid was filtered and washed with THF; yield, 0.22 g. An additional amount of the product (0.12 g) was recovered by condensation of the mother liquor *in vacuo* followed by removal of THF-soluble materials from the residue. The combined products were reprecipitated from dioxane-water; yield, 0.30 g (75%); mp 220 °C (dec); $[\alpha]_D^{25}$ + 56° (*c* 0.50, DMA).

Found: C, 63.37; H, 6.47; N, 7.70%. Calcd for $C_{66}H_{79}O_{17}N_7 \cdot 1/2H_2O$: C, 63.34; H, 6.44; N, 7.84%.

Boc–Gly–D-Glu(OBzl)–Gly–OBzl (**7a**). After removal of the Boc group in **12** (2.42 g, 5.0 mmol) with TFA (4 ml) in a usual way, the resulting TFA salt was dissolved in THF (30 ml). To the solution were added *N*-methylmorpholine (0.55 ml, 5.0 mmol), Boc–Gly–ONSu (1.50 g, 5.5 mmol) and K_2CO_3 (0.5 g) under ice-cooling. The mixture was worked up as usual and then subjected to silica gel column chromatography. Elution with benzene–ethyl acetate (3: 1) gave pure **7a** as a syrup; yield, 2.1 g (78%). The structure was confirmed by means of NMR spectrum.

H–Gly–D-Glu(OBzl)–Gly–OBzl. *i) HCl Salt*: Compound **7a** (80 mg, 0.15 mmol) was treated with 2 M dry HCl in ethyl acetate (1 ml) in a usual way and the product was recrystallized from methanol–ether; yield, 60 mg (84%); mp 133—134 °C; $[\alpha]_D^{30}$ +13.7° (*c* 1.74, methanol).

Found: C, 57.53; H, 5.88; N, 8.77; Cl, 7.38%. Calcd for $C_{23}H_{28}O_6N_3Cl$: C, 57.80; H, 5.91; N, 8.79; Cl, 7.42%.

ii) TFA Salt: Compound **7a** (80 mg, 0.15 mmol) was treated with TFA (1 ml), and the product was recrystallized from THF–abs ether; yield, 80 mg (97%); mp 84 °C (dec). This substance was identified with the HCl salt mentioned above on TLC.

1-α-O-Benzyl-4,6-O-benzylidene-MurNAc-Gly-D-Glu(OBzl)-Gly-OBzl (**7b**). This compound was prepared in a similar manner to that described for **6b** from H–Gly–D-Glu(OBzl)–Gly–OBzl TFA salt (0.56 g, 1.0 mmol) and **11** (0.68 g, 1.2 mmol), and recrystallized from dioxane–water; yield, 0.70 g (78%); mp 203 °C.

Found: C, 63.94; H, 6.03; N, 6.23%. Calcd for $C_{48}H_{54}O_{13}N_4 \cdot 1/2H_2O$: C, 63.77; H, 6.13; N, 6.20%.

Boc–D-Glu(OBzl)–D-Ala–NH₂. This compound was prepared from Boc–D-Glu(OBzl)–ONSu (3.04 g, 7.0 mmol) and H–D-Ala–$NH_2 \cdot$HCl (0.93 g, 7.5 mmol) and recrystallized from ethyl acetate–hexane; yield, 2.57 g (90%); mp 139.5—140.5 °C.

Found: C, 58.94; H, 7.20; N, 10.19%. Calcd for $C_{20}H_{29}O_6N_3$: C, 58.95; H, 7.17; N, 10.31%.

Boc–L-Ala–D-Glu(OBzl)–D-Ala–NH₂ (**8a**). After removal of the Boc group in Boc–D-Glu(OBzl)–D-Ala–NH_2 (2.42 g, 5.9 mmol) with TFA (6 ml), the resulting TFA salt was treated with triethylamine (0.83 ml, 5.9 mmol) and Boc–L-Ala–ONSu (1.43 g, 5.0 mmol) in THF. The product was recrystallized from $CHCl_3$–hexane; yield, 2.35 g (98%); mp 182.5—183.5 °C (dec); $[\alpha]_D^{28}$ −4.98° (*c* 2.03, DMF).

Found: C, 57.73; H, 7.14; N, 11.61%. Calcd for $C_{23}H_{34}O_7N_4$: C, 57.72; H, 7.16; N, 11.71%.

1-α-O-Benzyl-4,6-O-benzylidene-MurNAc-L-Ala-D-Glu(OBzl)-D-Ala-NH₂ (**8b**). Compound **8a** (0.57 g, 1.2 mmol) was treated with TFA (2 ml) in a usual manner, and the resulting TFA salt was neutralized with triethylamine and coupled with **11** (0.57 g, 1.0 mmol) in a mixture of DMF (8 ml) and THF (30 ml) overnight. Water was added to the mixture to afford gelatinous solid, which was filtered and then washed with 0.2 M citric acid and water. Reprecipitation was carried out from dioxane–water; yield, 0.60 g (72%); mp 275 °C (dec); $[\alpha]_D^{28}$ +83° (*c* 0.10, DMF).

Found: C, 61.77; H, 6.40; N, 8.31%. Calcd for $C_{43}H_{53}O_{12}N_5$: C, 62.28; H, 6.42; N, 8.42%.

Boc–D-Glu(OBzl)–Gly–OEt (**20**). This compound was prepared from H–Gly–OEt·HCl (1.66 g, 11.9 mmol) and Boc–D-Glu(OBzl)–ONSu (5.18 g, 11.9 mmol) in THF and was recrystallized from CH_2Cl_2–hexane; yield, 3.96 g (79%); mp 71—72 °C; $[\alpha]_D^{25}$ +6.20° (*c* 2.34, ethyl acetate).

Found: C, 59.79; H, 7.14; N, 6.59%. Calcd for $C_{21}H_{30}O_7N_2$: C, 59.70; H, 7.16; N, 6.63%.

Boc–L-Ala–D-Glu(OBzl)–Gly–OEt (**22**). Compound **20** (3.00 g, 7.1 mmol) was treated with 1 M dry HCl in ethyl acetate (50 ml), and the product was crystallized from $CHCl_3$–ether to give H–D-Glu(OBzl)–Gly–OEt (**21**) HCl salt (2.15 g, 84%). This salt (2.00 g, 5.6 mmol) was neutralized with triethylamine and coupled with Boc–L-Ala–ONSu (1.63 g, 5.7 mmol) in a usual manner. The product was recrystallized from ethyl acetate–hexane; yield, 2.55 g, (92%); mp 104—105 °C; $[\alpha]_D^{28}$ +7.19° (*c* 2.31, ethyl acetate).

Found: C, 58.37; H, 7.13; N, 8.45%. Calcd for $C_{24}H_{35}O_8N_3$: C, 58.40; H, 7.15; N, 8.51%.

*Boc–L-Ala–D-Glu(OH)–Gly–OEt (**23**) DCHA Salt.* Compound **22** (1.03 g, 2.1 mmol) was hydrogenolyzed in the presence of palladium black in ethanol (30 ml). The product was dissolved in ethyl acetate, and DCHA (0.42 ml, 2.1mmol) and ether were added to the solution to afford **23** DCHA salt as colorless crystals; yield, 1.04 g (83%); mp 138—139 °C; $[\alpha]_D^{26}$ +3.1° (*c* 2.06, methanol).

Found: C, 58.94; H, 9.05; N, 9.42%. Calcd for $C_{29}H_{52}O_8N_4 \cdot 1/2H_2O$: C, 58.66; H, 9.00; N, 9.44%.

*Boc–L-Ala–D-Glu(OH)–Gly–NH₂ (**9a**) DCHA Salt.* DCHA salt of **23** (0.29 g, 0.49 mmol) was dissolved in methanol (15 ml). The solution was saturated with NH_3 and left stand at room temperature in a pressure bottle for 3 days. After removal of the solvent *in vacuo*, the residue was triturated with ether to afford **9a** DCHA salt; yield, 0.28 g (quantitative). Recrystallization was effected from ethanol–ethyl acetate–ether; mp 183 °C (dec); $[\alpha]_D^{25}$ −10.5° (*c* 1.99, methanol).

Found: C, 57.61; H, 8.78; N, 12.57%. Calcd for $C_{27}H_{49}O_7N_5 \cdot 1/2H_2O$: C, 57.42; H, 8.92; N, 12.40%.

*1-α-O-Benzyl-4,6-O-benzylidene-MurNAc-*L*-Ala-*D*-Glu(OH)-Gly-NH$_2$ (**9b**).* A solution of **9a** DCHA salt (0.56 g, 1.0 mmol) in water was passed through a column of Dowex 50 (H^+ form). The column was washed with water, and the combined eluate and washings was condensed *in vacuo*. The residue was twice dissolved in abs ethanol and the solvent was evaporated *in vacuo* to remove water completely. The final residue was dissolved in TFA (2 ml) and allowed to stand at room temperature for 20 min. Addition of abs ether to the solution afforded white precipitate of H–L-Ala–D-Glu(OH)–Gly–NH$_2$ (**24**) TFA salt (0.35 g, 0.90 mmol). An aqueous solution of this TFA salt was adjusted to pH 8 by addition of aqueous 1 M K_2CO_3 solution and mixed with a solution of 1-*α*-*O*-benzyl-4,6-*O*-benzylidene-MurNAc-ONb (0.44 g, 0.70 mmol) in THF (10 ml). The reaction mixture was stirred at room temperature for 4.5 h, and then brought to pH 3 by addition of 2 M HCl. Water was added to the mixture to afford gelatinous solid, which was collected by filtration; yield, 0.39 g (76%). Reprecipitation was effected from DMF–ethanol; mp 240 °C (dec); $[\alpha]_D^{25}$ +100° (*c* 0.95, DMF).

Found: C, 57.10; H, 6.28; N, 9.55%. Calcd for $C_{35}H_{45}O_{12}N_5 \cdot 1/2H_2O$: C, 57.05; H, 6.29; N, 9.51%.

References

1) Abbreviations according to IUPAC-IUB comission, *J. Biol. Chem.*, **247**, 977 (1972), are used throughout. Boc: *t*-butoxycarbonyl, Z: benzyloxycarbonyl, Bzl: benzyl, DCC: dicyclohexylcarbodiimide, HONSu: *N*-hydroxysuccinimide, HOBt: 1-hydroxybenzotriazole, HONb: *N*-hydroxy-5-norbornene-2,3-dicarboximide, THF: tetrahydrofuran, DMF: *N,N*-dimethylformamide, DMA: *N,N*-dimethylacetamide, TFA: trifluoroacetic acid, DCHA: dicyclohexylamine.

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13) Samples of free *N*-acetylmuramyl peptides precipitated from ethanol–ether contained considerable amount of ethanol, which could not be otherwise removed.