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Chemical Synthesis and Biological Activities of Two Disaccharide Dipeptides Corresponding to the Repeating Units of Bacterial Peptidoglycan

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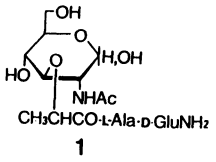
Two disaccharide dipeptides, i.e., *O*-(*N*-acetyl-β-D-glucosaminyl)-(1→4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2**) and *O*-(*N*-acetyl-β-muramyl-L-alanyl-D-isoglutamine)-(1→4)-*N*-acetyl-D-glucosamine (**3**), which correspond to partial structures of cell wall peptidoglycan were synthesized via the same glucosamine disaccharide as a common intermediate. Thus, selective introduction of lactic acid moiety on either one of the sugar moieties of the intermediate gave two disaccharides respectively which contain muramic acid on either reducing or nonreducing side. Coupling of the peptide moiety followed by deprotection afforded **2** and **3**. The results of biological tests showed that both **2** and **3** have the same magnitude of immunostimulating activities as those of *N*-acetylmuramyl-L-alanyl-D-isoglutamine which is the minimum structure required for expression of the activities.

Bacterial peptidoglycan is an integral part of the cell walls and known to exhibit a potent activity to modulate the defense mechanism of higher animals. Its structure is composed of glycan chains interlinked with peptide bridges. The former is β(1—4)glycan of alternating *N*-acetylglucosamine and *N*-acetylmuramic acid whose carboxyl group is the point of linkage to the peptide. It was independently demonstrated by us and a French group that the minimum structure required for the immunostimulation is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**1**), which is a common building block of the above architecture.[1,2] After this initial work, we prepared many derivatives and structural analogs of the muramyl peptide **1** and studied the relationship between their chemical structures and immunological activities.[3] In the meantime, comparison of immunopharmacological activities of various enzymatic digests of cell wall peptidoglycans has revealed that digests prepared with endopeptidases are generally more active than the muramyl dipeptide **1** as well as than digests obtained by use of glycosidases.[4] The endopeptidases are capable of hydrolyzing the cross linkages between the neighboring glycan chains, while the glycosidases split the glycan chain itself. These results seem to indicate that the presence of long glycan chain rather than the peptide network is required for efficient manifestation of these biological activities.

In view of the above findings, as the first step of a synthetic approach to a peptidoglycan polysaccharide peptides with stronger biological activities than the muramyl dipeptide **1**, we synthesized two disaccharide dipeptides, i.e., *O*-(*N*-acetyl-β-D-glucosaminyl)-(1→4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2**) and *O*-(*N*-acetyl-β-muramyl-L-alanyl-D-isoglutamine)-(1→4)-*N*-acetyl-D-glucosamine (**3**), which correspond to structures having an *N*-acetylglucosamine unit β(1—4)-linked on either side of **1**. In other words, **2** and **3** are larger fragments of cell wall than **1** by one glucosamine residue.



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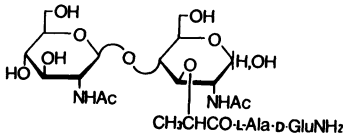

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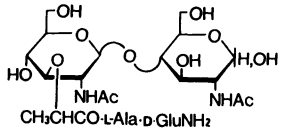

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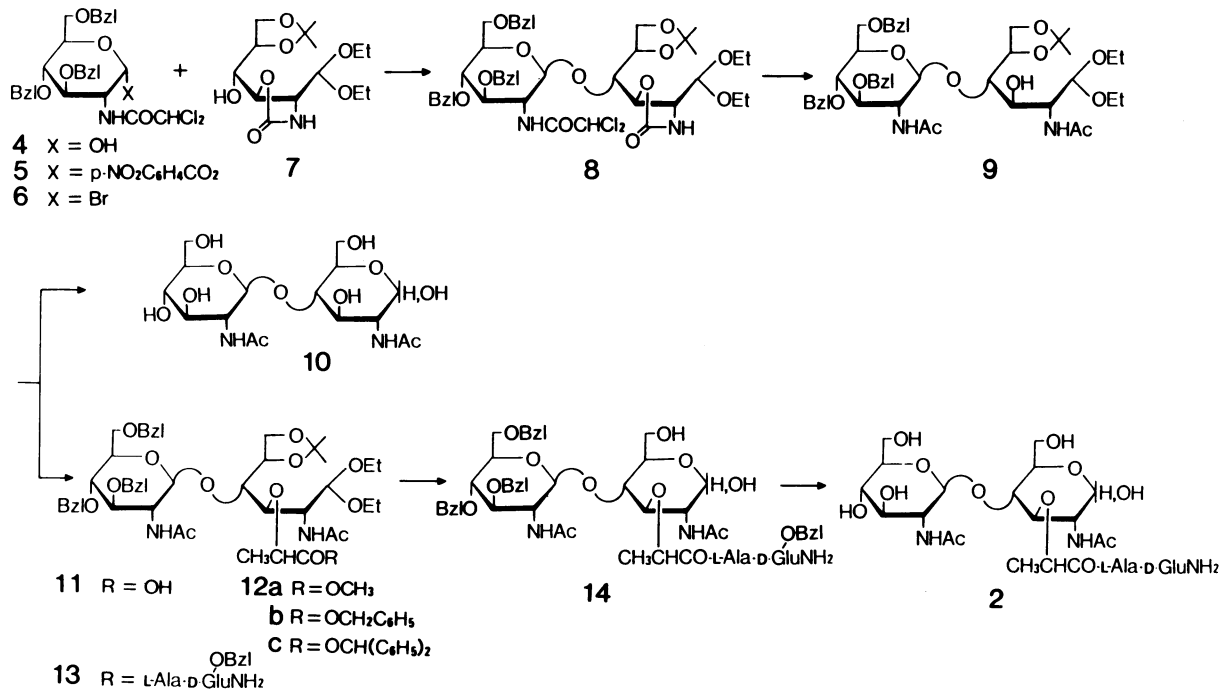
As reported in our preliminary communications,[5,6] the synthesis was performed via a common β(1—4)-linked disaccharide of glucosamine. One of its sugar residues was then selectively transformed into muramic acid to form the two alternative β(1—4)-linked disaccharides respectively. Coupling of the peptide moiety followed by deprotection afforded the desired products **2** and **3**. We describe in this paper the details of the synthesis. After our initial paper[5] on the synthesis of **2**, two other research groups also reported syntheses of both types of the disaccharide dipeptides via glucosamine disaccharides.[7,8] In the accompanying paper,[9] we also describe different approaches to form the corresponding disaccharide portions of **2** and **3** by means of a direct coupling of

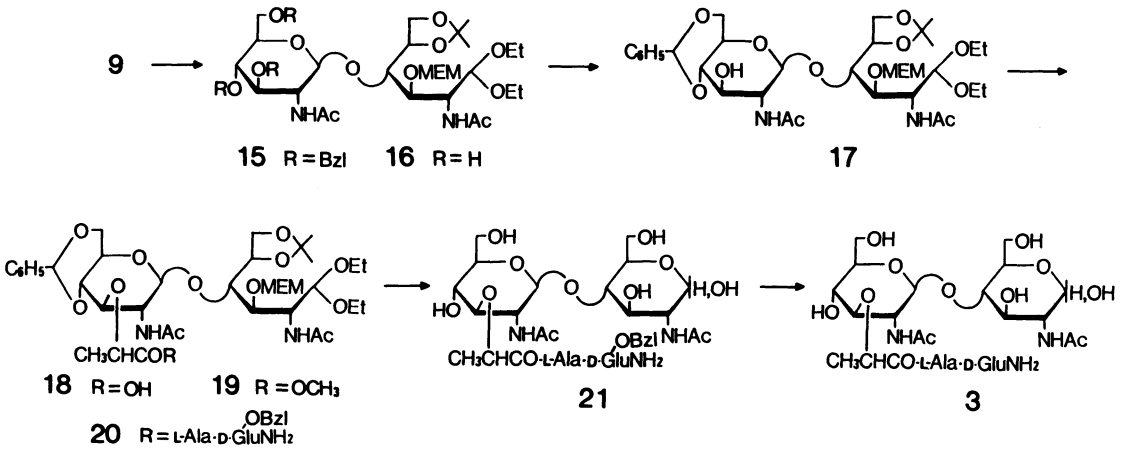


glucosamine and muramic acid residues.

In this investigation, an appropriate β(1—4)-linked glucosamine disaccharide which served as the common synthetic intermediate was prepared by coupling of the glycosyl bromide **6** with a known open-chain derivative of glucosamine, i.e., 2-amino-2-*N*: 3-*O*-carbonyl-2-deoxy-5,6-*O*-isopropylidene-D-glucose diethyl acetal (**7**).[10] The bromide **6** was prepared from 2-amino-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucose.[11] After dichloroacetylation[12] of its amino group, the product **4** was converted into 1-*p*-nitrobenzoate **5** and then treated with dry hydrogen bromide in dichloromethane to give **6**.[13] The condensation of **6** with **7** in the presence of mercury(II) cyanide in benzene afforded the disaccharide **8** as the main product. The β-configuration of the newly formed glycosidic bond was ascertained chemically by converting **8** into *N,N'*-diacetylchitobiose. Thus, cleavage of the oxazolidone and dichloroacetyl protecting groups in **8** by alkaline hydrolysis gave **9**. *N*-Acetylation followed by further deprotection afforded the disaccharide **10** which was identified with an authentic sample of *N,N'*-diacetylchitobiose obtained from natural chitin.

For the preparation of the disaccharide which has the muramic acid residue on the reducing side, **9** obtained above was used as an intermediate. In this compound only one hydroxyl group remains free on C-3 where lactic acid moiety should be introduced to form muramic acid. The hydroxyl group of **9** was thus converted into the sodium alkoxide and treated with (*S*)-2-chloropropionic acid as described by Matsushima and Park.[14] However, when only dioxane was used as solvent as described,[14] the yield of the product **11** was quite variable. This seemed to be due to the difficulty in formation of alkoxide anion in that solvent in which **9** was only poorly soluble. Use of DMF as co-solvent improved the situation and the desired disaccharide derivative **11** could be obtained in a reproducible yield. The product was purified and isolated after esterification with diazomethane, phenyldiazomethane, or diphenyldiazomethane as methyl, benzyl, or diphenylmethyl ester (**12a, b, c**), respectively. Among these, only the diphenylmethyl derivative was crystalline. The structure of **12** was confirmed by converting **12a** into a known peracetylated derivative of *O*-β-D-glucosaminyl-(1→4)-muramic acid methyl ester.[15,16] The ester group of **12** was again hydrolyzed after purification and the resultant, free carboxyl group was coupled with L-alanyl-D-isoglutamine benzyl ester[1b] by means of the dicyclohexylcarbodiimide (DCC)–*N*-hydroxysuccinimide procedure to give the fully protected disaccharide dipeptide **13**. Deprotection of **13** was performed in two steps. Thus, the two acetal groups were first removed with hot aqueous acetic acid to give **14**, which in turn was subjected to hydrogenolysis. The final product **2** was isolated as a homogeneous hygroscopic powder.

Another disaccharide having the muramic acid residue on the nonreducing end was also synthesized from **9**. After the free hydroxyl group on position 3 had been protected with 2-methoxyethoxymethyl (MEM) group, the benzyl groups were hydrogenolyzed and the two hydroxyl groups on positions 4' and 6' again protected as benzylidene derivative to give a disaccharide **17** to be used for introduction of lactic acid moiety at position 3'. It was condensed with



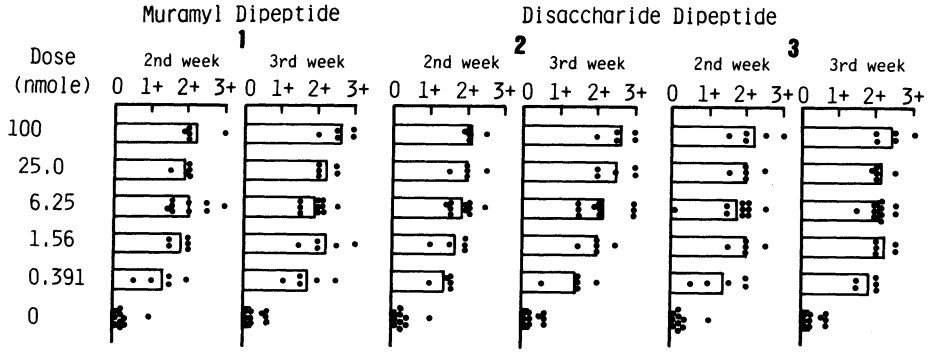


Fig. 1. Induction of delayed-type hypersensitivity against ovalbumin by **1**, **2**, and **3** in guinea pigs (corneal reaction). Each dot represents the score (arbitrarily graded with a maximum of 3+) for individual animal. Each column shows the mean score.

(*S*)-2-chloropropionic acid under similar conditions as above to afford the desired disaccharide **18** which was purified as the methyl ester **19** after esterification with diazomethane. Coupling of the dipeptide moiety as above gave a protected disaccharide dipeptide **20**, which was then subjected to deprotection reaction in two steps. In the first step when **20** was heated in aqueous acetic acid in order to remove all acetal-type protections, several by-products such as ethyl glycoside of the desired product were formed. However, by-products formation could be greatly suppressed by employing appropriate reaction conditions (see experimental section). The product **21** was isolated after purification with a column of porous polymer. It was then hydrogenolyzed to give the second disaccharide dipeptide **3**.

Immunoadjuvant activities of both disaccharide dipeptides prepared (**2** and **3**) were tested in guinea pigs by using ovalbumin as a test antigen. Their activities were compared with those of the muramyl dipeptide **1**. As shown in Figs. 1, 2, and 3, no significant differences were observed with the adjuvant activities among these compounds in terms of the induction of cellular immunity (as tested by corneal and tuberculin-type skin reactions) and the

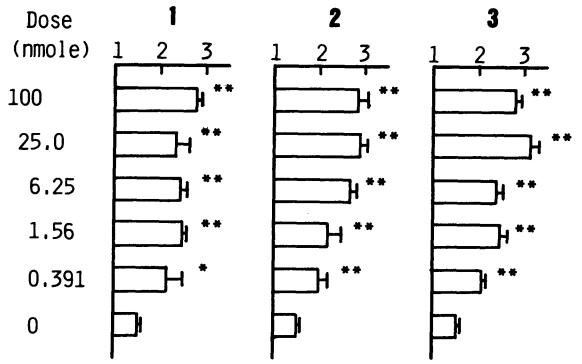


Fig. 2. Induction of delayed-type hypersensitivity against ovalbumin by **1**, **2**, and **3** in guinea pigs (tuberculin type of skin reaction). The extent of skin induration is expressed as ratios of the reading (48 h) at each test site to that at the control site. Significant difference from control group: $*P<0.05$, $**P<0.01$.

stimulation of humoral immunity (by determination of serum anti-ovalbumin level). We reported in a previous paper[17] that the glucosaminylmuramyl dipeptide **2** showed higher immunoadjuvant activities than **1**. Though discrepancy on this point might be due to the difference of antigen doses used for the

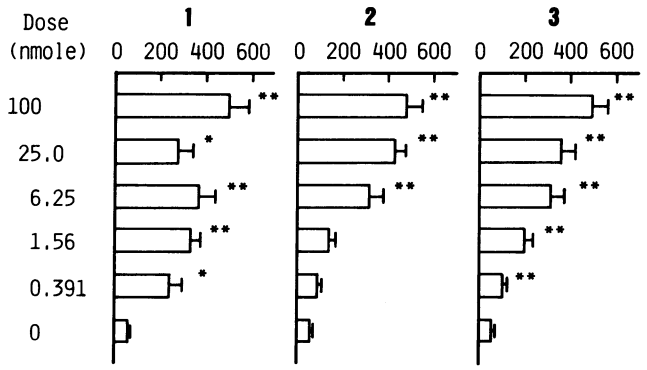


Fig. 3. Elevation of serum precipitating antibody levels against ovalbumin by **1**, **2**, and **3** in guinea pigs. Antibody level in serum is expressed as μg of antibody nitrogen per ml of serum. Significant difference from control group: $*P<0.05$, $**P<0.01$.

immunization (1 mg in the previous study and 0.1 mg in the present one), it should be concluded from the result obtained above that additional single glucosamine residue was not sufficient to induce a significant enhancement of the activities of **1**. Longer glycan chains might be required for effective potentiation. Synthesis of a peptidoglycan part structure with a longer saccharide is now being undertaken in our laboratory to confirm this assumption.

Experimental

All melting points are uncorrected. ^{1}H NMR spectra were measured for $CDCl_3$ solutions with tetramethylsilane as an internal standard (δ, ppm). FD-MS spectra were obtained with Si-emitter on JEOL OI-SG mass spectrometer. Optical rotations were measured with a Perkin-Elmer 141 polarimeter. Column chromatography and preparative thin-layer chromatography were carried out with Kieselgel 60 (0.063—0.2 mm) and with pre-coated Kieselgel F_{254} plates (0.5 mm thickness), E. Merck.

3,4,6-Tri-*O*-benzyl-2-deoxy-2-dichloroacetamido-D-glucopyranose (4). Pentachlorophenyl dichloroacetate (1.95 g, 5.16 mmol) and triethylamine (0.73 ml, 5.16 mmol) were added at room temperature to a stirred suspension of 2-amino-3,4,6-tri-*O*-benzyl-2-deoxy-D-glucose hydrochloride[11] (2.51 g, 5.16 mmol) in anhyd tetrahydrofuran (THF). After the mixture had been stirred at room temperature for 90 min, insoluble materials were filtered off, the filtrate evaporated in vacuo to give a gelatinous solid. It was purified by reprecipitation from dioxane–ether to give **4**; 2.38 g (82%); mp 217—219 °C(decomp). Found: C, 61.89; H, 5.56; N, 2.50; Cl, 12.50%. Calcd for $C_{29}H_{31}NO_6Cl_2$: C, 62.15; H, 5.58; N, 2.50; Cl, 12.56%.

3,4,6-Tri-*O*-benzyl-2-deoxy-2-dichloroacetamido-α-D-glucopyranosyl *p*-Nitrobenzoate (5). To an ice-cooled stirred solution of **4** (2.31 g, 4.12 mmol) in pyridine (50 ml) was added *p*-nitrobenzoyl chloride (2.30 g, 12.4 mmol) dissolved in a small amount of CH_2Cl_2. The mixture was stirred under ice-cooling for 2 h then at room temperature for 30 min. After usual work-up, the crude product was recrystallized from $CHCl_3$–ether; 2.50 g (86%); mp 173—174 °C; $[\alpha]_D^{20}$ +125° (*c* 1.00, $CHCl_3$). Found: C, 60.72; H, 4.82; N, 3.81; Cl, 9.89%. Calcd for $C_{36}H_{34}N_2O_9Cl_2$: C, 60.49; H, 4.83; N, 3.95; Cl, 9.99%.

3,4,6-Tri-*O*-benzyl-2-deoxy-2-dichloroacetamido-D-glucopyranosyl Bromide (6). A saturated solution of dry HBr in dry CH_2Cl_2 (5.5 ml) was added to a solution of **5** (2.00 g, 2.82 mmol) in dry CH_2Cl_2 (30 ml). The mixture was left stand at room temperature for 30 min. The precipitate of *p*-nitrobenzoic acid was removed by filtration. The residue obtained by evaporation of the solvent in vacuo at room temperature was dried over KOH and P_2O_5 in a desiccator overnight. It was used for the next coupling reaction without further purification.

2-Amino-4-*O*-(3,4,6-tri-*O*-benzyl-2-deoxy-2-dichloroacetamido-β-D-glucopyranosyl)-2-*N*: 3-*O*-carbonyl-2-deoxy-5,6-*O*-isopropylidene-D-glucose Diethyl Acetal (8). Dry benzene (30 ml) was added to 2-amino-2-*N*:3-*O*-carbonyl-2-deoxy-5,6-*O*-isopropylidene-D-glucose diethyl acetal (**7**) (0.90 g, 2.82 mmol) and powdered $Hg(CN)_2$ (2.14 g, 8.46 mmol). From this mixture, benzene (ca. 10 ml) was distilled off in order to remove the remaining trace of water. After cooling, a solution of the bromide **6** [obtained from **5** (2.00 g, 2.82 mmol)] in dry benzene (20 ml) was added and the mixture was stirred at room temperature for 24 h. The reaction mixture was diluted with $CHCl_3$, washed successively with 10% brine and H_2O, and dried ($MgSO_4$). The product was purified by chromatography on a silica-gel column ($CHCl_3$–acetone 6:1). Recrystallization from benzene–hexane gave **8**; 0.81 g (33%); mp 52—57 °C. $[\alpha]_D^{17}$ −11.0° (*c* 1.00, $CHCl_3$). Found: C, 59.74; H, 6.37; N, 3.23; Cl, 8.13%. Calcd for $C_{43}H_{54}N_2O_{11}Cl_2$: C, 59.92; H, 6.33; N, 3.25; Cl, 8.23%.

2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-2-deoxy-5,6-*O*-isopropylidene-D-glucose Diethyl Acetal (9). A mixture of **8** (4.25 g, 4.93 mmol) and $Ba(OH)_2 \cdot 8H_2O$ (18.6 g, 59.1 mmol) in dioxane (43 ml) and H_2O (43 ml) was heated at 100 °C with stirring for 16 h. The mixture was cooled, neutralized with CO_2 and filtered. The filtrate was concentrated in vacuo. The residue was dissolved in pyridine (50 ml), treated with acetic anhydride (13.9 ml), and worked up as usual. The crude acetylation product was dissolved in MeOH (80 ml), and 1M[††] NaOMe in MeOH (8 ml) was added to it. The mixture was stirred at room temperature for 1 h and neutralized with Dowex 50 (H^+). Evaporation of the solvent in vacuo and purification with silica-gel column chromatography ($CHCl_3$–MeOH 15:1) afforded **9** as colorless syrup; 3.56 g (89%). ^{1}H NMR: 1.13 (6H, t, *J*=6.8 Hz $OCH_2C\underline{H}_3$), 1.28 and 1.40 (each 3H, s, isopropylidene CH_3), 1.90 (6H, s, $COCH_3$), 7.37 (15H, s, $OCH_2C_6\underline{H}_5$).

***N,N'*-Diacetylchitobiose (10).** Compound **9** (310 mg) was heated at 80 °C in 60% aq AcOH (10 ml) with stirring for 1 h. After evaporation of the solvent in vacuo, the residue was recrystallized from ethanol–hexane and then subjected to hydrogenolysis on Pd-black in 50% AcOH. The product was purified with preparative TLC (phenol–H_2O 3:1) to give a solid, 40 mg (27%), which was recrystallized from H_2O–acetone; mp 242—243 °C(decomp);

[††] 1 M=1 mol dm^{-3}.

$[\alpha]_D^{22}$ +16.3° (*c* 0.40, H_2O; after equilibration). This product was identified with an authentic specimen of *N,N'*-diacetylchitobiose (mp 240—241 °C decomp, $[\alpha]_D^{22}$ +17.5°) by means of TLC and ^1H NMR.

2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-3-*O*-[(*R*)-1-(benzyloxycarbonyl)ethyl]-2-deoxy-5,6-*O*-isopropylidene-D-glucose Diethyl Acetal (12b). Sodium hydride (50% oil dispersion, 1.1 g, 23 mmol) was added to a solution of **9** (5.30 g, 6.56 mmol) in a mixture of dioxane (120 ml)–DMF(60 ml) at room temperature. After the mixture had been stirred at 60 °C for 20 min, (*S*)-2-chloropropionic acid (2.13 g, 20 mmol) and then NaH (1.3 g, 26 mmol) were added. The mixture was stirred at 60 °C for 8 h, cooled and the excess of NaH was decomposed by addition of ice. Acidification with 1 M HCl followed by extraction with $CHCl_3$ gave crude free acid **11**, which was treated with phenyldiazomethane in $CHCl_3$–hexane. The product was purified by silica-gel colunm chromatography (benzene–ethyl acetate 1:2) to give the benzyl ester **12b** as a pale yellow syrup; 3.0 g (47%); FD-MS: *m/z* 970 (M^+).

The corresponding methyl and diphenylmethyl esters **12a** and **c** were prepared in similar manners.

Compound 12a: Syrup; ^1H NMR: 1.12 (6H, t, *J*=7.0 Hz, $OCH_2C\underline{H}_3$), 1.25 and 1.38 (each 3H, s, isopropylidene CH_3), 1.25—1.38 (3H, $C\underline{H}_3CHCO_2$), 1.93 and 2.03 (each 3H, s, $COCH_3$), 3.73 (3H, s, CO_2CH_3), 7.33 (16H, C_6H_5 and $N\underline{H}COCH_3$).

Compound 12c: Mp 87—92 °C; $[\alpha]_D^{18}$ +21.0° (*c* 0.40, $CHCl_3$). Found: C, 68,99; H, 7.08; N, 2.56%. Calcd for $C_{60}H_{74}N_2O_{14}$: C, 69.05; H, 7.15; N, 2.68%.

2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranosyl)-1,4,6-tri-*O*-acetyl-2-deoxy-3-*O*-[(*R*)-1-(methoxycarbonyl)ethyl]-α-D-glucopyranose. Methyl ester (**12a**) (50 mg) was heated in 60% AcOH (1 ml) at 60 °C for 1 h then at 80 °C for 1 h. The main product was isolated by means of preparative TLC on silica gel ($CHCl_3$–MeOH 9:1). Hydrogenolysis followed by acetylation with acetic anhydride and pyridine afforded peracetate of methyl ester as a mixture of the α- and β-anomers. It was again dissolved in acetic anhydride and treated with $ZnCl_2$ at 40 °C for 14 h to enrich the α-acetate, which was isolated as needles through recrystallization from acetone-ether; mp 231—233 °C; $[\alpha]_D^{18.5}$ +42.9° (*c* 0.07, $CHCl_3$).

Reported values for the natural specimen isolated from bacterial cells: Mp 235—236 °C; $[\alpha]_D^{22}$ +40° (*c* 0.20, $CHCl_3$).[14] Those for a synthetic specimen: Mp 237—238 °C; $[\alpha]_D$ +38° (*c* 0.23, $CHCl_3$).[15]

Benzyl L-Alanyl-D-isoglutaminate *N*-Acylated with 2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-2-deoxy-5,6-*O*-isopropylidene-3-*O*-[(*R*)-1-carboxyethyl]-D-glucose Diethyl Acetal (13). A solution of methyl ester **12a** (0.63 g, 0.70 mmol) in 0.5 M KOH in MeOH (14 ml) was stirred at room temperature for 24 h. The mixture was acidified with 3M citric acid, diluted with water and extracted with CH_2Cl_2. The organic layer was washed with H_2O, dried ($MgSO_4$) and evaporated in vacuo to give **11** as a syrup. It was dried by coevaporation with benzene in vacuo and then dissolved in dry THF. Benzyl L-alanyl-D-isoglutaminate hydrochloride (0.36 g, 1.1 mmol) and triethylamine (0.15 ml, 1.1 mmol) was added to the solution and the mixture was stirred in an ice bath for 30 min. *N*-Hydroxysuccinimide (0.12 g, 1.1 mmol) and DCC (0.14 g, 0.70 mmol) were added. Stirring was continued under cooling overnight. After addition of DCC (0.03 g, 0.14 mmol), the mixture was stirred at room temperature for further 3 h. Usual work-up and purification by column chromatography on silica gel ($CHCl_3$–MeOH 9:1) followed by reprecipitation form benzene–hexane afforded **13** as amorphous solid; 0.48 g (59%); mp 55—65 °C; $[\alpha]_D^{18}$ +9.5° (*c* 0.21, $CHCl_3$). Found: C, 63.11; H, 7.33; N, 5.90%. Calcd for $C_{62}H_{83}N_5O_{17}\cdot 1/2H_2O$: C, 63.14; H, 7.18; N, 5.94%.

Benzyl L-Alanyl-D-isoglutaminate *N*-Acylated with 2-Acetamido-4-*O*-(2-acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-D-glucose (14). Compound **13** (0.35 g, 0.30 mmol) was heated in 60% AcOH at 60 °C for 70 min. The product was purified by preparative TLC on silica gel ($CHCl_3$–MeOH 9:1). Precipitation by addition of ethyl acetate–hexane to a $CHCl_3$–MeOH (2:1) solution afforded colorless solid; 0.18 g (57%); mp 187—194 °C(decomp); $[\alpha]_D^{18}$ +8.5° (*c* 0.13, pyridine). Found: C, 61.25; H, 6.55; N, 6.42%. Calcd for $C_{55}H_{69}N_5O_{16}\cdot H_2O$: C, 61.49; H, 6.66; N, 6.51%.

L-Alanyl-D-isoglutamine *N*-Acylated with 2-Acetamido-4-*O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-D-glucose (2). Compound **14** (135 mg, 0.13 mmol) was suspended in MeOH and hydrogenolyzed in the presence of Pd-black catalyst at room temperature. The product was precipitated from MeOH–acetone to give hygroscopic powder, 90 mg (quantitative); mp 170—178 °C (decomp); $[\alpha]_D^{24}$ +0.6° $[\alpha]_{365}^{24}$ −21.7° (*c* 1.0, H_2O after equilibration). Found: C, 45.36; H, 6.40; N, 9.66%. Calcd for $C_{27}H_{45}N_5O_{16}\cdot H_2O$: C, 45.44; H, 6.44, N, 9.81%.

4-*O*-(2-Acetamido-3,4,6-tri-*O*-benzyl-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-5,6-*O*-isopropylidene-3-*O*-(2-methoxyethoxymethyl)-D-glucose Diethyl Acetal (15). To a solution of **9** (1.60 g, 1.97 mmol) in a mixture of dioxane (32 ml)–DMF (16 ml) was added NaH (50% oil dispersion, 95 mg, 2.37 mmol). The mixture was stirred under Ar atmosphere at 60 °C for 30 min. The mixture was then cooled in an ice bath. 2-Methoxyethoxymethyl chloride (295 mg, 2.37 mmol) was added with stirring. After 70 min, a saturated aq solution of $NaHCO_3$ was added to the mixture. The solvent was removed in vacuo, the residue worked up as usual and subjected to silica-gel column chromatography ($CHCl_3$–acetone 2:1) to give **15** as colorless syrup; 1.00 g (57%); ^1H NMR: 4.75 (2H, s, OCH_2O) 3.80 (3H, s, OCH_3); FD-MS: *m/z* 919 $(M+Na)^+$. Further elution of the column afforded unchanged starting material (**9**); 0.65 g (41%).

4-*O*-(2-Acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranosyl)-2-acetamido-2-deoxy-5,6-*O*-isopropylidene-3-*O*-(2-methoxyethoxymethyl)-D-glucose Diethyl Acetal (17). Compound **15** (1.38 g, 1.54 mmol) was dissolved in EtOH (15 ml) and hydrogenolyzed at room temperature in the presence of Pd-black catalyst. The solid product **16** obtained by evaporation of the solvent in vacuo was used for the following reaction after drying by co-evaporation with benzene.

To a solution of **16** in DMF (20 ml) were added benzaldehyde dimethyl acetal (1.00 g, 6.61 mmol) and *p*-toluenesulfonic acid monohydrate (30 mg, 0.16 mmol). The mixture was kept at 40 °C and rotated on a rotary

evaporator in vacuo for 30 min. After usual work-up and purification with a silica-gel column ($CHCl_3$–acetone 1:2) afforded **17** as a pale yellow solid; 0.59 g (54% from **15**); FD-MS: *m/z* 737 $(M+Na)^+$.

4-*O*-[2-Acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-1-(methoxycarbonyl)ethyl]-β-D-glucopyranosyl]-2-acetamido-2-deoxy-5,6-*O*-isopropylidene-3-*O*-(2-methoxyethoxymethyl)-D-glucose Diethyl Acetal (19). To a solution of **17** (370 mg, 0.52 mmol) in a mixture of dioxane (8 ml)–DMF (4 ml) was added NaH (50% oil dispersion, 41 mg, 1.0 mmol). The mixture was stirred under Ar atmosphere at 60 °C for 30 min. Then (*S*)-2-chloropropionic acid (224 mg, 2.06 mmol) and NaH (103 mg, 2.6 mmol) were added. The mixture was treated and worked up as described for the preparation of **12b**. The crude product was treated with CH_2N_2 and purified by silica-gel column chromatography ($CHCl_3$-acetone 2:3). Lyophilization of a dioxane solution afforded a colorless solid; 274 mg (66%) $[\alpha]_D^{20}$ +0.4°, $[\alpha]_{365}^{20}$ −11.8° (*c* 1.00, MeOH), ^1H NMR: 3.90 (3H, s, CO_2CH_3), 1.20 (3H, d, J=7 Hz, CH_3 of lactyl residue); FD-MS: *m/z* 801 $(M+H)^+$, 823 $(M+Na)^+$. Found: C, 55.64; H, 7.33; N, 3.45%. Calcd for $C_{38}H_{60}N_2O_{16}\cdot H_2O$: C, 55.73; H, 7.63, N, 3.42%.

Benzyl L-Alanyl-D-isoglutaminate *N*-Acylated with 4-*O*-[2-Acetamido-4,6-*O*-benzylidene-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-β-D-glucopyranosyl]-2-acetamido-2-deoxy-5,6-*O*-isopropylidene-3-*O*-(2-methoxyethoxymethyl)-D-glucose Diethyl Acetal (20). To a solution of **19** (264 mg, 0.33 mmol) in MeOH (3.3 ml) was added 1 M KOH in MeOH (3.3 ml). The mixture was stirred at room temperature for 2 h, acidified with aq citric acid solution. After evaporation of the solvent, the residue was extracted with $CHCl_3$ and worked up as usual. The free acid obtained was dissolved in dry THF and cooled in an ice-bath. To this solution were added benzyl L-alanyl-D-isoglutaminate trifluoroacetate (209 mg, 0.50 mmol), triethylamine (51 mg, 0.50 mmol), *N*-hydroxysuccinimide (58 mg, 0.50 mmol), and DCC (82 mg, 0.40 mmol). The mixture was stirred at 0 °C for 30 min and then at room temperature for 4 h. Usual work-up and purification with a silica-gel column (acetone) followed by lyophilization from dioxane afforded colorless powder; 313 mg (88%); mp 78—85 °C (with sintering at 65 °C); $[\alpha]_D^{17}$ −1.5°, $[\alpha]_{365}^{17}$ −9.3° (*c* 1.00, MeOH); FD-MS: *m/z* 1076 $(M+H)^+$. Found: C, 56.63; H, 7.03; N, 6.38%. Calcd for $C_{52}H_{77}N_5O_{19}\cdot 1.5H_2O$: C, 56.61; H, 7.31; N, 6.35%.

Benzyl L-Alanyl-D-isoglutaminate *N*-Acylated with 2-Acetamido-4-*O*-[2-acetamido-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-β-D-glucopyranosyl]-2-deoxy-D-glucose (21). A solution of **20** (145 mg, 0.13 mmol) in 90% AcOH (5 ml) was heated at 100 °C for 30 min. After cooling and evaporation of the solvent, the residue was subjected to column chromatography on DIAION HP-20 (linear gradient from H_2O to MeOH) and lyophilized from water to give colorless powder; 42 mg (40%): $[\alpha]_D^{11}$ +18.6° (*c* 1.00, H_2O); FD-MS: *m/z* 786 $(M+H)^+$. Found: C, 50.80; H, 6.58; N, 8.47%. Calcd for $C_{34}H_{51}N_5O_{16}\cdot H_2O$: C, 50.80; H, 6.65; N, 8.71%.

L-Alanyl-D-isoglutamine *N*-Acylated with 2-Acetamido-4-*O*-[2-acetamido-2-deoxy-3-*O*-[(*R*)-1-carboxyethyl]-β-D-glucopyranosyl]-2-deoxy-D-glucose (3). Compound **21** (38 mg, 0.048 mmol) was dissolved in H_2O (4 ml) and hydrogenolyzed in the presence of Pd-black catalyst at room temperature. The product was lyophilized from water; 33 mg (quantitative); $[\alpha]_D^{11}$ +18.8° (*c* 1.00, H_2O); FD-MS: *m/z* 718 $(M+Na)^+$. Found: C, 46.50; H, 6.57; N, 9.67%. Calcd for $C_{27}H_{45}N_5O_{16}\cdot 1/4H_2O$: C, 46.32; H, 6.55; N, 10.00%.

Immunoadjuvant Activities. Immunoadjuvant activities of two disaccharide dipeptides, **2** and **3**, were tested in comparison with the muramyl dipeptide **1** by the methods previously described.[18,19] In brief, group of five guinea pigs [weight 300 to 400 g (strain Yodo, Nihon Rabbit Co., Osaka)] were immunized by intrafoot-pad injection of graded amount of test synthetic specimens or the muramyl dipeptide **1** and with 100 μg of ovalbumin (Sigma) incorporated into Freund incomplete adjuvant (Difco). Two and three weeks later, induction of delayed-type hypersensitivity was evaluated by corneal test, in which ovalbumin solution was injected intracorneally. The induction of delayed-type hypersensitivity was also determined by measuring the extent of induration of the skin site which received intracutaneous injection of 0.1 ml of ovalbumin solution (1 mg/ml) on day 26. Anti-ovalbumin precipitating antibody levels in serum were estimated with serum specimen drawn 4 weeks after the immunization by the quantitative precipitin reaction. The results are summarized in Figs. 1, 2, and 3.

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