Preparation of *N*-Acetylmuramyl-L-[U-^{14}C]alanyl-D-isoglutamine *via* a Novel Synthetic Route[1)]

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(Received October 16, 1978)

For the investigation on the action mechanism of immunoadjuvant active *N*-acetylmuramyl-L-alanyl-D-isoglutamine, its labeled compound, *i.e.*, *N*-acetylmuramyl-L-[U-^{14}C]alanyl-D-isoglutamine was synthesized. For this purpose, an entirely novel synthetic route for the muramyl dipeptide was exploited using cold materials and applied to the preparation of the labeled compound. A convenient synthetic procedure of isoglutamine derivatives is also described.

It has been known that the biologically important immune reactions, which participate in the self-defence mechanisms of living bodies against non-self materials, can be stimulated by immunoadjuvant substances such as bacterial cell walls. The common active structure in the cell walls was recently elucidated to be *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**1**) by the synthetic studies by us[2)] and Merser *et al.*[3)] The muramyl dipeptide (**1**) exhibited remarkable activities not only for antibody production and delayed-type hypersensitivity, but also for so many other immunological systems.[4)]

However, in spite of the current importance of the adjuvant activity in the immunological and therapeutic fields, little has been known about the mechanism for this biological action. This seems to be mainly due to the fact that all the hitherto known natural adjuvants are high molecular materials of unknown or complex structures. Now, we can replace the cell walls with synthetic *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**1**) as an effective adjuvant in many immuno reactions. Therefore, preparation of a labeled compound of the muramyl dipeptide (**1**) has been much urged for investigation of its action mechanism. We thus intended to prepare *N*-acetylmuramyl-L-[U-^{14}C]alanyl-D-isoglutamine (**1a**) of extremely high specific radioactivity, since we aimed a whole body autoradiographic study as a preliminary approach to obtain informations for its local distribution. For this reason, the synthesis must be carried out in μmol scale and preferably in a one-flask system starting from the commercial L-alanine labeled uniformly with ^{14}C with highest specific activity available. Our previous method[2)] can not be applied for this purpose because of several technical disadvantages. Thus, a novel synthetic route to the muramyl dipeptide (**1**) was established in this investigation and the labeled compound (**1a**) was successfully prepared by its application. It should be noted that this new route also serves as an alternative method for a facile and convenient preparation of cold muramyl dipeptide (**1**).

The new synthetic route designed for the preparation of *N*-acetylmuramyl-L-[U-^{14}C]alanyl-D-isoglutamine (**1a**) must fulfil the following requirements. i) The synthesis must be throughout performed in a μmol scale without experimental difficulties. ii) Excess reagents should be used in all reaction steps in order to utilize the radioactive fragments as effective as possible. iii) By-products and excess reagents are better removed by simple procedures such as evaporation or extraction so that the products can be subjected to the successive reactions without transfer into other vessels. Taking account of these principles, the basic strategy of the synthesis was adopted first to prepare a free dipeptide, L-alanyl-D-isoglutamine (**2**), and then to couple it with a muramic acid derivative by means of an active ester method. Only acid-labile protective groups were employed in order to avoid the procedure of hydrogenolysis where an adsorption to the catalyst might occur causing considerable loss of materials.

The synthetic route based on the above principles was first examined with cold materials in preparative scales. The dipeptide, L-alanyl-D-isoglutamine (**2**) was prepared from *t*-butoxycarbonyl(Boc)-L-alanine (**3**) and D-isoglutamine *t*-butyl ester (**5**). To avoid the undesirable dehydration of the isoglutamine moiety by excess condensation reagent, Boc-L-alanine was converted into 1-succinimidyl ester (**4**) in advance and then coupled with **5** giving Boc-L-alanyl-D-isoglutamine *t*-butyl ester (**6**). The product was then treated with trifluoroacetic acid (TFA) to afford pure dipeptide (**2**) TFA salt. This was identified with the authentic sample obtained previously.[2)]

The following alternative pathway for the preparation of the dipeptide (**2**) was also examined. Thus, an isoglutamine derivative in which 4,4′-dimethoxybenzhydryl group[5)] was employed for amide protection, *i.e.*, D-glutamic acid α-4,4′-dimethoxybenzhydrylamide γ-*t*-butyl ester (**7**), was condensed with Boc-L-alanine (**3**) by means of dicyclohexylcarbodiimide (DCC)–*N*-hydroxysuccinimide (HONSu). However, several unknown by-products were formed in this coupling reaction unexpectedly and the pure protected dipeptide, Boc-L-alanyl-D-glutamic acid α-4,4′-dimethoxybenzhydrylamide γ-*t*-butyl ester (**8**), could be obtained only after repeated recrystallization. Although **8** could be converted into the free dipeptide (**2**) by action of dry hydrogen chloride in acetic acid, this second route seemed not to be advantageous for the purpose of preparation of the labeled compound because of the difficulty of purification of **8**.

Nevertheless, the preparative methods of the D-isoglutamine derivatives (**5** and **7**) used in the above syntheses are worthwhile to be described here, since they were both prepared from benzyloxycarbonyl(Z)-D-isoglutamine (**9**) which was obtained *via* a new convenient procedure. Ammonia gas was introduced into a solution of Z-D-glutamic acid anhydride in THF, and the resulting mixture of α- and γ-amide was recrystallized

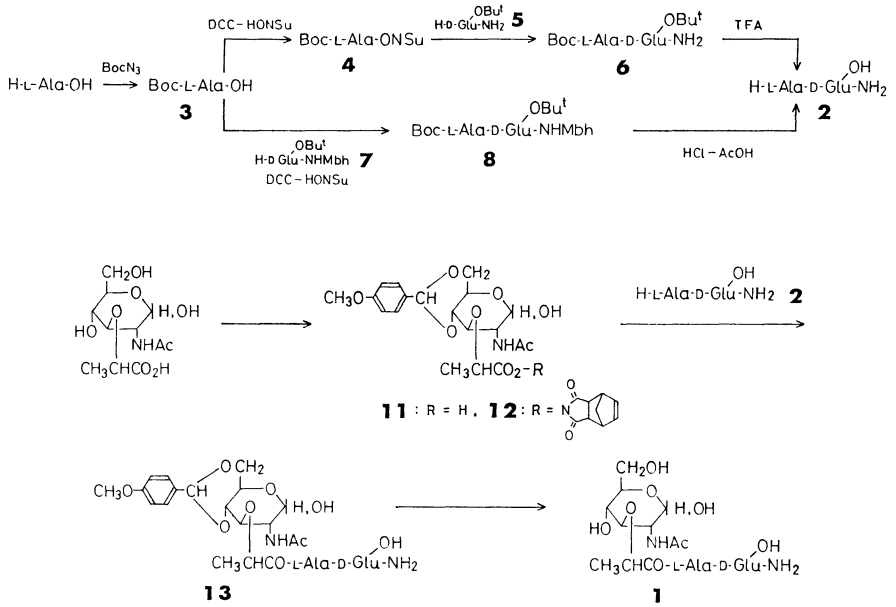
from methanol–ether. Pure Z-D-isoglutamine (**9**) ammonium salt was readily isolated in 64% yield after this simple recrystallization, while isomeric Z-D-glutamine remained in the mother liquor.[6] Hydrogenolysis of **9** followed by the perchloric acid-catalyzed transesterification with *t*-butyl acetate afforded D-isoglutamine *t*-butyl ester (**5**). D-Glutamic acid α-4,4′-dimethoxybenzhydrylamide γ-*t*-butyl ester (**7**) was also obtained as follows. Condensation of **9** with 4,4′-dimethoxybenzhydrol in the presence of sulfuric acid afforded Z-D-glutamic acid α-4,4′-dimethoxybenzhydrylamide (**10**). Hydrogenolytic removal of Z-group[7] in **10** followed by transesterification with *t*-butyl acetate afforded **7** in a good yield.

For the coupling of the muramic acid moiety with the dipeptide (**2**) obtained by the first method described above, *N*-hydroxy-5-norbornene-2,3-dicarboximide (HONb)[8] was employed as a carboxyl activating agent, which was known to give good condensation yields particularly in aqueous media.[8,9] In order to prevent a formation of the undesirable intramolecular ester in the muramic acid moiety, its 4-hydroxyl group was protected with the *p*-anisylidene group, which can be removed afterward under a very mild acidic conditions. Thus, *N*-acetylmuramic acid was treated with *p*-anisaldehyde dimethyl acetal in *N*,*N*-dimethylformamide (DMF) in the presence of *p*-toluenesulfonic acid to give 4,6-*O*-*p*-anisylidene-*N*-acetylmuramic acid (**11**), which was then converted into HONb ester (**12**) in a usual manner. It was found to be advantageous to transform **11** without isolation directly into the active ester (**12**), since the *p*-anisylidene group was so highly acid-labile that it was slowly but spontaneously cleaved at **11** by action of the free carboxyl group on the own molecule.

The coupling of the muramic acid active ester (**12**) with the peptide moiety and the successive deprotection to the free muramyl dipeptide (**1**) were performed as follows. A solution of L-alanyl-D-isoglutamine (**2**) in aqueous dioxane was treated with an excess of the *p*-anisylidenemuramic acid active ester (**12**) until all the dipeptide was consumed. Evaporation of the solvent afforded a mixture of 4,6-*O*-*p*-anisylidene-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**13**) and 4,6-*O*-*p*-anisylidene-*N*-acetylmuramic acid (**11**).[10] The mixture was then treated with aqueous acetic acid to remove the anisylidene group. After evaporation of the solvent, the residue was subjected to preparative TLC on silica gel, and the desired *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**1**) was separated from *N*-acetylmuramic acid and other by-products by developing with 1-butanol–acetic acid–butyl acetate–water (80: 20: 7: 40). The final product (**1**) thus obtained was identified with the sample prepared in the previous work[2] in respects of TLC, NMR, and $[\alpha]_D$. In this preparative scale synthesis, the muramyl dipeptide (**1**) was obtained in a fairly good overall yield (41%) from the Boc-L-alanine active ester (**4**).

An application of this novel route to a small scale synthesis was next examined using still cold materials. Thus, a pyrex test tube (15×130 mm) with a standard ground-glass joint was used as a reaction flask. Extraction and evaporation of solvents were carried out by the procedures described in the experimental section.

L-Alanine could be quantitatively converted into the Boc derivative in μmol scale by use of Boc azide. The transformation from Boc-L-alanine (**3**) (1.89 mg, 10 μmol) to L-alanyl-D-isoglutamine (**2**) was then investigated in a one-flask procedure *via* three steps. DCC was also used in this experiment for the preparation of the active ester (**4**). *N*,*N*′-Dicyclohexylurea formed in the reaction course was removed in the final step after deprotection of the dipeptide.[11] The all operations could be carried out without difficulty as described in



Boc = *t*-butoxycarbonyl
Mbh = 4,4′-dimethoxybenzhydryl
HONSu = *N*-hydroxysuccinimide
Bu^t = *t*-butyl
DCC = dicyclohexylcarbodiimide
Ac = acetyl

the experimental section. The chromatographically pure free dipeptide (**2**) was obtained in a total yield of 90% from Boc-L-alanine as calculated on the basis of amino acid analysis.

TLC examination revealed that the successive steps up to the muramyl dipeptide (**1**) from **2** could be also achieved satisfactorily even in a small scale experiment. Therefore, the new synthetic route became promissing for the preparation of labeled muramyl dipeptide (**1a**) in an extremely small scale. However, in order to apply the new procedure to a radioactive material, one more preliminary experiment prior to the final one was tried starting from diluted labeled L-alanine (10 μmol, 30 μCi) according to the same procedure. In this synthesis, overall 26% (7.7 μCi) of labeled muramyl dipeptide (**1a**) was obtained. The radiochemical purities of **2a** and **1a** were confirmed by a combined technique of TLC and a liquid scintillation counter.

On the basis of the successful results in the series of above experiments, the ultimate synthesis of the strongly labeled compound *via* the novel route was finally carried out. The commercial L-[U-^{14}C]alanine with highest specific activity available[12] was used as a starting material without dilution. All operations except the removal of *N,N'*-dicyclohexylurea were carried out in one test tube without transfer of labeled intermediates to other vessels. The procedures of actual synthesis are described in the experimental section. From 0.97 mCi of L-[U-^{14}C]alanine, *N*-acetylmuramyl-L-[U-^{14}C]alanyl-D-isoglutamine (**1a**) was obtained in a total yield of 46% (0.45 mCi) after purification with TLC.

Experimental

All melting points are uncorrected. TLC was performed on silica gel G, Merck, unless otherwise stated. For each synthesis of the labeled compound as well as the preliminary experiment with cold material in a μmol scale, all operations were carried out in a pyrex test tube (15×130 mm) with a standard ground-glass stopper. Extraction was carried out by mixing two phases in the test tube by means of an electric vibrator and then withdrawing the upper phase with a pipette. The solvent was evaporated by introduction of air stream through a capillary tube on surface of the solution in the tube under reduced pressure.

Boc-L-alanyl-D-isoglutamine t-*Butyl Ester (6).* *t*-Butoxycarbonyl(Boc)-L-alanine 1-succinimidyl ester (**4**) (0.86 g, 3.0 mmol) was added to an ice-cooled mixture of D-isoglutamine *t*-butyl ester (**5**) perchlorate[13] (0.96 g, 3.0 mmol) and triethylamine (0.42 ml, 3.0 mmol) in THF (20 ml). The mixture was stirred at room temperature overnight and then the solvent was evaporated *in vacuo*. The residue was taken up in ethyl acetate and worked up as usual. The product was crystallyzed from ethyl acetate–ether–hexane; yield, 0.93 g (83%); mp 132—134 °C. A sample for elemental analysis was obtained by further recrystallization from ethyl acetate–hexane; mp 132.5—133.5 °C; $[\alpha]_D^{26}$ −0.23°, $[\alpha]_{365}^{26}$ +3.94° (*c* 2.13, ethyl acetate).

Found: C, 54.75; H, 8.37; N, 11.11%. Calcd for $C_{17}H_{31}O_6N_3$: C, 54.67; H, 8.37; N, 11.25%.

L-Alanyl-D-isoglutamine (2) Trifluoroacetate from 6. Compound **6** (0.186 g, 0.50 mmol) was dissolved in trifluoroacetic acid (1.0 ml) and allowed to stand at room temperature for 30 min. Abs ether was added to the mixture to afford hygroscopic white powder, which was collected by filtration; yield, 166 mg (quantitative); mp 72 °C (dec) (with sintering at around 55 °C and resolidifying thereafter); $[\alpha]_D^{26}$ +18.7° (*c* 1.98, H_2O).[14]

Boc-L-alanyl-D-glutamic Acid α-4,4'-Dimethoxybenzhydrylamide γ-t-Butyl Ester (8). One molar solution of triethylamine in dioxane (0.45 ml, 0.45 mmol) was added to a stirred mixture of Boc-L-alanine (**3**) (0.076 g, 0.40 mmol), D-glutamic acid α-4,4'-dimethoxybenzhydrylamide γ-*t*-butyl ester (**7**) hydrochloride[13] (0.200 g, 0.43 mmol) and *N*-hydroxysuccinimide (HONSu) (0.058 g, 0.50 mmol) in dioxane (10 ml) and tetrahydrofuran (THF) (5 ml). After addition of dicyclohexylcarbodiimide (DCC) (0.103 g, 0.50 mmol) under ice-cooling, the mixture was stirred at room temperature overnight. Usual work-up and crystallization from methanol–acetone–water afforded a crude product, which was recrystallized twice from acetone–water; yield, 0.151 g (63%); mp 146—147 °C. A pure sample was only obtained after two more repetitions of recrystallization from ethyl acetate–hexane; mp 146.5—147.5 °C; $[\alpha]_D^{28}$ −3.3° (*c* 1.94, ethyl acetate).

Found: C, 64.18; H, 7.58; N, 7.01%. Calcd for $C_{33}H_{45}O_8N_3$: C, 64.09; H, 7.56; N, 7.01%.

L-Alanyl-D-isoglutamine (2) from 8. Compound **8** (6.0 mg, 0.01 mmol) was dissolved in 2 M dry HCl in acetic acid (1.0 ml, 2 mmol) and allowed to stand at room temperature for 40 h. After evaporation of the solvent *in vacuo*, the residue was dissolved in water and washed with ether. Evaporation of water *in vacuo* afforded **2** hydrochloride. This was identified with a sample obtained above from **6** by means of TLC (1-butanol–acetic acid–water, 4: 1: 2) and amino acid analysis.

Z-D-isoglutamine (9). DCC (5.49 g, 26.6 mmol) was added to an ice-cooled solution of Z-D-glutamic acid (6.23 g, 22.2 mmol) in THF (50 ml) with stirring. After the mixture had been stirred in an ice bath for 7 h, *N,N'*-dicyclohexylurea was filtered off. The filtrate was again cooled in an ice-salt bath, and ammonia gas was introduced with stirring for 15 min. After evaporation of the solvent *in vacuo*, the residue was crystallized from methanol–ether to give pure **9** ammonium salt; yield, 4.21 g (64%).

This ammonium salt (4.00 g, 13.5 mmol) was well ground and shaken with ethyl acetate (200 ml) and 1 M HCl (20 ml). The organic layer was washed with 1 M HCl and water, then dried over $MgSO_4$ and concentrated *in vacuo*. The colorless crystals were collected by filtration; yield, 3.29 g (87%); mp 174.5—175.5 °C. These were recrystallized from methanol–ether–hexane; mp 175—176 °C; $[\alpha]_D^{30}$ +5.61° (*c* 2.05, methanol)

Found: C, 55.60; H, 5.74; N, 9.95%. Calcd for $C_{13}H_{16}O_5N_2$: C, 55.71; H, 5.75; N, 10.00%.

D-Isoglutamine t-*Butyl Ester (5) Perchlorate.* Z-D-isoglutamine (**9**) (3.30 g, 11.8 mmol) was hydrogenolyzed in a mixture of methanol (100 ml) and water (60 ml) in the presence of palladium black catalyst. After usual work-up, recrystallization from water–acetone afforded D-isoglutamine; yield, 1.62 g (94%); mp 188.5—189 °C (dec). This compound (1.62 g, 11.1 mmol) was added to *t*-butyl acetate (150 ml) with stirring in the presence of 70% perchloric acid (1.5 ml, 13 mmol) at room temperature. As the starting material dissolved in about 30 min, another colorless crystals separated out slowly. After 18 h, the crystals were collected by filtration and recrystallized from methanol–ether; yield, 2.93 g (82% from **9**); mp 141—142 °C (dec); $[\alpha]_D^{26}$ −13.6° (*c* 2.00, H_2O).

Found: C, 35.65; H, 6.38; N, 9.17; Cl, 11.65%. Calcd for $C_9H_{18}O_3N_2\cdot HClO_4$: C, 35.71; H, 6.33; N, 9.25; Cl, 11.71%.

Z-D-glutamic Acid α-4,4'-Dimethoxybenzhydrylamide (10). To a solution of **9** (1.40 g, 5.0 mmol) in acetic acid (20 ml) was added 4,4'-dimethoxybenzhydrol (1.22 g, 5.0 mmol) and conc H_2SO_4 (0.02 ml). The mixture was stirred at room temperature overnight. Addition of abs ether afforded a

crystalline material, which was filtered and recrystallized from THF–hexane; yield, 2.04 g (81%); mp 209.5—210.5 °C; $[\alpha]_D^{26}$ −4.6° (*c* 2.05, THF).

Found: C, 66.51; H, 6.01; N, 5.46%. Calcd for $C_{28}H_{30}O_7N_2$: C, 66.39; H, 5.97; N, 5.53%.

D-Glutamic Acid α-4,4'-Dimethoxybenzhydrylamide γ-t-Butyl Ester (7) Hydrochloride. Compound **10** (2.53 g, 5.0 mmol) was suspended in acetic acid (25 ml) and hydrogenolyzed in the presence of palladium black catalyst. After evaporation of the solvent, the residue was crystallized from ethanol–ether to afford D-glutamic acid α-4,4'-dimethoxybenzhydrylamide as hygroscopic powder; yield, 1.55 g (83%).

This compound (0.74 g, 2.0 mmol) was dissolved in *t*-butyl acetate (20 ml) and stirred at room temperature in the presence of 70 % perchloric acid (0.24 ml, 2.1 mmol) for 3 days. After addition of ethyl acetate, the mixture was washed successively with saturated aqueous solution of $NaHCO_3$ and NaCl, dried and evaporated *in vacuo*. The residual syrup was dissolved in abs ether, and 2 M dry HCl in ethyl acetate (1.0 ml, 2.0 mmol) was added. Addition of hexane to the mixture afforded **7** hydrochloride, which was recrystallized from methanol–ether–hexane; yield, 0.31 g (33%); mp 210 °C (dec); $[\alpha]_D^{26}$ −6.0° (*c* 1.01, methanol).

Found: C, 61.37; H, 7.16; N, 5.86; Cl, 7.60%. Calcd for $C_{24}H_{32}O_5N_2 \cdot HCl \cdot 1/4H_2O$: C, 61.40; H, 6.98; N, 5.97; Cl, 7.55%.

4,6-O-p-Anisylidene-N-acetylmuramic Acid (11). *p*-Toluenesulfonic acid (27 mg, 0.14 mmol) was dissolved in a mixture of DMF and benzene (1: 1, 10 ml). From this solution, benzene was evaporated *in vacuo* to remove water completely. To the remaining solution, *N*-acetylmuramic acid[15] (0.40 g, 1.4 mmol) and *p*-anisaldehyde dimethyl acetal (0.95 g, 5.2 mmol) were added. The mixture was kept on a rotary evaporator at 15 mmHg and 40 °C for 2.5 h to remove resulting water, and then most of DMF was evaporated at 3 mmHg. The residue was dissolved in ether, washed with water and dried over $MgSO_4$. After evaporation of the solvent *in vacuo*, benzene was added to the residue. The gelatinous solid formed was filtered and recrystallized from methanol–ether–hexane; yield, 0.31 g (54%); mp 192 °C (dec).

Found: C, 55.65; H, 6.15; N, 3.31%. Calcd for $C_{19}H_{25}O_9N$: C, 55.47; H, 6.13; N, 3.40%.

4,6-O-p-Anisylidene-N-acetylmuramic Acid N-Hydroxy-5-norbornene-2,3-dicarboximide (HONb) Ester (12). DCC (0.17 g, 0.83 mmol) was added to an ice-cooled solution of **11** (0.28 g, 0.68 mmol) and HONb (0.15 g, 0.84 mmol) in THF (10 ml). The mixture was stirred overnight at room temperature. *N,N'*-dicyclohexylurea was filtered off and the solvent was evaporated *in vacuo*. The residue was dissolved in ethyl acetate, washed successively with saturated aqueous solution of $NaHCO_3$ and NaCl, and then dried over $MgSO_4$. After evaporation of the solvent, the residue was subjected to silica gel column chromatography. Elution with $CHCl_3$–acetone (4: 1) and complete evaporation of the solvent afforded pure **12** as white powder; yield, 0.17 g (44%); mp 115 °C.

In an alternative way, the same compound (**12**) was prepared directly from *N*-acetylmuramic acid without isolation of the anisylidene derivative (**11**) as the intermediate. Thus, after formation of **11**, the reaction mixture was neutralized with triethylamine, and then treated with HONb and DCC. In this case, starting from *N*-acetylmuramic acid (0.15 g, 0.51 mmol), **12** was obtained in a rather better yield (0.17 g, 58 %).

N-Acetylmuramyl-L-alanyl-D-isoglutamine (1). L-Alanyl-D-isoglutamine (**2**) trifluoroacetate (54 mg, 0.16 mmol) was dissolved in water (0.5 ml). To this solution were added 1 M triethylamine in dioxane (0.32 ml, 0.32 mmol) and **12** (92 mg, 0.16 mmol) in dioxane (0.6 ml). The mixture was allowed to stand at room temperature for 20 h, then 1 M triethylamine in dioxane (0.30 ml, 0.30 mmol) and **12** (79 mg, 0.14 mmol) in dioxane (0.5 ml) were again added. After the mixture had been allowed to stand at room temperature for further 20 h, the solvent was removed *in vacuo*. The residue was dissolved in a mixture of acetic acid (6 ml) and water (3 ml) and left stand at room temperature for 18 h. After removal of the solvent *in vacuo*, the residue was dissolved in water and washed with ether. Evaporation of the aqueous phase *in vacuo* afforded a pale yellow syrup, which was subjected to preparative TLC; commercial pre-coated silica gel plates were used (seven plates of Wako-gel F_{254}, 0.25 mm thickness, 20×20 cm, Wako Pure Chemicals Co., Ltd.)[16] with a solvent system of 1-butanol–acetic acid–butyl acetate–water (80:20:7:40). The main band was extracted with 80% ethanol, the solvent was evaporated *in vacuo* and the residue was triturated with 99% ethanol. After removal of the insoluble material, ethanol was again evaporated *in vacuo*. The remaining substance was dissolved in water and passed through a short column of Amberlite IRC50 (H^+ form). Lyophilization of the eluate afforded **1**; yield, 39 mg (50% from **2**); $[\alpha]_D^{25}$ +34.4° (*c* 0.550, H_2O after equilibration). This product was identified by TLC and NMR spectrum with the sample ($[\alpha]_D^{15}$ +33.1° (*c* 0.51, H_2O)) obtained previously.[2]

Small Scale Preparation of L-Alanyl-D-isoglutamine (2) from Boc-L-alanine. To a solution of Boc-L-alanine (1.89 mg, 10 μmol) and HONSu (1.96 mg, 17 μmol) in THF (0.27 ml) was added DCC (3.57 mg, 17 μmol) in THF (0.17 ml) under ice-cooling. After the mixture had been allowed to stand overnight, the solvent was evaporated *in vacuo*. The residue was dissolved in $CHCl_3$ (1.5 ml) and the excess DCC was decomposed with acetic acid. The solution was then washed with aqueous $NaHCO_3$ and water. After evaporation of the solvent *in vacuo*, $CHCl_3$ (1 ml) was added to the residue and evaporated again *in vacuo* to remove the remaining water. To the residue were added D-isoglutamine *t*-butyl ester (**5**) perchlorate (5.6 mg, 18.5 μmol) and 0.1 M solution of triethylamine in $CHCl_3$ (0.20 ml, 20 μmol). The mixture was allowed to stand at room temperature for 20 h, then diluted with $CHCl_3$ (1.5 ml) and washed successively with aqueous $NaHCO_3$, 1 M HCl and water. The residue obtained after evaporation of the solvent *in vacuo* was dissolved in TFA (1 ml). After 1.5 h at room temperature, TFA was removed *in vacuo*, the residue was dissolved in water, and insoluble *N,N'*-dicyclohexylurea was removed by filtration through a sintered glass filter. Evaporation of water from the filtrate afforded **2** TFA salt, which showed a single ninhydrin-positive spot on TLC. The yield was deduced to be 90% by means of amino acid analyser.

N-Acetylmuramyl-L-[U-^{14}C]alanyl-D-isoglutamine (1a). To a solution of L-[U-^{14}C]alanine (0.97 mCi, 5.9 μmol)[12] in water (0.5 ml) were added 0.1 M solution of triethylamine in dioxane (0.5 ml, 50 μmol) and Boc azide (10 μl, 74 μmol). While the mixture was allowed to stand at room temperature for 50 h, Boc azide (74 μmol×2) and triethylamine (20 μmol) were again added. After evaporation of the solvent, the residue was dissolved in water (1 ml) and washed with ether. After complete removal of water by coevaporation with ethanol and $CHCl_3$, dry HCl (4.8 μM) in THF (0.12 ml) and HONSu (1.15 mg, 10 μmol) in THF (0.1 ml) were added to the residue. To this mixture was added DCC (2.1 mg, 10 μmol) in THF (0.1 ml) under cooling in an ice bath. Thereafter, the mixture was treated in the same manner as described above for the cold material. Thus, the active ester (**4a**) was coupled with **5** HCl salt (2.4 mg, 10 μmol) in $CHCl_3$ in the presence of triethylamine and the resulting protected dipeptide (**6a**) was

treated with TFA (1 ml). After *N,N'*-dicyclohexylurea had been removed by filtration, the filtrate was condensed to give the free dipeptide (**2a**), which was again dissolved in water (0.3 ml). To this solution were added 4,6-*O*-*p*-anisylidene-*N*-acetylmuramic acid HONb ester (**12**) (3.6 mg, 6.3 μmol) in dioxane (0.3 ml) and triethylamine (60 μmol). During next 26 h, two portions of **12** (6.1 and 3.2 μmol) were added and the final mixture was left to stand overnight. The solvent was then removed by evaporation, and the residue was treated with 66 % aqueous acetic acid (1 ml) for 6 h. After evaporation, the residue was dissolved in water (1 ml), washed with ether and again evaporated. The residue was dissolved in methanol and subjected to preparative TLC as described for the preparation of cold **1** using one 20×20 cm plate. The location of the radioactive substance on the plate was detected with a Geiger-Müller counter and the corresponding band was extracted with 80% ethanol to give chromatographically pure **1a**; total activity 0.45 mCi (overall 46 %).

References

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6) We also observed a preferential crystallization of Boc-α-glutamylglycine benzyl ester dicyclohexylammonium salt to the corresponding γ-peptide derivative.[9]

7) Almost no transesterification occurred when **10** itself was treated with *t*-butyl acetate and perchloric acid probably due to steric hindrance of the two voluminous protective groups.

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10) Because of the high acid-lability of the *p*-anisylidene group (see above in the text), attempts to isolate pure **11** led to loss of the material. Separation of the excess muramic acid component was readily accomplished by preparative TLC after deprotection.

11) Preliminary experiments indicated that use of DCC gave the best result for this reaction even though the insoluble *N,N'*-dicyclohexylurea had to be anyhow removed. With the water-soluble carbodiimide, *i.e.*, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide, the yield of the active ester (**4**) was very low presumably due to the acylurea formation. Other coupling methods such as mixed anhydride were not suitable actually for small scale experiments.

12) 164 mCi/mmol, 87 % isotopic abundance in all carbon atoms; supplied by The Radiochemical Centre, Amersham.

13) Preparation of this compound is described below.

14) The same compound, **2** TFA salt, was obtained by action of TFA on Boc-L-alanyl-D-isoglutamine;[2b] mp 72 °C (dec) (with sintering at 52 °C and resolidifying); $[\alpha]_D^{26}$ +18.4° (*c* 1.96, H_2O).

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16) Good separation of **1** and *N*-acetylmuramic acid was not achieved with pre-coated silica gel 60 plate, Merck.