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SYNTHESIS OF *N*-ACETYLMURAMYL-L-[U-^{14}C]ALANYL-D-ISOGLUTAMINE[1)]

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Bacterial cell wall has a unique immuno potentiating ability, *i.e.*, "immuno-adjuvant" activity, whether it is a Gram-positive or -negative. As results of synthetic studies by us[2)] as well as Meser *et al.*[3)], it was revealed that *N*-acetylmuramyl-L-alanyl-D-isoglutamine (1) involved in cell wall peptidoglycan of many bacteria as common moiety is the smallest structure required for the activity. Thus, synthetic muramyl dipeptide (1) distinctly stimulates the antibody production and induces delayed hypersensitivity to protein antigens. Meanwhile so many derivatives and analogs of 1 were prepared to investigate the significance of the structure.[2b)] Among them, 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine was found to possess a remarkable antitumor activity.[4)]

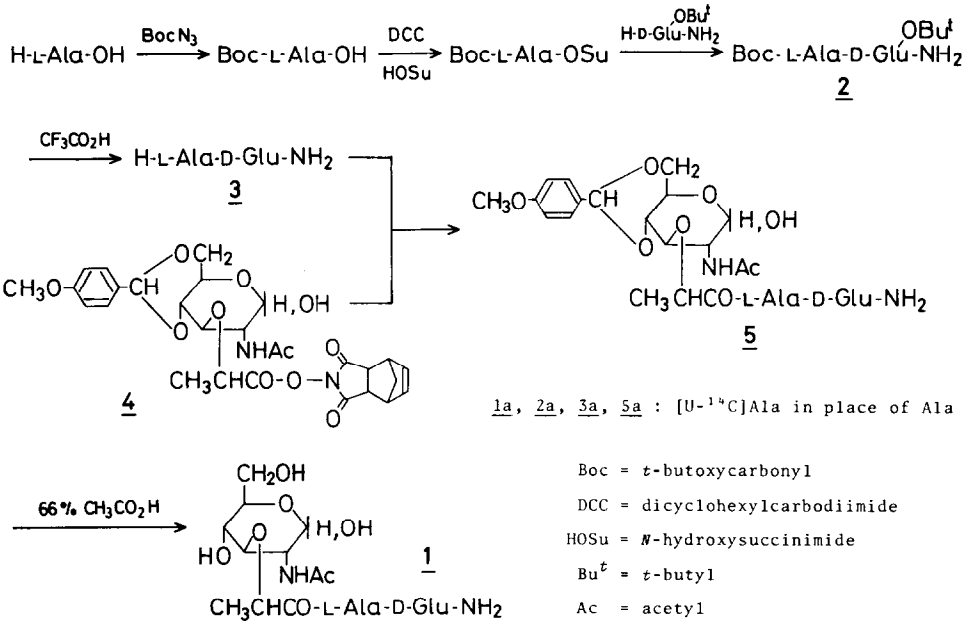
In view of importance of the activities, we were urged to elucidate the action mechanism of such adjuvant substances which has been not yet clarified at all. For this purpose, we synthesized a labelled compound of 1, *i.e.*, *N*-acetylmuramyl-L-[U-^{14}C]alanyl-D-isoglutamine (1a), in order to investigate its fate *in vivo* after administration in living body. The synthesis starting from L-[U-^{14}C]-alanine of commercially highest specific activity[5)] must be carried out without dilution and in μmole scale to maintain a sufficient radioactivity to the whole body autoradiography in the final product.

Such special requirement forced us to exploit a new preparative method for 1 in the following principles, since the previous method[2b)] could not be applied to a small scale preparation: 1) In all reaction steps excess reagents should be used to utilize the radioactive fragment as effectively as possible, unless they

cause side reactions. 2) The remaining reagents and by-products must be removed by simple procedure such as evaporation or extraction in order to subject the product directly to the successive step. 3) The all reactions are better carried out in one flask without transfer of the contents, to minimize the loss of materials and avoid the danger of contamination. Therefore, for instance, the process of hydrogenolysis could not be used.

Preliminary experiments to fulfil the above requirements were performed with cold materials first in a preparative scale and then in μmole scale. As a result, a novel synthetic method, which is along this line and enough applicable to extremely hot material, was now established as shown in the scheme.[6,7]

Thus, for synthesis of the labelled compound (1a), all operations were carried out in a pyrex test tube (15 x 130 mm) with a standard ground-glass stopper without transfer of labelled intermediates except the removal of *N*,*N*'-dicyclohexylurea. Extraction was carried out by mixing two phases in a test tube by means of an electric vibrator and then withdrawing the upper phase with a pipette. Solvents were evaporated by introduction of air stream through a capillary tube on the surface of solutions under reduced pressure.



Actual synthesis was performed as follows. To a solution of L-[U-^{14}C]-alanine (0.97 mCi, 5.9 μmol) in water (0.5 ml) were added triethylamine (50 μmol) and *t*-butoxycarbonyl azide ($BocN_3$) (10 μl, 74 μmol) in dioxane (0.5 ml). Additional portions of $BocN_3$ (74 μmol x 2) and triethylamine (20 μmol) were supplied within 50 hr. After the solvent had been evaporated, the residue was dissolved in water (1 ml) and washed with ether to remove excess $BocN_3$. After complete removal of water, dry HCl (4.8 μmol) in tetrahydrofuran (THF) (0.12 ml) and *N*-hydroxysuccinimide (10 μmol) in THF (0.1 ml) were added to the residue. To the resultant solution, dicyclohexylcarbodiimide (10 μmol) in THF (0.1 ml) was added under ice-cooling and the mixture was set aside overnight. After evaporation of the solvent, the residue was dissolved in $CHCl_3$ (1.5 ml) and treated with acetic acid (2 drops). The solution was then washed successively with water, aqueous $NaHCO_3$ and water. Evaporation gave a mixture of labelled Boc-L-alanine 1-succinimydyl ester and *N*,*N*'-dicyclohexylurea, to which triethylamine (24 μmol) in $CHCl_3$ (0.24 ml) and D-isoglutamine *t*-butyl ester hydrochloride (2.4 mg, 10 μmol) were added. After 20 hr, the mixture was diluted with $CHCl_3$ (1.1 ml) and washed with aqueous $NaHCO_3$, 1 M HCl and water. The residue obtained after evaporation of the solvent was treated with trifluoroacetic acid (TFA) (1.0 ml) for 1.5 hr. TFA was removed by evaporation and water was added to the residue. The insoluble *N*,*N*'-dicyclohexylurea was filtered off through a sintered glass and the filtrate was evaporated to give the labelled L-alanyl-D-isoglutamine (3a). The active ester of muramic acid, *i.e.*, 4,6-*O*-anisilidene-*N*-acetylmuramic acid 5-norbornene-2,3-dicarboxyimidyl ester (4), (3.6 mg, 6.3 μmol) in dioxane (0.3 ml) and triethylamine (60 μmol) were added to a solution of 3a in water (0.3 ml). During next 26 hr, two portions of 4 (6.1 and 3.2 μmol) were again added and finally the mixture was set aside overnight. The solvent was evaporated, and the residue was treated with 66% acetic acid (1 ml) for 6 hr to remove the anisilidene group. After evaporation, the residue was dissolved in water (1 ml), washed with ether and again evaporated. The final residue was subjected to preparative TLC[8] on a pre-coated silica gel plate (Wako-gel F_{254}, 20 x 20 cm, 0.25 mm thickness) and developed with butanol - acetic acid - water - butyl acetate (80 : 20 : 40 : 7). The position of the radioactive substance on

the plate was detected with a Geiger-Müller counter and the corresponding band (shaded part in Fig. 1) was scraped and extracted with 80% ethanol to give 0.45 mCi (overall 46%) of chromatographically pure *N*-acetylmuramyl-L-[U-^{14}C]alanyl-D-isoglutamine (1a).

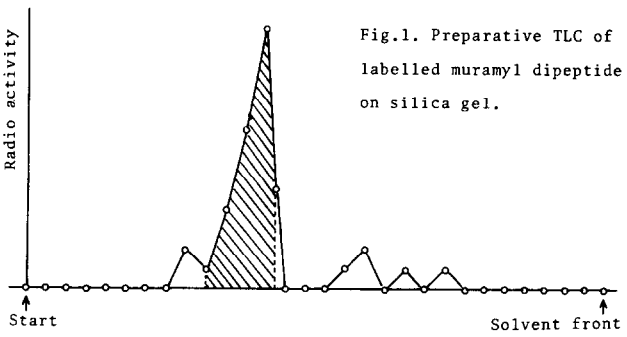


Fig.1. Preparative TLC of labelled muramyl dipeptide on silica gel.

Autoradiographic study on the fate of this substance in living body is now in progress and will be reported elswhere.

References and Footnotes

1) This work was supported by a Grant-in-Aid for Scientific Research from the Ministry of Education and presented at the 36th Annual Meeting of the Chemical Society of Japan, Osaka, April, 1977; p.1233.

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5) 164 mCi/mmol, 87% isotopic abundance in all carbon atoms; supplied by The Radiochemical Centre, Amsterdam.

6) Details of the new synthetic method of the muramyl dipeptide (1) in a preparative scale will be reported elswhere. Both the dipeptide (3) and the final product (1) were identified with the corresponding samples obtained previously.[2b] Satisfactory elemental analyses were obtained for the all intermediates.

7) In a preliminary experiment starting from diluted hot L-alanine (10 μmol, 30 μCi), overall 26% (7.7 μCi) of the labelled muramyl dipeptide (1a) was obtained. The high radiochemical as well as chemical purities of 3a and 1a were confirmed by combination of TLC and liquid scintilation counter.

8) Although the main purpose of the preparative TLC was removal of *N*-acetylmuramic acid and *N*-hydroxy-5-norbornene-2,3-dicarboxyimide arizing from 4, some other impurities with weak activity could be also eliminated by this procedure (see Fig. 1).