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SYNTHESIS OF 6-*O*-MYCOLOYL-*N*-ACETYLMURAMYL-L-ALANYL-D-ISOGLUTAMINE WITH IMMUNOADJUVANT ACTIVITY

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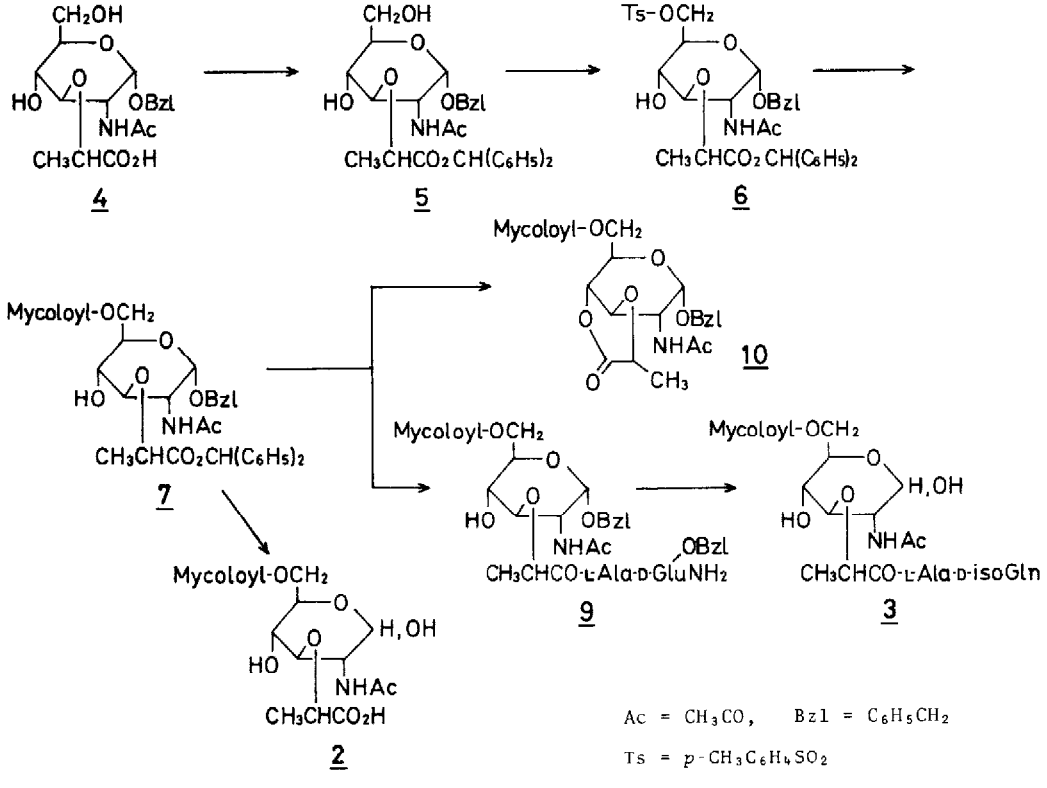
Cell walls of a variety of bacterial species are known to show immunoadjuvant activity. They markedly enhance both humoral and cellular immune responses against various antigens when they are administered together with the antigen. Several investigators have attempted to elucidate the structural entity responsible for this activity by means of enzymatic degradation of cell walls. Ellouz *et al.*[1] and Kotani *et al.*[2] revealed that mono or disaccharide oligopeptide fraction in the degradation products of cell wall peptidoglycan is essential for exhibition of the activity. Subsequently, our group[3] and Meser *et al.*[4] demonstrated independently, introducing the chemical synthesis into this problem, that the minimum structure required for the adjuvant activity is *N*-acetylmuramyl-L-alanyl-D-isoglutamine (1) of rather small but common fragment of cell walls. Combination of the chemical synthesis and immunological test manifested a significant contribution to clarify relationships between the structure and the activity by making possible to employ so many structural analogs of the muramyl peptide (1).[3,5,6]

On the other hand, the BCG cell wall has been often used for immunotherapy of cancer. This material is known to be peptidoglycan linked with mycoloyl arabinogalactan.[7] In our assumption, the antitumor effect of this cell wall

might be attributed mainly to the unique adjuvant activity of the peptidoglycan modified with high lipophilicity of mycolic acids, α-branched β-hydroxy fatty acids of high molecular weight.[8] On the basis of this consideration, we synthesized 6-*O*-mycoloyl-*N*-acetylmuramic acid (2) and 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (3) to examine immunological properties particularly antitumor activities.

Mycolic acid isolated from cells of *Mycobacterium tuberculosis* strain Aoyama B was used in this study without separation into subgroups.[8] An average molecular formula $C_{80}H_{158}O_{3.5}$ was deduced on the basis of acid titration and elemental analysis.

The starting muramic acid derivative, benzyl *N*-acetylmuramide (4),[9] was converted into its diphenylmethyl ester (5) with diphenyldiazomethane[10] (91%; mp 155-156°C; $[\alpha]_D^{22}$ +122° (*c* 1.0, $CHCl_3$)). Such protection of the carboxyl group of muramic acid was preferable not only to prevent undesirable side reactions but



also to facilitate chromatographic purification of the products on subsequent synthetic steps. For the introduction of the mycoloyl group, the simple acid chloride and the trifluoroacetic anhydride methods, which were actually employed in the preparations of other 6-*O*-acyl derivatives,[5] could not be applied since mycolic acid has a β-hydroxyl group sensitive to these reagents. Therefore, we chose the exchange reaction of the tosylate with potassium mycolate.[11] Thus, the diphenylmethyl ester (5) was treated with tosyl chloride (11 mol equivalent) in pyridine under ice cooling for 1 hr and the product was purified by means of column chromatography on silica gel (benzene – ethyl acetate 5 : 1) to afford pure mono-6-*O*-tosylate (6), *i.e.*, 1-α-*O*-benzyl-6-*O*-tosyl-*N*-acetylmuramic acid diphenylmethyl ester, (88%; mp 68-73°C; $[\alpha]_D^{22}$ +84.4° (*c* 0.5, $CHCl_3$)). When the tosylate (6) was treated with potassium mycolate in DMF at 125°C for 19 hr, substitution occurred to afford 1-α-*O*-benzyl-6-*O*-mycoloyl-*N*-acetylmuramic acid diphenylmethyl ester (7) (41%) which was isolated after chromatographic purification on silica gel. This reaction could be appreciably improved by use of crown ether.[12] Thus, by refluxing a benzene solution of the tosylate (6) and potassium mycolate in the presence of a catalytic amount of 18-crown-6 for 3 hr, the mycoloyl diphenylmethyl ester (7) was obtained in 60% yield (mp 54-57°C; $[\alpha]_D^{22}$ +32.6° (*c* 0.5, $CHCl_3$)). Catalytic hydrogenolysis of 7 afforded 6-*O*-mycoloyl-*N*-acetylmuramic acid (2) (87%; mp 49-52°C; $[\alpha]_D^{22}$ +23.4° (after 20 hr, *c* 0.5, THF – H_2O 50 : 1).

The diphenylmethyl group of 7 was selectively removed with trifluoroacetic acid in chloroform to give 1-α-*O*-benzyl-6-*O*-mycoloyl-*N*-acetylmuramic acid (8). This product was then coupled with L-alanyl-D-isoglutamine benzyl ester by means of so-called "Eintopf" method[13] using *N*,*N*'-dicyclohexylcarbodiimide and *N*-hydroxysuccinimide. The desired product, *i.e.*, 1-α-*O*-benzyl-6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine benzyl ester (9) (mp 171-172°C; $[\alpha]_D^{22}$ +30.2° (*c* 0.5, $CHCl_3$)), was obtained in a rather low yield (38%) because of a competitive cyclization of 8 to an internal ester (10) (26%; mp 46-51°C) during the condensation reaction, though this yield could be somewhat improved as follows. When the pentachlorophenyl ester of 8 prepared *in situ* with pentachlorophenyl trichloroacetate[14] was treated with L-alanyl-D-isoglutamine benzyl

ester under ice cooling, formation of the internal ester (10) was suppressed (17%) and the desired product (9) was obtained in 50% yield. The final deprotection of 9 was effected by catalytic hydrogenolysis to afford 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (3), which was reprecipitated from ether – ethanol (92%; mp 137-160°C; $[\alpha]_D^{22}$ +25.8° (after 20 hr, *c* 0.4, THF – H_2O 50 : 1).

Although immunological properties of 2 and 3 are now still under investigation, some important activities relating to antitumor effect were recognized at 3, the details of which were already submitted for publication.[15)]

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