Synthesis of 6-*O*-Mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine with Antitumor Activity[1)]

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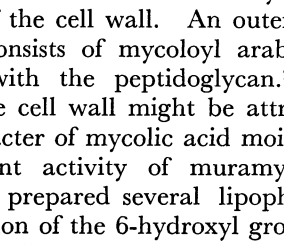
N-Acetylmuramyl-L-alanyl-D-isoglutamine corresponding to the minimum structure responsible for the immunoadjuvant activity of bacterial cell walls was acylated at the 6-hydroxyl group with various mycolic acids of long carbon chains in order to test their possible antitumor effects. Mycolic acids used were extracted from cell walls of *Mycobacterium*, *Nocardia*, and *Corynebacterium*. All the mycoloyl derivatives synthesized show antitumor activity based on a principle of immunotherapy.

In the course of synthetic studies on immunoadjuvant active *N*-acetylmuramyl-L-alanyl-D-isoglutamine (**1**), we attempted to synthesize a novel compound which would exhibit antitumor activity based on the principle of immunotherapy of cancer. Since *Mycobacterium bovis* Bacille de Calmette-Guérin (BCG) cell wall is highly effective in tumor immunotherapy, it was expected that a modification of the structure of **1**, the key substance in immuno reactions, might turn out to be desirable substance by simulation of the structure of the cell wall. An outer layer of the BCG cell wall consists of mycoloyl arabinogalactan which is linked with the peptidoglycan.[2)] The antitumor effect of the cell wall might be attributed to the lipophilic character of mycolic acid moiety connected with the adjuvant activity of muramyl peptide moiety.

We have prepared several lipophilic derivatives of **1** by acylation of the 6-hydroxyl group of the muramic acid residue.[3)] However, no antitumor effect was observed even in 6-*O*-stearoyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine,[4)] which possesses the maximum adjuvant activity among 6-*O*-acyl derivatives prepared so far.[5)] We have thus extended our synthetic program to preparation of more lipophilic derivative coupled with the natural mycolic acid isolated from *Mycobacterium*. 6-*O*-Mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2a**) thus obtained[1)] showed distinct activity in a suppression test of hepatoma in mice.[6)] In the present paper, we report the details of the synthesis of the mycoloyl derivative (**2a**) as well as two other 6-*O*-acyl derivatives of **1** prepared by using mycolic acid analogs originated from cells of *Nocardia* and *Corynebacterium*.

The general name "mycolic acid" represents α-branched β-hydroxylated fatty acids of high molecular weight (Fig. 1). They are involved in mycobacterial cell walls as typical constituents, being always present as a homologous mixture of some subgroups.[7)] The mycolic acid from *Mycobacterium tuberculosis* strain Aoyama B[8)] was found to have an average molecular formula $C_{80}H_{158}O_{3.5}$ on the basis of acid titration and elemental analysis.

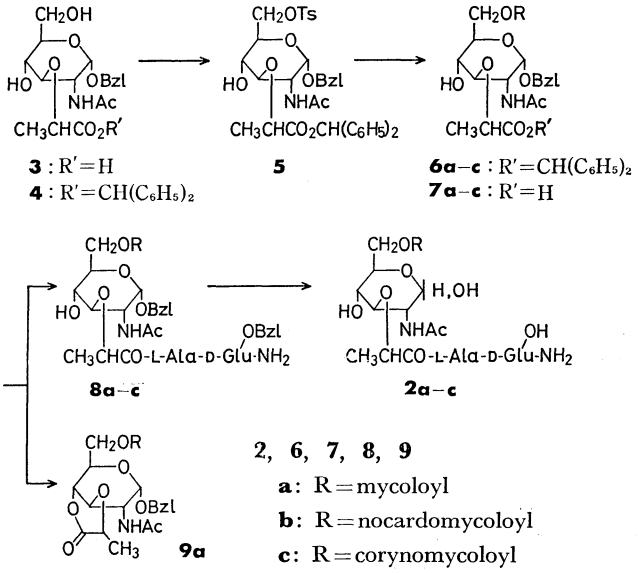


mycolic acid :	m=42—56	n=19—23
nocardomycolic acid :	m=30—40	n= 7—13
corynomycolic acid :	m=10—16	n= 9—15

These values are representative for each group.

Fig. 1. The basic structure of mycolic acid analogs.

The synthetic route for 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2a**) is depicted in the scheme. It differs from the previous method for



the 6-*O*-stearoyl derivative in the following points. For introduction of the mycoloyl group into the muramic acid moiety, the acid chloride method, which was successfully used for the stearoyl derivative, should be avoided because of the presence of β-hydroxyl function in mycolic acid. An exchange reaction of 6-*O*-tosylate of a muramic acid derivative with potassium mycolate was employed for this purpose. Since the reaction conditions in this step are drastic, using a strongly basic reagent at high temperature, the peptide moiety had to be coupled after the reaction.

Benzyl *N*-acetyl-α-muramide (**3**)[3)] was converted with diphenyldiazomethane into the diphenylmethyl ester (**4**), which was then treated with excess tosyl chloride (11 mol equivalent) in pyridine under ice-cooling. Tosylation occurred almost exclusively at

TABLE 1. AVERAGE MOLECULAR WEIGHTS AND MOLECULAR FORMULAE OF MYCOLIC ACID ANALOGS USED IN THIS STUDY

	Average molecular weight[a]	Elemental analysis[b]		Average molecular formula calculated			
		C%	H%		C%	H%	mol wt
Mycolic acid	1180	81.45	13.55	$C_{80}H_{158}O_{3.5}$	81.70	13.54	1176
Nocardomycolic acid	766	79.84	12.76	$C_{51}H_{97}O_{3.6}$	79.77	12.73	768
Corynomycolic acid	483	76.97	12.38	$C_{31}H_{59}O_{3.3}$	76.83	12.27	485

a) Determined by acid titration, see Experimental. b) Mean values of two individual analyses.

the primary hydroxyl group, so that 6-*O*-monotosylate (**5**) was isolated in 89% yield after chromatographic purification. The exchange reaction of the tosylate with potassium mycolate proceeded smoothly in boiling benzene in the presence of 18-crown-6 to afford 1-α-*O*-benzyl-6-*O*-mycoloyl-*N*-acetylmuramic acid diphenylmethyl ester (**6a**). Although this reaction can also be performed without the crown ether in *N*,*N*-dimethylformamide (DMF) at higher temperature,[9] the yield became appreciably low. The diphenylmethyl group in the ester **6a** was then selectively removed with trifluoroacetic acid to give 1-α-*O*-benzyl-6-*O*-mycoloyl-*N*-acetylmuramic acid (**7a**), which was then subjected to the successive coupling reaction with the dipeptide moiety.

When the mycoloyl muramic acid (**7a**) was coupled with L-alanyl-D-isoglutamine benzyl ester[10] by means of the dicyclohexylcarbodiimide (DCC)–*N*-hydroxysuccinimide (HONSu) method, the desired product, 1-α-*O*-benzyl-6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine benzyl ester (**8a**), was obtained. However, the yield was rather low (38%) because of a competitive formation of an intramolecular ester (**9a**) (26%). The situation was somewhat improved by employing the following alternative procedure, in which an active ester of **7a** was prepared by ester-exchange reaction without use of dehydrating agent.[11] Thus, when **7a** was converted with pentachlorophenyl trichloroacetate into the pentachlorophenyl ester and then treated with the dipeptide benzyl ester under ice-cooling, formation of the internal ester (**9a**) was suppressed (17%) and the desired product (**8a**) was obtained in 47% yield. The final hydrogenolytic deprotection of **8a** was carried out in tetrahydrofuran (THF) in the presence of palladium black catalyst to afford 6-*O*-mycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2a**) in a good yield.

The mycoloyl-*N*-acetylmuramyl dipeptide (**2a**) thus obtained gave a positive antitumor effect.[6] When a suspension of **2a** in phosphate-buffered saline was administered intradermally with MH134 hepatoma cells to syngeneic mice, a distinct suppression of the tumor growth was observed. Accordingly, we synthesized two more analogs of **2a** with use of nocardomycolic acid and corynomycolic acid with different ranges of carbon numbers in order to estimate the influence of chain length in fatty acids on the antitumor activity.

Cells of *Nocardia* and *Corynebacterium*, closely related to *Mycobacterium* taxonomically, contain nocardomycolic (or nocardic) and corynomycolic acid, respectively, which have similar α-branched β-hydroxylated structures but with smaller carbon numbers than in the mycolic acid from *Mycobacterium*. Extraction of cells of *Nocardia asteroides* 131 and *Corynebacterium diphtheriae* PW8 afforded the corresponding nocardomycolic and corynomycolic acid, whose average molecular formulae were deduced to be $C_{51}H_{97}O_{3.6}$ and $C_{31}H_{59}O_{3.3}$, respectively, on the basis of elemental analyses and acid titrations. Average molecular weights and molecular formulae of the mycolic acid analogs are given in Table 1.

TABLE 2. SUPPRESSION OF MH-134 HEPATOMA WITH 6-*O*-MYCOLOYL-*N*-ACETYLMURAMYL DIPEPTIDES (**2a**—**c**)[6]

Compound	Dose (μg)	No. of mice tested	Tumor-free mice /mice survived[a]
2a	20	10	3/8
2b	20	10	5/9
2c	20	10	6/7
Control[b]	—	10	0/1

a) At 49 days. b) Only tumor cells were inoculated.

The syntheses of nocardomycoloyl- and corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2b** and **c**) were performed in a similar way to that for **2a**. In the coupling reaction of nocardomycoloyl- and corynomycoloyl-*N*-acetylmuramic acid (**7b** and **c**) with the dipeptide moiety, 1-α-*O*-benzyl-6-*O*-nocardomycoloyl- and corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine benzyl ester (**8b** and **c**) could be obtained in better yields as compared to that for mycoloyl derivative (**8a**). In these cases, the intramolecular esters of type **9** were not formed from **7b** and **c**, although the DCC–HONSu method was employed under similar conditions to those for the coupling of mycoloyl derivative (**7a**).[12] Hydrogenolysis of **8b** and **c** afforded the final products, 6-*O*-nocardomycoloyl and corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine (**2b** and **c**). However, one of the two benzyl groups in **8b** and **c** resisted the cleavage at room temperature below 20 °C in this reaction. Thus, hydrogenolysis was carried out at 28—30 °C for 3 or more days to complete deprotection.[13,14]

The results of an antitumor test of the compounds synthesized in this study are summarized in Table 2.[6] Nocardomycoloyl and corynomycoloyl muramyl dipeptides (**2b** and **c**) also showed similar or even stronger activity to mycoloyl derivative (**2a**). Although the activity seemed to be less significant than natural BCG cell wall and not sufficient for practical use, this is the first case in which synthetic substances suppressed growth of a tumor on the basis of their im-

munoadjuvant activities. In order to clarify the role of the unique structure of mycolic acid, we are now preparing other acyl derivatives of *N*-acetylmuramyl dipeptide (**1**) using various kinds of synthetic fatty acids of high molecular weights.

Experimental

All melting points are uncorrected. Silica gel 60 (0.063-0.2 mm) and silica gel G, Merck, were used for column chromatography and TLC, respectively.

Preparation of Mycolic Acid, Nocardomycolic Acid, and Corynomycolic Acid from the Cells of Mycobacterium tuberculosis *Aoyama B*, Nocardia asteroides *131, and* Corynebacterium diphtheriae *PW8*. The cells[15] were defatted by repeated extraction with ether–ethanol (1:1) and $CHCl_3$–methanol (2:1), and then hydrolyzed by heating with 2.5% KOH in benzene–methanol (1:1) under reflux for 23 h. After the reaction mixture had been filtered, the filtrate was concentrated to dryness *in vacuo*, acidified with HCl and extracted with hexane. The solvent was removed from the extract by evaporation and the residue was heated in 2.5% HCl–methanol for 5 h. The products were subjected to column chromatography on silica gel and eluted with hexane–ether to afford methyl mycolates (1.7—2.2% from defatted cells). The free acids could be regenerated by hydrolysis of the corresponding methyl esters with 3 M KOH in methanol–benzene under reflux for 3 h.

Determination of the Average Molecular Formulae of Mycolic Acids and Preparation of Their Potassium Salts. i) Mycolic Acid: Mycolic acid (0.500 g) from *Mycobacterium* was dissolved in $CHCl_3$ (5 ml) and titrated with 0.176M KOH in methanol against phenolphthalein. After the solvent had been evaporated *in vacuo*, the residual potassium mycolate was washed with methanol and filtered; yield, 0.51 g (99%).

From the amount of alkali consumed (2.41 ml), the average molecular weight was deduced to be 1180. The average molecular formula $C_{80}H_{158}O_{3.5}$ was obtained from this molecular weight and the results of elemental analyses (Table 1).

ii) Nocardomycolic Acid: Nocardomycolic acid (0.870 g) in $CHCl_3$ (10ml) was titrated with 0.46 M KOH in methanol. The consumed volume of the solution to neutrality was 2.47 ml. After evaporation of the solvent *in vacuo*, the residue was treated with ether and insoluble material was filtered off. Evaporation of the solvent afforded the potassium salt as waxy residue; yield, 0.88 g (96%) (Table 1).

iii) Corynomycolic Acid: Corynomycolic acid (0.420 g) in $CHCl_3$ (10 ml) was titrated with 3.41 ml of 0.255M KOH in methanol and the resulting potassium salt was isolated as described in (ii); yield, 0.44 g (97%) (Table 1).

1-α-O-Benzyl-N-acetylmuramic Acid Diphenylmethyl Ester (4). Diphenyldiazomethane (0.80 g, 4.1 mmol) in THF (5 ml) was added to a stirred solution of benzyl *N*-acetyl-α-muramide (**3**) (1.00 g, 2.6 mmol) in THF (10 ml). After being left to stand at room temperature overnight, the solvent was evaporated *in vacuo*. The residue crystallized on trituration with hexane. The crystals were washed with hexane to remove excess diphenyldiazomethane and then recrystallized from ethyl acetate–hexane: yield, 1.30 g (91%); mp 151—153 °C. An analytical sample was again recrystallized from the same solvent system; mp 155—156°C; $[\alpha]_D^{22}$ +122° (*c* 1, $CHCl_3$).

Found: C, 67.62; H, 6.50; N, 2.52%. Calcd for $C_{31}H_{35}O_8N$: C, 67.74; H, 6.42; N, 2.55%.

1-α-O-Benzyl-6-O-tosyl-N-acetylmuramic Acid Diphenylmethyl Ester (5). Tosyl chloride (1.20 g, 6.3 mmol) was added to an ice-cooled solution of **4** (0.30 g, 0.55 mmol) in pyridine (3 ml) with stirring. After 1 h, excess reagent was decomposed with ice-water and the mixture was extracted with ethyl acetate. The organic layer was washed successively with 0.3 M NaOH, H_2O, 1 M HCl, and H_2O, dried ($MgSO_4$) and evaporated *in vacuo*. The residue was purified with silica gel column chromatography (benzene–ethyl acetate, 5:1). Fractions containing only the main product (monitored with TLC) were collected and evaporated *in vacuo* to afford colorless powder; yield, 0.34 g, (89%); mp 68—73 °C; $[\alpha]_D^{22}$ +84.4° (*c* 0.5, $CHCl_3$).

Found: C, 64.68; H, 5.92; N, 1.93; S, 4.31%. Calcd for $C_{38}H_{41}O_{10}NS$: C, 64.85; H, 5.87; N, 1.99; S, 4.56%.

1-α-O-Benzyl-6-O-mycoloyl-N-acetylmuramic Acid Diphenylmethyl Ester (6a). i) In Benzene and in the Presence of 18-Crown-6: A mixture of **5** (325 mg, 0.46 mmol), potassium mycolate (375 mg, 0.31 mmol) and 18-crown-6 (18 mg, 0.07 mmol) in anhydrous benzene (15 ml) was heated under reflux for 3 h. After evaporation of the solvent *in vacuo*, acetone-soluble material in the residue was subjected to silica gel column chromatography. Elution with hexane–ethyl acetate (5:1) gave a mixture of the product (**6a**) and a small amount of recovered mycolic acid. The mixture was treated with diazomethane and again chromatographed on silica gel. Elution with benzene–ethyl acetate (10:1) afforded pure **6a**; yield, 0.32 g (60%); mp 55—56 °C; $[\alpha]_D^{22}$ +32.6° (*c* 0.5, $CHCl_3$).

Found: C, 78.34; H, 11.48; N, 0.85%. Calcd for $C_{111}H_{191}O_{10.5}N$: C, 78.07; H, 11.27; N, 0.82%.

ii) In DMF: A mixture of potassium mycolate (49 mg, 0.040 mmol) and **5** (39 mg, 0.055 mmol) in DMF (1 ml) was heated at 125 °C for 19 h. The mixture was acidified with 0.5M H_2SO_4 under ice-coling and then neutralized with aqueous $NaHCO_3$. After evaporation of the solvent *in vacuo*, the product was isolated by means of preparative TLC on silica gel (benzene–ethyl acetate, 2 : 1), the main band of R_f 0.6—0.8 was extracted with ethyl acetate. The residue obtained by evaporation of the solvent was triturated with methanol and filtered; yield, 28 mg (41%); mp 50—56 °C. This product was identified with **6a** obtained in (i) on TLC.

1-α-O-Benzyl-6-O-mycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine Benzyl Ester (8a). i) The DCC–HONSu Method: Trifluoroacetic acid (3 ml) was added to an ice-cooled solution of **6a** (0.30 g, 0.18 mmol) and anisole (1 ml) in $CHCl_3$ (20 ml) with stirring . After being stirred in an ice bath for 1 h, the mixture was diluted with acetone (100 ml) and the solvent was removed *in vacuo*. Trituration of the resulting syrup with ethanol gave **7a** as white powder, which was collected by filtration. To a solution of **7a** thus obtained in THF (10 ml) were added L-alanyl-D-isoglutamine benzyl ester hydrochloride (65 mg, 0.19 mmol), triethylamine (0.024 ml, 0.18 ml) and HONSu (75 mg, 0.65 mmol). To the mixture cooled in an ice bath was added DCC (37 mg, 0.18 mmol). After being stirred overnight, *N,N'*-dicyclohexylurea and triethylamine hydrochloride were filtered off; the filtrate was evaporated *in vacuo*, and the residue was triturated with methanol. The insoluble materials were subjected to silica gel column chromatography. A mixture of benzene–acetone (1:1) eluted the intramolecular ester (**9a**) in an early fraction; yield, 0.07 g (26%); mp 46—51 °C (Found: N, 0.86%. Calcd for $C_{98}H_{179}O_{9.5}N$: N, 0.92%). Further elution with the same solvent and recrystallization from benzene–methanol afforded **8a**; yield, 0.12 g (36%); mp 171—172 °C; $[\alpha]_D^{22}$ +30.2° (*c* 0.5, $CHCl_3$).

Found: C, 73.63; H, 11.05; N, 3.18%. Calcd for $C_{113}H_{200}O_{13.5}N_4$: C, 74.13; H, 11.01; N, 3.06%.

ii) The Pentachlorophenyl Ester Method: A solution of **6a**

(0.33 g, 0.19 mmol) and anisole (1 ml) in $CHCl_3$ (20 ml) was treated with trifluoroacetic acid (3 ml) and worked up as in (i). Resulting **7a** and pentachlorophenyl trichloroacetate[11] (0.20 g, 0.49 mmol) were dissolved in a mixture of DMF and THF (1:1, 15 ml), triethylamine (0.038 ml, 0.27 mmol) being added under ice-cooling. After being stirred at this temperature for 2.5 h, benzene was added and the mixture was washed with water, dried ($MgSO_4$) and evaporated *in vacuo*. Trituration of the residue with acetone gave the pentachlorophenyl ester of **7a** as powder. This was dissolved in THF (10 ml) and added to a mixture of L-alanyl-D-isoglutamine benzyl ester hydrochloride (76 mg, 0.22 mmol) and triethylamine (0.031 ml, 0.22 mmol) in THF (3 ml). After being stirred at room temperature for 3 h, triethylamine hydrochloride was filtered off, the filtrate was concentrated *in vacuo*, and the residue was chromatographed on silica gel as in (i). After **9a** (yield, 0.05 g (17%); mp 45—50 °C), **8a** was eluted. This was recrystallized from ether–methanol; yield, 0.17 g (47%); mp 171—172 °C.

6-O-Mycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine (2a). Compound **8a** (76 mg, 0.04 mmol) in THF (20 ml) was hydrogenolyzed in the presence of palladium black catalyst. After removal of the catalyst and evaporation of the solvent *in vacuo*, the residue was recrystallized from ether–ethanol; yield, 64 mg (93%); mp 137—160 °C (dec); $[\alpha]_D^{22}$ +25.8° (*c* 0.4, THF–H_2O, 50: 1, after 20 h).

Found: C, 71.08; H, 11.40; N, 3.26%. Calcd for $C_{99}H_{188}O_{13.5}N_4 \cdot H_2O$: C, 71.26; H, 11.48; N, 3.36%.

1-α-O-Benzyl-6-O-nocardomycoloyl-N-acetylmuramic Acid Diphenylmethyl Ester (6b). A mixture of **5** (0.35 g, 0.50 mmol), potassium nocardomycolate (0.30 g, 0.37 mmol) and 18-crown-6 (19 mg, 0.07 mmol) in anhydrous benzene (15 ml) was heated under reflux for 3 h. After addition of benzene (15 ml), the resulting solution was washed with 0.1 M HCl and water, dried (Na_2SO_4) and then concentrated *in vacuo*. The residue was chromatographed on silica gel. Elution with benzene–ethyl acetate (5:1) and complete evaporation of the solvent afforded **6b** as waxy residue; yield, 0.34 g (70%); $[\alpha]_D^{27}$ +46.7° (*c* 1, $CHCl_3$).

Found: C, 75.38; H, 10.15; N, 1.03%. Calcd for $C_{82}H_{130}O_{10.6}N$: C, 75.79; H, 10.08; N, 1.08%.

1-α-O-Benzyl-6-O-nocardomycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine Benzyl Ester (8b). To an ice-cooled solution of **6b** (0.24 g, 0.18 mmol) and anisole (0.1 ml) in CH_2Cl_2 (10 ml) was added trifluoroacetic acid (1.5 ml) with stirring. After 30 min, the solvent was removed *in vacuo* and the residue was chromatographed on a silica gel column ($CHCl_3$–methanol, 5:1) to afford **7b**. This was added to a suspension of L-alanyl-D-isoglutamine benzyl ester hydrochloride (75 mg, 0.22 mmol) and triethylamine (0.030 ml, 0.22 mmol) in THF (5 ml). To the mixture cooled in an ice-salt bath (−13 °C) were added HONSu (36 mg, 0.31 mmol) and DCC (39 mg, 0.19 mmol) with stirring. After 1 h, the cold bath was removed and the mixture was stirred at room temperature overnight. The insoluble materials were filtered off, the filtrate was evaporated *in vacuo* and the residue was chromatographed on silica gel. Elution with $CHCl_3$–methanol (30:1) and recrystallization from methanol–water gave **8b**; yield, 0.14 g (53%); mp 164—167 °C; $[\alpha]_D^{17}$ +44.7 ° (*c* 1, $CHCl_3$).

Found: C, 71.05; H, 9.87; N, 3.65%. Calcd for $C_{84}H_{139}O_{13.6}N_4$: C, 70.91; H, 9.85; N, 3.94%.

6-O-Nocardomycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine (2b). Compound **8b** (84 mg, 0.06 mmol) in THF (5 ml) was hydrogenolyzed in the presence of palladium black at 28 °C for 3 days. After the usual work-up, the product was recrystallized from $CHCl_3$–acetone; yield, 51 mg (68%); mp 154—157 °C (dec); $[\alpha]_D^{20}$ +30.0° (*c* 1, THF–H_2O, 50: 1, after 24 h).

Found: C, 66.07; H, 10.58; N, 4.26%. Calcd for $C_{70}H_{127}O_{13.6}N_4 \cdot 1.5H_2O$: C, 66.23; H, 10.32; N, 4.41%.

1-α-O-Benzyl-6-O-corynomycoloyl-N-acetylmuramic Acid Diphenylmethyl Ester (6c). A solution of **5** (0.48 g, 0.68 mmol), potassium corynomycolate (0.31 g, 0.59 mmol), and 18-crown-6 (30 mg, 0.11 mmol) in anhydrous benzene (15 ml) was heated under reflux for 3 h. The mixture was then treated as for **6b** and subjected to column chromatography on silica gel. Elution with benzene–ethyl acetate (5 : 1) yielded **6c** as waxy solid; yield, 0.30 g (50%); $[\alpha]_D^{25}$ +58.4° (*c* 1, $CHCl_3$).

Found: C, 72.85; H, 9.27; N, 1.40%. Calcd for $C_{62}H_{92}O_{10.3}N$: C, 73.28; H, 9.13; N, 1.38%.

1-α-O-Benzyl-6-O-corynomycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine Benzyl Ester (8c). This compound was prepared in a similar way to that for nocardomycoloyl derivative (**8b**). After the diphenylmethyl group of **6c** (0.52 g, 0.51 mmol) had been removed with trifluoroacetic acid, the resulting **7c** was mixed with L-alanyl-D-isoglutamine benzyl ester hydrochloride (0.21 g, 0.61 mmol) and triethylamine (0.087 ml, 0.62 mmol) in THF (10 ml). HONSu (101 mg, 0.88 mmol) and DCC (100 mg, 0.49 mmol) were added to the mixture under cooling and worked up as above. The product (**8c**) was isolated and purified by silica gel column chromatography followed by recrystallization from methanol–water; yield, 0.28 g (48%); mp 172—175 °C; $[\alpha]_D^{14}$ +53.7° (*c* 1, $CHCl_3$).

Found: C, 67.16; H, 9.08; N, 4.99%. Calcd for $C_{64}H_{101}O_{13.3}N_4$: C, 67.47; H, 8.94; N, 4.92%.

6-O-Corynomycoloyl-N-acetylmuramyl-L-alanyl-D-isoglutamine (2c). Compound **8c** (127 mg, 0.11 mmol) in THF (10 ml) was subjected to hydrogenolysis in the presence of palladium black at 28 °C for 3 days. After evaporation of the solvent, the residue was dissolved in a mixture of ether–methanol (1:1). On addition of acetone, white powder precipitated; yield, 77 mg (71%); mp 156—159 °C (dec); $[\alpha]_D^{18}$ +33.0° (*c* 1, THF–H_2O, 50:1, after 24 h).

Found: C, 61.64; H, 9.48; N, 5.59%. Calcd for $C_{50}H_{89}O_{13.3}N_4 \cdot H_2O$: C, 61.46; H, 9.39; N, 5.73%.

Determination of Antitumor Activity.[6] A mixture of tumor cells of MH 134 hepatoma (1×10^5) and **2a**, **b**, or **c** (20 μg) suspended in phosphate-buffered saline was inoculated intradermally into C3H mice and tumor growth was observed in inoculated sites.

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7) Mycolic acids can be classified according to the type of modification in the α-branched β-hydroxylated structure. Thus, typical subgroups contain double bonds, cyclopropane rings, or methoxy substituents in the main chains. Each subgroup exists again as a mixture of homologs with various chain lengths. J. Asselineau, "The Bacterial Lipids," Herman, Paris (1966).

8) The natural mycolic acid was used without separation into subgroups.[7]

9) These reaction conditions were described by G. Brochere-Ferreol and J. Polonsky in the synthesis of trehalose 6,6′-di-*O*-mycolate (the so-called cord factor) *via* the same type of substitution. *Bull. Soc. Chim. Fr.*, **1958**, 714.

10) S. Kusumoto, Y. Tarumi, K. Ikenaka, and T. Shiba, *Bull. Chem. Soc. Jpn.*, **49**, 533 (1976).

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12) Internal ester formation was observed in the preparation of neither 6-*O*-stearoyl-,[3] nocardomycoloyl- nor corynomycoloyl-*N*-acetylmuramyl-L-alanyl-D-isoglutamine derivatives. It seems that the ease of ester (**9a**) formation from **7a** can be attributed to the length of mycolic acid, which keeps the molecule in a certain conformation in the solution to make contact between the active ester and the dipeptide difficult.

13) The trouble was not encountered in the case of preparation of **2a**, when the ambient temperature was over 25 °C.

14) When the reaction was interrupted before completion, the product had to be purified by means of column chromatography first on silica gel ($CHCl_3$–methanol–acetic acid, 5:1:0.01) then on Sephadex LH20 (THF). The latter column was necessary to remove the trace of inorganic materials eluted from silica gel.

15) Cells of *M. tuberculosis* and *N. asteroides* were cultured in Sauton's synthetic medium, while those of *C. diphtheriae* were in the medium of Mueller and Miller. For details, see: I. Azuma, F. Kanetsuna, T. Taniyama, Y. Yamamura, H. Hori, and Y. Tanaka, *Biken J.*, **18**, 1 (1975).