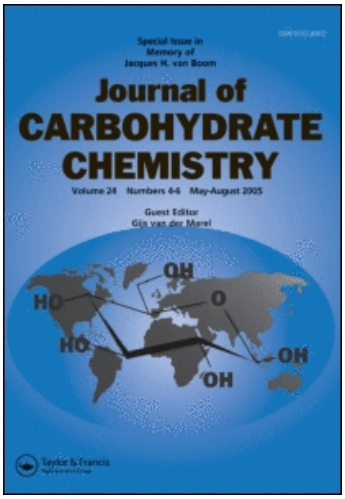


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# SYNTHESES OF TREHALOSE MONOMYCOLATE AND RELATED COMPOUNDS, AND THEIR LETHAL TOXICITY AND ADJUVANT ACTIVITY

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## ABSTRACT

Five monoesters, 6-O-mycoloyl-α,α-trehalose (TMM), 6-O-mycoloyl-D-glucose (GlcM), 6-O-mycoloyl-N-acetyl-D-glucosamine (GlcNAcM), 5-O-mycoloyl-D-arabinose (AraM) and 6-O-mycoloyl-D-galactose (GalM), were synthesized by use of mycolic acid isolated from Mycobacterium tuberculosis strain Aoyama B. Their toxicity and macrophage activating ability were examined in mice. A single intravenous administration of 400 μg of TMM in 9% oil-in-water emulsion killed 8 of 8 treated mice. The other analogs showed less lethal toxicity to mice at the same dose. Tumoricidal activity of mouse peritoneal macrophages was induced by intraperitoneal injection of TMM, GlcM, and GlcNAcM, respectively.

## INTRODUCTION

In the previous paper,[1] we reported the synthesis and biological activities of trehalose-6,6'-dimycolate (TDM) and its analogs. TDM and its lower homologs, in which $C_{80}$-mycolic acid are replaced by various shorter chain fatty acids, were shown to



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have potent macrophage activating ability in mice. In addition, 6,6'-di-N-acyl derivatives of 6,6'-diamino-6,6'-dideoxy-α,α-trehalose were slightly more effective than the corresponding trehalose-6,6'-diesters on macrophage activation. On the other hand, 6,6'-dideoxy-6,6'-bis-mycoloylamino-α,α-trehalose (TDNM) and TDM were lethal to mice when injected intravenously as 9% oil-in-water emulsions at a dose of 150 μg, whereas the other analogs showed no lethal toxicity to mice at the same dose. TDNM was less toxic than TDM. These results suggest that the macrophage activating ability of TDM analogs may not be related to their toxicity to mice. In the present study, we describe lethal toxicity and macrophage activating ability in mice of five esters of mycolic acid having various sugar residues, such as α,α-trehalose, D-glucose, N-acetyl-D-glucosamine, D-galactose and D-arabinose.



## RESULTS AND DISCUSSION

### Synthesis

The five monomycolates were synthesized according to the procedure in Fig. 1. Introduction of mycoloyl group into the sugar moiety was performed by an exchange reaction of 6(or 5)-O-tosylate of sugar derivative with potassium mycolate.

Treatment of 1,2-O-isopropylidene-6-O-tosyl-3,5-bis-O-trimethylsilyl-α-D-glucofuranose (2), which was derived from 1,2-O-isopropylidene-6-O-tosyl-α-D-glucofuranose[2] (1) by trimethylsilylation, with potassium mycolate in toluene containing 18-crown-6 at 90°C gave the corresponding 6-O-mycoloyl derivative (2) in 80% yield. On hydrolytic removal of the trimethylsilyl and isopropylidene groups with 6M-HCl, compound 2 yielded 6-O-mycoloyl-D-glucose (3). The IR spectrum of 3 showed absorption bands due to a mycolic acid moiety (3350, 1465, and 720 $cm^{-1}$) and an ester linkage (1720 $cm^{-1}$).

Benzyl 2-acetamido-3-O-benzyl-2-deoxy-6-O-tosyl-α-D-glucopyranoside[3] (4) was converted into the 6-O-mycolate (5) by the same procedure as described for 2, and reductive removal of the benzyl

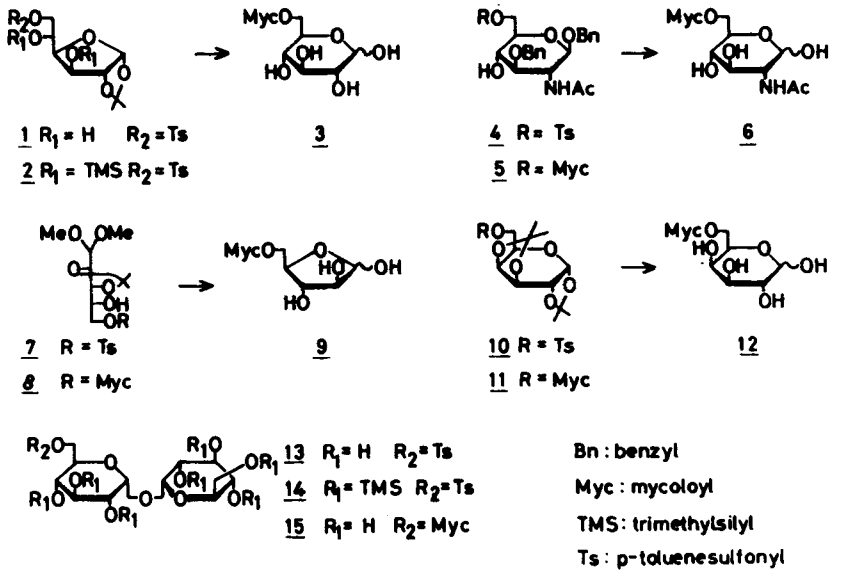


Fig. 1. Syntheses of trehalose monomycolate and related compounds.



groups in 5 by hydrogenolysis in the presence of 10% Pd-C as catalyst gave the desired 6-O-mycoloyl-N-acetyl-D-glucosamine (6) in 53% yield based on 4.

2,3-O-Isopropylidene-5-O-tosyl-aldehydo-D-arabinose dimethyl acetal (7), convenient material for the synthesis of 5-O-mycoloyl-D-arabinofuranose (9), was prepared from D-arabinose as follows: dimethyl acetalation, complete isopropylidenation of the hydroxyl groups at C-2, 3, 4 and 5 positions, selective hydrolysis of the 4,5-O-isopropylidene group, and selective tosylation of the hydroxyl group at C-5 position. After introduction of mycoloyl group into the 7, protecting groups were removed by acid hydrolysis with 6M-HCl to give 5-O-mycoloyl-D-arabinose (9).

1,2:3,4-Di-O-isopropylidene-6-O-mycoloyl-α-D-galactopyranose (11) was likewise prepared from 6-O-tosyl derivative[4] (10), which, on acid hydrolysis of isopropylidene groups with aqueous trifluoroacetic acid, gave the desired 6-O-mycoloyl-D-galactose (12).

6-O-Tosyl-α,α-trehalose[5] (13) was converted into the trimethylsilylated derivative (14) to avoid the formation of 3,6-anhydro

derivative in the next reaction. Esterification of 14 with potassium mycolate (78% yield), followed by hydrolytic removal of the trimethylsilyl groups (71% yield), afforded 6-O-mycoloyl-α,α-trehalose (15).

## Toxicity in Mice

TABLE 1 shows that 400 μg of TMM was lethal when it was injected intravenously into mice as 9% oil-in-water emulsions. TMM was shown to be less toxic than TDM, for 150 μg of TMM showed no lethal toxicity to mice, whereas the same dose of TDM killed 8 of 10 treated mice. Kato et al.[6] reported that methyl 6-O-mycoloyl-α-D-glucopyranoside, which was almost regarded as a half molecule of TDM, was still lethal and more toxic than TMM. However, 6-O-mycoloyl-D-glucose (GlcM) had no toxicity as shown in TABLE 1. These facts suggest that glycoside structure is one of the important factors for the manifestation of toxicity.

## Effect of Monomycolates on Peritoneal Macrophage Activation in Mice

The tumoricidal activity of mouse peritoneal macrophages induced by intraperitoneal injection of five monomycolates was investigated (TABLE 2). TMM, GlcM and GlcNAcM were shown to induce cytolytic activity against syngeneic tumor cells. The same activity was not induced with either AraM or GalM. These results indicate that a restricted structure of sugar residue is essential to manifestation of tumoricidal activity in macrophages. The configuration of hydroxyl group in sugar moiety seemed to be important for the manifestation of activity, for glucose can not be replaced without loss of activity by galactose.[7] In the present study, only D-glucose derivatives induced tumoricidal activity in macrophages, however, it is too early to suggest the relationship between structure and activity.

# EXPERIMENTAL

## SYNTHESIS

General Methods Melting points were determined with a Yamato micro melting-point apparatus and are uncorrected. Specific rota-

TABLE 1

Toxicity of TDM and Monomycolates in Mice

| Treatment | Number of death/number treated | |
|---|---|---|
| | 150 μg/mouse | 400 μg/mouse |
| TDM | 8/10 | 8/8 |
| TMM (15) | 0/10 | 8/8 |
| GlcM (3) | 0/10 | 2/8 |
| GlcNAcM (6) | — | 1/8 |
| AraM (9) | 0/10 | 0/10 |
| GalM (12) | — | 2/8 |
| Control (emulsion alone) | 0/10 | 0/10 |

Oil(9%)-in-water emulsion of TDM and monomycolates or emulsions alone (Control) were prepared by grinding. They were injected intravenously into C57BL/6 mice in volumes of 0.1 ml.



tions were determined with a Union PM-101 polarimeter, and IR spectra were recorded with a Shimadzu IR-27G spectrophotometer. Preparative chromatography was performed on silica gel (Merk Co., 200 mesh) with the solvent systems specified. All evaporations were conducted *in vacuo*.

6-O-Mycoloyl-D-glucose (3)

1,1,1,3,3,3-Hexamethyldisilazane (2 mL) and trimethylchlorosilane (1 mL) were added to a solution of 1[2] (1.0g) in pyridine (6 mL), and the mixture was kept for 50 min at room temperature. Ice was then added and the mixture was extracted with $CHCl_3$. The extract was successively washed with 2M-HCl, saturated $NaHCO_3$, and water. It was dried over $Na_2SO_4$ and evaporated to a syrup (homogeneous on TLC), which was crystallized from hexane, to give pure sample of 2; yield, 1.20g (80%).

TABLE 2

Effect of TDM and Monomycolates on Peritoneal Macrophage in Mice

| Treatment[a] | Timing (days) | Dose (μg) | % Cytolysis[b] 3LL | % Cytolysis[b] FBL-3 |
|---|---|---|---|---|
| Expt. 1 | | | | |
| TDM | -7 | 50 | 13.0 ± 1.0[c] | 33.7 ± 3.0[c] |
| TMM (15) | -7 | 50 | 12.5 ± 1.3[c] | 27.2 ± 3.7[c] |
| GlcM (3) | -7 | 50 | 19.9 ± 1.9[c] | 29.1 ± 3.1[c] |
| AraM (9) | -7 | 50 | 0.5 ± 1.1 | 9.3 ± 2.7 |
| Control (saline) | -7 | — | 0.7 ± 1.3 | 7.0 ± 1.8 |
| MVE-2 | -3 | 500 | 18.3 ± 1.3 | 18.7 ± 3.6 |
| Expt. 2 | | | | |
| TDM | -7 | 50 | 35.3 ± 2.3[c] | 42.6 ± 2.6[c] |
| GlcM (15) | -7 | 50 | 38.1 ± 1.9[c] | 42.6 ± 2.0[c] |
| GlcNAcM (6) | -7 | 50 | 36.8 ± 1.6[c] | 37.0 ± 1.7[c] |
| GalM (12) | -7 | 50 | 4.9 ± 1.1 | 2.2 ± 1.9 |
| Control (saline) | -7 | — | 1.4 ± 0.9 | 1.0 ± 1.4 |
| MVE-2 | -3 | 500 | 50.6 ± 1.7 | 49.3 ± 1.1 |

a) C57BL/6 mice were injected intraperitoneally with TDM and monomycolates in saline at 7 days before harvest of macrophages.

b) Each value is the mean ± standard error of 6 wells in each group. 3LL and FBL-3 were used as target cells.

c) Significant difference from the control group by Student's t-test ($P<0.005$).

A mixture of potassium mycolate (0.9g), compound 2 (0.4g) and 18-crown-6 (0.2g) in toluene (20 mL) was stirred for 24 h at 90°C. The reaction mixture was evaporated and the residue was extracted with $CHCl_3$. The extract was washed with water, dried over $Na_2SO_4$, and evaporated to a syrup, which was chromatographed on silica gel. Elution with 100:1 $CHCl_3$-MeOH gave a white solid residue. This residue was treated with 6M-HCl (8 mL) in 1,4-dioxane (12 mL) for 1.5 h at 75°C. Ice was added to the reaction mixture, and it was extracted with $CHCl_3$. The extract was washed with water, dried over $Na_2SO_4$, and evaporated to a syrup, which was chromatographed on silica gel with (a) 100:1, (b) 20:1 $CHCl_3$-MeOH. Eluant b gave the pure product; yield, 0.14g (18.4%), m.p. 50-52°C ($Et_2O$-MeOH), $[\alpha]_D^{25}$ + 13.1° (c 0.5, $CHCl_3$); IR (KBr): 3350, 1720, 1465, and 720 $cm^{-1}$. Anal. Calcd for $C_{86}H_{168}O_{8.5}\cdot H_2O$: C, 76.16; H, 12.63. Found: C, 76.03; H, 12.63.

6-O-Mycoloyl-N-acetyl-D-glucosamine (6)

A mixture of potassium mycolate (0.71g), compound 4[3] (0.25g) and 18-crown-6 (0.15g) in toluene (15 mL) was stirred for 12 h at 100°C. The solvent was evaporated off and the residue was extracted with $CHCl_3$. The extract was washed with 2M-HCl and water successively, dried over $Na_2SO_4$ and evaporated to a syrup, which was applied to a column of silica gel. Elution with 400:1 $CHCl_3$-MeOH gave the mixture of the compound 5 and mycolic acid, which was subject to the next reaction without further purification. The above syrup was dissolved in a mixture of $CH_2Cl_2$ (7 mL), MeOH (6 mL) and AcOH (1 mL), and hydrogenolyzed in the presence of palladium carbon catalyst for 2 days. After removal of the catalyst and evaporation of the solvent, the residue was chromatographed on silica gel with (a) 150:1, (b) 20:1 $CHCl_3$-MeOH. Eluant b gave the compound 6; yield, 0.33g (53.2% based on 4), m.p. 150-153°C ($CHCl_3$-MeOH), $[\alpha]_D^{25}$ + 13.1° (c 0.5, $CHCl_3$); IR (KBr): 3350, 1720, 1640, 1550, 1460, and 720 $cm^{-1}$. Anal. Calcd for $C_{88}H_{171}NO_{8.5}\cdot 3H_2O$: C, 73.74; H, 12.44; N, 0.97. Found: C, 73.98; H, 12.30; N, 1.05.

2,3-O-Isopropylidene-5-O-tosyl-aldehydo-D-arabinose dimethyl acetal (7)

Conc. $H_2SO_4$ (3 mL) was added to a stirred suspension of D-arabinose (10g) in MeOH (150 mL), and the mixture was stirred for 17.5 h at room temperature. 2,2-Dimethoxypropane (100 mL) and conc. $H_2SO_4$ (12 mL) were added to the reaction mixture, and the reaction mixture was stirred for 4.5 h at room temperature, and then neutrallized with $NH_4OH$ in an ice-water bath, and concentrated. The residue was extracted with $CHCl_3$, washed with water and dried over $Na_2SO_4$, and the solvent was evaporated. The crude product was purified by column chromatography on silica gel. Elution with 100:1 $CHCl_3$-MeOH gave 2,3:4,5-di-O-isopropylidene-aldehydo-D-arabinose dimethyl acetal as a syrup; yield, 8.36g (45%), $[\alpha]_D^{25}$ + 4.5° (c 1.6, $CHCl_3$).

The dimethyl acetal (2.29g) was treated with 70% aqueous acetic acid (20 mL) for 1.2 h at 40°C, the mixture was evaporated, and the residue was chromatographed on silica gel. Elution with 100:1 $CHCl_3$-MeOH gave 2,3-O-isopropylidene-aldehydo-D-arabinose dimethyl acetal as a syrup; yield, 1.70g (87%), $[\alpha]_D^{25}$ - 13.9° (c 1.3, $CHCl_3$).

p-Toluenesulfonyl chloride (1.3g) was added to an ice-cooled solution of the above compound (1.33g) in pyridine (10 mL), and the mixture was stirred for 0.5 h in an ice-water bath and for additional 1 h at room temperature. The excess reagent was decomposed with ice-water and the mixture was extracted with $CHCl_3$. The extract was successively washed with 2M-HCl, saturated $NaHCO_3$, and water, dried over $Na_2SO_4$, and evaporated to a syrup which was applied to a column of silica gel. Elution with 200:1 $CHCl_3$-MeOH gave 7; yield, 1.60g (73%), $[\alpha]_D^{23}$ + 9.7° (c 0.8, $CHCl_3$); IR (film): 3350, 1590, 1350, 1170, 860, and 660 $cm^{-1}$. Anal. Calcd for $C_{17}H_{26}O_8S$: C, 52,30; H, 6.71. Found: C, 52.54; H, 7.00.

5-O-Mycoloyl-D-arabinose (9)

A mixture of potassium mycolate (0.60g), compound 7 (0.40g) and 18-crown-6 (0.20g) in toluene (20 mL) was stirred for 3.5 h

at 90°C. After work-up as just described for the preparation of 5, the syrup obtained was chromatographed on a column of silica gel. Elution with 200:1 $CHCl_3$-MeOH gave 8; yield, 0.93g (64.7%).

Compound 8 (0.57g) was treated with 6M-HCl (4 mL) in 1,4-dioxane (6 mL) for 3 h at 70°C. Ice was added to the reaction mixture, and the mixture was extracted with $CHCl_3$. The extract was washed with water, dried over $Na_2SO_4$, and evaporated to a syrup, which was chromatographed on silica gel. Elution with 50:1 $CHCl_3$-MeOH gave 12; yield, 0.125g (23.3%), m.p. 45°C sinter 62°C ($Et_2O$-MeOH), $[\alpha]_D^{25}$ + 1.7° (c 0.3, $CHCl_3$); IR (KBr): 3350, 1715, 1460, and 715 $cm^{-1}$. Anal. Calcd for $C_{85}H_{106}O_{7.5}\cdot\frac{3}{2}H_2O$: C, 76,57; H, 12.62. Found: C, 76.35; H, 12.64.

6-O-Mycoloyl-D-galactose (12)

Compound 10[4] (0.20g) was esterified as described for 5 and the crude product was chromatographed on silica gel. Elution with $CHCl_3$ gave compound 11; yield, 0.35g (51.0%).

Compound 11 (0.28g) was suspended in a mixture of trifluoroacetic acid (5 mL) and water (0.5 mL), and kept for 3.5 h at 40°C. After evaporation of the solvent, the residue was chromatographed on silica gel. Elution with 50:1 $CHCl_3$-MeOH gave the pure product; yield, 0.10g (37.9%), m.p. 42-45°C ($Et_2O$-MeOH), $[\alpha]_D^{25}$ + 13.1° (c 0.5, $CHCl_3$); IR (KBr): 3400, 1780, 1725, 1460, and 720 $cm^{-1}$. Anal. Calcd for $C_{86}H_{168}O_{8.5}\cdot\frac{3}{4}CF_3CO_2H$: C, 73.65; H, 11.94. Found: C, 73.67; H, 11.79.

6-O-Mycoloyl-α,α-trehalose (15)

Compound 13[5] (0.16g) was trimethylsilylated as described for 2 and the crude product was purified by column chromatography on silica gel. Elution with $CHCl_3$ gave 14 as the pure product; yield, 0.25g (77.4%).

A mixture of potassium mycolate (0.30g), compound 14 (0.18g) and 18-crown-6 (0.06g) in toluene (10 mL) was stirred for 4 days at 90°C. The solvent was evaporated off, and the residue was triturated with hexane. The insoluble materials were filtered off, and the

filtrate was evaporated to a syrup. This syrup was dissolved in a mixture of $CHCl_3$, MeOH, AcOH and $H_2O$, and kept for 8 h at 45°C. The solvent was evaporated off, and the residue was chromatographed on silica gel. Elution with 20:1 $CHCl_3$-MeOH removed by-products, and elution with 10:1 $CHCl_3$-MeOH gave 15; yield, 0.19g (70.5%), m.p. 160°C sinter 179°C ($Et_2O$-MeOH), $[\alpha]_D^{25}$ + 31.2° (c 0.5, $CHCl_3$), IR (KBr): 3350, 1720, 1465, and 720 $cm^{-1}$. Anal. Calcd for $C_{92}H_{178}O_{13.5}\cdot CH_3CO_2H$: C, 70.19; H, 11.40. Found: C, 70.07; H, 11.28.

## BIOLOGICAL TEST

Materials Maleic anhydride divinyl ether polymer (MVE-2, M.W. 15,500) was kindly supplied from Dr. Michael Chirigos, Immunopharmacology Section, NCI-FCRF, Frederick MD 21701.

Animals Seven-week-old female C57BL/6 mice were obtained from Shizuoka Agricultural Co-operations for Experimental Aniamls, Hamamatsu, Japan.

Toxicity test The details were given in the previous paper.[1] Briefly, TDM and five monomycolates were dissolved in Drakeol 6-VR and emulsified in 1% Tween 80 in saline by grinding with a Potter homogenizer. The final concentration of glycolipid was 150 or 400 μg/0.1 ml and of oil 9% (w/w). The emulsions were injected into the tail vein of female C57BL/6 mice in 0.1 ml volumes, and the mice were observed for 30 days.

Culture Medium Hank's balanced salt solution (HBSS) (Nissui Seiyaku Co., Ltd., Tokyo, Japan) was supplemented with 100 units of penicillin per milliliter and 100 μg of streptomycin per milliliter. RPMI 1640 medium (Nissui Seiyaku Co., Ltd., Tokyo, Japan) was supplemented with 10% heat-inactivated fetal bovine serum (FBS) (lot 27N4133, GIBCO Laboratories, Grand Island, N.Y., USA), 2 mM L-glutamine, 100 units of penicillin per milliliter, and 100 μg of streptomycin per millilieter (RPMI-FBS).

Macrophages TDM and five monomycolates were suspended in saline as previously described,[1] and they were injected intraperitoneally into C57BL/6 mice at a dose of 50 μg per mouse in groups of 4 each. Seven days later, mice were killed and the peritoneal exudate cells were obtained by washing out peritoneal cavities with 10 ml of HBSS containing 10 units of heparin per milliliter. The cells were pooled in each group, resuspended in RPMI-FBS, and plated to result in uniform densities of adherent cells (macrophages) into 96-well micro tissue culture plate (Corning Cell Wells 25860, Corning, New York 14831) according to the procedure described previously.[8] The plates were incubated for 2 h at 37°C and then washed with RPMI-FBS to remove nonadherent cells.



Assay for Cytolytic Activity of Macrophage FBL-3 leukemia cells (FBL-3) and Lewis lung carcinoma (3LL) were maintained *in vitro* culture. Cytolysis of target cells was quantitated as previously described.[4] In brief, $^{51}Cr$-labeled tumor cells ($5 \times 10^3$) were cultured with peritoneal macrophages ($2.5 \times 10^5$) for 18-20 h at 37°C. The radioactivity in the culture supernatant was determined $\gamma$-counting to estimate target cytolysis by the formula:

$$\% \text{ Cytolysis} = \frac{\text{experimental release - spontaneous release}}{\text{maximum release - spontaneous release}} \times 100$$

Maximum release of $^{51}Cr$ was determined by freezing and thawing of labeled target cells three times. The spontaneous release was measured as the radioactivity released from labeled cells in the absence of macrophages.

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