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Chemical synthesis and biological activities of 6,6′-di-*O*-mycoloyl-β,β- and -α, β-trehalose

Ichiro Azuma, Takuma Sakurai, Hideharu Ishida*,
Institute of Immunological Science, Hokkaido University, Sapporo 060 (Japan)

Toru Kitajima, and Masayoshi Yamazaki
Fuji Laboratory, Towa Chemical Industry Co., Ltd., Shizuoka 417 (Japan)

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ABSTRACT

6,6′-Di-*O*-mycoloyl-β,β-trehalose (β,β-TDM) and 6,6′-di-*O*-mycoloyl-α,β-trehalose (α,β-TDM) were synthesized and their toxicity and ability to activate peritoneal macrophages *in situ* were examined in mice, in comparison with 6,6′-di-*O*-mycoloyl-α,α-trehalose (TDM). Both β,β-TDM and α,β-TDM caused a decrease in body weight two days after injection, however the weights reverted to a normal level. No deaths were caused by either analog. On the other hand, TDM showed potent toxicity, causing decrease in body weight and death of all animals injected. β,β-TDM and α,β-TDM were effective in the *in situ* activation of mouse peritoneal macrophages.

INTRODUCTION

6,6′-Di-*O*-mycoloyl-α,α-trehalose (trehalose 6,6′-dimycolate, TDM) was first identified as a glycolipid putatively responsible for the pathogenecity of virulent mycobacteria[1,2]. However, TDM and related glycolipids have been obtained not only from pathogenic mycobacteria but also from the cells of nonpathogenic mycobacteria, nocardiae, corynebacteria, and related organisms.

Regardless, it has been shown that TDM exhibits a variety of biological activities, including lethal toxicity to mice and also immunoadjuvant activities and antitumor activity in experimental models[3]. To elucidate the structure-activity relationships of compounds of this class, many kinds of TDM analogs have been synthesized and their biological properties investigated. Some of the analogs are trehalose esters of short-chain fatty acids instead of natural mycolic acid[4–6], and others are mycolate esters of various disaccharides[7,8] and monosaccharides[9,12]. Biological studies[9,10,13] of the latter compounds demonstrated that a restricted range of structures of the sugar moiety, with particular regard to the configuration of the hydroxyl groups, is compatible with the biological activities of mycolate esters. Recently, Gillois *et al.*[14] reported the greatly attenuated biological activities of the 6,6′-dicorynomycolate of β,β-trehalose in contrast to the α,α-trehalose derivative.

* Present address: Department of Applied Bioorganic Chemistry, Gifu University, Gifu 501-11 (Japan)

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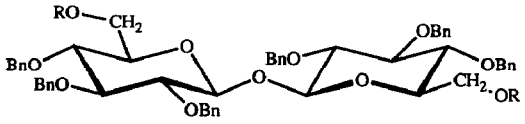
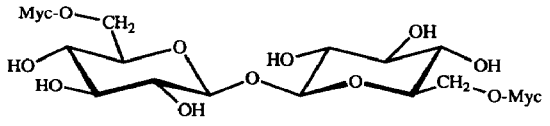
In this study, as part of our continuing research on the structure–activity relationships of trehalose dimycolates[6,11–13], we have examined the toxicity of, and the *in situ* activation of murine peritoneal macrophages by, synthetic 6,6′-di-*O*-mycoloyl-β,β-trehalose (β,β-TDM) and 6,6′-di-*O*-mycoloyl-α,β-trehalose (α,β-TDM), in comparison with 6,6′-di-*O*-mycoloyl-α,α-trehalose (TDM).

RESULTS AND DISCUSSION

Chemical synthesis of β,β-TDM (**5**) *and α,β-TDM* (**10**). — So far, many efforts have been made to synthesize α,α-trehalose and its anomeric isomers from D-glucose derivatives[15,16]. The β,β- and α,β-trehalose employed in this study were prepared *via* the corresponding orthoesters by the reaction of 2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl bromide with 2,3,4,6-tetra-*O*-acetyl-D-glucopyranose[17]. The details will be presented



1 R = trityl **3** R = p-tolylsulfonyl (Ts)
2 R = H **4** R = mycoloyl (Myc)



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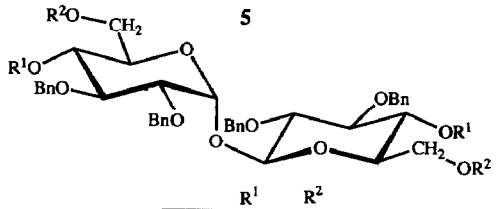
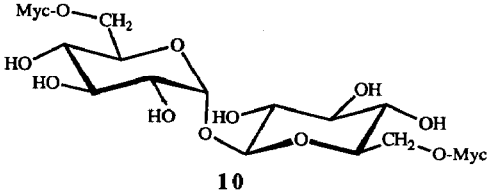


	R^1	R^2
6	—Bzd—	
7	H	H
8	H	Ts
9	H	Myc



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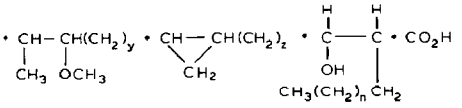
Mycolic acid (representative): 

elsewhere. β,β-TDM (**5**) was prepared by employing 2,3,4,2',3',4'-hexa-*O*-benzyl-β,β-trehalose (**2**) as described by Liav *et al.*[18] The precursor **1** was obtained in 82% yield by successive tritylation of β,β-trehalose with trityl chloride and per-*O*-benzylation with benzyl bromide in the presence of sodium hydride. Hydrolysis of the trityl ether groups in **1** gave the key intermediate **2**. Treatment of **2** with *p*-toluenesulfonyl chloride then afforded 2,3,4,2',3',4'-hexa-*O*-benzyl-6,6'-di-*O*-*p*-tolylsulfonyl-β,β-trehalose (**3**) in 86% yield. Displacement of the tosyl groups in **3** with potassium mycolate[19] gave the 6,6'-di-*O*-mycoloyl derivative (**4**) in 62% yield. Catalytic hydrogenolysis of **4** in the presence of 10% palladium on carbon (Pd–C) then afforded 38% of the desired 6,6'-di-*O*-mycoloyl-β,β-trehalose (**5**). The poor yield in the debenzylation step may be explained by the poor solubility of the product in the polar reaction solvent (ethanol–oxolane), which was chosen in the interest of promoting Pd–C catalysis.

For the synthesis of 6,6'-di-*O*-mycoloyl-α,β-trehalose, we employed 2,3,2',3'-tetra-*O*-benzyl-α,β-trehalose as an intermediate to make the debenzylation easy. 4:6,4':6'-Di-*O*-benzylidene-α,β-trehalose was prepared from α,β-trehalose by treatment with α,α-dimethoxytoluene in the presence of *p*-toluenesulfonic acid. Per-*O*-benzylation of the benzylidene derivative then gave compound **6** in 89% yield. Compound **6** was debenzylidenated in 80% aqueous acetic acid to yield 65% of **7**. With *p*-toluenesulfonyl chloride (2.5 molar equiv.) in pyridine, **7** was regioselectively tosylated[20], giving the 6,6'-disulfonate **8** in 67% yield. Condensation of **8** with the potassium salt of mycolic acid gave 61% of 2,3,2',3'-tetra-*O*-benzyl-6,6'-di-*O*-mycoloyl-α,β-trehalose (**9**). Hydrogenolysis of the benzyl groups in **9** was accomplished over palladium black instead of 10% Pd–C in 1:1 chloroform–oxolane, to afford the desired 6,6'-di-*O*-mycoloyl-α,β-trehalose (**10**) in 80% yield.

Compounds **5** and **10** were obtained as white powders by precipitation from methanol with ether, and employed for the biological tests.

Lethal toxicity of β,β-TDM and α,β-TDM. — Bloch[1] has reported that the repeated intraperitoneal injection, at 2- to 3-day intervals, of TDM (called cord factor in his paper) dissolved in mineral oil caused loss of body weight and death in mice. It was

TABLE I

Lethal toxicity of TDM analogs in mice

Compound[a]	*Dose*[b] *(μg)*	*Number of deaths by day 21/ number of treated animals*	*Body weight (g) on day 2*[c]
β,β-TDM (**5**)	400	0/5	14.9 (−2.1)
α,β-TDM (**10**)	400	0/5	13.5 (−3.0)
TDM	400	5/5	14.1 (−2.7)
Control (emulsion alone)		0/5	17.0 (+0.1)

[a] Oil-in-water emulsions were prepared by grinding the compounds indicated with mineral oil (Drakeol 6 VR) and water containing 1% of Tween 80. The final concentrations were: TDM and its analogs, 400 μg/0.1 mL; mineral oil, 9 mg/0.1 mL. [b] Administered intravenously to C57BL/6 mice in a volume of 0.1 mL. [c] In parentheses, changes in body weight compared to day 0.

TABLE II

In situ activation of mouse peritoneal macrophages by TDM analogs

Compound	*Dose*[a] *(μg)*	*Radioactivity in viable target cells (c.p.m. ± SD)*[b,c]
β,β-TDM (**5**)	50	3004 ± 315 (55%)
α,β-TDM (**10**)	50	3626 ± 438 (46%)
TDM	50	1878 ± 467 (72%)
Tumor cells alone		6581 ± 549

[a] Compounds were suspended in phosphate-buffered saline (PBS). Mice were injected i.p. 5 d before harvest of the macrophages. [b] Target cells (B16-BL6) labeled with [^{125}I]iododeoxyuridine were plated into culture wells to obtain an initial target:effector cell ratio of 1:10. After 72 h the culture wells were washed twice with PBS and the adherent viable cells were lysed with 0.1 mL of 0.5M NaOH. The radioactivity of the lysate was measured in a gamma counter. [c] The values in parentheses are % decrease with respect to tumor cells alone.

later shown that a single intravenous injection of 5–100 μg of TDM, as an emulsified mineral-oil solution, similarly caused the loss of body weight and death in mice[21]. In the present study, we used 400 μg of synthetic TDM or its analogs as an emulsified mineral-oil solution. The lethal toxicity of TDM varied with variation in the form and route of administration and strain of mice. In this experimental system, 100 or 400 μg of TDM was required to kill 100% of the mice.

As shown in Table I, the single injection of emulsified TDM (α,α-TDM) killed all mice within 21 days after injection. These mice suffered a continuous decrease of body weight. However, β,β-TDM and α,β-TDM caused only a transient loss of body weight on days 1 and 2 after the injection. On day 21 after injection, the body weight of mice treated had recovered to the same level as that of the control mice, and no mice had died. The above result indicates clearly that the α,α anomeric configuration of the trehalose moiety is essential for the manifestation of the lethal toxicity of TDM at the dose administered.

In situ activation of peritoneal macrophages in mice. — Lepoivre *et al.*[22] have reported that intraperitoneal injection of natural TDM into mice induced peritoneal macrophages cytoxic to syngeneic tumor cells.

As shown in Table II, β,β-TDM and α,β-TDM were effective for the activation *in situ* of mouse peritoneal macrophages for cytolysis of tumor cells, but not as effective as α,α-TDM.

EXPERIMENTAL

General methods. — Optical rotations were determined with a HORIBA SE-PA-200 high sensitivity polarimeter, at 25° unless noted otherwise. Column chromatography was performed on silica gel (Wako Gel C-200). T.l.c. and high-performance (h.p.) t.l.c. were performed on Silica Gel 60 F_{254} (Merck). Molecular sieves were purchased from Nakarai Chemicals. N.m.r. spectra were recorded with a Jeol GSX-270 (270 MHz for ^{1}H, 67.5 MHz for ^{13}C) spectrometer, for solutions in chloroform-*d* unless noted

otherwise. Values of δ_C and δ_H are given relative to the signal for internal tetramethylsilane.

2,3,4,2',3',4'-Hexa-O-*benzyl-6,6'-di*-O-*trityl-β,β-trehalose* (**1**). — To a stirred solution of β-D-glucopyranosyl β-D-glucopyranoside[17] (β,β-trehalose; 304 mg, 0.89 mmol) in pyridine (6 mL) was added trityl chloride (594 mg, 2.13 mmol). The mixture was stirred for 39 h at 50°, and for another 23 h at 50° after a further addition of trityl chloride (148 mg, 0.53 mmol). After evaporation of the solvent, the residue was chromatographed on silica gel (80 g) with 10:1 chloroform–methanol containing 1% of triethylamine as eluent, to give 6,6'-di-*O*-trityl-β,β-trehalose (R_F 0.53 in 5:1 chloroform–methanol). To a solution of the eluted ditrityl compound in *N,N*-dimethylformamide (DMF; 20 mL), cooled to 20°, was added a 60% suspension of sodium hydride in oil (192 mg of NaH, 8.0 mmol). The mixture was stirred for 30 min at 20°, and then benzyl bromide (0.95 mL, 8.0 mmol) was added. Stirring was continued for 17 h at 20°, the reaction was quenched with methanol (2 mL), and after a further 30 min of stirring the reaction mixture was concentrated. The residue was chromatographed on silica gel (80 g) with toluene containing 1% of triethylamine as eluent, to afford **1** (999 mg, 82%); R_F 0.32 (toluene); $[\alpha]_D^{25}$ −12.4° (*c* 1.2, $CHCl_3$).

Anal. Calc. for $C_{92}H_{86}O_{11}\cdot H_2O$ (1367.6): C, 79.74; H, 6.40. Found: C, 79.74; H, 6.36.

2,3,4,2',3',4'-Hexa-O-*benzyl-β,β-trehalose* (**2**). — A solution of **1** (939 mg) in 80% aqueous acetic acid (25 mL) was stirred for 2 h at 80°, and for an additional 1 h at 80° after the addition of oxolane (tetrahydrofuran, THF; 10 mL), then concentrated. The residue was chromatographed on silica gel (80 g) with 2:1 toluene–ethyl acetate, to yield compound **2** (314 mg, 52%); R_F 0.49 (1:1 toluene–ethyl acetate); $[\alpha]_D^{25}$ +10.1° (*c* 0.6, $CHCl_3$); ^{13}C-n.m.r. ($CDCl_3$): 61.7 (C-6,6'), 75.2 (C-5,5'), 77.3 (C-4,4'), 82.1 (C-2,2'), 84.4 (C-3,3'), and 100.1 (d, $J_{C,H}$ 162.4 Hz, C-1,1').

Anal. Calc. for $C_{54}H_{58}O_{11}$ (883.0): C, 73.45; H, 6.62. Found: C, 73.28; H, 6.63.

2,3,4,2',3',4'-Hexa-O-*benzyl-6,6'-di*-O-p-*tolylsulfonyl-β,β-trehalose* (**3**). — To a cooled (0°) solution of **2** (224 mg, 0.25 mmol) in pyridine (2 mL) was added, with stirring, *p*-toluenesulfonyl chloride (145 mg, 0.76 mmol), then the mixture was stirred for 19 h at room temperature. The solution was diluted with ethyl acetate and water and partitioned, and the organic layer was washed successively with 5% hydrochloric acid, brine, saturated sodium hydrogencarbonate, and brine. The ethyl acetate solution was dried (magnesium sulfate) and concentrated to a syrup, which was chromatographed on silica gel (25 g) with 10:1 toluene–ethyl acetate to give **3** (260 mg, 86%); R_F 0.57 (6:1 toluene–ethyl acetate); $[\alpha]_D^{25}$ −4.1° (*c* 0.2, $CHCl_3$).

Anal. Calc. for $C_{68}H_{70}O_{15}S_2$ (1191.4): C, 68.55; H, 5.92. Found: C, 68.47; H, 5.96.

2,3,4,2',3',4'-Hexa-O-*benzyl-6,6'-di*-O-*mycoloyl-β,β-trehalose* (**4**). — Mycolic acid was prepared from the cell wall of *Mycobacterium tuberculosis* Aoyama B by alkaline hydrolysis. The average molecular formula of the product was calculated as $C_{80}H_{158}O_{3.5}$. A mixture of the potassium salt of the mycolic acid[8] (241 mg, 0.20 mmol), compound **3** (117 mg, 0.10 mmol), and 18-crown-6 (32 mg, 0.12 mmol) in toluene (10 mL) was stirred for 16 h at 100°. After concentration of the reaction mixture the residue

was triturated with hexane. Insoluble materials were filtered off and the filtrate was concentrated. The syrupy residue was chromatographed on silica gel (30 g) with 7:4 hexane–ether to afford **4** (193 mg, 62%); R_F 0.41 (10:1 toluene–ethyl acetate); $[\alpha]_D^{25}$ +2.2° (*c* 0.4, $CHCl_3$).

Anal. Calc. for $C_{214}H_{370}O_{16}$ (3199.1): C, 80.34; H, 11.66. Found: C, 80.10; H, 11.64.

*6,6'-Di-*O*-mycoloyl-β,β-trehalose* (**5**). — To a solution of **4** (155 mg) in THF (10 mL) were added ethanol (10 mL) and a suspension of 10% Pd–C (150 mg) in water (0.5 mL). The mixture was hydrogenated for 19 h at 50° under atmospheric pressure. Catalyst was filtered off, and the filtrate was concentrated. The residue obtained was again hydrogenated in the same manner over 10% Pd–C (140 mg) in THF (15 mL) and ethanol (15 mL). After removal of the catalyst by filtration, the filtrate was concentrated and chromatographed on silica gel (10 g) with 25:1 chloroform–methanol, to give **5** as a wax. The compound (49 mg, 38%) was obtained as a white powder by precipitation from methanol with ether, R_F 0.46 (15:1 chloroform–methanol); $[\alpha]_D^{25}$ +32.0° (*c* 0.4, $CHCl_3$); ^{1}H-n.m.r. (30:1 $CDCl_3$–CD_3OD): δ 4.55 (d, 2 H, $J_{1,2} = J_{1',2'} = 9.2$ Hz, H-1,1').

Anal. Calc. for $C_{172}H_{334}O_{16}$ (2658.4): C, 77.71; H, 12.66. Found: C, 77.40; H, 12.39.

*4,6:4',6'-Di-*O*-benzylidene-α,β-trehalose* (**6**). — To a solution of α-D-glucopyranosyl β-D-glucopyranoside[17] (α,β-trehalose; 263 mg, 0.77 mmol) in DMF (5 mL) were added α,α-dimethoxytoluene (0.26 mL, 1.7 mmol) and a little *p*-toluenesulfonic acid, and the mixture was stirred for 1 h at 60° under reduced pressure. After cooling the solution to 20°, a 60% suspension of sodium hydride in oil (89 mg of NaH, 3.7 mmol) was added with stirring. After 15 min benzyl bromide (0.44 mL, 3.7 mmol) was added, and stirring was continued for 23 h at 20°, and for an additional 1 h after the addition of more sodium hydride suspension (74 mg of NaH) and benzyl bromide (0.22 mL). Finally, methanol (2 mL) was added, and the reaction mixture was stirred for 30 min at 20°, then concentrated. The residue was chromatographed on silica gel (50 g) with 10:1 toluene–ethyl acetate containing 1% triethylamine to give compound **6** (602 mg, 89%); R_F 0.32 (10:1 toluene–ethyl acetate); $[\alpha]_D^{25}$ +10.0° (*c* 0.6, $CHCl_3$); ^{1}H-n.m.r. ($CDCl_3$): δ 5.55 (s, 2 H, 2 C*H*Ph) and 7.16–7.52 (m, 30 H, Ph-H).

Anal. Calc. for $C_{54}H_{54}O_{11}$ (879.0): C, 73.78; H, 6.19. Found: C, 73.75; H, 6.21.

*2,3,2',3'-Tetra-*O*-benzyl-α,β-trehalose* (**7**). — Compound **6** (562 mg) in 80% aqueous acetic acid (15 mL) was stirred for 1 h at 80°, and the solution was then concentrated. The residue was chromatographed on silica gel (50 g) with 10:1 chloroform–methanol to afford compound **7** (294 mg, 65%); R_F 0.32 (10:1 chloroform–methanol); $[\alpha]_D^{25}$ +13.2° (*c* 0.3, $CHCl_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 99.3 ($J_{C,H}$ 168.2 Hz, C-1α) and 104.6 ($J_{C,H}$ 158.5 Hz, C-1β).

Anal. Calc. for $C_{40}H_{46}O_{11}$ (702.8): C, 68.36; H, 6.60. Found: C, 67.93; H, 6.59.

*2,3,2',3'-Tetra-*O*-benzyl-6,6'-di-*O*-p-tolylsulfonyl-α,β-trehalose* (**8**). — Compound **8** was synthesized from **7** by the same procedure as described for the preparation of **3**. The yield was 67%, R_F 0.50 (3:1 toluene–ethyl acetate); $[\alpha]_D^{25}$ −44.0° (*c* 0.2, $CHCl_3$); ^{1}H-n.m.r. ($CDCl_3$): δ 7.16–7.35 (24 H, Ph-H) and 7.71–7.80 (4 H, Ph-H); ^{13}C-n.m.r. ($CDCl_3$): δ 99.3 (d, $J_{C,H}$ 170.2 Hz, C-1α) and 103.5 (d, $J_{C,H}$ 161.4 Hz, C-1β).

Anal. Calc. for $C_{54}H_{58}O_{15}S_2 \cdot 0.33\ C_6H_5CH_3$ (1011.1): C, 64.94; H, 5.87. Found: C, 65.11; H, 5.82.

2,3,2',3'-Tetra-O-*benzyl-6,6'-di*-O-*mycoloyl-α,β-trehalose* (**9**). — Compound **9** was obtained from **8** by the same procedure as described for compound **4**. The yield was 60%, R_F 0.54 (4:1 toluene–ethyl acetate); $[\alpha]_D^{25}$ +18.6° after 42 h (*c* 0.4, $CHCl_3$).

Anal. Calc. for $C_{200}H_{358}O_{16}$ (3018.9): C, 79.57; H, 11.95. Found: C, 79.63; H, 11.76.

6,6'-Di-O-*mycoloyl-α,β-trehalose* (**10**). — Compound **9** (175 mg) was dissolved in chloroform (5 mL) and THF (5 mL), and the solution was hydrogenated in the presence of palladium black (35 mg) for 4 h at 50° under atmospheric pressure. After removal of the catalyst by filtration, the filtrate was concentrated. The residue was chromatographed on silica gel with 10:1 chloroform–methanol to yield **10**. The compound (123 mg, 80%) was obtained as white powder as described for **5**, R_F 0.38 (10:1 chloroform–methanol); $[\alpha]_D^{25}$ −19.8° (*c* 0.3, $CHCl_3$).

Anal. Calc. for $C_{172}H_{334}O_{16}$ (2658.4): C, 77.71; H, 12.66. Found: C, 77.49; H, 12.51.

Biological tests. — The determination of lethal toxicity in mice and the *in situ* activation of mouse peritoneal macrophages were carried out according to the method reported previously[6].

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