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Brief Article

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Development of Vizantin, a Safe Immunostimulant, Based on the Structure–Activity Relationship of Trehalose-6,6′-dicorynomycolate

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Supporting Information

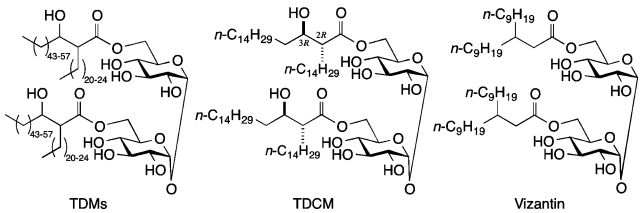
ABSTRACT: Vizantin, 6,6′-bis-*O*-(3-nonyldodecanoyl)-α,α′-trehalose, was developed as a safe immunostimulator on the basis of a structure–activity relationship (SAR) study with trehalose 6,6′-dicorynomycolate (TDCM). It was possible to synthesize vizantin on a large scale more easily than in the case of TDCM, and the compound exhibited more potent prophylactic effect on experimental lung metastasis of B16–F0 melanoma cells. Because vizantin stimulated human macrophages, it is a promising candidate for clinical application.

INTRODUCTION

Trehalose 6,6′-dimycolates (TDMs) (formerly called cord factor) are well-known structural components of the outer surface membrane of *Mycobacterium tuberculosis*.[1−3] TDMs trigger innate immune responses through Toll-like receptor 2 (TLR2)[4] and show a variety of significant properties such as potent antitumor activity against in vivo models of several murine tumor cells,[5−8] enhancement of nonspecific host resistance to microorganisms,[9,10] and a number of others.[11−15]

In recent years, bacterial components, including TDMs, have attracted considerable attention as lead compounds for adjuvant development.[16−23] However, a primary concern over the use of these compounds is that they can overactivate immune responses, leading to clinical symptoms of septic shock. Therefore, it is an imperative for the design of safe immunostimulants that detailed knowledge of structure–activity relationships (SARs) is gathered in order to harness the beneficial effects without causing toxicity.

Chart 1. TDMs, TDCM, and Vizantin



It was hypothesized that a shorter carbon chain analogue of TDM, trehalose 6,6′-dicorynomycolate (TDCM), might be useful in the development of a safer adjuvant because its lethal toxicity to mice is known to be much lower than that of TDMs.[24] Thus, we have synthesized a variety of TDCM analogues in order to identify potential candidates that display increased immunological activity with minimal associated toxicity. In this report, we describe the relationship between the immunological activity and structure of these molecules, focusing on the fatty acid residue. We also investigated in detail the most promising candidate (which is designated vizantin) for clinical application using several assay systems.

In 1994, our project started with a study involving the synthesis of TDCM previously isolated from *Corynebacterium* spp.[25−27] During this initial stage, the stereochemistry of the fatty acid residues of TDCM was established to be 2*R*,3*R*.[28,29] Furthermore, it was found that the lethal toxicity in mice displayed by TDM was no longer observed using synthetic TDCM.[24] Twenty-five different TDCM analogues with a variety of chain lengths were then prepared, with 2*R*,3*R*,2′*R*,3′*R*- (hereafter abbreviated *RRRR*-), *SSSS*-, and *RRSS*-stereochemistry and monoesters with *RR*- and *SS*-stereochemistry, in order to study their SARs.[30] As a result, it was found that the chain length of the fatty acid residue significantly affected the immunostimulating activity while the stereochemistry had little impact. We subsequently focused on the contribution of the intramolecular hydrogen bond between the C3-hydroxyl group and the carbonyl group in the fatty acid residue on the immune response. The formation of hydrogen bond locked the conformation of the mycolic motif (α-substituted β-hydroxyester) in the fatty acid residue.[31−33] Thus, the presence of a hydroxyl group, which favorably interacted with the carbonyl moiety, was considered to be an essential factor for eliciting a variety of biological responses. To evaluate the effect of the hydrogen bond, we designed methyl ether analogue **8**, which masked the hydroxyl group and had two successive chiral centers in 2*R*,3*R* configurations.

RESULTS AND DISCUSSION

The synthetic route to **8** is illustrated in Scheme 1. (2*R*,3*R*)-Hydroxyester **2** was prepared from commercially available **1** in three steps according to the synthetic procedure for TDCM.[30] The optical purity of **2** was estimated to be over 98% ee based

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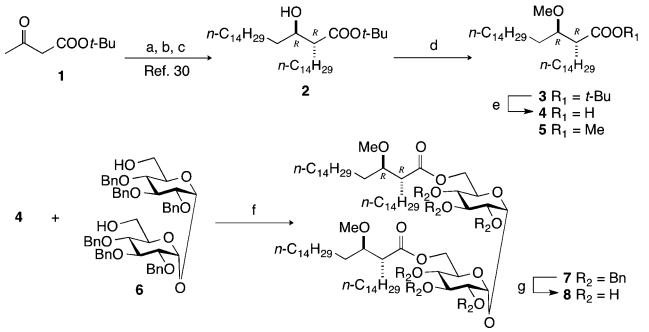
Scheme 1. Synthesis of C3-Methoxy Derivative 8[a]



[a]Reagents and conditions: (a) NaH, *n*-BuLi, *n*-$C_{14}H_{29}I$, THF, 0 °C, 0.5 h, 78%; (b) (*R*)-BINAPRuCl$_2$, H_2 (70 atm), MeOH, rt, 72 h, 94%, >98% ee; (c) LDA, HMPA, $C_{14}H_{29}I$, −48 °C, 6 h, 55%; (d) MeOTf, DTBP, CH_2Cl_2, rt, 30 h, 80%; (e) TMSOTf, CH_2Cl_2, 0 °C to rt, 1 h, 85%; (f) EDCI, DMAP, MS 4A powder, CH_2Cl_2, reflux, 4 h, 90%; (g) Pd(OH)$_2$, $CHCl_3$/MeOH (2:1), rt, 3 h, 87%.

on the ^{19}F NMR of the MTPA derivative. The hydroxyl group on **2** was converted to methyl ether using excess MeOTf and DTBP in CH_2Cl_2 at room temperature for 30 h. In addition, this methylation was sensitive to reaction temperature as transesterification took place under reflux, giving **5** as the major product. The cleavage of the *tert*-butyl ester was carried out using TMSOTf, and the resulting carboxylic acid **4** was condensed with hexabenzyl trehalose **6**[34] using EDCI and DMAP to afford **7**. Finally, hydrogenolysis over 20 mol % of Pd(OH)$_2$ resulted in the deprotection of all benzyl ethers to provide the desired **8**.

To examine the immunological properties of the hydroxy-masking analogue **8**, mouse macrophages (5.0×10^5 cells) were stimulated using **8** (50 μM) in the presence of fluorescent microspheres (Fluoresbrite carboxylate microspheres, 1.75 μm in diameter, Polysciences).[35] After 2 h, phagocytic activity was determined by measuring fluorescence intensity in the cells. As can be seen in Figure 1A, **8** had comparable potency to TDCM, although the LAU value (efficacy) of **8** was slightly lower. The supernatant was also harvested and investigated for the presence of macrophage-produced cytokines such as IL-6 and MIP-1β using commercial capture ELISAs,[36] with both exhibiting similar trends in potency and efficacy as were observed for the response of phagocytosis (Figure 1B,C).

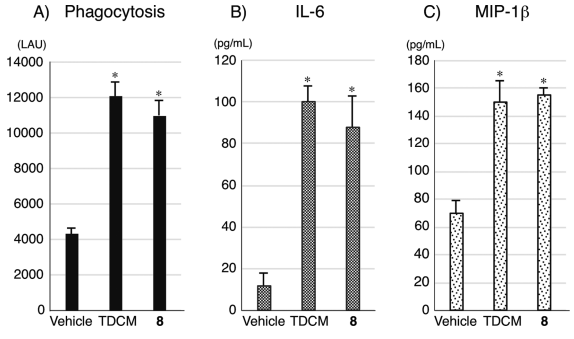
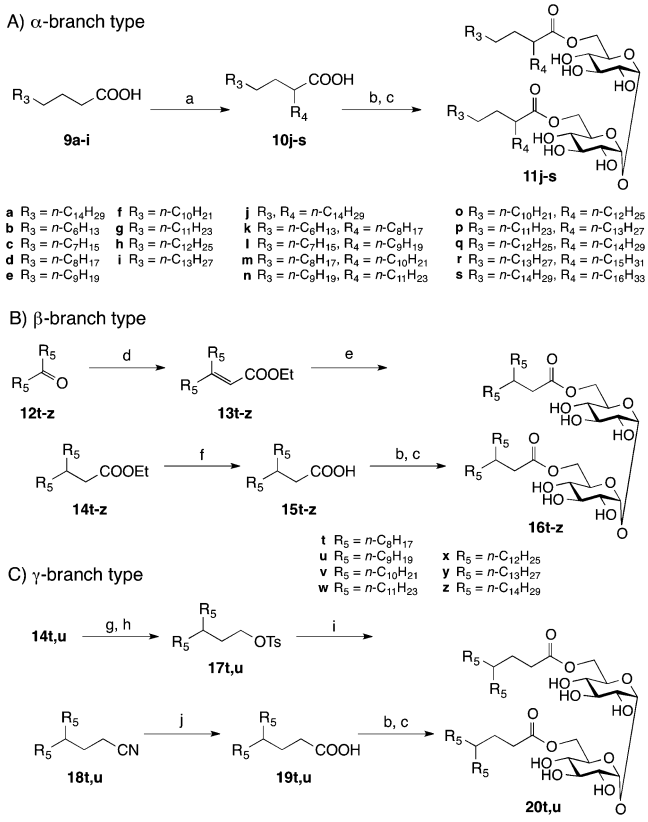


Figure 1. Immunological properties of TDCM and **8**. Mouse macrophages were incubated at 37 °C for 2 h. Phagocytic activity was determined by measuring the uptake of fluorescent microspheres as described in the Experimental Section. IL-6 and MIP-1β in culture supernatants were determined by ELISA kit. Values represent mean ± SD (n = 5). *P < 0.01, compared with vehicle (DMSO).

The observation that hydroxy-masking analogue **8** showed a significantly high level of macrophage activation suggested that the presence of the hydroxyl group might not be necessary for the induction of an immune response. Thus, dehydroxy derivative **11j** was designed in addition to its mimetic analogues with achiral fatty acid chains such as α-branched **11k−s**, β-branched **16t−z**, and γ-branched **20t** and **20u** in order to eliminate the effect of the chiral centers (Scheme 2).

Scheme 2. Preparations of Dehydroxy Derivative 11j and the Mimetic Analogues[a]



[a]Reagents and conditions: (a) NaH, LDA, R_4I, THF, 0 °C to rt; (b) **6**, EDCI, DMAP, MS 4A powder, CH_2Cl_2, reflux; (c) Pd(OH)$_2$, $CHCl_3$/MeOH (2:1), rt; (d) NaH, $(EtO)_2POCH_2CO_2Et$, THF, reflux; (e) PtO_2, H_2 (1 atm), $CHCl_3$/MeOH (5:1), rt; (f) KOH, *n*-BuOH/H_2O (2:1), 100 °C; (g) $LiAlH_4$, THF, rt; (h) TsCl, Et_3N, DMAP, CH_2Cl_2, rt; (i) KCN, DMF, 80 °C; (j) KOH, *n*-BuOH, 100 °C.

Dehydroxy fatty acid **10j** was prepared by the alkylation of commercially available stearic acid (**9a**) with 1-iodotetradecane using NaH and then LDA. The α-branched fatty acids **10k−s** were synthesized by the same procedure. Preparations of β-branched **15t−z** were achieved from ketones **12t−z**. The HWE reaction with $(EtO)_2POCH_2COOEt$ was carried out under reflux conditions, giving the conjugated esters **13t−z** in good yields. Hydrogenation of the double bond using a catalytic amount of PtO_2 proceeded smoothly, and the resulting **14t−z** were hydrolyzed to afford **15t−z**. Synthetic intermediates **14t** and **14u** were utilized for producing γ-branched **19t** and **19u**. Reductions of **14t** and **14u** with $LiAlH_4$ followed by cyanations of tosylate **17t** and **17u** gave **18t** and **18u**, which were hydrolyzed with excess KOH to yield **19t** and **19u**, respectively. Condensations of trehalose derivative **6** with the prepared fatty acids were performed using EDCI and DMAP to give the

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corresponding diesters. Finally deprotection of the benzyl ethers using $Pd(OH)_2$ afforded the desired trehalolipids **11j–s**, **16t–z**, **20t**, and **20u**.

The synthesized trehalolipids were evaluated for their phagocytosis inducing activities (Figure 2). In this assay,

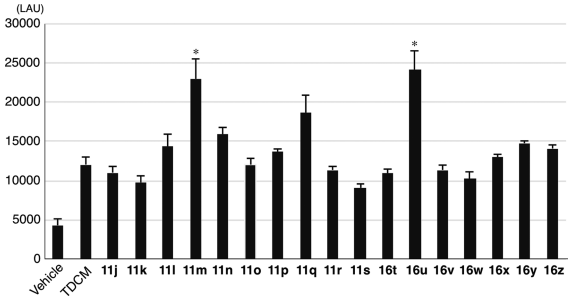

Figure 2. Phagocytic activities of TDCM, **11j–s**, and **16t–z**. Mouse macrophages (5.0×10^5 cells) were stimulated using 50 μM TDCM and analogues in the presence of fluorescent microspheres at 37 °C for 2 h. Phagocytic activities were determined by measuring the uptake of fluorescent microspheres as described in the Experimental Section. Values represent mean ± SD (n = 5). *P < 0.01, compared with the vehicle group (DMSO).

dehydroxy derivative **11j** showed comparable activity to that of TDCM. Among the synthesized compounds, the β-branched type derivative with C-9 side chains, compound **16u**, showed the highest activity followed by the α-branched **11m** with C-10 side chains. γ-Branched analogues **20t**, **20u**, and linear chain analogue with dodecanoic acid residue **9d** were practically inactive (data not shown). From these findings, it was concluded that the presence of a hydroxyl group on the fatty acid chain is not required for phagocytosis inducing activity. Although the exact role of the fatty acid chain is unclear, it is apparent that an α- or a β-branched structure is essential for inducing the activity and that the optimal chain length for the carbon atom of the ester moiety is C-12. IL-6 and MIP-1β inductions also showed analogous results (see Supporting Information (SI)).

The effect on human-derived immune cells was subsequently investigated with the aim of developing a new immune activating drug for humans. As seen in Figure 3A, release of MIP-1β from human monocyte macrophage cells (THP-1) was dramatically increased on treatment with **16u**, but very little release was observed for other human cell lines A595 cell and

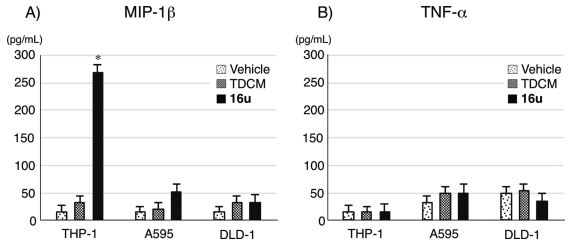

Figure 3. MIP-1β and TNF-α released from human cells treated with TDCM and **16u**. The THP-1, A549, and DLD-1 (5.0×10^5 cells) were incubated with 100 μM TDCM or **16u** at 37 °C for 3 h. MIP-1β and TNF-α in culture supernatants were determined using ELISA kits. In (B), *Escherichia coli* LPS (0.5 μg) was employed as a positive control that caused the release of 830 ± 34 pg/mL of TNF-α from THP-1. Values represent mean ± SD (n = 5). *P < 0.01, compared with the vehicle group (DMSO).

DLD-1 cell. TDCM and compound **11m** did not show such specificity (data not shown). Among a variety of cytokines, TNF-α has significant high proinflammatory activity, which may lead to serious complications such as a cytokine storm.[37] Interestingly, **16u** did not induce TNF-α (Figure 4B);

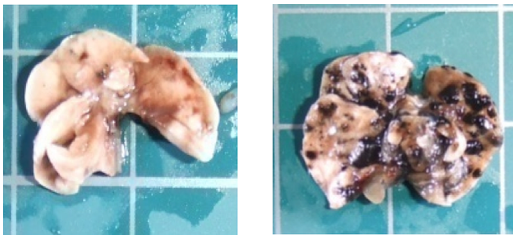

Figure 4. Prophylactic effect of vizantin (**16u**) on experimental lung metastasis. Left picture is a lung treated with **16u** and right is the vehicle control (saline including 1.1% Tween 80, 5.6% mannitol, and 10% soy oil). The lung metastasis was evaluated at 21 days after tumor inoculation.

therefore, it is conceivable that **16u** would not induce a cytokine storm, a factor which contributes to its very low toxicity. The lethal toxicity of **16u** in mice was found to be incredibly low. All mice (n = 5) survived until day 20 even when doses of 50 mg of **16u** were injected (corresponding to 2500 mg/kg) intraperitoneally at 7-day intervals (see SI). Moreover, an Ames test demonstrated that **16u** is totally free from mutagenesis[38] (see SI). Owing to these results, **16u** was selected as the most suitable candidate for clinical application, and it was named vizantin after the symbolic hill Bizan of Tokushima city.

The antitumor activity of vizantin (**16u**) was examined with respect to its prophylactic effect on distant metastasis of tumor cells (B16–F0 melanoma cells) in accordance with the protocol reported by Azuma and co-workers.[24] When 100 μg (4.0 mg/kg) of **16u** was administered intravenously in mice (C57BL/6 mice, 6 week old, male) at 7-day intervals over 14 days before tumor inoculation (5×10^5 cells), vizantin showed strong inhibition of lung metastasis, with an inhibition ratio of more than 98% (Figure 4).

Furthermore, it was found that oral administration of **16u** exhibited a significant inhibition of lung metastasis without the loss of body weight and death in mice. As shown in Table 1, multiple (6 times) administrations of **16u** at 7-day intervals from 14 days before tumor inoculation inhibited spontaneous

Table 1. Dose Dependency of Prophylactic Inhibition by Oral Administration of Vizantin (16u)[a]

		no. of lung metastases[b]	
	treatment	mean ± SD (% inhibition)	range
control	(vehicle)[c]	197.5 ± 5	92–267
16u	100 μg	85.5 ± 3 (57)*[d]	1–234
16u	500 μg	75.5 ± 6 (62)*[d]	6–138
16u	1000 μg	68.0 ± 4 (66)*[d]	17–130
TDCM	1000 μg	117 ± 4 (41)*[d]	32–187

[a]Groups of C57BL/6 mice (n = 8) intravenously inoculated with B16–F0 melanoma cells (5×10^5 cells) and orally administered with the indicated doses of **16u** and TDCM from 14 days before tumor inoculation. [b]The lung metastasis was evaluated at 28 days after tumor inoculation. [c]Saline containing 1.1% Tween 80, 5.6% glucose, and 10% soy oil. [d]P < 0.01, compared with the vehicle group.

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lung metastasis of B16−F0 melanoma cells, with a clear dose response and higher observed potency than TDCM. Because **16u** was not detected in blood sera even when using supercritical HPLC, orally administered **16u** may act upon gut immunity (e.g., Peyer's patches[39,40]) and induce antitumor activity without being absorbed. However, more detailed investigation is required for full elucidation of the effect.

CONCLUSION

On the basis of the knowledge on SAR of the C3-methoxy derivative **8** and dehydro derivative **11j**, we developed vizantin (**16u**) as a novel immunostimulating compound. Because **16u** showed potent antitumor activity and stimulated mouse macrophages, it was considered to be a nonspecific immunostimulant. In addition, **16u** also stimulated human immune cells such as THP-1 cells, whereas TDCM had no effect. These findings demonstrate that **16u** may be a clinically useful agent for the prevention and/or treatment of cancer. To explore its practical use, its detailed action mechanism and other adjuvant properties are now under investigation.

EXPERIMENTAL SECTION

According to Scheme 2B, vizantin, 6,6′-bis-*O*-(3-nonyldodecanoyl)-*α*,*α*′-trehalose (**16u**) was synthesized in five steps with a 59% overall yield. This section describes experimental procedure for the synthesis of **16** from **15u**.

6,6′-Bis-*O*-(3-nonyldodecanoyl)-*α*,*α*′-trehalose (16u). The mixture of **15u** (462 mg, 1.41 mmol), 2,3,4,2′,3′,4′-hexabenzyl-*α*,*α*′-trehalose **6** (500 mg, 0.566 mmol), EDCI (434 mg, 2.27 mmol), DMAP (69 mg, 0.566 mmol), and MS 4A powder (500 mg) in CH_2Cl_2 (5 mL) was stirred for 6 h under reflux. The resulting mixture was filtered using Celite 535RVS, and the filtrate was washed with brine. The separated organic layer was dried over anhydrous $MgSO_4$ and concentrated under reduced pressure. The residue was purified by flash chromatography (silica gel, hexane:EtOAc, 20:1) to give 6,6′-bis-*O*-(3-nonyldodecanoyl)-2,3,4,2′,3′,4′-hexabenzyl-*α*,*α*′-trehalose (724 mg, 85%) as a colorless syrup. $[\alpha]_D^{21}$ +65.3° (*c* 1.0 $CHCl_3$). FT IR (neat) 3088, 3063, 3031, 2925, 2853, 1944, 1871, 1806, 1739 cm^{-1}. ^1H NMR (300 MHz in $CDCl_3$) *δ* 0.87 (12H, t, *J* = 5.1 Hz), 1.20 (64H, m), 1.81 (2H, m), 2.20 (4H, d, *J* = 6.9 Hz), 3.54 (2H, t, *J* = 9.3 Hz), 3.56 (2H, m), 4.04 (2H, t, *J* = 9.3 Hz), 4.09 (4H, m), 4.23 (2H, m), 4.51 (2H, d, *J* = 10.5 Hz), 4.67 (2H, d, *J* = 12.0 Hz), 4.72 (2H, d, *J* = 12.0 Hz), 4.86 (4H, d, *J* = 10.5 Hz), 5.00 (2H, d, *J* = 10.5 Hz), 5.17 (2H, d, *J* = 3.6 Hz), 7.23−7.37 (30H, m). ^{13}C NMR (75 MHz in $CDCl_3$) *δ* 14.13, 22.68, 26.51, 29.33, 29.56, 29.64, 29.92, 31.89, 33.65, 33.76, 34.89, 39.08, 62.35, 69.12, 72.94, 75.30, 75.70, 77.60, 79.38, 81.56, 94.02, 127.44, 127.63, 127.78, 127.92, 128.09, 128.41, 128.47, 137.78, 137.84, 138.60, 173.24. HRMS (FAB$^+$) *m*/*z* calcd for $C_{96}H_{138}O_{13}Na$ (M^+ + Na) 1522.0036, found 1522.0020.

To a solution (10 mL) of 6,6′-bis-(3-nonyldodecanoyl)-2,3,4,2′,3′,4′-hexabenzyl trehalose (724 mg, 0.483 mmol) in $CHCl_3$ and MeOH (2:1) was added $Pd(OH)_2/C$ (145 mg, 20 w/w%). The mixture was stirred vigorously for 8 h at room temperature under H_2 (1 atm), filtered, and the concentrated residue purified by flash chromatography (silica gel, CH_2Cl_2:MeOH, 20:1) to give **16u** (356 mg, 77%) as a colorless syrup. $[\alpha]_D^{21}$ +62.8° (*c* 0.7 $CHCl_3$). FT IR (neat) 3316, 2926, 2854, 1743 cm^{-1}. ^1H NMR (300 MHz in C_5D_5N) *δ* 0.86 (12H, t, *J* = 6.3 Hz), 1.24 (64H, m), 2.03 (2H, m), 2.37 (4H, d, *J* = 6.6 Hz), 4.19 (2H, t, *J* = 9.3 Hz), 4.31 (2H, dd, *J* = 9.3, 3.9 Hz), 4.74 (2H, t, *J* = 9.3 Hz), 4.85 (2H, dd, *J* = 11.7, 5.1 Hz), 5.01 (2H, dd, *J* = 11.7, 1.8 Hz), 5.10 (2H, m), 5.89 (2H, d, *J* = 3.9 Hz). ^{13}C NMR (75 MHz in C_5D_5N) *δ* 14.29, 22.94, 29.84, 29.62, 29.91, 29.94, 30.22, 32.12, 34.10, 35.23, 39.33, 64.26, 71.48, 71.96, 73.35, 74.82, 95.82, 173.51. HRMS (FAB$^+$) *m*/*z* calcd for $C_{54}H_{102}O_{13}Na$ (M^+ + Na) 981.7218, found 981.7198.

Assay of Phagocytosis. Mouse macrophages (80% confluent in 48-well plates) were stimulated with 50 *μ*M concentration of compound in the presence of 5.0×10^5 fluorescent microspheres/mL. After 2 h incubation, cells were washed and the fluorescence intensity in the cells was determined using a fluorescent imaging analyzer (FLA-1000, Fujifilm, Japan).

Statistics. Results were expressed as the mean ± SD, with *n* representing the sample size. Statistical comparisons were performed using an unpaired *t* test or one-way analysis of variance (ANOVA) with Bonferroni correction. *P* values of less than 0.05 were considered statistically signification.

ASSOCIATED CONTENT

Supporting Information

Experimental procedures concerning the synthesis, spectral characterization data (^1H and ^{13}C NMR) of **16u**, and a description of biological tests. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

†These authors contributed to this work equally. All authors have given approval to the final version of the manuscript.

Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

MTPA, *α*-methoxy-*α*-(trifluoromethyl)-phenylacetyl; DTBP, 2,6-di-*tert*-butylpyridine; BINAP, 2,2′-bis(diphenylphosphino)-1,1′-binaphthyl; EDCI, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide hydrochloride; MS 4A, molecular sieves 4 Å; LAU, linear arbitrary unit; IL-6, interleukin-6; MIP-1*β*, macrophage inflammatory protein-1*β*; SD, standard deviation; HWE, Horner−Wadsworth−Emmons; LPS, lipopolysaccharide

REFERENCES

(1) Bloch, H. Studies on the virulence of tubercle bacilli; the relationship of the physiological state of the organisms to their pathogenicity. *J. Exp. Med.* **1950**, *92*, 507−526.

(2) Noll, H.; Bloch, H.; Asselineau, J.; Lederer, E. The chemical structure of the cord factor of *Mycobacterium tuberculosis*. *Biochim. Biophys. Acta* **1956**, *20*, 299−309.

(3) Middlebrook, G.; Dubos, R. J.; Pierce, C. Virulence and morphological characteristics of mammalian tubercle bacilli. *J. Exp. Med.* **1947**, *86*, 175−183.

(4) Bowdish, D. M.; Sakamoto, K.; Kim, M. J.; Kroos, M.; Mukhopadhyay, S.; Leifer, C. A.; Tryggvason, K.; Gordon, S.; Russell, D. G. MARCO, TLR2, and CD14 are required for macrophage cytokine responses to mycobacterial trehalose dimycolate and *Mycobacterium tuberculosis*. *PLoS Pathog.* **2009**, *5*, e1000474.

(5) Bekierkunst, A.; Levij, I. S.; Yarkoni, E. Suppression of urethane-induced lung adenomas in mice treated with trehalose-6,6-dimycolate

(cord factor) and living bacillus Calmette Guerin. *Science* **1971**, *174*, 1240−1242.
(6) Furukawa, M.; Ohtsubo, Y.; Sugimoto, N.; Katoh, Y.; Dohi, Y. Induction of tumoricidal activated macrophages by a liposome-encapsulated glycolipid, trehalose 2,3,6′-trimycolate from *Gordona aurantiaca*. *FEMS Microbiol. Immunol.* **1990**, *2*, 83−88.
(7) Natsuhara, Y.; Oka, S.; Kaneda, K.; Kato, Y.; Yano, I. Parallel antitumor, granuloma-forming and tumor-necrosis-factor-priming activities of mycoloyl glycolipids from *Nocardia rubra* that differ in carbohydrate moiety: structure−activity relationships. *Cancer Immunol. Immunother* **1990**, *31*, 99−106.
(8) Orbach-Arbouys, S.; Tenu, J. P.; Petit, J. F. Enhancement of in vitro and in vivo antitumor activity by cord factor (6,6-dimycolate of trehalose) administered suspended in saline. *Int. Arch. Allergy Appl. Immunol.* **1983**, *71*, 67−73.
(9) Ribi, E.; Granger, D. L.; Milner, K. C.; Yamamoto, K.; Strain, S. M.; Parker, R.; Smith, R. W.; Brehmer, W.; Azuma, I. Induction of resistance to tuberculosis in mice with defined components of mycobacteria and with some unrelated materials. *Immunology* **1982**, *46*, 297−305.
(10) Sharma, A.; Upadhyay, S. N. Cellular immune responsiveness in rabbits with *Setaria digitata* filarial antigen and TDM adjuvant. *Int. J. Immunopharmacol.* **1993**, *15*, 395−400.
(11) Sakaguchi, I.; Ikeda, N.; Nakayama, M.; Kato, Y.; Yano, I.; Kaneda, K. Trehalose 6,6-dimycolate (cord factor) enhances neovascularization through vascular endothelial growth factor production by neutrophils and macrophages. *Infect. Immun.* **2000**, *68*, 2043−2052.
(12) Saita, N.; Fujiwara, N.; Yano, I.; Soejima, K.; Kobayashi, K. Trehalose 6,6-dimycolate (cord factor) of *Mycobacterium tuberculosis* induces corneal angiogenesis in rats. *Infect. Immun.* **2000**, *68*, 5991−5997.
(13) Kunkel, S. L.; Chensue, S. W.; Strieter, R. M.; Lynch, J. P.; Remick, D. G. Cellular and molecular aspects of granulomatous inflammation. *Am. J. Respir. Cell Mol. Biol.* **1989**, *1*, 439−447.
(14) Orme, I. M. The immunopathogenesis of tuberculosis: a new working hypothesis. *Trends Microbiol.* **1998**, *6*, 94−97.
(15) Yarkoni, E.; Rapp, H. J. Granuloma formation in lungs of mice after intravenous administration of emulsified trehalose-6,6′-dimycolate (cord factor): reaction intensity depends on size distribution of the oil droplets. *Infect. Immun.* **1977**, *18*, 552−554.
(16) Andersen, C. S.; Agger, E. M.; Rosenkrands, I.; Gomes, J. M.; Bhowruth, V.; Gibson, K. J. C.; Petersen, R. V.; Minnikin, D. E.; Besra, G. S.; Andersen, P. A simple mycobacterial monomycolated glycerol lipid has potent immunostimulatory activity. *J. Immunol.* **2009**, *182*, 424−432.
(17) Didierlaurent, A. M.; Morel, S.; Lockman, L.; Giannini, S. L.; Bisteau, M.; Carlsen, H.; Kielland, A.; Vosters, O.; Vanderheyde, N.; Schiavetti, F.; Larocque, D.; Van Mechelen, M.; Garçon, N. AS04, an aluminum salt- and TLR4 agonist-based adjuvant system, induces a transient localized innate immune response leading to enhanced adaptive immunity. *J. Immunol.* **2009**, *183*, 6186−6197.
(18) Garçon, N.; Chomez, P.; Van Mechelen, M. GlaxoSmithKline adjuvant systems in vaccines: concepts, achievements and perspectives. *Expert Rev. Vaccines* **2007**, *6*, 723−739.
(19) Martin, O. R.; Zhou, W.; Wu, X.; Front-Deschamps, S.; Moutel, S.; Schindl, K.; Jeandet, P.; Zbaeren, C.; Bauer, J. A. Synthesis and immunobiological activity of an original series of acyclic lipid a mimics based on a pseudodipeptide backbone. *J. Med. Chem.* **2006**, *49*, 6000−6014.
(20) Yang, H.-Z.; Xu, S.; Liao, X.-Y.; Zhang, S.-D.; Liang, Z.-L.; Liu, B.-H.; Bai, J.-Y.; Jiang, C.; Ding, J.; Cheng, G.-F.; Liu, G. A novel immunostimulator, N^2-[α-*O*-benzyl-*N*-(acetylmuramyl)-L-alanyl-D-isoglutaminyl]-N^6-*trans*-(*m*-nitrocinnamoyl)-L-lysine, and its adjuvancy on the hepatitis B surface antigen. *J. Med. Chem.* **2005**, *48*, 5112−5122.
(21) Brandenburg, K.; Hawkins, L.; Garidel, P.; Andrä, J.; Müller, M.; Heine, H.; Koch, M. H. J.; Seydel, U. Structural polymorphism and endotoxic activity of synthetic phospholipid-like amphiphiles. *Biochemistry* **2004**, *43*, 4039−4046.
(22) Ishihara, C.; Miyazawa, M.; Nishio, J.; Azuma, I.; Chesebro, B. Use of low toxicity adjuvants and killed virus to induce protective immunity against the Friend murine leukaemia retrovirus-induced disease. *Vaccine* **1992**, *10*, 353−356.
(23) Saito, R.; Tanaka, A.; Sugiyama, K.; Azuma, I.; Yamamura, Y. Adjuvant effect of cord factor, a mycobacterial lipid. *Infect. Immun.* **1976**, *13*, 776−781.
(24) Watanabe, R.; Yoo, Y. C.; Hata, K.; Motobe, M.; Koike, Y.; Nishizawa, M.; García, D. M.; Noguchi, Y.; Imagawa, H.; Yamada, H.; Azuma, I. Inhibitory effect of trehalose dimycolate (TDM) and (TDCMs), with low toxicity on lung metastasis of tumour cells in mice. *Vaccine* **1999**, *17*, 1484−1492.
(25) Ioneda, T.; Lenz, M.; Pudles, J. Chemical constitution of a glycolipid from *C. diphtheriae* P.W.B. *Biochem. Biophys. Res. Commun.* **1963**, *13*, 110−114.
(26) Senn, M.; Ioneda, T.; Pudles, J.; Lederer, E. Spectrométrie de masse de glycolipides 1. Structure du *"cord factor"* de *Corynebacterium diphtheriae*. *Eur. J. Biochem.* **1967**, *1*, 353−356.
(27) Datta, A. K.; Takayama, K. Isolation and purification of trehalose 6-mono- and 6,6-di-corynomycolates from *Corynebacterium matruchotii*. Structural characterization by ^{1}H NMR. *Carbohydr. Res.* **1993**, *245*, 151−158.
(28) Nishizawa, M.; Minagawa, R.; García, D. M.; Hatakeyama, S.; Yamada, H. Syntheses and characterization of four diastereomers of trehalose-6,6′-dicorynomycolates (TD BH32). *Tetrahedron Lett.* **1994**, *35*, 5891−5894.
(29) Nishizawa, M.; García, D. M.; Minagawa, R.; Noguchi, Y.; Imagawa, H.; Yamada, H.; Watanabe, R.; Yoo, Y. C.; Azuma, I. Effective synthesis of four isomeric trehalose dicorynomycolates (TDCMs) and their immunoadjuvant activities. *Synlett* **1996**, 452−454.
(30) Nishizawa, M.; Yamamoto, H.; Imagawa, H.; Chassefière, V. B.; Petit, E.; Azuma, I.; Papy-García, D. Efficient syntheses of a series of trehalose dimycolate (TDM)/trehalose dicorynomycolate (TDCM) analogues and their interleukin-6 level enhancement activity in mice sera. *J. Org. Chem.* **2007**, *72*, 1627−1633.
(31) Durand, H.; Welby, M.; Lanéelle, G.; Tocanne, J.-F. Phase behaviour of cord factor and related bacterial glycolipid toxins. *Eur. J. Biochem.* **1979**, *93*, 103−112.
(32) Durand, H.; Gillois, M.; Tocanne, J.-F.; Lanéelle, G. Property and activity of mycoloyl esters of methyl glucoside and trehalose. *Eur. J. Biochem.* **1979**, *94*, 109−118.
(33) Moody, D. B.; Guy, M. R.; Grant, E.; Cheng, T.-Y.; Brenner, M. B.; Besra, G. S.; Porcelli, S. A. CD1b-mediated T cell recognition of a glycolipid antigen generated from mycobacterial lipid and host carbohydrate during Infection. *J. Exp. Med.* **2000**, *192*, 965−976.
(34) Imagawa, H.; Tsuchihashi, T.; Singh, R. K.; Yamamoto, H.; Sugihara, T.; Nishizawa, M. Triethyl- (or trimethyl-) silyl triflate-catalyzed reductive cleavage of triphenylmethyl (trityl) ethers with triethylsilane. *Org. Lett.* **2003**, *5*, 153−155.
(35) Callegan, M. C.; Jett, B. D.; Hancock, L. E.; Gilmore, M. S. Role of hemolysin BL in the pathogenesis of extra-intestinal *Bacillus cereus* infection assessed in an endophthalmitis model. *Infect. Immun.* **1999**, *67*, 3357−3366.
(36) We found that IL-6 and MIP-1β are released from macrophages treated with TDCM.
(37) Beutler, B.; Greenwald, D.; Hulmes, J. D.; Chang, M.; Pan, Y. C.; Mathison, J.; Ulevitch, R.; Cerami, A. Identity of tumour necrosis factor and the macrophage-secreted factor cachectin. *Nature* **1985**, *316*, 552−554.
(38) Mortelmans, K.; Zeiger, E. The Ames Salmonella/microsome mutagenicity assay. *Mutat. Res.* **2000**, *455*, 29−60.
(39) Nagler-Anderson, C. Man the barrier! strategic defences in the intestinal mucosa. *Nature Rev. Immunol.* **2001**, *1*, 59−67.
(40) Neutra, M. R.; Frey, A.; Kraehenbuhl, J.-P. Epithelial M cells: Gateways for mucosal infection and immunization. *Cell* **1996**, *86*, 345−348.