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Simplified Deoxypropionate Acyl Chains for *Mycobacterium tuberculosis* Sulfoglycolipid Analogues: Chain Length is Essential for High Antigenicity

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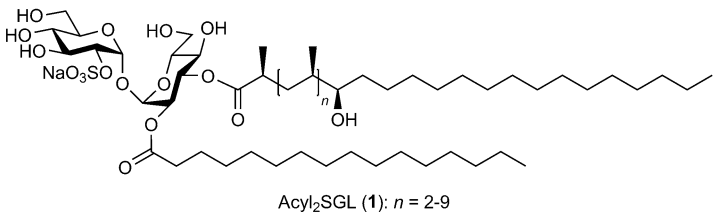
Human tuberculosis remains a major worldwide health problem, with about 1.5 million casualties every year. Despite numerous efforts over decades to implement better diagnosis and extensive availability of antibiotic treatments, this number is only slowly decreasing, making tuberculosis still one of the most lethal infectious diseases in the world.[1] New antibiotic treatments against *Mycobacterium tuberculosis*, the etiologic agent of human tuberculosis, are needed.[2] A new vaccinal approach is also mandatory, because the bacillus of Calmette and Guérin (BCG), the only existing vaccine against tuberculosis, does not afford protection in adults.[3]



Scheme 1. Structures of the natural $Acyl_2SGLs$ **1** from *M. tuberculosis*.

In this context, lipidic antigens presented by the CD1 (cluster of differentiation 1) family of proteins might be worth consideration as potential candidates for a subunit vaccine.[4] Indeed, experimental demonstration of the vaccinal interest of lipidic antigens in the case of mycobacterial infections was provided in 2003, when it was shown that immunization of guinea pigs with an uncharacterized mixture of mycobacterial lipids improved the pulmonary pathology of the animals after *M. tuberculosis* infection.[5] We found strong responses to diacylsulfoglycolipids ($Acyl_2SGLs$, Scheme 1) only in patients with clinical histories of mycobacterial infection; this supported the conclusion that lipid-specific T-cells are primed during infection and persist in patients with latent tuberculosis.[6]

Most of the mycobacterial lipidic antigens characterized so far are presented by the CD1b protein. Among these antigens, $Acyl_2SGLs$ **1** (Scheme 1) stimulated human CD1b-restricted $\alpha\beta$ T-cells to release interferon-γ (IFN-γ) and to kill intracellular mycobacteria residing in *M. tuberculosis*-infected antigen-presenting cells (APCs).[6] The scarcity of this lipid antigen in mycobacterial cells prompted us to synthesize $Acyl_2SGL$ analogues in which the hydroxyphthioceranoic acyl unit (Scheme 1) had been replaced by chiral multiply methylated saturated and α,β-unsaturated fatty acyl chains.[7]

Some of these analogues were able to stimulate the $Acyl_2SGL$-specific T-cell clone, with the T-cell response governed by the presence of the sulfate group,[6] the location of the fatty acyl chains on the trehalose core, and the fine structure of the multiply methylated fatty acyl chain, including chirality and number of methyl groups.[7,8]

The very recent total synthesis of an $Acyl_2SGL$ with a specific hydroxyphthioceranoic acyl side chain (**1**, $n=7$, Scheme 1) revealed antigenic properties of the synthetic glycolipid identical to those of the natural mixture of antigens.[9] We show here that synthetic structures much simpler in their 1,3-methyl-branched lipid part are as potent as or more potent than the natural antigens **1**. Their synthesis is based on an efficient preparation of long-chain chiral 1,3-methyl-branched fatty acids of very high optical purity and on a new sequence of regioselective steps initiated by a tandem catalytic approach for the one-pot regioselective protection of trehalose. We further demonstrate the crucial role of the length of the lipid chain in the antigenic properties of synthetic SGLs.

We devised a new route to the deoxypropionate fatty acids that overcome the drawbacks (purification and solubility problems) of our previous synthesis[7] (Scheme 2).

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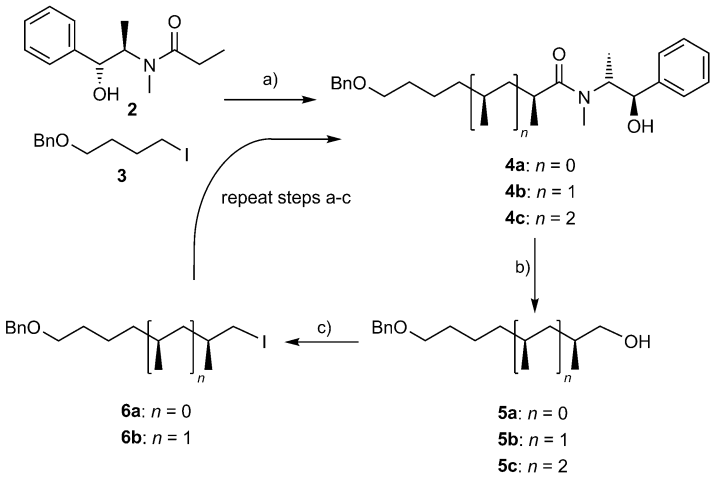


Scheme 2. Synthesis of chiral 1,3-methyl-branched alcohols **5a–c**. a) LDA (3.0 equiv), LiCl (7.0 equiv), THF, −78 °C, then iodide (1.0 equiv), 0 °C, 1 h for **3**, RT, 20 h for **6a** and **6b**, 85–99%; b) $LiNH_2BH_3$ (4.0 equiv), 0 °C to RT, 2 h, 90%; c) I_2 (1.2 equiv), PPh_3 (1.3 equiv), toluene, RT, 6 h, 90%.

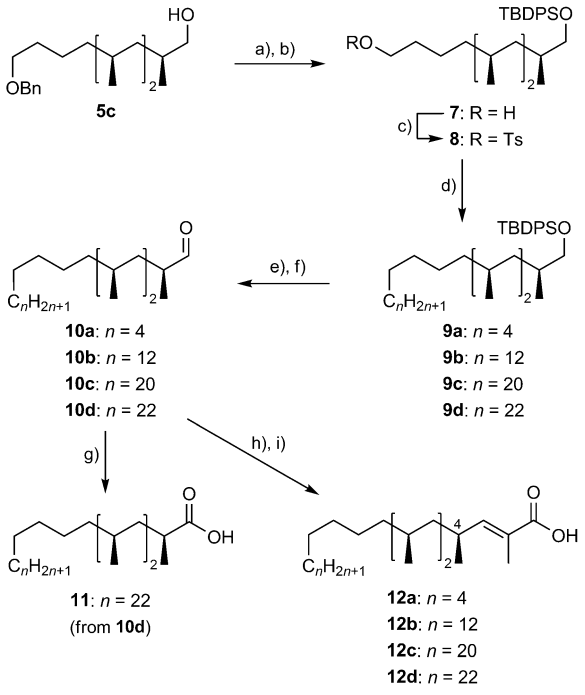
It started with the Myers alkylation of the lithium enolate of (1*R*,2*R*)-(−)-pseudoephedrine derivative **2**[10] with iodide **3**[11] in the presence of lithium chloride, which provided derivative **4a** in high yield. Evaluation of the diastereoselectivity of the reaction was difficult at this step because of the presence of rotamers around the amide bond in the NMR spectrum. Amide **4a** was thus reduced with an excess of lithium amidotrihydridoborate to give alcohol **5a** in 90% yield, and its optical purity (>98% *ee*) was established by derivatization of a sample of the alcohol to the (1*S*)-camphanate ester.[12] Conversion of **5a** into iodide **6a** was carried out in a 90% yield under standard Garreg conditions.[13]

The same sequence was repeated starting from **6a** for further introduction of methyl groups on the chain. When using more sterically hindered iodides **6a** and **6b**, the alkylation reaction had to be warmed to room temperature and left for 20 h to ensure high yields of alkylation products **4b** and **4c** (90 to 98%). The overall yield for each iteration from **6a** to **6b** was reproducibly over 70%, and the whole cycle could be run on multigram quantities in three days.

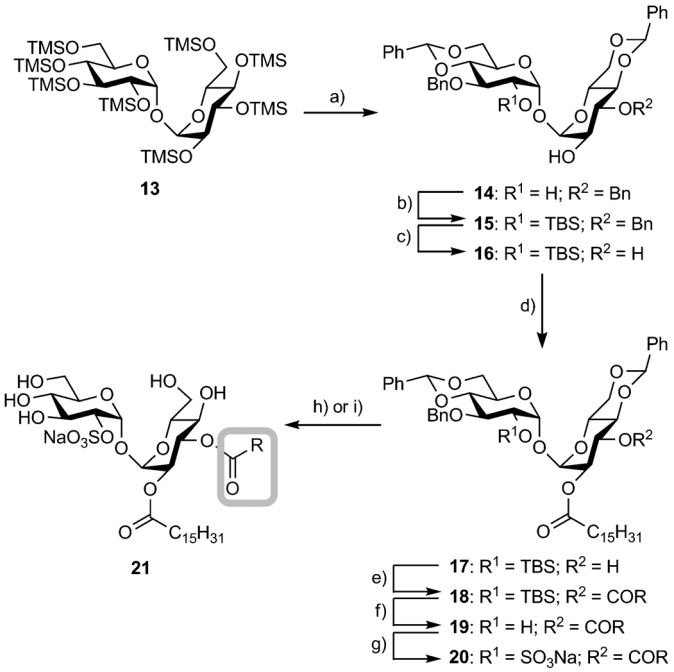
Once the required number of methyl groups had been incorporated, alcohol **5c** was protected as a *tert*-butyldiphenyl silyl ether (95%), and the benzyl ether was removed by hydrogenolysis to give alcohol **7** in high yield (Scheme 3). Tosylate **8** (90%) was obtained from **7** and subjected to copper-catalyzed nucleophilic displacement with suitable Grignard reagents[14] ($C_nH_{2n+1}MgBr$, THF, $n=4$, 12, 20, 22) to give the corresponding silyl ethers **9a–d** in moderate to good yields (45–60%). Removal of the silyl ether in **9** (90%) followed by PCC oxidation gave aldehydes **10a–d**. Carboxylic acid **11** was obtained from **10d** by using a slight excess of Jones's reagent in acetone, whereas unsaturated acids **12a–c** were prepared by coupling of **10a–c** with the Wittig reagent (carbethoxyethylidene)triphenylphosphorane, followed by saponification as previously described.[7,15]

The other major challenge in the synthesis of the SGLs is the rapid construction of a trehalose-based core structure suitably protected to differentiate the 2-, 2′-, 3-, and 3′-positions. Solutions to the problem have usually resulted in a lengthy generation of the appropriately protected disaccharide building block.[16] To simplify synthetic sequential methods, we recently developed one-pot regioselective protection approaches to mono- and disaccharides through Lewis-acid-catalyzed transformations on per-*O*-trimethylsilylated glycosides.[17,18] This approach was applied to the regioselective protection of trehalose with use of $FeCl_3{\cdot}6H_2O$ as an inexpensive and environmentally friendly catalyst.[18,19] In this procedure, two catalyzed acetalations and two regioselective reductive etherifications yielded C_2-symmetrical **14** in a good 61% yield[18] (Scheme 4). Desymmetrization[20] of diol **14** by monosilylation at a low temperature (→**15**) was followed by regioselective mono-3′-de-*O*-benzylation under hydrogen transfer conditions on the least hindered unit to afford diol **16**, leaving the benzylidene acetals unaffected.[21] Finally, regioselective esterification at the C-2 position of diol **16** with palmitic acid led to the key common intermediate **17** in good yield.

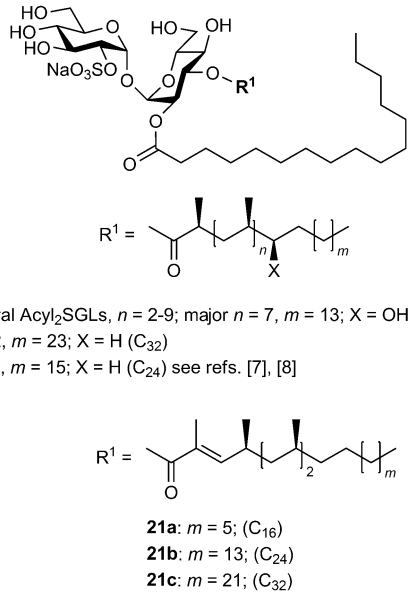
The synthesis of the diacylated SLGs was completed by a standard acylation of alcohol **17** (1–1.5 equiv) with the saturated and unsaturated chiral fatty acids to provide diacyl derivatives **18**. Quantitative desilylation to afford **19** and sulfation



Scheme 3. Synthesis of the chiral 1,3-methyl-branched fatty acids. a) *t*$BuPh_2SiCl$, NEt_3, CH_2Cl_2, RT, 10 h; b) H_2, cat. $Pd(OH)_2$/C, RT, 20 h, 81% over two steps; c) TsCl, NEt_3, CH_2Cl_2, RT, 4 h, 90%; d) $C_nH_{2n+1}MgBr$, $CuBr{\cdot}SMe_2$, THF, RT, 20 h, 45–60%; e) NBu_4F, THF, RT, 10 h; f) PCC, AcONa, CH_2Cl_2, RT, 2 h; g) Jones reagent, acetone/water, RT, 30 min; h) $Ph_3P{=}C(Me){-}COOEt$, CH_2Cl_2, RT, 4 d; i) KOH (2 M), ethanol/water, 80 °C.



Scheme 4. Preparation of the sulfoglycolipids. a) PhCHO, Et_3SiH, cat. $FeCl_3 \cdot 6H_2O$, 61% (see ref. [17]); b) TBSOTf (1.1 equiv), 2,6-lutidine (2 equiv), CH_2Cl_2, −78 °C, 3.5 h, 74%; c) HCO_2NH_4 (20 equiv), Pd (10%)/C, MeOH, 65 °C, 45 min, 65%; d) $C_{15}H_{31}CO_2H$ (1.1 equiv), DCC (1.5 equiv), DMAP (1.7 equiv), CH_2Cl_2, RT, 4 h, 85%; e) RCO_2H (0.7 to 1 equiv), DCC (0.7 to 1 equiv), DMAP (1 to 2 equiv), CH_2Cl_2, RT, 6 to 16 h, 32 to 64%; f) Bu_4NF (2 to 2.6 equiv), THF, RT, 2.0 h; 76 to 97%; g) i: SO_3·pyr (5 equiv), pyridine, 90 °C, 3 h; ii: Amberlite IR120 Na, 81 to 91%; h) for saturated acyl chains: H_2 (1 atm), Pd (5%)/C, MeOH/CH_2Cl_2, RT, 2 h, then Amberlite IR120 Na, 70 to 72%; i) for unsaturated acyl chains: $FeCl_3$, 15 equiv, CH_2Cl_2, 0 °C, 2.5 h, then Amberlite IR120 Na, 47 to 51%. TBS = *tert*-butyldimethylsilyl; TMS = trimethylsilyl.



Scheme 5. Structures of the natural $Acyl_2SGLs$ (**1**) and of the synthetic analogues **21a–d**. The lengths of the R^1 acyl chains are given in parentheses.

provided the 2′-*O*-sulfated derivatives **20** in high yields. The final complete deprotection was achieved by hydrogenolysis for SGL analogues **21d** and **21e**, each substituted at O-3 with a saturated chiral lipid, or by treatment with an excess of anhydrous $FeCl_3$ in dichloromethane, a procedure that preserves the conjugated unsaturation of chiral lipids in SGL analogues **21a–c**.[22] Some of the new SGLs are presented in Scheme 5.

The antigenicities of these new $Acyl_2SGLs$ were then assessed with the aid of the $Acyl_2SGL$-specific T-cells.[6–8] GM-CSF (granulocyte-macrophage colony-stimulating factor) released by the T-cells was quantified, and the results are summarized in Figure 1. We had demonstrated earlier[7,8] that the number of methyl groups on the 1,3-methyl-branched fatty acyl chain of the SGL was an important structural element, with increasing antigenicity of the analogues as the number of methyl groups increased. Furthermore, with acyl chains no longer that 26 carbon atoms, when the same number of methyl groups was present on the fatty acyl chain, analogues containing α,β-unsaturated acyl groups—structures not found in the natural SGLs—were more active than analogues with saturated acyl groups of the same chain length.[7,8] We show here that the chain length of the unsaturated fatty acyl chain is also decisive for the efficient activation of the T-cells (Figure 1). Synthetic SGLs **21a–c** differ only in the lengths of the chains of the tetramethylated unsaturated fatty esters on position 3 of the trehalose (see Scheme 5). They are substituted by fatty esters with chain lengths of 16, 24, and 32 carbon atoms, respectively. However, these SGLs show strong differences in their abilities to activate T-cells: the longer the chain, the more stimulatory the analogue (Figure 1A).

This was clearly indicated by comparison of the potencies (doses of antigen giving 50% of maximum activation) and of the efficacies (maximum quantities of released cytokines) of the different sulfoglycolipids. Interestingly, **21a**, with a short C_{16} alkyl chain, showed lower potency and efficacy than the other analogues (Figure 1B). Compounds **21b** and **21c** showed similar potencies, but efficacies that were clearly different from one another, with **21c** being more active than **21b** (Figure 1B). The potency differences suggest that whereas **21a** forms smaller numbers of active CD1b-antigen complexes than the other analogues, **21b** and **21c** form similar numbers of complexes with CD1b. This issue was addressed through CD1b-plate-bound experiments in which recombinant soluble CD1b molecules were immobilized on the plastic, loaded with different doses of **21b**, **21c**, or natural **1**, and then incubated with T-cells.

Also in the absence of APCs, **21b** and **21c** stimulated T-cells with comparable potencies, which were dramatically higher than that of **1** (Figure 1C). In another set of experiments the stabilities of the stimulatory complexes formed by these two analogues with CD1b were evaluated in vivo by pulse-chase experiments. Dendritic cells (DCs) were separately pulsed with the sulfoglycolipids, washed, and then used to stimulate CD1b-restricted T-cells after different chase times. Because the extent of T-cell activation is directly correlated with the amounts of stimulatory Ag-CD1b complexes present on the surfaces of the APCs at each time point, these experiments are

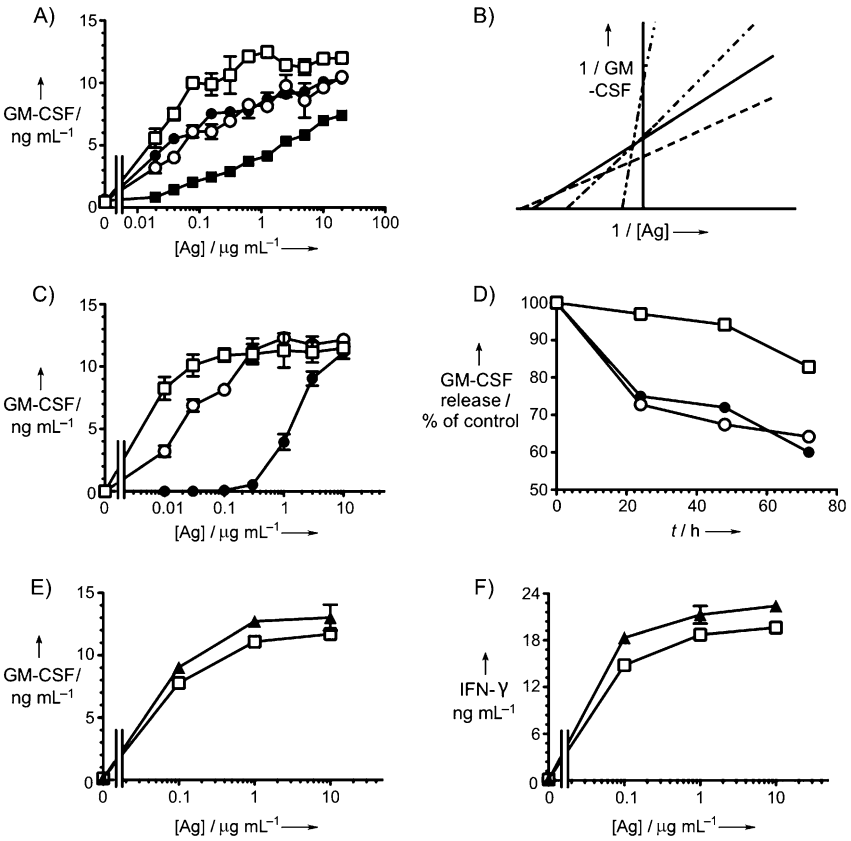


Figure 1. Immunological activities of synthetic sulfoglycolipid analogues. Antigenicities of $Acyl_2SGLs$ **1** and compounds **21 a**–**d**. A) GM-CSF release by T-cells activated by $Acyl_2SGLs$ **1** and compounds **21 a**–**c**. B) Lineweaver–Burk plot representing efficacies and potencies of $Acyl_2SGLs$ (–·–) and compounds **21 a** (–··–), **21 b** (—) and **21 c** (– –). C) Plate-bound assay of sulfoglycolipid complexes **21 b** and **21 c** with the recombinant soluble CD1b, GM-CSF release by T-cells;. D) Persistence of the stimulatory activity of DCs pulsed with sulfoglycolipids $Acyl_2SGLs$, **21 b** and **21 c** over time. E) GM-CSF release by T-cells activated by compounds **21 c** and **21 d**. F) IFN-γ release by T-cells activated by compounds **21 c** and **21 d**. ●: $Acyl_2SGLs$, ■: **21 a**, ○: **21 b**, □: **21 c**, ▲: **21 d**.

informative about the stabilities of active complexes over time in living cells. DCs pulsed with **21 b** and with natural $Acyl_2SGLs$ **1** induced 70% of the maximum T-cell GM-CSF release after 24 h, dropping to about 60% after 72 h (Figure 1 D). In contrast, DCs pulsed with **21 c** were significantly more efficient, their stimulatory capacity being almost unaffected in the first 48 h of chasing and still 85% after 72 h (Figure 1 D). These findings indicate that the presence of the long acyl chain stabilizes the immune-active CD1b-sulfoglycolipid complexes, prolonging their availability within living cells.

Compound **21 c** also showed higher efficacy than **21 b**; this suggests that it induces a more productive triggering of T-cells than **21 b**, ultimately resulting in higher cytokine release. Analogue **21 c** differs from **21 b** in the presence of a longer (C_{32} vs. C_{24}) alkyl chain, so this structural difference might be important for more efficient interaction with the T-cell receptors (TCRs) of responding cells.

Another striking example of the importance of the chain length for the antigenicity of a SGL is emphasized by comparison of **21 d**, which carries a saturated trimethylated C_{32} chain, with **21 e**, with a saturated trimethylated C_{24} chain (Scheme 5). We had shown earlier that **21 e** was very weakly antigenic.[7,8] In contrast, **21 d** is highly antigenic, and it is as active as the natural product. Moreover, **21 d**, substituted with a C_{32} saturated fatty acyl chain with only three methyl groups, and **21 c**, which carries a C_{32} unsaturated fatty acyl chain with four methyl groups, were found to be equally antigenic (Figure 1 E and 1 F). This shows that the unsaturation of the acyl chain has secondary functional effects. These findings, together with our previous studies, confirm that the major features controlling SGL antigenicity were indeed the length of the deoxypropionate fatty acyl chain and the presence of methyl groups on this lipid chain.[8]

The relevance of the methyl groups was also highlighted by the crystal structure of the soluble CD1b·**21 b** complex,[23] which showed that the first three methyl groups of the 1,3-methyl-branched fatty acyl chain of **21 b** are located outside the hydrophobic A′ channel of CD1b. This unique localization suggested that they might interact with the TCR; this would thus explain their absolute requirement for antigenicity. Finally, an in-depth reevaluation of the structures of natural $Acyl_2SGLs$ showed that some phthioceranoic-acid-substituted SGLs were also present in the natural mixture.[24] Compound **21 d** can thus be considered a true analogue of these phthioceranoic-acid-substituted SGLs that have high antigenic capacities. The presence of a hydroxy group on the fatty acyl chain is thus not critical for the antigenicity of a SGL towards CD1b-restricted T-cells. These results seem to contradict modeling studies predicting that the hydroxy groups in the hydroxyphthioceranoic side chains of the natural $Acyl_2SGLs$ contribute to the binding mode and play a key role in the chain positioning.[9]

In conclusion, we have developed an efficient synthetic route to a collection of $Acyl_2SGL$ analogues; it includes regioselective steps for the desymmetrization of the trehalose core, combined with highly diastereoselective access to chiral polymethylated fatty acids of any chain length. This new synthetic route is compatible with the incorporation of very-long-chain fatty acyl chains (length $> C_{30}$) in the SGLs. These long chains have been shown to be of utmost importance for high antigenicities of the SGL analogues. Products **21 c** and **21 d** showed the same potencies as natural $Acyl_2SGLs$, together—surprisingly—with higher efficacies. These unique features suggest they

should be good candidates as components of anti-tuberculosis subunit vaccines.

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