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Synthetic Arabinomannan Heptasaccharide Glycolipids Inhibit Biofilm Growth and Supplements Isoniazid Effects in *Mycobacterium smegmatis*

Krishnagopal Maiti,[a,c] Kirtimaan Syal,[b,c] Dipankar Chatterji,*[b] and Narayanaswamy Jayaraman*[a]

Abstract: Biofilm formation is a critical survival strategy of mycobacterial colonies in hostile environmental conditions involving attachment to an adherent surface. In this article, we report synthesis of heptasaccharide glycolipids, constituted with mannopyranosides anchored on to a branched arabinofuranoside core. Two types of glycolipids, 2,3-branched and 2,5-branched, are synthesized and evaluated for their efficacies to inhibit biofilm growth on the non-pathogenic mycobacterium variant *Mycobacterium smegmatis*. The biofilm formation is inhibited at a minimal biofilm growth inhibition concentration (mbic) of 100 μg/mL, in the case of the 2,5-branched heptasaccharide glycolipid. Further, we identify that a combination of the drug isoniazid with the branched heptasaccharide glycolipid (50 μg/mL) potentiates the drug to be three-fold more effective to reach the mbic, at 30 μg/mL. The studies herein establish that synthetic glycolipids act as not only inhibitors of biofilm growth, but also provide a synergistic effect when combined with significantly lowered concentrations of isoniazid drug to disrupt the biofilm structures of the mycobacterium.

1. Introduction

Sessile growth phase of a mycobacterium onto surfaces include colonization and biofilm matrix formation. Colony and biofilm matrix formation provide an ability to the mycobacterium to overcome harsh environmental conditions. Switching from the free-living planktonic form to a surface-attached, multi-cellular organized colony enables bacterial cells to survive not only a nutrient depleted environment, through altered genomic and metabolic activities, but also provide the cells a resistance to antibacterial and antimicrobial agents.[1] Such resistance by micro-colonized bacterial phenotypes also leads to aberrations in host cell survivability due to the mycobacterial virulence.[2,3] Planktonic cells expressing glycopeptidolipids are known to be essential for the biofilm formation in the case of acid-fast mycobacterial species, such as, *Mycobacterium smegmatis*.[4]

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The biofilm matrix in itself is composed of a heterogeneous mixture of extracellular polymeric substances, namely, polysaccharides, proteins, nucleic acids, lipids and humic substances.[5] Due to the detrimental effects caused by virulent mycobacterium colonies to the host cells, there are sustained efforts to develop anti-biofilm causative agents. Antimicrobial peptides, such as, LL-37 exemplify a class of agents that act against biofilms, by preventing surface adhesion or twitching mobility or late stage development or by down-regulation of genes responsible for biofilm formation.[6,7] *N*-Acylhomoserine lactone derivatives represent a major development to identify small molecules that modulate quorum sensing, which refers to a coordinated response of the bacterial population density and its associated processes including biofilm formation.[8,9] A number of natural products and their analogues act as anti-biofilm agents.[10] Marine natural product scaffolds formed a basis to develop a novel class of 2-aminoimidazole derivatives that exhibit potent anti-biofilm activities on *P. aeruginosa* biofilms, with no direct antibiotic effect.[11] Realizing that virulence of a bacterium includes its pili-mediated adhesion onto cells, tissues and macromolecular surfaces, synthetic GalNAcβ(1→4)Galβ disaccharide-containing glycolipids were designed and studied as inhibitors of *P. aeruginosa* adhesion to solid surfaces.[12] Maltose-containing glycolipids were demonstrated by Luk and co-workers to modulate quorum sensing circuits, leading to activation of swarming motility in a non-swarming mutant of *P. aeruginosa*, even when the glycolipids inhibited the adhesion and biofilm formation.[13] Similarly, the authors identified recently that synthetic rhamnolipids could either promote or inhibit biofilm formation in a non-rhamnolipid producing mutant strain of *P. aeruginosa*, depending on the concentration of the synthetic rhamnolipids.[14] Cluster galabioside-based and mannoside-based inhibitors that prevent adhesion of bacteria onto supports through adhesion proteins were developed.[15,16] These advancements illustrate efforts to overcome adhesion of bacterial cells on to surfaces and subsequent transformation in to persistent biofilm forms.[17]

Our sustained earlier studies demonstrated that synthetic glycolipids based on mannopyranoside-arabinofuranoside moieties, which we shortly represent as synthetic arabinomannan glycolipids, act as a new class of potent inhibitors of biofilm growth.[18-20] Naturally-occurring lipoarabinomannan glycolipids constitute as one of the major components of mycobacterial cell wall. Abundance of these sugars provided a clue to the critical roles originating from these glycolipids in the life cycle and virulence of a mycobacterial species. For example, virulent *M. tuberculosis* possesses mannose caps in the lipoarabinomannan component, whereas in avirulent *M. smegmatis*, the mannose caps are replaced by phosphatidylinositol in the same component. Arabinomannan biosynthesis was thus a target to develop inhibitors.[21] A number of synthetic arabinofuranoside glycolipids demonstrated inhibition of arabinofuranosyl transferase enzyme activities, required for the biosynthetic assembly of arabinan

chains of cell wall glycolipids.[22-26] We observed earlier that di- to pentasaccharide-based synthetic arabinomannan glycolipids are potent inhibitors of the phenotype functions of mycobacterial stationary phase processes, particularly, sliding motility and biofilm activity.[18-20] Further, the synthetic glycolipids also showed a considerable effect on the mycobacterial growth. A systematic lipodomics and chromatographic analyses of the regulatory pathways enabled an understanding of the key pathways that are affected in the presence of non-cytotoxic glycolipids.[20] These synthetic glycolipids were found to interfere with the biosynthesis of epoxy-, ketomycolic acids and phosphatidylinositol mannosides, and a significant down-regulation of these components occurred in the presence of the glycolipids. On the basis of potent mycobacterial sliding motility and biofilm growth inhibition activities exhibited by the synthetic arabinomannan glycolipids, identification of such activities arising from larger oligosaccharide glycolipids consisting of arabinofuranosyl core and mannopyranosyl caps was envisaged to provide a clue to the effectiveness of the glycolipid constitution. With this motivation, we undertook specifically to identify the inhibition potential of synthetic arabinomannan heptasaccharides, in a 2,3- and 2,5-branched fashions at the core moiety. The studies were undertaken in two directions: (i) identification of the inhibitory effects of the synthetic glycolipids on mycobacterial biofilm growth and (ii) supplementation of the clinically relevant antibiotics, namely, isoniazid with synthetic glycolipids. Supplementation of the drug may help to contain the emergence of drug resistance, and identify a synergistic effect arising from a combination of these two types of inhibitors. Synthesis and the bioactivity studies of the arabinomannan heptasaccharide glycolipids are presented herein.

2. Results and Discussion

2.1. Synthesis

Molecular structures of target branched synthetic arabinomannan heptasaccharide glycolipids, having an arabinofuranoside core and mannopyranoside caps, are shown in **Figure 1**. Two types of anomeric linkages, α-(1→2), α-(1→3) and α-(1→2), α-(1→5) are present in the ara*f* moieties, whereas α-(1→6) Man*p* linkages are present between mannopyranosides. Each of the heptasaccharide glycolipid consists of an arabinofuranoside trisaccharide core, on to which mannopyraoside disaccharides are installed as the non- reducing ends. Further, a lipidic portion constitutes the aglycon portion of the glycolipids. Synthesis of glycolipids was initiated, through appropriate glycosyl donors and acceptors and as many as 35 consecutive steps enabled securing the heptasaccharide glycolipids **1** and **2** (**Figure 1**), starting from commercially available starting materials and reagents.

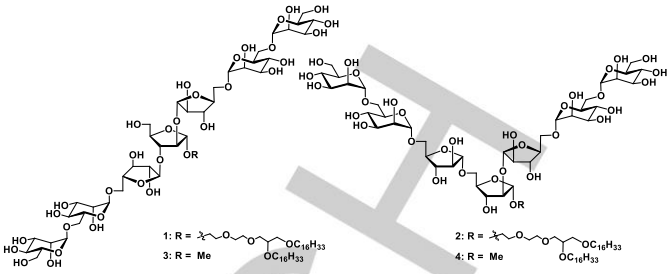
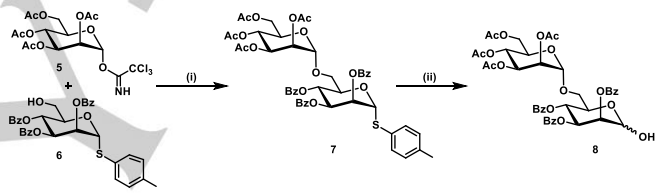


Figure 1. Molecular structures of synthetic arabinomannan heptasaccharide glycolipids **1**, **2** and the corresponding heptasaccharides **3** and **4**, without the lipidic chain.

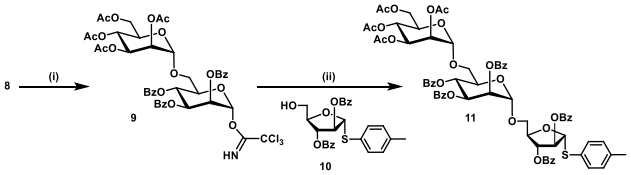
(i) Synthesis of trisaccharide donor: Synthesis of heptasaccharides was planned in a convergent manner, requiring a trisaccharide donor and a mannopyranoside disaccharide acceptor initially. Synthesis of the trisaccharide donor was initiated with the preparation of suitably protected disaccharide derivative **7** (**Scheme 1**).



Scheme 1. *Reagents and conditions:* (i) $BF_3.Et_2O$, CH_2Cl_2, molecular sieves (4 Å), rt, 30 min., 78%; (ii) NIS, THF/water (1:1), rt, 2 h, 81%.

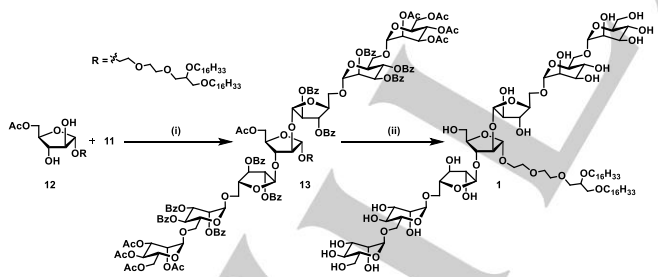
Glycosylation of acceptor **6**[27,28] with trichloroacetimidate **5**, in the presence of $BF_3.OEt_2$ afforded disaccharide thioglycoside **7**, in a good yield (**Scheme 1**). The newly formed α-anomeric linkage in the disaccharide **7** was confirmed by the appearance of a peak at 4.84 ppm, as an apparent singlet for H-1' in ^{1}H NMR spectrum and a resonance at 97.9 ppm for *C*-1' nucleus at the non-reducing end of **7**, in ^{13}C NMR spectrum. The appearance of molecular ion peak in the ESI-mass spectrum further confirmed the constitution of **7**. Thioglycoside **7** was treated with *N*-iodosuccinimide (NIS) to afford disaccharide hemiacetal **8**, in 81% yield (**Scheme 1**). Appearance of resonances at 92.5 and 97.8 ppm in ^{13}C NMR spectrum, for anomeric C_α-1 and C_β-1 at the reducing end of **8**, confirmed the formation of hemiacetal. Hemiacetal **8** was converted to the trichloroacetimidate **9**,[29] using trichloroacetonitrile (Cl_3CCN) and 1,8-diazabiclo[5.4.0]undec-7-ene (DBU), which was further glycosylated with acceptor alcohol **10**,[18] in the presence of $BF_3.OEt_2$ to afford trisaccharide thioglycoside donor **11**, in 64% yield (**Scheme 2**). In ^{13}C NMR spectrum, appearance of a peak at 91.2 ppm was attributed to the C_α-1 nucleus attached through a thio-linkage, whereas resonance at 98.1 and 97.5 ppm corresponded to α-anomeric *O*-glycosidic linkages at the non-reducing end of **11**. The appearance of the molecular ion peak at

as the base peak (*m/z* 1291.3457 [M + Na]$^+$), in ESI-mass spectrum, further confirmed the formation of **11**.



Scheme 2. *Reagents and conditions:* (i) Cl_3CCN, 1,8-DBU, CH_2Cl_2, 0 ^oC, 1 h, 69%; (ii) $BF_3.Et_2O$, CH_2Cl_2, molecular sieves (4 Å), rt, 30 min, 64%.

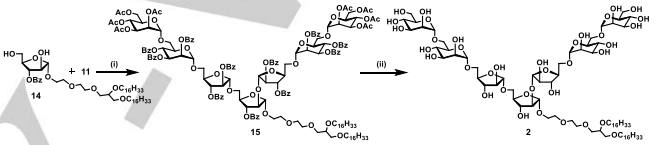
(i) *Synthesis of heptasaccharide glycolipids*: Synthesis of oligosaccharides was initiated after securing the trisaccharide donor and suitably protected monosaccharide acceptors. The general strategy for the synthesis of heptasaccharides involved two-fold glycosylations of monosaccharide diol acceptor with trisaccharide thioglycoside donor in the presence of a promoter. Double glycosylation of the diol **12**,[19] tethered with lipidic portion, with thioglycoside donor **11**, in the presence of promoter NIS and silver trifluoromethanesulfonate (AgOTf) afforded protected heptasaccharide glycolipid **13**, containing α-(1→2) and α-(1→3) linkages between furanoside units (**Scheme 3**). Deprotection of ester functionalities in **13** was performed under Zemplén condition to afford 2,3-branched heptasaccharide glycolipid **1**, in a good yield. The constitutions of protected and deprotected glycolipids were confirmed by NMR spectrocopies and mass spectrometry. In ^{13}C NMR spectrum of **13**, appearance of peak at 106.6 ppm attributed to the α-anomeric linkage of core arabinofuranoside unit, attached to the lipidic portion. In ESI-mass spectrum of **13**, appearance of molecular ion peak as the base peak (*m/z* 3114.5381 [M + Na]$^+$), further confirmed the formation of protected glycolipid.



Scheme 3. Reagents and conditions: (i) NIS, AgOTf, CH_2Cl_2, molecular sieves (4 Å), 0 ^oC, 30 min., 54%; (ii) NaOMe, MeOH/THF (1:1), rt, 12 h, 88%.

In ^1H NMR spectrum of **1**, α-anomeric proton of core arabinofuranoside appeared at 5.18 ppm, as a singlet, whereas anomeric protons of mannopyranosyl units merged at 4.92 ppm, as a singlet. In ^{13}C NMR spectrum of **1**, α-anomeric carbons of arabinofuranoside units appeared in the region of 106.5-106.8 ppm, whereas the same for mannopyranosyl units resonated in the region of 99.4-99.9 ppm. The appearance of molecular ion peak at 1695.9067 [M + Na]$^+$, as the base peak in ESI-mass spectrum, confirmed the formation of glycolipid **1**.

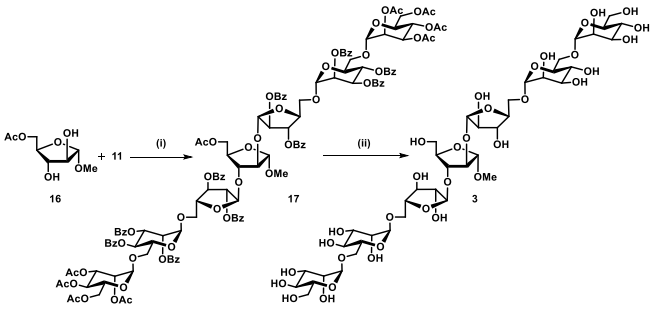
In order to synthesize 2,5-branched glycolipid **2**, installation of trisaccharide moieties onto the monosaccharide diol was performed by glycosylation between **14**[19] and **11**, in the presence of the promoter to afford protected glycolipid **15**, having α-(1→2) and α-(1→5) linkages between furanoside moieties (**Scheme 4**). Removal of the ester groups in **15** under Zemplén condition led to the formation of 2,5-branched heptasaccharide glycolipid **2**, in 92% yield. Structural identities of protected and deprotected glycolipids were established by physical techniques. In ^{13}C NMR spectrum of **15**, α-anomeric carbon of core furanoside moiety resonated at 106.8 ppm, whereas the same for mannopyranosyl moieties appeared in the region of 97.6-98.3 ppm. The formation of **15** was further confirmed by the appearance of molecular ion peak at 3177.3050 [M + Na]$^+$, as the base peak in MALDI-TOF mass spectrum.



Scheme 4. *Reagents and conditions:* (i) NIS, AgOTf, CH_2Cl_2, molecular sieves (4 Å), 0 ^oC, 30 min, 51%; (ii) NaOMe, MeOH/THF (1:1), rt 12 h, 92%.

In ^1H NMR spectrum of **2**, H-1 of core arabinofuranoside moiety appeared at 5.03 ppm, as a singlet, whereas α-anomeric protons of mannopyranosyl moieties appeared at 4.91 ppm, as a singlet. The resonance of *C*-1 nucleus of reducing end of **2** was observed at 107.8 ppm, whereas carbon nucleus of mannopyranosides appeared in the region of 99.4-100.0 ppm in ^{13}C NMR spectrum. The formation of glycolipid **2** was further confirmed by the appearance of molecular ion peak at 1695.9478 [M + Na]$^+$, as the base peak in ESI-mass spectrum.

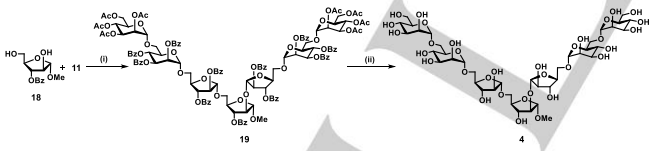
Towards the synthesis of heptasaccharides without lipid chain, suitably protected monosaccharide acceptors were chosen for the glycosylations. Glycosylation of diol acceptor **16**[19] with glycosyl donor **11**, in the presence of NIS and AgOTf afforded protected heptasaccharide **17** with α-(1→2) and α-(1→3) linkages between furanoside moieties (**Scheme 5**). Global deprotection of ester groups in **17** afforded heptasaccharide **3**, in 90% yield. The formation of protected and deprotected heptasaccharides was confirmed by NMR spectroscopies and mass spectrometry. The appearance of peak at 107.6 ppm in ^{13}C NMR spectrum, attributed to the α-anomeric carbon of core arabinofuranoside moiety of **17**. In ESI-mass spectrum, peak at 2517.4609 [M + Na]$^+$, corresponded to the molecular ion peak.



Scheme 5. *Reagents and conditions:* (i) NIS, AgOTf, CH_2Cl_2, molecular sieves (4 Å), 0 °C, 30 min, 56%; (ii) NaOMe, MeOH/THF (1:1), rt 12 h, 90%.

In ^{1}H NMR spectrum of heptasaccharide **3**, α-anomeric H-1 protons of arabinofuranoside moieties appeared at 5.03 ppm, as a singlet, whereas in ^{13}C NMR spectrum, *C*-1 of core arabinofuranoside appeared at 109.3 ppm and anomeric carbon nucleus of mannopyranosides merged in the region of 101.0-101.6 ppm. In ESI-mass spectrum, appearance of molecular ion peak at 1099.3541 $[M + Na]^+$, as the base peak, further confirmed the formation of **3**.

Synthesis of 2,5-branched heptasaccharide **4** was initiated by the formation of protected heptasaccharide **19** (**Scheme 6**). Glycosylation between diol **18**[19] and thioglycoside donor **11**, in the presence of promoter furnished protected heptasaccharide **19**, containing α-(1→2) and α-(1→5) linkages between furanoside units, in 52% yield. Removal of ester functionalities in **19** under Zemplén condition afforded branched heptasaccharide **4**, in 94% yield. The constitutions of protected and deprotected heptasaccharides were established by physical methods. In ESI-mass spectrum of **19**, molecular ion peak appeared as the base peak at 2579.7002 $[M + Na]^+$. Anomeric *C*-1 nucleus at the reducing end of **19** appeared at 107.9 ppm in ^{13}C NMR spectrum, whereas anomeric carbons of mannopyranoside moieties appeared in the region of 97.6-98.3 ppm.



Scheme 6. *Reagents and conditions:* (i) NIS, AgOTf, CH_2Cl_2, molecular sieves (4 Å), 0 °C, 30 min, 52%; (ii) NaOMe, MeOH/THF (1:1), rt, 12 h, 94%.

In ^{1}H NMR spectrum of branched heptasaccharide **4**, anomeric H-1 of core arabinofuranoside appeared at 5.13 ppm, as a singlet, whereas anomeric protons of mannopyranosides merged as a singlet at 4.90 ppm. In ^{13}C NMR spectrum, carbon nucleus of arabinofuranosides appeared in the region of 107.3-107.6 ppm and the same for mannopyranosides resonated in the region of 99.4-99.9 ppm. The formation of **4** was further confirmed by ESI-mass spectral analysis, where the molecular ion peak appeared as a base peak at 1099.3541 $[M + Na]^+$.

Growth Inhibition Assay: Synthetic glycolipids and oligosaccharides **1** - **4** were studied for their effects in the mycobacterial growth initially. Earlier studies demonstrated that synthetic glycolipids interfere the cellular growth.[20] The model non-pathogenic mycobacterial organism, namely, *M. smegmatis*, was chosen for the studies, for reasons that our earlier studies demonstrated that glycolipids in the form of glycopeptidolipids is critical to the emergence of smooth phenotype functions, such as, the colony morphology,[30] thereby revealing importance of glycolipids in the outer cell wall phenotype functions. This bacterium is considered generally as a surrogate to pathogenic *M. tuberculosis*, due to its persistence under nutrient starvation conditions, coupled with stress resistance, characteristic of the stationary-phase of the bacterium.[31,32] The *Mycobacterium smegmatis* strain mc^{2}155, which is a fast growing bacterium exhibiting a smooth colony morphology, was undertaken for the studies herein.[33] The mycobacterial growth was followed by measuring optical density changes of the culture (600 nm) for up to 180 h. Glycolipids **1** and **2** exhibited the most effect in the growth function. A significant reduction or slowness of the growth in the exponential and lag phases was observed, which indicated that the glycolipids interfered with the cell division of the mycobacterium (**Figure 2**). A two-fold reduction in the

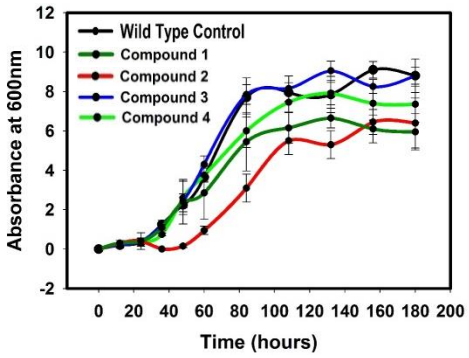


Figure 2. Bacterial growth in the presence of synthetic glycolipids at the concentration of 100 μg/mL in comparison to the wild type control (without any compound).

growth was observed in the cell viability assay with the glycolipid **2** than the remaining compounds. On the other hand, the cells were non-susceptible to oligosaccharides alone and the growth was comparable to that of the wild-type. The trend in the growth inhibition further indicates that the molecular structure of the glycolipid is critical. Thus, glycolipid **2** shows more effective inhibition of growth than **1**, even when both glycolipids are constituted with equal number of arabinofuranoside and mannopyranoside moieties. In the absence of any aqueous solubility of the lipid alone, without the sugar moieties, assessing the effect of lipid component alone could not be conducted. The glycolipid concentrations at 50 $\mu g/mL^{-1}$ were also assayed, growth reduction was ~30 ± 3%, in comparison to the wild-type bacterial growth. Lag phase was observed to be longer in case

of bacterial cells treated with glycolipid **2** in comparison to the untreated control. Furthermore, the slope in exponential phase was found to be reduced suggesting more doubling time in the presence of the compounds. Additionally, bacterial cells treated with the glycolipids reached the stationary phase at lower optical density. These results are in line with lower homologues of glycolipids studied earlier, but for variations in the extent of mycobacterial growth inhibitions. The mechanism of growth reduction in the presence of glycolipids is unclear. We premise that the growth inhibition might arise due to synthetic glycolipids interfering with the multiple diverse roles of mycolic acids and associated lipid metabolism.[34]

2.2. Quantification of Biofilm

M. smegmatis, an environmental mycobacterium, is known to colonize and form biofilm, upon attachment on to a surface, from a free-floating planktonic state.[34] Primary outer cell wall being the interface between the bacterium and the surface, an effect on the outer cell wall components has the potential to affect colonization and biofilm matrix formation. The bacterium entering into the biofilm envelop exerts remarkable resistance to antibiotics and antimicrobials. The exopolysaccharide production and changes in the gene expression contribute to such resistivity by biofilm forming bacterium.[34] Luk and co-workers demonstrated that maltose-containing alkyl glycosides inhibit bacterial adhesion and biofilm in the case of *P. aeruginosa*.[13,14] Our earlier studies on arabinomannan pentasaccharide glycolipids inhibited *M. smegmatis* biofilm by ~80%. The effect of synthetic glycolipids of the present study on the biofilm-formed bacterium was assayed on PVC cell culture plate (Laxbro) in Sauton's medium supplemented with 2% glycerol and 0.05% Tween-80. The quantification of the biofilm was evaluated in 96 well plates. Briefly, primary *M. smegmatis* culture was pelleted, washed, diluted in Sauton's medium, the glycolipid inhibitors at varying concentrations added into the culture at zero time point and the experiments were conducted in three biological replicates. Inoculum culture was added in each well and biofilm formation was monitored up to 196 h. The media was removed from the wells which were washed thoroughly with water and assayed using 1% crystal violet solution.[35] The dye was quantitated after incubating each well with DMSO for an hour and subsequently measuring the absorption at 590 nm using a microplate reader.

The results of the above biofilm assay are shown in **Figure 3**. Biofilm morphology was affected to varying extent in the presence of compounds **1**-**4**. Among synthetic derivatives, heptasaccharide glycolipid **2** was found to be the most effective in biofilm growth inhibition. Morphology of biofilms treated with compounds **1** - **4** was evaluated at different time points, where reference pictures of time point 108 h and 144 h are shown in **Figure 3**. Further, a dose dependence of the inhibition of biofilm was assessed at concentrations 1 – 100 μg/mL. The biofilm growth inhibition was observed progressively higher as the glycolipid concentration increased and at 100 μg/mL, biofilm growth inhibition was nearly complete as that of the negative

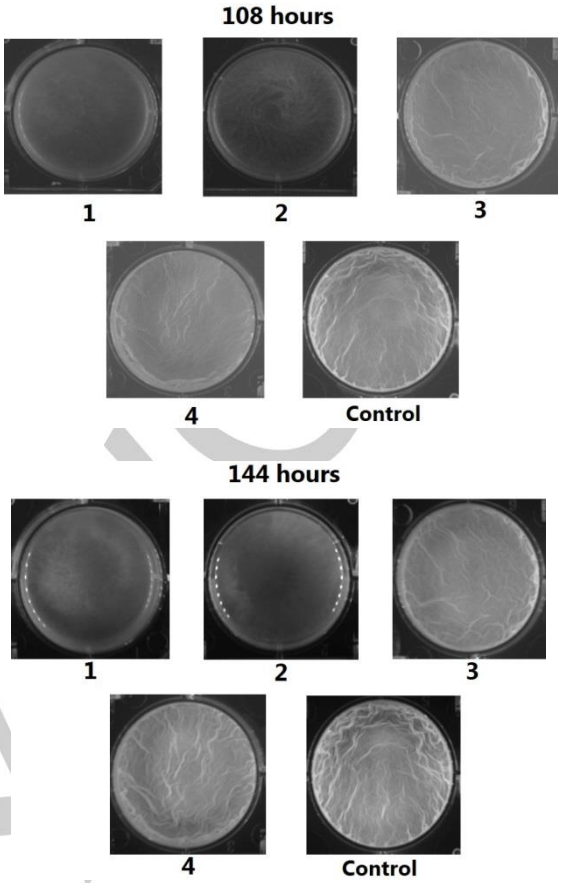


Figure 3. Biofilm formation assay of **1** – **4**, at 100 μg/mL. Images were recorded at 108 h and 144 h time points.

control (**Fig. 4**), referred to as the minimum biofilm growth inhibition concentration (mbic). Among the synthetic compounds, glycolipids **1** and **2** exhibited a significant reduction in the biofilm, to as much as, 85 ± 5%, in comparison to biofilm formation in the presence of oligosaccharides and the wild type control. Further, we quantified the biofilms at different time points in the presence of the glycolipids, in order to confirm the inhibition and check whether the bacterial biofilm has an ability to recover the growth after the inhibition event. However, biofilm formation was not observed till 180 h by both glycolipids **1** and **2** (**Fig. 5**). Compounds **1** and **2** showed remarkable inhibition of the biofilm growth in comparison to the control. In the presence of **1** and **2**, bacterial cell aggregates were observed at the bottom of the plate (**Fig. 3**), whereas the biofilm layer was not visible even after longer incubation time at 37 °C in humidified conditions (Lower panel of **Fig. 3**, **Fig. 5**). Compound **2** achieved such an inhibition at a concentration lower than compound **1** (**Fig. 4a,b**). Taken together, these results illustrate that synthetic glycolipids interfere significantly with the biofilm structure and morphology. In comparison to glycolipids having smaller oligosaccharide segments, the branched heptasaccharide containing glycolipids studied herein exhibit the most biofilm growth inhibition effect. Compounds **3** and **4**, *i.e.* oligosaccharides alone, showed biofilm growth inhibition at ~10-15%.

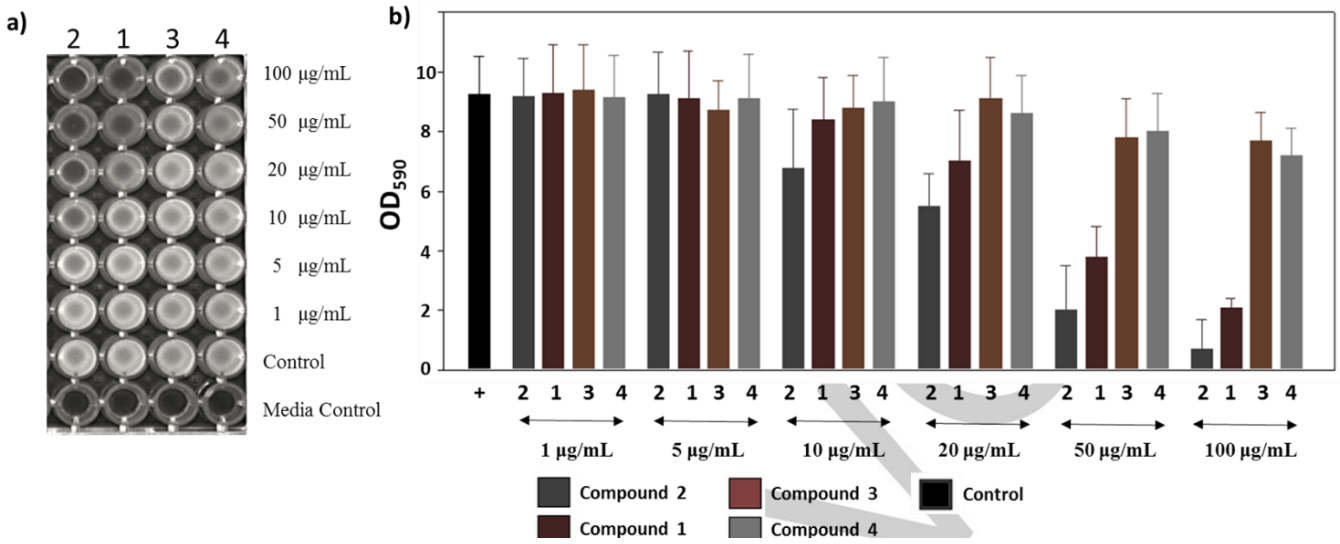


Figure 4. Concentration dependent inhibition of biofilm growth by compounds. a) Picture View; b) Crystal violet assay based quantification of biofilm growth.

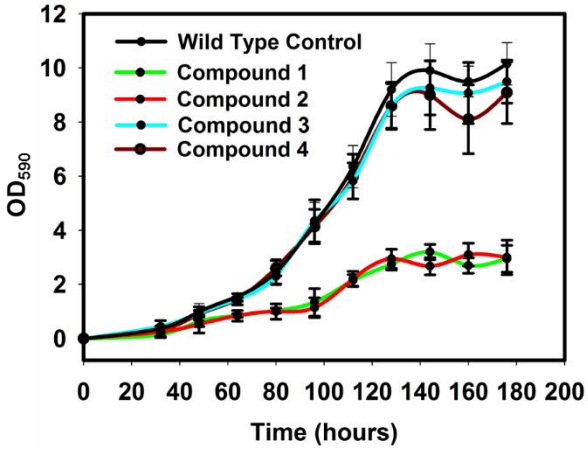


Figure 5. Biofilm quantification by crystal violet assay in the presence of 100 µg/mL compounds in comparison to the control.

Comparing to our earlier studies on many lower homologues of the oligosaccharide glycolipids that showed biofilm growth inhibition to varying extent, we observe in the present study that the mycobacterial biofilm growth inhibition is strongly correlated with the oligosaccharide composition. Higher the mannopyranoside component in the arabinomannan oligosaccharide structure, higher the inhibition and an inhibition to as much as 85 ± 5% is observed with the heptasaccharide glycolipid studied herein. A surfactant-like activity as a possible source to biofilm activity by glycolipids is ruled out, for reason that the assayed concentrations of up to 100 µg/mL is far below the critical aggregation concentration, which is typically ~three times more. As observed in our earlier studies of the lipodomics profile,[20] we premise that mycolic acids and phosphatidylinositol mannosides and related components are down-regulated severely in the presence of the glycolipids, leading to inhibition of the biofilm.

2.3. Glycolipid 2 Supplements the Activity of Isoniazid for Biofilm Growth Inhibition

Acquired tolerance of antibiotics by pathogens is a major challenge currently, leading to ineffectiveness of antibiotics and the necessity to increased concentrations of the antibiotic to overcome pathogenic effects on to the host.[34] Biofilm formation is a protective strategy of the pathogens to overcome antibiotic effects. For example, there is ~ four orders of magnitude increase in the number of viable biofilm-cultured mycobacterial cells when treated with rifampicin, in comparison to viable planktonic cell culture for the same antibiotic treatment.[36] Similarly, isoniazid has been reported to inhibit the biofilm growth at 80 µg/mL, as opposed to MIC of 10 µg/mL for the planktonic culture, clearly illustrating antibiotic resistance enabled by biofilm cultures.[37] Combination antibiotics and non-anibiotics are used to treat many pathogenic infection originated from pathogens, such as, *M. tuberculosis* and *M. laprae*.[38] The synergistic effect of a non-antibiotic molecule as an adjuvant was demonstrated recently to reduce the antibiotic dosages to mitigate bacterial infections by Zhang and co-workers.[39] We undertook an effort to identify an effect of the synthetic glycolipids of the present study in combination with a known antibiotic, namely, isoniazid. This important drug exhibits an action through inhibition of mycolic acid biosynthesis.[40] This antibiotic is shown to inhibit the biofilm growth by the bacterial cultures at 80 to 100 µg/mL in *M. smegmatis*.[37] In context of this paper, MIC of biofilm refers to the minimal concentration of antimicrobial required to inhibit the visible biofilm growth.

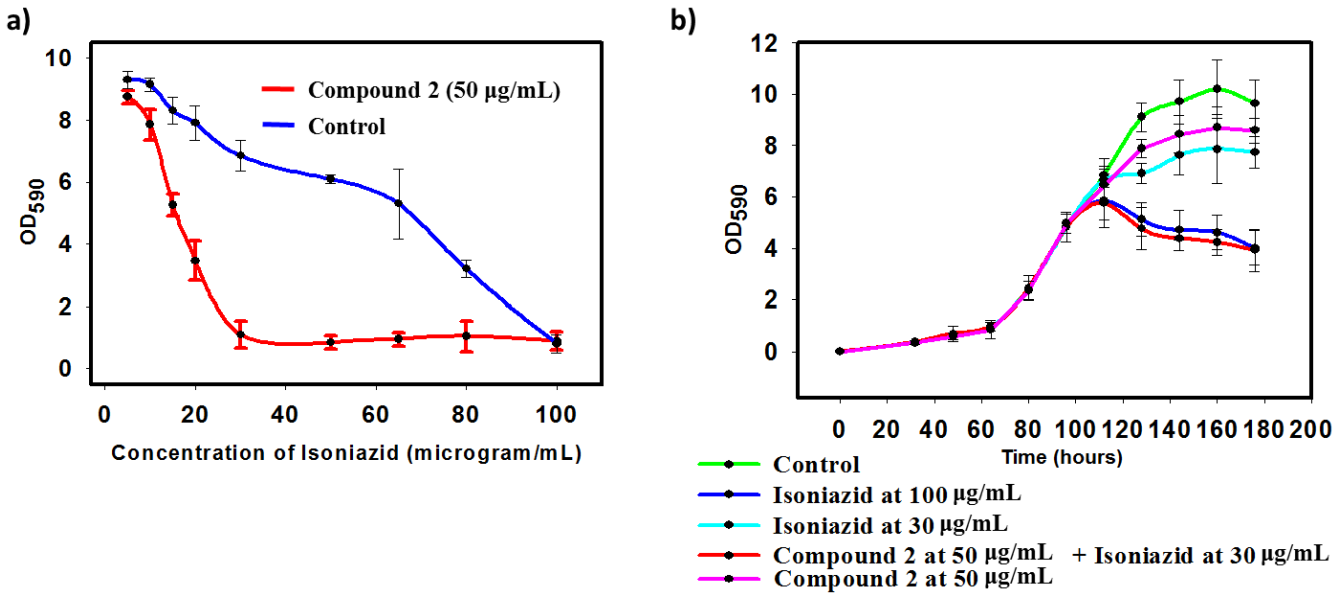


Figure 6. a) Crystal violet assay based quantification of biofilm at varying concentrations of isoniazid in the absence (control, blue) and presence of compound **2** (red). At 100 µg/mL, isoniazid achieved the maximum biofilm inhibition and increasing it further did not showed any change in the level of inhibition (data not shown). b) Biofilm was quantified at various time points before and after the addition of isoniazid and glycolipid **2**. Isoniazid or **2** or isoniazid-**2** mixture were added at 92 h time point just below the biofilm layer by the application of 1 mL syringe without disturbing the formed biofilm layer.

Studies of biofilm growth inhibition in the presence of isoniazid and glycolipid **2** were conducted in 96-well PVC plates as described previously. Biofilm grown *M. smegmatis* mc^2155 bacterial cultures in Sauton's medium were inoculated with glycolipid, isoniazid and combination of these two constituents at different concentrations at the early exponential phase. **Figure 6a** shows the results of growth inhibition effect mediated by the addition of heptasaccharide glycolipid **2** and isoniazid. We find initially that isoniazid alone required a concentration of 100 µg/mL for the biofilm growth inhibition to occur at more than 90% level. Such a biofilm growth inhibition was observed when the culture was inoculated with a mixture of isoniazid at 30 µg/mL and glycolipid **2** at 50 µg/mL.

Having identified that glycolipid **2** inhibited both planktonic and biofilm growth, we were interested to verify whether a pre-formed biofilm be disrupted by the inhibitors. To find out whether compound **2** can also inhibit formed biofilm and cause its disruption, biofilm growth was allowed for 92 h, followed by the addition of the inhibitors just below the formed biofilm layer. More than 90% disruption of the formed biofilm was observed at the isoniazid concentration of 100 µg/mL. Such an extent of biofilm disruption was searched subsequently through supplementation of the drug with glycolipid **2**. This effort showed that a concentration of the drug at 30 µg/mL, in combination with 50 µg/mL of glycolipid **2**, led to more than 90% disruption of the formed biofilm. **Figure 6b** also shows the effect of disruption by isoniazid and glycolipid **2** individually, that are ~20 and 15%, respectively. These experiments show that it is feasible to reduce isoniazid concentration by more than three-fold in the presence of glycolipid **2** at 50 µg/mL. Thus, a synergistic effect appears to arise from the combination of the antibiotic and glycolipid, an observation hitherto unknown in the case of isoniazid to an acid-fast mycobacterial biofilm culture.

Conclusion

The present study establishes that synthetic Ara*f*-Man*p*-containing heptasaccharide glycolipids are potent mycobacterial biofilm growth inhibitors. Identification of effective inhibitors for recalcitrant biofilm-protected colonies of a acid-fast mycobacterium is challenging, given the biofilm matrix being heterogeneous, capable of protecting the mycobacterial colonies against neutralizations by antibacterials and antimicrobials.[17] Concentration dependent studies show that nearly complete biofilm growth inhibition of *M. smegmatis* colonies is observed at glycolipid concentration of 100 µg/mL. The 2,5-branched glycolipid possesses higher inhibition potential than 2,3-branched glycolipid, illustrating that structural features of glycolipids are important to biofilm growth inhibition. In addition, a significant reduction of the minimum biofilm growth inhibition concentration of drug isoniazid is observed when the drug is supplemented with the synthetic glycolipid, in order to achieve biofilm growth or disruption. The synergistic effect could thus be promoted on to a drug in the presence of non-cytotoxic synthetic glycolipids. These observations illustrate that synthetic glycolipids are not only inhibitors of biofilms, but also enable a synergistic effect to reduce the minimum biofilm growth inhibition concentration of isoniazid.

3. Experimental Section

General Information: Solvents were dried and distilled according to literature procedures. Chemicals were purchased from commercial sources and were used without further purifications. Silica gel (100-200 mesh) was used for column chromatography and TLC analysis was performed on commercial plates coated with silica gel 60 F_{254}. Visualization of the spots on

TLC plates was achieved by UV radiation or spraying 5% sulphuric acid in ethanol. High-resolution mass spectra were obtained from Q-TOF instrument by electrospray ionization (ESI). ^{1}H and ^{13}C NMR spectral analyses were performed on a spectrometer operating at 300 MHz, 400 MHz, and 75 MHz, 100 MHz, respectively in $CDCl_3$ solutions unless otherwise stated. Chemical shifts are reported with respect to tetra methyl silane (TMS) for ^{1}H NMR and the central line (77.0 ppm) of $CDCl_3$ for ^{13}C NMR. Coupling constants (*J*) are reported in Hz. Standard abbreviations s, d, t, dd, br s, app s, m refer to singlet, doublet, triplet, doublet of doublet, broad singlet, apparent singlet, multiplet, respectively.

*p-Tolyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4,-tri-O-benzoyl-1-thio-α-D-mannopyranoside (**7**)*: A solution of **6** (1.0 g, 1.67 mmol), **5** (0.98 g, 1.99 mmol) and MS (4 Å) (1.0 g) in CH_2Cl_2 (30 mL) was stirred for 15 min., $BF_3.OEt_2$ (0.25 mL, 1.99 mmol) was added drop-wise at rt, under N_2 atmosphere. The reaction mixture was stirred at rt for 30 min., neutralized with Et_3N, filtered and filtrate concentrated *in vacuo* and purified (SiO_2, pet. ether:EtOAc = 3:1) to afford **7**, as a foamy solid. Yield: 1.2 g (78%); R_f (pet. ether:EtOAc = 7:3) 0.14; $[\alpha]_D$ +27.0 (*c* = 0.5, $CHCl_3$); ^{1}H NMR ($CDCl_3$, 400 MHz) δ 1.91 (s, 3 H), 2.01 (s, 3 H), 2.07 (s, 3 H), 2.11 (s, 3 H), 2.34 (s, 3 H), 3.65 (d, *J* = 11.2 Hz, 1 H), 3.83-3.91 (m, 2 H), 4.01 (dd, *J* = 5.6, 11.2 Hz, 1 H), 4.09 (dd, *J* = 5.0, 11.8 Hz, 1 H), 4.84 (s, 1 H), 4.86 (dd, *J* = 4.4, 10.4 Hz, 1 H), 5.26 (t, *J* = 9.8 Hz, 1 H), 5.35 (s, 1 H), 5.39 (dd, *J* = 3.4, 9.8 Hz, 1 H), 5.66 (s, 1 H), 5.84 (dd, *J* = 3.0, 10.2 Hz, 1 H), 5.96 (app s, 1 H), 6.05 (t, *J* = 10 Hz, 1 H), 7.18 (d, *J* = 8 Hz, 2 H), 7.27 (t, *J* = 7.6 Hz, 2 H), 7.38-7.43 (m, 3 H), 7.47 (d, *J* = 7.6 Hz, 2 H), 7.51-7.56 (m, 3 H), 7.60 (t, *J* = 7.0 Hz, 1 H), 7.84 (d, *J* = 8.4 Hz, 2 H), 8.01 (d, *J* = 8.4 Hz, 2 H), 8.11 (d, *J* = 8 Hz, 2 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 20.5, 20.6, 20.7, 20.8, 21.1, 62.1, 65.8, 66.7, 66.9, 68.5, 69.1, 69.2, 70.3, 70.4, 71.9, 86.5, 97.9, 128.3, 128.5, 128.7, 128.8, 128.9, 129.1, 129.2, 129.7, 129.8, 129.9, 130.1, 132.6, 133.2, 133.5, 133.6, 138.3, 165.3, 165.4, 165.5, 169.3, 169.4, 169.6, 169.7, 170.4; ESI-MS Calcd. for $C_{48}H_{48}O_{17}SNa$ $[M+Na]^+$: 951.2510, found 951.2509.

*p-Tolyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4,-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-1-thio-α-D-arabinofuranoside (**11**)*: A solution of **9**[29] (1.2 g, 1.21 mmol), **10**[17] (0.38 g, 0.81 mmol) and MS (4 Å) (2 g) in CH_2Cl_2 (30 mL) was stirred for 15 min. $BF_3.OEt_2$ (0.15 mL, 1.21 mmol) was added drop-wise at rt, under N_2 atmosphere. The reaction mixture was stirred at room temperature for 30 min., neutralized with Et_3N, filtered and filtrate concentrated *in vacuo* and purified (pet. ether:EtOAc = 3:1) to afford **11**, as a gum. Yield: 0.66 g (64%); R_f (pet.ether:EtOAc = 3:2) 0.2; ^{1}H NMR ($CDCl_3$, 400 MHz) δ 8.13 (d, *J* = 6.4 Hz, 5 H), 7.94 (d, *J* = 6.4 Hz, 5 H), 7.94 (d, *J* = 7.2 Hz, 2 H), 7.78 (d, *J* = 7.6 Hz, 2 H), 7.62-7.24 (m, 13 H), 7.15 (d, *J* = 8 Hz, 2 H), 5.98 (t, *J* = 9.8 Hz, 1 H), 5.92 (dd, *J* = 3.2 10 Hz, 1 H), 5.82-5.76 (m, 4 H), 5.49 (dd, *J* = 3.2 Hz, 10 Hz, 1 H), 5.34 (app s, 1 H), 5.25 (t, *J* = 10.2 Hz, 1 H), 5.20 (s, 1 H), 4.86 (s, 1 H), 4.76 (app s, 1 H), 4.50 (dd, *J* = 3.8 Hz, 9.4 Hz, 1 H), 4.29 (dd, *J* = 4.8 Hz, 11.2 Hz, 1 H), 4.13-3.87 (m, 5 H), 3.65 (d, *J* = 10.8 Hz, 1 H), 2.28 (s, 3 H), 2.07 (s, 3 H), 2.04 (s, 3 H), 1.92 (s, 6 H); ^{13}C NMR ($CDCl_3$, 100 MHz) δ 170.5, 169.8, 169.4, 165.7, 165.6, 165.4, 165.0, 138.0, 133.5, 133.4, 132.9, 130.0, 129.9, 129.8, 129.7, 128.7, 128.5, 128.4, 128.2, 98.1, 97.5, 91.2, 82.2, 81.5, 70.3, 70.0, 69.5, 69.4, 69.0, 68.6, 66.9, 66.1, 65.9, 62.2, 21.1, 20.7, 20.6, 20.5; ESI-MS Calcd. for $C_{67}H_{64}O_{23}SNa$ $[M + Na]^+$: 1291.3457, found 1291.3496.

*8-O-Hexadecyl-3,6,10-trioxa-1,8-hexacosanedioyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-α-D-arabinofuranosyl-(1→2)-[2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-α-D-arabinofuranosyl-(1→3)]-5-O-acetyl-α-D-arabinofuranoside (**13**)*: A solution of **11** (0.48 g, 0.38 mmol), **12** (0.1 g, 0.12 mmol) and MS 4 Å (0.6 g) in dichloromethane (5 mL) was stirred for 10 min., *N*-iodosuccinimide (0.12 g, 0.54 mmol) and silver trifluoromethanesulfonate (0.038 g, 0.15 mmol) were added into it at 0 °C, under N_2 atmosphere. The reaction mixture was stirred at the same temperature for another 30 min., neutralized with Et_3N, filtered and filtrate diluted with dichloromethane (50 mL), washed with satd. aq. sodium thiosulphate (2 x 20 mL) and water (1 x 20 mL). The organic portion was dried (Na_2SO_4), filtered and filtrate concentrated *in vacuo* and purified (pet. ether/EtOAc = 3:2) to afford **13**, as a gum. Yield: 0.20 g (54%); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.13 (d, *J* = 7.6 Hz, 4 H), 8.09 (t, *J* = 7.2 H, 3 H), 8.03 (d, *J* = 7.2 Hz, 4 H), 7.96 (d, *J* = 7.2 Hz, 4 H), 7.74 (d, *J* = 7.6 Hz, 4 H), 7.62-7.39 (m, 16 H), 7.33 (t, *J* = 7.6 Hz, 5 H), 7.29-7.22 (m, 10 H), 6.04-5.97 (m, 2 H), 5.93-5.88 (m, 2 H), 5.82 (s, 2 H), 5.75 (dd, *J* = 5.8 Hz, 13 Hz, 2 H), 5.61 (s, 4 H), 5.44 (d, *J* = 10 Hz, 2 H), 5.36 (s, 2 H), 5.28-5.16 (m, 5 H), 4.89 (s, 2 H), 4.53-4.44 (m, 7 H), 4.34 (s, 2 H), 4.27 (d, *J* = 11.2 Hz, 2 H), 4.10-4.04 (m, 2 H), 3.99-3.82 (m, 9 H), 3.71-3.40 (m, 18 H), 2.07 (s, 6 H), 2.06 (s, 3 H), 2.05 (s, 6 H), 1.93 (s, 3 H), 1.91 (s, 6 H), 1.90 (s, 3 H), 1.54-1.51 (m, 4 H), 1.24 (br s, 52 H), 0.88 (t, *J* = 6.8 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.6, 170.5, 170.4, 169.8, 169.7, 169.3, 165.8, 165.7, 165.6, 165.5, 165.4, 165.3, 164.9, 133.4, 133.2, 132.9, 130.0, 129.9, 129.8, 129.7, 129.3, 129.2, 129.1, 129.0, 128.9, 128.7, 128.5, 128.4, 128.2, 106.6, 105.8, 105.7, 98.2, 97.6, 86.8, 82.9, 82.5, 81.9, 81.6, 79.2, 77.9, 77.2, 71.6, 71.3, 71.0, 70.8, 70.6, 70.4, 70.3, 70.2, 70.1, 69.5, 69.4, 69.0, 68.6, 67.1, 66.8, 66.7, 66.1, 65.9, 63.1, 62.2, 31.9, 30.1, 29.7, 29.6, 29.5, 29.3, 26.1, 26.0, 22.7, 20.8, 20.7, 20.6, 20.5, 14.1; ESI-MS Calcd. for $C_{166}H_{202}O_{56}Na$ $[M + Na]^+$: 3114.2856, found 3114.5381.

*8-O-Hexadecyl-3,6,10-trioxa-1,8-hexacosanedioyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→5)-α-D-arabinofuranosyl-(1→2)-[α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→5)-α-D-arabinofuranosyl-(1→3)]-α-D-arabinofuranoside (**1**)*: Sodium methoxide in methanol (1 M) (0.15 mL) was added to a solution of **13** (0.15 g, 0.048 mmol) in methanol/tetrahydrofuran (1:1) (5 mL) at room temperature and stirred for 12 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated *in vacuo* to afford **1**, as a solid. Yield: 0.07 g, (88%); $[\alpha]_D$ +98.68 (*c* 0.4, D_2O); ^{1}H NMR (D_2O, 400 MHz): δ 5.18 (s, 1 H), 5.14 (s, 2 H), 4.92 (s, 4 H), 4.27-3.69 (m, 56 H), 1.57 (br s, 4 H), 1.31 (br s, 52 H), 0.89 (s, 6 H);

^{13}C NMR (D_2O, 100 MHz): δ 106.8, 106.6, 106.5, 99.9, 99.5, 99.4, 82.5, 82.2, 81.9, 81.5, 78.0, 76.8, 76.5, 72.8, 71.4, 71.2, 70.9, 70.7, 70.3, 70.1, 70.0, 66.8, 66.6, 65.5, 61.1, 32.1, 30.3, 30.1, 29.7, 26.4, 26.3, 22.7, 13.9; ESI-MS Calcd. for $C_{78}H_{144}O_{37}Na$ [M + Na]$^+$: 1695.9284, found 1695.9067.

*8-O-Hexadecyl-3,6,10-trioxa-1,8-hexacosanedioyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-α-D-arabinofuranosyl-(1→5)-[2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-α-D-arabinofuranosyl-(1→2)]-3-O-benzoyl-α-D-arabinofuranoside (***15***)*: A solution of **11** (0.44 g, 0.35 mmol), **14** (0.1 g, 0.12 mmol) and MS 4 Å (0.6 g) in dichloromethane (5 mL) was stirred for 10 min., *N*-iodosuccinimide (0.11 g, 0.50 mmol) and silver trifluoromethanesulfonate (0.035 g, 0.14 mmol) were added into it at 0 °C, under N_2 atmosphere. The reaction mixture was stirred at the same temperature for another 30 min., neutralized with Et_3N, filtered and filtrate diluted with dichloromethane (50 mL), washed with satd. aq. sodium thiosulphate (2 x 20 mL) and water (1 x 20 mL). The organic portion was dried (Na_2SO_4), filtered and filtrate concentrated *in vacuo* and purified (pet. ether/EtOAc = 7:3) to afford **15**, as a gum. Yield: 0.19 g (51%); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.14 (d, *J* = 7.2 Hz, 4 H), 8.11-8.09 (m, 3 H), 8.05 (d, *J* = 8 Hz, 4 H), 8.01 (d, *J* = 8 Hz, 2 H), 7.94 (d, *J* = 7.2 Hz, 4 H), 7.75 (d, *J* = 7.6 Hz, 4 H), 7.62-7.28 (m, 31 H), 7.23 (d, *J* = 7.6 Hz, 3 H), 6.05-5.99 (m, 2 H), 5.90 (d, *J* = 10.4 Hz, 2 H), 5.84 (s, 2 H), 5.74 (d, *J* = 4.4 Hz, 3 H), 5.68 (d, *J* = 5.6 Hz, 1 H), 5.66 (s, 1 H), 5.51 (s, 1 H), 5.46-5.43 (m, 3 H), 5.38-5.36 (m, 2 H), 5.33 (s, 1 H), 5.29-5.24 (m, 2 H), 5.20 (s, 2 H), 4.88 (s, 2 H), 4.57-4.54 (m, 6 H), 4.29-3.94 (m, 13 H), 3.82 (d, *J* = 12 Hz, 2 H), 3.68 (d, *J* = 11.2 Hz, 5 H), 3.60-3.42 (m, 11 H), 2.07 (s, 3 H), 2.06 (s, 3 H), 2.04 (s, 6 H), 1.94 (s, 3 H), 1.93 (s, 3 H), 1.90 (s, 6 H), 1.54-1.51 (m, 4 H), 1.24 (br s, 50 H), 0.88 (t, *J* = 6.8 Hz, 6 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.4, 169.8, 169.7, 169.3, 165.9, 165.8, 165.7, 165.6, 165.5, 165.4, 165.3, 164.9, 139.2, 133.4, 133.2, 133.1, 133.0, 132.9, 130.0, 129.9, 129.8, 129.7, 129.4, 129.3, 129.2, 129.1, 129.0, 128.9, 128.8, 128.7, 128.5, 128.4, 128.3, 128.2, 128.1, 106.8, 106.0, 104.9, 98.3, 98.2, 97.7, 97.6, 84.1, 82.6, 82.2, 82.1, 81.9, 81.5, 78.1, 77.9, 77.2, 71.6, 71.4, 71.0, 70.8, 70.6, 70.5, 70.4, 70.3, 70.1, 69.5, 69.4, 69.3, 69.1, 68.6, 67.4, 66.8, 66.6, 66.1, 65.9, 62.2, 31.9, 31.6, 30.3, 30.1, 29.7, 29.6, 29.5, 29.3, 26.1, 26.0, 22.6, 20.7, 20.6, 20.5, 20.4, 14.1; MALDI-TOF-MS Calcd. for $C_{171}H_{204}O_{56}Na$ [M+Na]$^+$: 3177.3047 (100%), found 3177.3050.

*8-O-Hexadecyl-3,6,10-trioxa-1,8-hexacosanedioyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→5)-α-D-arabinofuranosyl-(1→5)-[α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→5)-α-D-arabinofuranosyl-(1→2)]-α-D-arabinofuranoside (***2***)*: Sodium methoxide in methanol (1 M) (0.15 mL) was added to a solution of **15** (0.15 g, 0.048 mmol) in methanol/tetrahydrofuran (1:1) (5 mL) at room temperature and stirred for 12 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated *in vacuo* to afford **2**, as a solid. Yield: 0.073 g (92%); $[\alpha]_D$ +22.82 (*c* 0.5, D_2O); ^{1}H NMR (D_2O, 400 MHz): δ 5.16 (s, 2 H), 5.03 (s, 1 H), 4.91 (s, 4 H), 4.13-3.56 (m, 56 H), 1.57 (br s, 4 H), 1.31 (br s, 52 H), 0.89 (s, 6 H); ^{13}C NMR (D_2O, 100 MHz): 107.8, 106.9, 106.6, 100.0, 99.9, 99.5, 99.4, 81.8, 81.6, 81.3, 78.0, 76.8, 76.6, 72.7, 71.4, 71.1, 70.7, 70.2, 70.1, 69.9, 66.8, 66.6, 65.6, 61.6, 61.0, 32.1, 30.3, 30.0, 29.8, 29.7, 26.4, 26.3, 22.7, 13.9; ESI-MS Calcd. for $C_{78}H_{144}O_{37}Na$ [M + Na]$^+$: 1695.9284, found 1695.9478.

*Methyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-α-D-arabinofuranosyl-(1→2)-[2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-α-D-arabinofuranosyl-(1→3)]-5-O-acetyl-α-D-arabinofuranoside (***17***)*: A solution of **11** (0.55 g, 0.44 mmol), **16** (0.03 g, 0.146 mmol) and MS 4 Å (0.6 g) in dichloromethane (5 mL) was stirred for 10 min., *N*-iodosuccinimide (0.12 g, 0.53 mmol) and silver trifluoromethanesulfonate (0.045 g, 0.18 mmol) were added into it at 0 °C, under N_2 atmosphere. The reaction mixture was stirred at the same temperature for another 30 min., neutralized with Et_3N, filtered and filtrate diluted with dichloromethane (50 mL), washed with satd. aq. sodium thiosulphate (2 x 20 mL) and water (1 x 20 mL). The organic portion was dried (Na_2SO_4), filtered and filtrate concentrated *in vacuo* and purified (pet. ether/EtOAc = 1:1) to afford **17**, as a gum. Yield: 0.203 g (56%); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.13 (d, *J* = 7.2 Hz, 4 H), 8.08 (dd, *J* = 2.8 Hz, 7.6 Hz, 4 H), 8.04-8.01 (m, 3 H), 7.96 (d, *J* = 7.6 Hz, 4 H), 7.75 (d, *J* = 7.6 Hz, 4 H), 7.62-7.39 (m, 17 H), 7.36-7.22 (m, 14 H), 5.98 (t, *J* = 10 Hz, 2 H), 5.93-5.88 (m, 2 H), 5.82 (s, 2 H), 5.72 (d, *J* = 5.2 Hz, 2 H), 5.61 (d, *J* = 4.8 Hz, 3 H), 5.44 (dd, *J* = 3.2 Hz, 10 Hz, 2 H), 5.35 (d, *J* = 3.2 Hz, 2 H), 5.25 (t, *J* = 10.2 Hz, 2 H), 5.18 (d, *J* = 8 Hz, 2 H), 5.12 (s, 1 H), 4.88 (s, 2 H), 4.54-4.49 (m, 5 H), 4.44 (dd, *J* = 5.2 Hz, 12.4 Hz, 1 H), 4.33-4.26 (m, 4 H), 4.15-3.95 (m, 10 H), 3.86 (d, *J* = 12 Hz, 2 H), 3.69 (d, *J* = 10.8 Hz, 2 H), 3.47 (s, 3 H), 2.07 (s, 3 H), 2.06 (s, 3 H), 2.05 (s, 3 H), 2.04 (s, 9 H), 1.91 (s, 9 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 171.1, 170.7, 170.5, 169.8, 169.7, 169.3, 165.8, 165.7, 165.6, 165.4, 165.3, 164.9, 133.4, 133.3, 133.2, 132.9, 130.0, 129.9, 129.8, 129.7, 129.3, 129.2, 129.1, 129.0, 128.9, 128.7, 128.5, 128.4, 128.1, 107.6, 106.1, 105.9, 98.2, 97.6, 97.5, 87.1, 82.8, 82.4, 81.9, 79.3, 77.2, 70.3, 70.1, 69.5, 69.4, 69.0, 68.6, 66.8, 66.1, 65.9, 62.2, 60.4, 55.3, 21.0, 20.8, 20.7, 20.5; ESI-MS Calcd. for $C_{128}H_{126}O_{52}Na$ [M + Na]$^+$: 2517.7113, found 2517.7609.

*Methyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→5)-α-D-arabinofuranosyl-(1→2)-[α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→5)-α-D-arabinofuranosyl-(1→3)]-α-D-arabinofuranoside (***3***)*: Sodium methoxide in methanol (1 M) (0.15 mL) was added to a solution of **17** (0.15 g, 0.06 mmol) in methanol/tetrahydrofuran (1:1) (5 mL) at room temperature and stirred for 12 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated *in vacuo* to afford **3**, as a solid. Yield: 0.058 g, (90%); $[\alpha]_D$ +91.98 (*c* 1, D_2O); ^{1}H NMR (CD_3OD, 400 MHz): δ 5.03 (s, 3 H), 4.96 (s, 1 H), 4.80 (d, *J* = 3.2 Hz, 3 H), 4.13-4.00 (m, 7 H), 3.87-3.64 (m, 32 H), 3.38 (s, 3 H); ^{13}C NMR (CD_3OD, 100 MHz): 109.3, 108.8, 108.7, 101.6, 101.4, 101.1, 101.0, 87.6, 83.8, 83.7, 83.5, 83.2, 82.2, 79.1, 79.0, 74.3,

72.9, 72.7, 72.6, 72.5, 72.1, 71.9, 68.6, 68.5, 68.0, 67.9, 67.2, 62.7, 62.6, 55.2; ESI-MS Calcd. for $C_{40}H_{68}O_{33}Na$ $[M + Na]^+$: 1099.3541, found 1099.3541.

*Methyl 2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-α-D-arabinofuranosyl-(1→5)-[2,3,4,6-tetra-O-acetyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzoyl-α-D-mannopyranosyl-(1→5)-2,3-di-O-benzoyl-α-D-arabinofuranosyl-(1→2)]-3-O-benzoyl-α-D-arabinofuranoside (**19**)*: A solution of **11** (0.42 g, 0.34 mmol), **18**[18] (0.03 g, 0.11 mmol) and MS 4 Å (0.6 g) in dichloromethane (5 mL) was stirred for 10 min., *N*-iodosuccinimide (0.092 g, 0.41 mmol) and silver trifluoromethanesulfonate (0.036 g, 0.14 mmol) were added into it at 0 °C, under N_2 atmosphere. The reaction mixture was stirred at the same temperature for another 30 min., neutralized with Et_3N, filtered and filtrate diluted with dichloromethane (50 mL), washed with satd. aq. sodium thiosulphate (2 x 20 mL) and water (1 x 20 mL). The organic portion was dried (Na_2SO_4), filtered and filtrate concentrated *in vacuo* and purified (pet. ether/EtOAc = 1:1) to afford **19**, as a gum. Yield: 0.146 g (52%); ^{1}H NMR ($CDCl_3$, 400 MHz): δ 8.15-8.04 (m, 12 H), 8.00 (d, *J* = 7.6 Hz, 2 H), 7.94 (d, *J* = 7.6 Hz, 4 H), 7.75 (d, *J* = 8 Hz, 4 H), 7.63-7.45 (m, 11 H), 7.41 (t, *J* = 7.6 Hz, 4 H), 7.37-7.22 (m, 18 H), 6.05-5.96 (m, 2 H), 5.92-5.88 (m, 2 H), 5.83 (d, *J* = 10.4 Hz, 2 H), 5.72 (d, *J* = 4.8 Hz, 2 H), 5.68 (d, *J* = 10.4 Hz, 3 H), 5.50 (s, 1 H), 5.46-5.42 (m, 3 H), 5.36 (d, *J* = 11.2 Hz, 2 H), 5.26 (dd, *J* = 3.8 Hz, 9.8 Hz, 2 H), 5.20 (s, 3 H), 4.88 (s, 2 H), 4.57-4.52 (m, 6 H), 4.28-4.23 (m, 2 H), 4.17 (dd, *J* = 5.8 Hz, 11 Hz, 1 H), 4.13-3.94 (m, 9 H), 3.84 (t, *J* = 10.4 Hz, 2 H), 3.69 (d, *J* = 10.8 Hz, 2 H), 3.44 (s, 3 H), 2.07 (s, 3 H), 2.06 (s, 3 H), 2.04 (s, 3 H), 2.03 (s, 3 H), 1.94 (s, 3 H), 1.90 (s, 9 H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 170.5, 169.8, 169.7, 169.3, 165.9, 165.8, 165.5, 165.4, 165.3, 164.9, 133.4, 133.2, 133.1, 132.9, 130.1, 130.0, 129.9, 129.8, 129.7, 129.5, 129.3, 129.2, 129.1, 129.0, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.1, 107.9, 106.0, 105.1, 98.3, 98.2, 97.7, 97.6, 84.4, 82.5, 82.2, 82.0, 81.9, 81.0, 78.4, 77.2, 70.3, 70.1, 70.0, 69.5, 69.4, 69.3, 69.1, 69.0, 68.6, 67.4, 67.0, 66.8, 66.1, 65.9, 65.8, 62.2, 54.9, 20.7, 20.6, 20.5, 20.4; ESI-MS Calcd. for $C_{133}H_{128}O_{52}Na$ $[M + Na]^+$: 2579.7269, found 2579.7002.

*Methyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→5)-α-D-arabinofuranosyl-(1→5)-[α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→5)-α-D-arabinofuranosyl-(1→2)]-α-D-arabinofuranoside (**4**)*: Sodium methoxide in methanol (1 M) (0.15 mL) was added to a solution of **19** (0.12 g, 0.047 mmol) in methanol/tetrahydrofuran (1:1) (5 mL) at room temperature and stirred for 12 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated *in vacuo* to afford **4**, as a solid. Yield: 0.048 g (94%); $[\alpha]_D$ +94.63 (*c* 1, D_2O); ^{1}H NMR (D_2O, 400 MHz): δ 5.13 (s, 1 H), 5.05 (s, 1 H), 5.03 (s, 1 H), 4.90 (s, 4 H), 4.13-4.11 (m, 4 H), 3.98-3.62 (m, 35 H), 3.42 (s, 3 H); ^{13}C NMR (D_2O, 100 MHz): 107.6, 107.3, 99.9, 99.4, 87.3, 81.8, 81.7, 81.6, 81.4, 81.2, 76.8, 75.2, 72.7, 70.9, 70.8, 70.7, 70.6, 70.0, 69.9, 66.8, 66.6, 66.3, 65.6, 60.9, 55.2; ESI-MS Calcd. for $C_{40}H_{68}O_{33}Na$ $[M + Na]^+$: 1099.3541, found 1099.3541.

Bacterial strains and chemicals: *M. smegmatis* mc^2155 strain was used for estimating the inhibitory effects of the compounds on cell and biofilm growth. Bacterial strain was cultured in Middlebrook 7H9 broth (MB7H9) minimal medium manufactured by DIFCO. Syringe filters (0.2 micron-pore size) were used for filter sterilization of Tween 80 and other solutions. We purchased the filters from Millipore. Tween 80, NaCl, KCl, Na_2HPO_4, EDTA and KH_2PO_4 were purchased from Sigma Aldrich and were of analytical grade. All solutions were made in MilliQ water (unless specified otherwise).

Mycobacterial growth inhibition assay: Mycobacterial growth was followed in the presence of synthesized compounds and growth profile of *M. smegmatis* was derived. *M. smegmatis* strain mc^2155 is a rapidly growing non-pathogenic mycobacterium widely used as model organism. *M. smegmatis* was grown in Middlebrook 7H9 broth (Difco) supplemented with 2% glucose as carbon source and 0.05% tween 80 in shaker at 37°C. *M. smegmatis* growth was studied in the presence of newly synthetic glycolipids (100 µg/mL) and variations in mycobacterial growth were analyzed. The optical density (O.D.) of the culture was recorded at 600 nm for 96 hours at the interval of 12 hours and growth curve was plotted on Sigma Plot 10 software.

Biofilm formation assay: Biofilm was grown in Sauton's media supplemented with 2% Glycerol and 0.05% Tween-80. The procedure was followed as described elsewhere.[43] Briefly, primary culture was washed with Sauton's media and diluted in Sauton's media to the OD 0.005. Biofilms were grown in 6-well cell culture plate (Laxbro) where 5 mL diluted culture was added to the each well. Inhibitors were added at zero time point at 100 µg/mL. Biofilm culture plates were stored in a humidified incubator set at 37°C and evaluated at different time points.

Biofilm quantitation: The biofilm quantification was evaluated in 96 well plates and protocol was studied as described previously. Briefly, primary *M. smegmatis* culture was pelleted, washed and diluted in Sauton's medium to an optical density (O.D.) of 0.005 at 600 nm.[35] The inhibitors were added into the culture at zero time point. Experiment was conducted in three biological replicates. Inoculum culture was 200 µL which was added in each well of 96-well plates and biofilm formation was monitored up to the 196 h. The media was removed from the wells which were washed thoroughly with water and stained by the addition of 1% crystal violet solution (200 µL) followed by incubation for 20 min. The wells were again washed and allowed to dry. The dye was quantitated after incubating each well with DMSO (200 µL) for an hour and subsequently measuring the absorption at 590 nm using a microplate reader.

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Keywords: biofilms, isoniazid, glycolipids, mycobacteria, oligosaccharides

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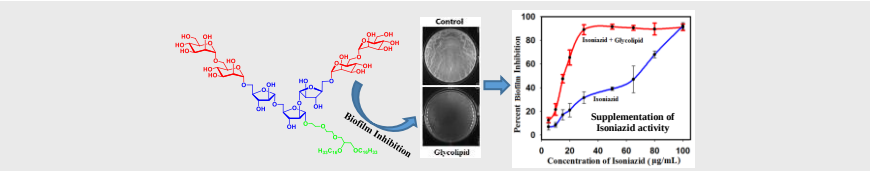

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Synthetic Arabinomannan Heptasaccharide Glycolipids Inhibit Biofilm Growth and Supplements Isoniazid Effects in Mycobacterium smegmatis

Non-cytotoxic synthetic mannopyranosyl-arabinofuranoside heptasaccharide glycolipids are identified as potent inhibitors of the biofilms of *M. smegmatis* mycobacterial strain, in a dose-depedent manner, with minimal biofilm growth inhibition concentration of 100 μg/mL. These glycolipids also supplement, in a synergistic manner, the minimal biofilm growth inhibition concentration of life-line drug isoniazid by more than 3 times, at 30 μg/mL.