Synthetic arabinomannan glycolipids and their effects on growth and motility of the *Mycobacterium smegmatis*†

Kottari Naresh,[a] Binod Kumar Bharati,[b] Prakash Gouda Avaji,[a] Narayanaswamy Jayaraman*[a] and Dipankar Chatterji*[b]

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Arabinomannan-containing glycolipids, relevant to the mycobacterial cell-wall component lipoarabinomannan, were synthesized by chemical methods. The glycolipids were presented with tri- and tetrasaccharide arabinomannans as the sugar portion and a double alkyl chain as the lyophilic portion. Following synthesis, systematic biological and biophysical studies were undertaken in order to identify the effects of the glycolipids during mycobacterium growth. The studies included mycobacterial growth, biofilm formation and motility assays. From the studies, it was observed that the synthetic glycolipid with higher arabinan residues inhibited the mycobacterial growth, lessened the biofilm formation and impaired the motility of mycobacteria. A surface plasmon resonance study involving the immobilized glycan surface and the mycobacterial crude lysates as analytes showed specificities of the interactions. Further, it was found that cell lysates from motile bacteria bound oligosaccharide with higher affinity than non-motile bacteria.

Introduction

There are as many as 70 different mycobacterium strains known currently and several of them are pathogenic. A morphological feature common to all mycobacterial strains is the highly orchestrated cell-wall, which provides protection against environmental stresses.[1] The cell-walls of mycobacterial strains are composed largely of two glycolipids, namely, lipoarabinomannan and lipoarabinogalactan.[2–6] These glycolipids play critical roles in the survival of organisms within the host.[7,8] In this context, compounds that inhibit the synthesis of the cell-wall components have potential as drugs.[9] Antibiotics such as isoniazid and ethambutol inhibit the biosynthesis of mycobacterial cell-wall by preventing the assembly of mycolyl-arabinogalactan (mAG).[10,11] Studies of the effects of enzymes responsible for the biosynthesis of cell-wall have been reported, using synthetic arabinogalactan and arabinomannans.[12–15]

In our studies of synthetic glycolipid derivatives, we reported recently glycolipids containing arabinofuranose trisaccharides and their inhibitory effects on the growth of *M. smegmatis* strain.[16] The inhibitory effect was found to be specific with arabinofuranoside-containing glycolipids. Continuing these efforts, we herein report synthesis of arabinomannan glycolipids, followed by biological and biophysical studies of these new glycolipids with a mycobacterial strain.

Results and discussion

The molecular structures of tri- and tetra-saccharide glycolipids of the present study are shown in Fig. 1. Both tri- and tetrasaccharide glycolipids are built up with an arabinofuranoside core moiety, which connects both the lipidic portion and the sugar moieties of glycolipids. The mannopyranosyl-arabinofuranoside glycolipids were chosen, as this combination of sugar moieties are implicated to act as the capping region in native lipoarabinomannan glycolipids, and the presence of the capping region is important for the virulence properties of the species.

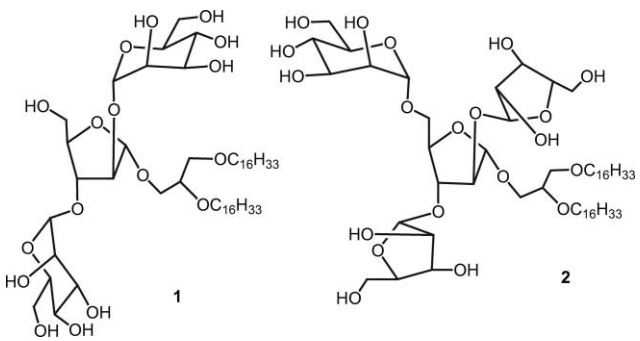


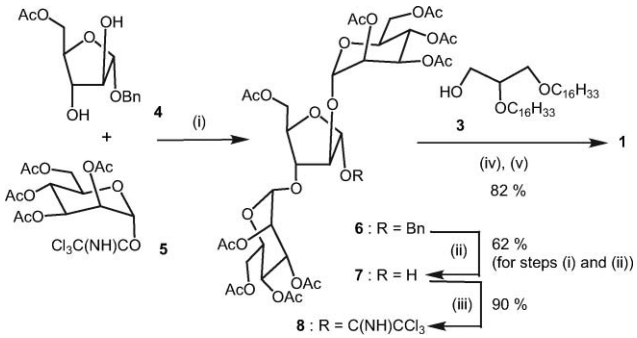
Fig. 1 Molecular structures of synthetic glycolipids.

Synthesis

Glycolipids **1** and **2** were synthesized by assembling individual constituents, namely, the alkyl chain portion and the sugar portion. The bis-*O*-alkylated glycerol **3** (Scheme 1) was synthesized from solketal through: (i) *O*-benzylation of solketal; (ii) deprotection of ketal protecting group; (iii) bis-*O*-alkylation of the resulting diol with a long alkyl chain bromide and (iv) deprotection of the

[a]*Department of Organic Chemistry, Indian Institute of Science, Bangalore 560 012, India. E-mail: jayaraman@orgchem.iisc.ernet.in; Fax: +91-80-2360-0529*
[b]*Molecular Biophysics Unit, Indian Institute of Science, Bangalore 560 012, India*

† Electronic supplementary information (ESI) available: ES-MS and NMR spectra of compounds **1**, **2**, **7**, **10**, **14**, **16** and **19**. See DOI: 10.1039/b917070g

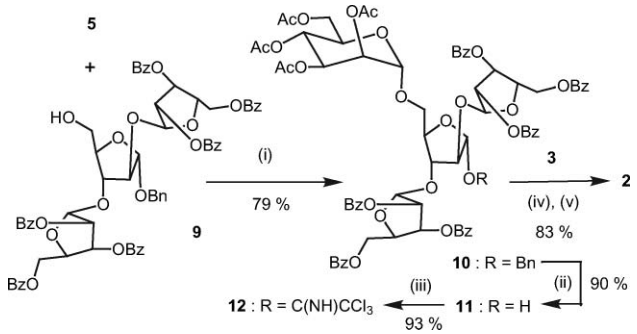


Scheme 1 *Reagents and conditions*: (i) $BF_3{\cdot}OEt_2$, rt, 2 h, CH_2Cl_2, MS 4 Å; (ii) H_2, Pd–C, EtOAc–MeOH (1 : 1), rt, 18 h; (iii) CCl_3CN, DBU, CH_2Cl_2, 0 °C, 20 min; (iv) TMSOTf, rt, 30 min, CH_2Cl_2, MS 4 Å; (v) NaOMe–MeOH, rt, 2 h.

benzyl group.[17] Derivative **3** was racemic and was used as such in the preparation of glycolipids.

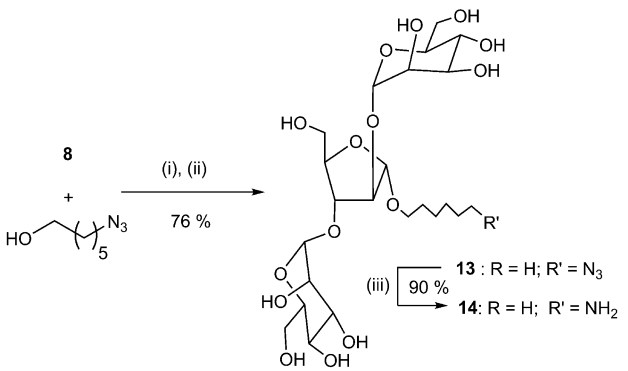
The tri- and tetrasaccharides were synthesized, using suitably protected arabinofuranosides and mannopyranosides. The orthogonally protected sugar derivative **4** and the activated glycosyl donor **5** were prepared by known methods.[16,18] A glycosylation of acceptor diol **4** with trichloroacetimidate donor **5**, in the presence of $BF_3{\cdot}OEt_2$, led to the formation of trisaccharide **6**, which upon hydrogenolysis (H_2, Pd–C) afforded lactol **7**. Activation of lactol **7** to trichloroacetimidate **8**, followed by glycosylation of acceptor **3** with **8**, in the presence of TMSOTf, afforded the protected derivative of glycolipid **1**, in 88% yield. The anomeric configuration of the protected derivative of **1** was ascertained from the ^{13}C NMR spectrum of the protected derivative of **1**, wherein the carbon nucleus of the newly formed glycosidic center was observed at 105.6 ppm and 100.5 ppm, values typical of α- and β-anomeric configurations of the arabinofuranosides (α : β ≈ 1 : 1).[19] Subsequent deprotection, under Zemplén conditions, led to the isolation of glycolipid **1**, in 94% yield. NMR spectroscopic and mass spectrometric analyses confirmed the structure of glycolipid **1**.

Preparation of tetrasaccharide **11** (Scheme 2) was initiated using arabinofuranosyl trisaccharide **9**.[16] The glycosylation of **9** with trichloroacetimidate **5**, mediated by $BF_3{\cdot}OEt_2$, led to the formation of tetrasaccharide **10**, in 79% yield. The appearance of an apparent singlet at 4.9 ppm in the ^1H NMR spectrum and a resonance at 97.7 ppm in ^{13}C NMR spectrum confirmed the α-anomeric configuration of the newly formed glycosidic bond. De-*O*-benzylation at the reducing end of tetrasaccharide **10** afforded lactol **11**, which was converted subsequently to an activated trichloroacetimidate glycosyl donor **12**. The preparation of tetrasaccharide glycolipid **2** was accomplished by glycosylation of **3** with **12**, followed by deprotection to afford **2**, in an overall 83% yield. The ^{13}C NMR spectra of the protected derivative of **2** exhibited peaks at 97.9, 102.4, 105.3, 105.5 and 106.7 ppm. Whereas peaks at 97.9, 105.3 and 105.5 ppm corresponded to the α-anomeric carbon of the glycosyl moieties at the non-reducing end, that of 102.4 and 106.7 ppm corresponded to β- and α-arabinofuranosyl moiety,[19] respectively, coupled with the aglycone lipid moiety. Further, mass spectrometric characterization confirmed the structure of **2**.



Scheme 2 *Reagents and conditions*: (i) $BF_3{\cdot}OEt_2$, rt, 30 min, CH_2Cl_2, MS 4 Å; (ii) H_2, Pd–C, EtOAc–MeOH (1 : 1), rt, 18 h; (iii) CCl_3CN, DBU, CH_2Cl_2, 0 °C, 20 min; (iv) TMSOTf, rt, 30 min, CH_2Cl_2, MS 4 Å; (v) NaOMe–MeOH, rt, 4 h.

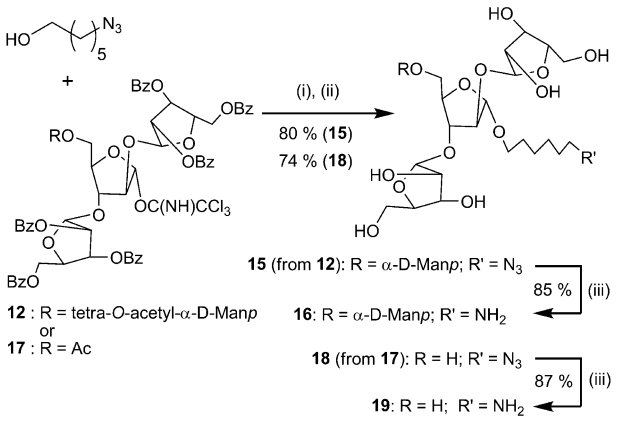
In order to facilitate further biophysical studies on tri- and tetrasaccharides with cell extracts of a mycobacterial strain, the sugar derivatives functionalized with an amine were also synthesized. Syntheses of amine tethered compounds **14**, **16** and **19** were accomplished accordingly through glycosylation of 6-azido-hexan-1-ol with tri- and tetrasaccharide glycoside donors. Thus, glycosylation of trichloroacetimidates **8**, **12** and **17**[16] with donor 6-azido-hexan-1-ol, in the presence of $BF_3{\cdot}OEt_2$, followed by deprotection of the protecting groups and a reduction of azide to amine group (H_2, Pd–C), afforded amine tethered derivatives **14**, **16** and **19**, in good yields (Schemes 3 and 4). The glycosylation led to the formation of an anomeric mixture of products, which was evident from the resonances for anomeric carbons in the ^{13}C NMR spectra. Thus, in the case of **14** in addition to peaks at 98.8, 99.0, 100.2 and 105.9 ppm for the α-anomeric carbons of the glycosyl moieties, a resonance at 101.8 ppm indicated the presence of the β-anomer of the arabinofuranoside moiety linked to the aglycan portion of the molecule. The anomeric protons in the corresponding ^1H NMR spectra could not be identified due to several overlapping resonances. The structures of **14**, **16** and **19** were confirmed further through mass spectrometry.



Scheme 3 *Reagents and conditions*: (i) $BF_3{\cdot}OEt_2$, rt, 30 min, CH_2Cl_2, MS 4 Å; (ii) NaOMe–MeOH, rt, 2 h; (iii) H_2, Pd–C, EtOAc–MeOH (1 : 1), rt, 6 h.

Effect of the arabinomannan glycolipids on growth, biofilm formation and sliding motility in *Mycobacterium smegmatis*

Growth inhibition studies. Glycolipids **1** and **2**, having moieties that constitute natural lipoarabinomannan cell-wall polysaccharides, were evaluated for their biological efficacies. The experiments performed were mostly on the biological properties of



Scheme 4 *Reagents and conditions*: (i) $BF_3{\cdot}OEt_2$, rt, 30 min., CH_2Cl_2, MS 4 Å; (ii) NaOMe–MeOH, rt, 4 h; (iii) H_2, Pd–C, EtOAc–MeOH (1 : 1), rt, 6 h.

Mycobacterium smegmatis. Our earlier study on the monovalent and bivalent arabinofuranoside glycolipids showed that these glycolipids inhibited the growth of mycobacteria.[16] Thus, we undertook to evaluate the effects of glycolipids **1** and **2** on the growth of the bacterial strain. Microbial growth is a global indicator for metabolic regulations occurring inside the cell. The growth of the bacteria is quantitated as a function of increase in the cell density by spectrophotometric measurement. In the present case, the cells of *M. smegmatis* were grown in a primary culture and a secondary inoculation was carried out from a well grown stock of primary culture. The growth profile of *M. smegmatis* was subsequently followed in the presence of varying concentrations of a solution **1** and **2** in MeOH. The growth rate in triplicate was followed by measuring the optical density at 600 nm at different time intervals. Growth of the bacteria without inhibitor or in MeOH was used as a control. Fig. 2 shows the effects observed on the growth profile of the strain in the presence of **1** and **2**. It was observed that whereas **1** did not affect the growth, glycolipid **2** inhibited the growth by about 65%, at a concentration 200 μg mL^{-1}. Comparing this observation with our earlier report on the arabinan glycolipid,[16] it appears that the presence of a larger proportion of arabinofuranose moiety in the glycolipid is required for inhibiting the bacterial growth. It is unclear at present how the inhibition of the bacterial growth occurs in the presence of the glycolipids. In order to identify the effects of **2**, further studies were performed, using biofilm formation assays and sliding motility tests.

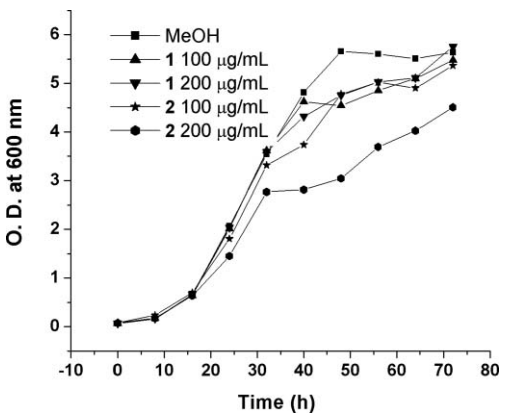


Fig. 2 Effect of **1** and **2** on the growth of *M. smegmatis*.

Biofilm formation and quantitation studies. Bacterial stationary phase is an interesting biological system to study, as the organism undergoes several metabolic changes during this period and new molecules are generated to support its survival. For pathogenic microorganisms such as mycobacteria, the stationary phase adds a further complication, as many antibacterial compounds become less effective.[20,21] Work on planktonic cultures under laboratory conditions showed that bacteria formed as adherent multicellular aggregates called biofilms,[22] which can form on any moist surface exposed to biofilm-forming bacteria. Biofilms are formed to protect the bacteria from host defenses, antibiotics and harsh environmental conditions. The studies on biofilms thus explore the possibilities for the development of antibiofilm therapies. The formation of biofilm proceeds through different steps. The first and most crucial step for biofilm formation is the initial attachment of bacteria to an abiotic surface, followed by multiplication and aggregate formation. The next step is the formation of structured and matured biofilm. The final step is the dispersion and reinitiation of the cycle.[23] As biofilm forms a complex of cell surface networks, we envisaged that it would be worth studying the effect of **2** on them. Fig. 3 and Fig. 4 show the effect of **2** (200 μg ml^{-1}) on the formation and the quantitation of biofilm, respectively. It can be seen from Fig. 3 that the biofilm formation is affected significantly in the presence of **2**, as compared to the untreated bacteria (Wt). The biofilm formation is regulated by several genes and their up and down regulation results in over-expression and diminished expression of proteins, respectively. The wild-type mycobacterium cultures started forming pellicles at the air–liquid interface which can be seen as microcolonies floating on the surface after 24 h of growth. These microcolonies spread

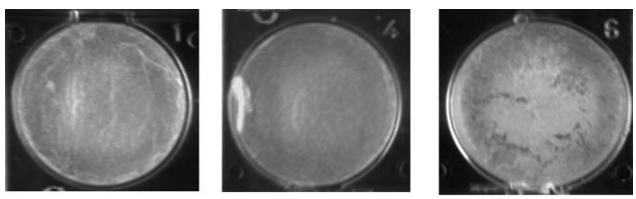


Fig. 3 Effect of **2** on biofilm formation of *M. smegmatis*.

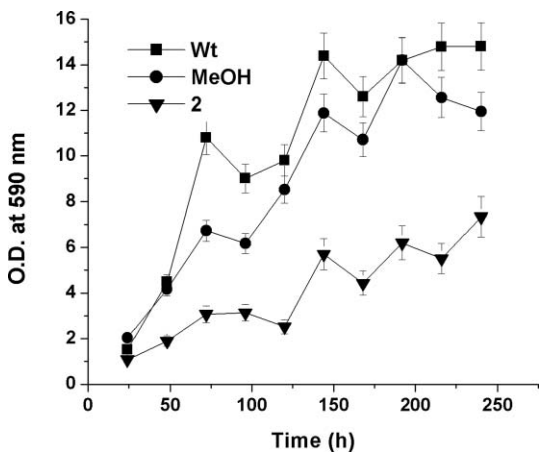


Fig. 4 Quantitation of biofilm formation.

over the liquid surface to form a film covering the entire interface surface in 5 to 7 days after inoculation (Fig. 3). It is also noticed from Fig. 4 that the cultures treated with the inhibitor show a slow growth of the biofilm, as compared to the untreated cultures or those treated with MeOH. Following this observation, we allowed the biofilm formation up to 240 h. It can be seen from Fig. 4 that the formation of biofilm was reduced substantially in the presence of **2** (200 μg ml^{-1}), when compared to the untreated culture or that in MeOH. The degree of inhibition of biofilm formation by *M. smegmatis* with compound **2** is significant, in comparison to our previous studies, where a gene was deleted and biofilm formation was impaired.[30] As such no other organic compound is known to inhibit mycobacterial biofilms.

Sliding motility inhibition studies. The mycobacterial cell membrane is surrounded by a thick and waxy envelope that contains diverse lipids.[24] The envelope is made up of three types of insoluble macromolecular components: (i) heteropolysaccharide arabinogalactan; (ii) peptidoglycan and (iii) mycolic acid.[25] It was demonstrated earlier that the glycolipids containing arabinofuranose trisaccharide inhibited the growth of mycobacteria.[16] Further, data presented herein show that an additional mannan portion in the arabinomannan glycolipids does not alter the growth pattern of the mycobacteria. The biofilm formation was also affected in the presence of arabinomannan glycolipid **2**. We were interested to identify other phenotypic behaviors, such as sliding motility. The sliding motility test was carried out to check the spreading ability of mycobacteria on the surface. The motile *M. smegmatis* was tested on the soft and moist surface of the Petri-plates. The sliding motility was assayed in the presence of 2% glucose as a carbon source. Glycolipid **2** was added in the MB7H9 base medium, supplemented with 0.3% high grade agarose. A relationship between the defect in biofilm formation and sliding motility was shown in *M. smegmatis* mutants defective in GPL synthesis by Kolter and Recht.[26] As the biofilm formation was defective in our case, we were interested to look at the sliding motility of mycobacteria. It was observed that the motility of the bacteria was highly diminished in the presence of **2**. Fig. 5 shows the sliding motility of mycobacteria in the presence of different concentrations of **2** up to 7 days. Whereas MeOH appeared to show little inhibition of the motility, glycolipid **2**, at concentrations of 100 μg mL^{-1} and 200 μg mL^{-1}, affected the sliding motility remarkably. Thus, the glycolipid **2** not only exhibited the effect of reducing the biofilm formation, but also induced an impaired mobility of mycobacteria on the moist surface, as compared to the untreated bacteria.

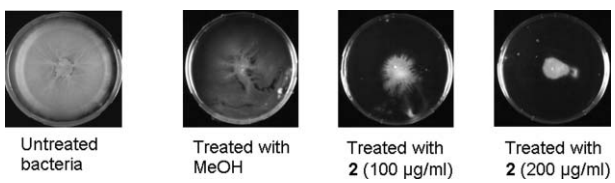


Fig. 5 Effect of **2** on the motility of *M. smegmatis* (7 days old).

The mechanism of sliding in mycobacteria has not been characterized in detail. It is known that the acetylation of glycopeptidolipids affects the biofilm formation and sliding motility in mycobacteria.[27] It appears that these synthetic compounds may be playing a role with the normal distribution of glycopeptidolipids (GPLs) or the level of proteins which regulate the synthesis of GPLs and results in the impaired sliding motility and biofilm formation.

Surface plasmon resonance studies. Further to the observations that the glycolipid **2** inhibited mycobacterial growth, lessened biofilm formation and impaired the motility of mycobacteria, we were interested to study glycolipids **1** and **2** with cell lysates from the motile and non-motile strain of mycobacteria, through surface plasmon resonance (SPR) biophysical methods. SPR provides the ability to evaluate the interactions between a receptor and a ligand, under non-labeling and non-invasive conditions.[28] The technique allows the studies to be performed under flowing conditions, over an immobilized surface, thereby mimicking the cell-surface environments. Initially, glycolipids **1** and **2** were immobilized on to the sensor chip, wherein the glycolipids interdigitated with long alkyl chain functionalized HPA sensor chip. Although interdigitation of the glycolipids occurred, interactions of mycobacterial cell lysates were unsuccessful, possibly due to removal of the glycolipids along with the flowing cell lysates. This difficulty led us to adopt an alternative method of immobilization, wherein a covalent functionalization of the surface was undertaken. Accordingly, amine tethered oligosaccharides **14**, **16** and **19** were used for immobilization over the C1 sensor chip, presenting carboxylic acid functionalities. The amine coupling methodology allowed immobilization of **14**, **16** and **19**. Immobilization of the arabinomannans was performed uniformly to ~200 RU, corresponding to a loading of ~200 pg mm^{-2} of the oligosaccharides immobilized on the surface. The remaining activated succinimide esters, unbound by oligosaccharides, were blocked using ethanolamine. In order to eliminate the bulk refractive change and non-specific interactions, ethanolamine was immobilized in one of the flow cells. After immobilization of **14**, **16** and **19**, physically adsorbed materials were removed through purging with the buffer. Upon immobilization, cell lysates from different types of bacteria were used as analytes. Cell lysates from motile (mc^2155) and non-motile (mcdrz) bacteria, with 2 and 0.02% glucose, respectively, were chosen.[30] Cell lysates were extracted at two stages of the growth: one was at the exponential phase of the growth, *i.e.*, after 36 h of the cell growth, and other at the late stationary phase of the growth, *i.e.*, after 88 h of the growth. The collected cell lysates were dissolved in the running buffer (HEPES), and passed over the flow cells. The cell lysates were passed for 60 s during the association phase. The dissociation phase was monitored during subsequent washing of the surface with buffer alone. Proteins remaining after the dissociation phase were removed from the chip, through an injection of an aqueous solution of sodium dodecyl sulfate (SDS) (0.03%). Fig. 6 shows binding of the cell lysate of the motile strain (wild type) in the presence of **19**. Different concentrations of the cell lysates were passed over the flow cells containing trisaccharide **19** and ethanolamine alone. Response units from the flow cell containing **19** were subtracted from the flow cell containing ethanol amine, in order to eliminate the refractive index change arising from the change in the buffer and the buffer containing cell lysates, as well as the non-specific interactions.

With the increase in the cell lysate concentration, *i.e.*, from 0.16 to 0.4, an increase in the response units was observed, indicating

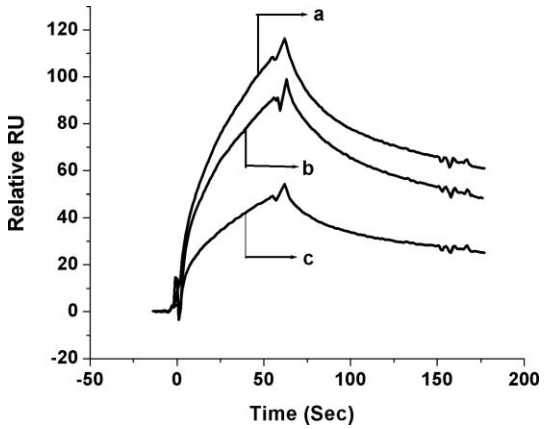


Fig. 6 Sensorgrams of the binding of varying concentrations of the cell lysates motile strain, after 36 h, and **19**. (a) Cell lysates with the protein absorbance 0.4; (b) cell lysates with absorbance 0.26 and (c) cell lysates with absorbance 0.16.

that the interactions between the compound **19** and cell lysates were specific.

Binding of the cell lysates extracted from the motile (wild type) and non-motile (mcdrz) bacteria is shown in Fig. 7. All the cell lysates were passed in a uniform concentration, in order to find the relative affinity between different types of strains. In general, motile bacteria showed stronger binding than the non-motile strain of the mycobacteria. In the case of non-motile bacteria, the cell extracts of the stationary phase exhibited a higher affinity than the cell extracts of the exponential phase. On the other hand, the motile bacterial cell extracts showed an almost similar binding behavior for both the phases. Binding of cell lysates from motile and non-motile strains, grown with a glucose concentration of 0.02%, with **19** was found to be low.

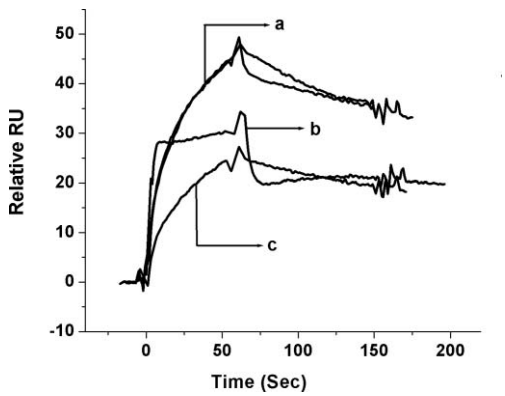


Fig. 7 Sensorgrams of the binding of the protein extracts from the wild-type and the mcdrz bacteria, with glucose (2%) after 36 h and 88 h of the growth, with **19**. (a) Wild-type strain with glucose (2%), after 36 h and 88 h of the growth; (b) mcdrz strain with glucose (2%), after 88 h of the growth and (c) mcdrz strain with glucose (2%), after 36 h of the growth.

Fig. 8 shows the relative binding of the cell lysates of the motile strain, with 2% glucose after 36 h with different arabinomannan compounds. From the sensorgrams, it is clear that the binding is relatively higher for the mannopyranosyl arabinofuranoside (**14**)

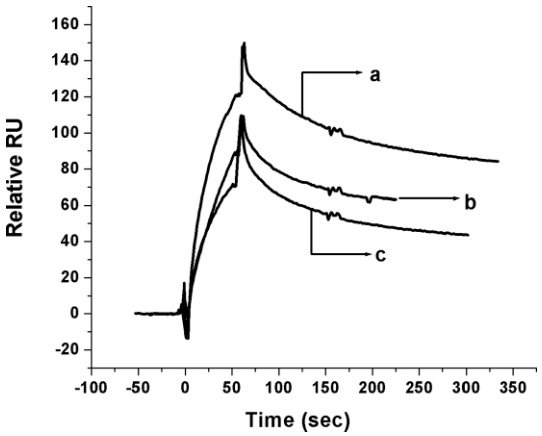


Fig. 8 Sensorgrams of the binding of the cell extracts of WT, with the glucose (2%) after 36 h with (a) **14**; (b) **16** and (c) **19**.

than the arabinofuranosides alone (**19**), or the mannopyranosyl arabinofuranoside, having one mannopyranosyl unit (**16**).

Conclusion

In conclusion, we have synthesized arabinomannan containing glycolipids and studied their effects on the growth, biofilm formation and motility of *M. smegmatis*. It was observed that the synthetic glycolipid with higher arabinofuranoside composition inhibited the growth, lessened the biofilm formation and impaired the motility of the mycobacteria significantly, when compared to the wild type. It appears that the synthetic glycolipids may interrupt the normal profile of the glycopeptidolipids. An effort to evaluate the interactions of the immobilized arabinomannan oligosaccharide with mycobacterial cell lysates was undertaken. It was found that the cell lysates from motile bacteria bound oligosaccharide with higher affinity than the non-motile bacteria. Future work will involve higher oligosaccharides of arabinofuranosides and mannopyranosyl arabinofuranosides, and studies of their biological profiles.

Experimental section

General information

Solvents were dried and distilled according to literature procedures. All chemicals were purchased from commercial sources and were used without further purification. Silica gel (100–200 mesh) was used for column chromatography and TLC analysis was performed on commercial plates coated with silica gel 60 F254. Visualization of the spots on TLC plates was achieved by UV radiation or spraying with 5% sulfuric acid in ethanol. High-resolution mass spectra were obtained from a 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 tetramethylsilane (TMS) for ^{1}H NMR and the central line (77.0 ppm) of $CDCl_3$ for ^{13}C NMR. Coupling constants are reported in Hz. Standard abbreviations s, app s, d, t, dd, br s, m refer to singlet, apparent singlet, doublet, triplet, doublet of doublets, broad singlet, multiplet.

5-*O*-Acetyl-[2,3,4,6-tetra-*O*-acetyl-α-D-(1→2)-mannopyranosyl]-[2,3,4,6-tetra-*O*-acetyl-α-D-(1→3)-mannopyranosyl]-D-arabinofuranose (7). A solution of **4**[16] (0.21 g, 0.75 mmol), **5**[18] (0.81 g, 1.66 mmol) and MS 4 Å (0.5 g) in CH_2Cl_2 (60 mL) was stirred for 15 min, $BF_3{\cdot}OEt_2$ (0.23 mL, 1.66 mmol) was added, under N_2 atmosphere and the reaction mixture was stirred at room temperature for 2 h, filtered and the filtrate washed with the saturated aq. $NaHCO_3$ solution and with water. The organic phase was separated, dried (Na_2SO_4), filtered and the filtrate concentrated to afford **6**. A solution of the crude reaction mixture of **6** in EtOAc–MeOH (1 : 1) (50 mL) was treated with Pd–C (10%) (0.45 g) and stirred under H_2 (1 bar) for 18 h. The reaction mixture was filtered, solvent removed *in vacuo*, the crude reaction mixture purified (pet. ether–EtOAc 3 : 2), to afford **7**, as a foamy solid. Yield: 0.4 g (62%); R_f (pet. ether–EtOAc 3 : 7) 0.34; $[\alpha]_D$ +41.2 (*c* 0.5, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.90–2.19 (m, 27 H), 3.88–4.09 (m, 5 H), 4.13–4.38 (m, 7 H), 4.92–4.96 (m, 1 H), 5.19 (br s, 1 H), 5.22–5.40 (m, 5 H), 5.45 (app s, 1 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.5, 20.6, 20.7, 62.3, 62.4, 62.8, 63.1, 63.5, 65.9, 66.1, 68.4, 68.7, 69.1, 69.4, 75.8, 79.0, 79.5, 80.8, 84.7, 85.9, 95.3, 96.9, 97.2, 99.2, 100.4, 169.5-171.7. ES-MS Calcd. for $C_{35}H_{48}O_{24}Na$: 875.2433 [M + Na]; found: 875.2427.

2-*O*-Hexadecyl-4-oxa-1,2-eicosanedioyl-[α-D-(1→2)-mannopyranosyl]-[α-D-(1→2)-mannopyranosyl]-D-arabinofuranoside (1). To a stirred solution of trisaccharide **7** (0.06 g, 0.068 mmol) and CCl_3CN (0. 068 mL, 0.68 mmol) in CH_2Cl_2 (10 mL), DBU (10 μL, 0.068 μmol) was added at 0 °C and stirred for 20 min. The crude reaction mixture was purified using pet. ether–EtOAc (1 : 1), to afford the trichloroacetimidate **8**, as a foamy solid. Yield (0.061 g, 90%). A solution of **8** (0.04 g, 0.039 mmol), **3** (0.02 g, 0.039 mmol) and MS 4 Å (0.1 g) in CH_2Cl_2 (10 mL) was stirred for 15 min, TMSOTf (7.1 μL, 10% solution in CH_2Cl_2, 4 μM) was added at −10 °C, 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 2 : 3), to afford the protected derivative of **1** (α : β ≈ 1 : 1); Yield: 0.047 g (88%); R_f (pet. ether–EtOAc 1 : 1) 0.5; ^{1}H NMR (400 MHz, $CDCl_3$) δ 0.88 (t, J = 6.8 Hz, 6 H), 1.25 (br s, 52 H), 1.53–1.60 (m, 4 H), 1.93–2.2 (m, 27 H), 3.36–3.60 (m, 7 H), 3.77–3.83 (m, 1 H), 3.90 (dd, J = 3.2 Hz, J = 12 Hz, 1 H), 3.96–3.99 (m, 1 H), 4.09–4.16 (m, 4 H), 4.18–4.4 (m, 6 H), 4.93–4.99 (m, 2 H), 5.13–5.19 (m, 2 H), 5.22–5.31 (m, 5 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 20.6, 20.8, 21.0, 22.6, 26.1, 29.3-29.6, 30.1, 31.8, 60.3, 62.4, 65.7, 65.9, 66.0, 68.4, 68.8, 69.1, 69.2, 69.4, 70.4, 70.8, 71.7, 77.5, 80.4, 84.7, 85.0, 97.3, 99.5, 99.8, 100.5, 105.6, 165.5–171.1; ES-MS Calcd. for $C_{70}H_{118}O_{26}Na$: 1397.7809 [M + Na]; found: 1397.7802.

To a solution of protected derivative of **1** (0.05 g, 0.037 mmol) in THF–MeOH (1 : 1) (5 mL), NaOMe in MeOH (0.010 mL) was added at room temperature and stirred for 2 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated to afford **1**, as a foamy solid. Yield: 0.035 g (94%); ^{1}H NMR (400 MHz, CD_3OD) δ 0.90 (t, J = 6.4 Hz, 6 H), 1.29 (br s, 52 H), 1.55–1.58 (m, 4 H), 3.47–3.87 (m, 24 H), 4.05–4.28 (m, 2 H), 4.94–5.01 (m, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 14.4, 23.7, 27.3, 30.4-31.0, 33.1, 62.8, 63.2, 64.9, 68.5, 71.5, 72.1, 72.3, 72.5, 72.6, 75.3, 79.1, 80.7, 82.0, 86.0, 101.9, 102.1, 103.8. ES-MS Calcd. for $C_{52}H_{100}O_{17}Na$ 1019.6858 [M + Na]; found: 1019.6881.

Benzyl-[2,3,4,6-tetra-*O*-acetyl-α-D-(1→5)-mannopyranosyl]-[2,3,5-tri-*O*-benzoyl-α-D-(1→2)-arabinofuranosyl]-[2,3,5-tri-*O*-benzoyl-α-D-(1→3)-arabinofuranosyl]-α-D-arabinofuranoside (10). A solution of **9**[16] (0.45 g, 0.40 mmol), **5** (0.23 g, 0.48 mmol) and MS 4 Å (0.5 g) in CH_2Cl_2 (30 mL) was stirred for 15 min., $BF_3{\cdot}OEt_2$ (0.07 mL, 0.476 mmol) was added, under N_2 atmosphere. The reaction mixture was stirred at room temperature for 30 min, filtered and filtrate washed with the saturated aq. $NaHCO_3$ solution and with water. The organic phase was separated, dried (Na_2SO_4), filtered and the filtrate concentrated and purified (pet. ether–EtOAc 3 : 2), to afford **10**, as a foamy solid. Yield: 0.45 g (79%); R_f (pet. ether–EtOAc 3 : 2) 0.42; $[\alpha]_D$ +25.7 (*c* 1, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 1.86–2.11 (m, 12 H), 3.82 (d, J = 10.4 Hz, 1 H), 3.94 (dd, J = 4.8 Hz, J = 10.4 Hz, 1 H), 4.09–4.16 (m, 3 H), 4.28 (dd, J = 4.8 Hz, J = 7.6 Hz, 1 H), 4.35 (s, 2 H), 4.48–4.77 (m, 9 H), 4.90 (app s, 1 H), 5.19 (br s, 1 H), 5.28 (t, J = 10 Hz, 1 H), 5.35 (s, 1 H), 5.38 (dd, J = 6.4 Hz, J = 10 Hz, 1 H), 5.51–5.64 (m, 5 H), 7.18–7.37 (m, 14 H), 7.4–7.6 (m, 10 H), 7.88 (dd, J = 7.2 Hz, J = 4 Hz, 3 H), 7.95–8.06 (m, 8 H); ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.4, 20.6, 20.7, 62.4, 63.5, 63.7, 66.0, 68.5, 69.0, 69.2, 69.3, 77.2, 77.4, 80.1, 81.0, 81.4, 82.3, 82.7, 86.5, 97.7, 105.5, 125.2-129.8, 132.9-133.5, 137.3, 165.3-169.7, 170.5, 170.6; ES-MS Calcd. for $C_{78}H_{74}O_{28}Na$: 1481.4264 [M + Na]; found: 1481.4232.

2,3,4,6-Tetra-*O*-acetyl-α-D-(1→5)-mannopyranosyl-[2,3,5-tri-*O*-benzoyl-α-D-(1→2)-arabinofuranosyl]-[2,3,5-tri-*O*-benzoyl-α-D-(1→3)-arabinofuranosyl]-D-arabinofuranose (11). A solution of **10** (0.32 g, 0.217 mmol) in EtOAc–MeOH (1 : 1) (20 mL) was treated with Pd–C (10%) (0.1 g) and stirred under H_2 (1 bar) for 18 h. The reaction mixture was filtered, solvent removed *in vacuo*, the crude reaction mixture purified (pet. ether–EtOAc 1 : 1), to afford **11**, as a foamy solid. Yield: 0.28 g (90%); R_f (pet. ether–EtOAc 1 : 1) 0.45; ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.90–2.11 (m, 12 H), 3.75–3.94 (m, 2 H), 4.03–4.18 (m, 4 H), 4.23–4.44 (m, 2 H), 4.51–4.98 (m, 8 H), 5.27–5.38 (m, 2 H), 5.39 (dd, J = 3.6 Hz, J = 9.9 Hz, 1 H), 5.46–5.68 (m, 7 H), 7.24–734 (m, 7 H), 7.42–7.61 (m, 12 H), 7.84–7.94 (m, 3 H), 8.0–8.09 (m, 8 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 20.4, 20.6, 20.8, 21.0, 60.4, 62.8, 63.6, 66.0, 66.6, 67.8, 68.1, 68.8, 69.1, 69.7, 77.6, 79.1, 81.2, 81.4, 82.4, 82.7, 96.2, 97.7, 101.6, 105.2, 105.6, 128.2-129.9, 133.0, 133.4, 133.5, 133.6, 165.3–166.1, 169.6–171.1; ES-MS Calcd. for $C_{71}H_{68}O_{28}Na$: 1391.3795 [M + Na]; found: 1391.3731.

2-*O*-Hexadecyl-4-oxa-1,2-eicosanedioyl-[2,3,4,6-tetra-*O*-acetyl-α-D-(1→5)-mannopyranosyl]-[2,3,5-tri-*O*-benzoyl-α-D-(1→2)-arabinofuranosyl]-[2,3,5-tri-*O*-benzoyl-α-D-(1→3)-arabinofuranosyl]-D-arabinofuranoside (2). To a stirred solution of tetrasaccharide **11** (0.06 g, 0.043 mmol) and CCl_3CN (0. 043 mL, 0.43 mmol) in CH_2Cl_2 (8 mL), DBU (7 μL, 0.043 mmol) was added at 0 °C and stirred for 20 min. The crude reaction mixture was purified using pet. ether–EtOAc (1 : 1), to afford the trichloroacetimidate **12**, as a foamy solid. Yield (0.06, 93%). A solution of **12** (0.057 g, 0.038 mmol), **3** (0.02 g, 0.04 mmol) and MS 4 Å (0.1 g) in CH_2Cl_2 (10 mL) was stirred for 15 min. TMSOTf (6.7 μL, 0.004 mmol) was added at −10 °C, 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 1 : 1), to afford the protected derivative of **2** (α : β ≈ 9 : 1); Yield: 0.057 g (87%); R_f (pet. ether–EtOAc 6.5 : 3.5) 0.57; ^{1}H NMR (400 MHz, $CDCl_3$) 0.87 (t, J = 6.8 Hz, 6 H), 1.25

(br s, 52 H), 1.4–1.53 (m, 4 H), 1.89–2.12 (m, 12 H), 3.22–3.57 (m, 9 H), 3.74–3.86 (m, 2 H), 3.96 (dd, $J = 11.2$ Hz, $J = 4.8$ Hz, 1 H) 4.04–4.14 (m, 3 H), 4.29 (dt, $J = 12$ Hz, $J = 4.4$ Hz, 1 H), 4.39–4.40 (m, 1 H), 4.48 (s, 1 H), 4.52–4.87 (m, 5 H), 4.91 (s, 1 H), 5.15 (s, 1 H), 5.22–5.37 (m, 3 H), 5.44–5.72 (m, 6 H), 7.25–7.56 (m, 18 H), 7.89–8.07 (m, 12 H). ^{13}C NMR (100 MHz, $CDCl_3$) δ 14.1, 20.4, 20.5, 20.6, 20.8, 21.0, 22.6, 26.1, 29.3-29.6, 30.1, 31.9, 50.8, 60.3, 62.2, 63.6, 66.1, 66.9, 68.6, 69.1, 69.4, 69.8, 70.1, 70.6, 70.9, 71.7, 71.8, 77.8, 80.1, 80.4, 81.2, 82.4, 82.9, 86.1, 97.9, 102.4, 105.3, 105.5, 106.7, 128.3–130.0, 133.3, 133.7, 165.3–171.1; ES-MS Calcd. for $C_{106}H_{138}O_{30}Na$: 1913.9171 [M + Na]; found 1913.9277.

To a solution of protected derivative of **2** (0.04 g, 0.02 mmol) in THF–MeOH (1 : 1) (5 mL), NaOMe in MeOH (0.010 mL) was added at room temperature and stirred for 4 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated to afford **2**, as a foamy solid. Yield: 0.017 g (95%); ^{1}H NMR (400 MHz, CD_3OD) δ 0.89 (t, $J = 6.8$ Hz, 6 H), 1.28 (br s, 52 H), 1.53–1.60 (m, 4 H), 3.43–3.50 (m, 4 H), 3.52–3.65 (m, 8 H), 3.67–3.79 (m, 7 H), 3.81–3.86 (m, 4 H), 3.90–3.95 (m, 2 H), 3.97–4.04 (m, 3 H), 4.09–4.22 (m, 2 H), 4.80–4.83 (m, 1 H), 4.96–5.12 (m, 3 H); ^{13}C NMR (100 MHz, CD_3OD) δ 14.5, 23.7, 27.3, 30.1-31.2, 33.1, 62.9, 67.2, 68.6, 71.6, 72.0, 72.5, 72.6, 74.7, 78.7, 79.0, 82.4, 82.6, 82.9, 83.8, 85.6, 101.8, 108.4, 108.8, 109.7, 110.5; ES-MS Calcd. for $C_{56}H_{106}O_{20}Na$: 1121.7175 [M + Na]; found: 1121.7164.

6-Aminohexyl-[α-D-(1→2)-mannopyranosyl]-[α-D-(1→2)-mannopyranosyl]-D-arabinofuranoside (14). A solution of **8** (0.2 g, 0.2 mmol), 6-azido-1-hexanol (0.028 g, 0.2 mmol) and MS 4 Å (0.1 g) in CH_2Cl_2 (20 mL) was stirred for 15 min, $BF_3{\cdot}OEt_2$ (26 μL, 0.2 mmol) was added dropwise at −10 °C, 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 2 : 3), to afford the protected derivative of **13**. To a solution of the crude protected derivative of **13** in THF–MeOH (1 : 1) (5 mL), NaOMe in MeOH (0.010 mL) was added at room temperature and stirred for 2 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated to afford **13**, as a foamy solid. Yield: 0.12 g (76%). A solution of **13** in EtOAc–MeOH (1 : 1) (20 mL) was treated with Pd–C (10%) (0.1 g) and stirred under H_2 (1 bar) for 6 h. The reaction mixture was filtered, solvent removed *in vacuo*, to afford **14** (α : β ≈ 1 : 1), as a foamy solid. Yield (0.080 g, 90%); ^{1}H NMR (D_2O, 400 MHz) δ 1.24 (br s, 4 H), 1.45–1.51 (m, 4 H), 2.83 (t, $J = 7.2$ Hz, 2 H), 3.33–4.17 (m, 19 H), 4.71–5.1 (m, 3 H); ^{13}C NMR (D_2O, 100 MHz) δ 24.7, 24.9, 25.2, 26.5, 28.3, 39.3, 60.7, 61.3, 63.6, 66.5, 67.4, 69.9, 70.1, 70.3, 72.6, 73.4, 76.3, 79.4, 79.6, 79.9, 83.2, 98.8, 99.0, 100.2, 101.8, 105.9; ES-MS Calcd. for $C_{23}H_{43}NO_{15}Na$ [M + Na]: 596.3, found 596.2.

6-Aminohexyl-[α-D-(1→5)-mannopyranosyl]-[α-D-(1→2)-arabinofuranosyl]-[α-D-(1→3)-arabinofuranosyl]-D-arabinofuranoside (16). A solution of **12** (0.24 g, 0.15 mmol), 6-azido-1-hexanol (0.02 g, 0.15 mmol) and MS 4 Å (0.1 g) in CH_2Cl_2 (20 mL) was stirred for 15 min, $BF_3{\cdot}OEt_2$ (20 μL, 0.15 mmol) was added dropwise at −10 °C, 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 : 2), to afford the protected derivative of **15**. To a solution of crude protected derivative of **15** in THF–MeOH (1 : 1) (5 mL), NaOMe in MeOH (0.010 mL) was added at room temperature and stirred for 4 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated to afford **15**, as a foamy solid. Yield: 0.08 g (80%). A solution of **15** in EtOAc–MeOH (1 : 1) (20 mL) was treated with Pd–C (10%) (0.1 g) and stirred under H_2 (1 bar) for 6 h. The reaction mixture was filtered, solvent removed *in vacuo*, to afford **16** (α : β ≈ 1 : 1), as a foamy solid. Yield: 0.066 g (85%); ^{1}H NMR (D_2O, 300 MHz) δ 1.25 (br s, 4 H), 1.49 (br s, 4 H), 2.82 (t, $J = 7.8$ Hz, 2 H), 3.32–4.06 (m, 23 H), 4.76 (s, 1 H), 4.9–5.08 (m, 3 H); ^{13}C NMR (D_2O, 75 MHz) δ 25.7, 26.4, 26.7, 29.2, 29.4, 62.0, 67.4, 69.2, 70.8, 71.3, 73.6, 77.4, 78.9, 82.3, 82.9, 84.9, 85.0, 100.7, 101.3, 107.8, 108.5, 109.3. ES-MS Calcd. for $C_{27}H_{50}NO_{18}$ [M + H]: 676.3, found 676.3.

6-Aminohexyl-[α-D-(1→2)-arabinofuranosyl]-[α-D-(1→3)-arabinofuranosyl]-D-arabinofuranoside (19). A solution of **17**[16] (0.133 g, 0.12 mmol), 6-azido1-hexanol (0.017 g, 0.12 mmol) and MS 4 Å (0.1 g) in CH_2Cl_2 (20 mL) was stirred for 15 min, $BF_3{\cdot}OEt_2$ (15 μL, 0.12 mmol) was added dropwise at −10 °C, 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 1 : 1), to afford the protected derivative of **18**. To a solution of protected derivative of **18** in THF–MeOH (1 : 1) (5 mL), NaOMe in MeOH (0.010 mL) was added at room temperature and stirred for 4 h, neutralized with Amberlite ion exchange (H^+) resin, filtered and filtrate concentrated to afford **18** (α : β ≈ 1 : 1), as a foamy solid. Yield: 0.045 g (74%). A solution of the in EtOAc–MeOH (1 : 1) (20 mL) was treated with Pd–C (10%) (0.1 g) and stirred under H_2 (1 bar) for 6 h. The reaction mixture was filtered, solvent removed *in vacuo*, to afford **19**, as a foamy solid. Yield: 0.04 g (87%); ^{1}H NMR (D_2O, 400 MHz) δ 1.21 (br s, 4 H), 1.41–1.48 (m, 4 H), 2.81 (t, $J = 7.6$ Hz, 2 H), 3.32–4.06 (m, 17 H), 4.8–5.08 (m, 3 H); ^{13}C NMR (D_2O, 100 MHz) δ 24.6, 24.8, 25.2, 26.5, 28.4, 39.4, 60.9, 61.1, 63.4, 68.0, 76.5, 79.6, 80.2, 81.1, 81.3, 82.4, 83.8, 84.0, 84.3, 85.3, 100.7, 106.1, 106.9, 107.6, 108.5; ES-MS Calcd. for $C_{21}H_{40}NO_{13}$ [M + H]: 514.2499, found 514.2492.

Bacterial strains, media, and growth conditions. Wild type *M. smegmatis* mc^{2}155 and mcdrz (*rpoZ* mutant) strains were grown in Middlebrook 7H9 broth (MB7H9, Difco) supplemented with 2% glucose, 0.05% Tween 80 and MB7H9 agar medium supplemented with 2% glucose at 37 °C with agitation as and when required. Kanamycin (25μg ml^{-1}) was used as and when required. The cells of *M. smegmatis* were grown in a primary culture and secondary inoculation was carried out from a well grown stock of primary culture. The growth profile of *M. smegmatis* was followed at different concentrations of **1** and **2** and the growth rate in triplicate was followed by measuring the optical density at 600 nm, at different durations. Growth of the bacteria without the glycolipids or in MeOH was used as the control. The inhibitors were dissolved in MeOH before addition to the *M. smegmatis* culture.

Biofilm formation assay and quantitation. Inoculum of *Mycobacterium smegmatis* mc^{2}155 was added into primary cultures containing 2% Glycerol and 0.05% Tween-80 in Sauton's medium.[24,29] The well-grown culture was washed with Sauton's medium before secondary inoculation. For the development of

biofilm, Sauton's medium (5 mL) was added to the 6-well cell culture plate (Laxbro) and inoculated with 1 : 100 dilutions from a well grown primary culture and inhibitors were added in different concentrations. Petri-plates were incubated in a humidified incubator at 37 °C and monitored at different points of time.

The quantitation of biofilm formation was done as described earlier.[24,30] The well-grown culture was washed with Sauton's medium and diluted up to a final optical density (O.D.) of 0.0025 at 600 nm in Sauton's medium. The inhibitors were added in different concentration at zero time point and distributed into 96-well polystyrene plates. Eight wells were assayed for each inhibitor at each time point and experiment was repeated thrice. Inoculum (250 μL) was added in each well and biofilm formation was monitored up to 240 h. The samples were emptied from the wells, and the wells were washed twice thoroughly with water and stained by the addition of 1% crystal violet solution (300 μL) and incubated for 20 min. The wells were again washed thoroughly with water and allowed to dry. The dye was quantitated after incubating each well with aq. ethanol (80%) (300 μL) for an hour and subsequently measuring the absorption at 590 nm using a microplate reader (Spectra Max 340PC^{384}, Molecular devices).

Sliding motility tests. Sliding motility tests were carried out to check the spreading ability of mycobacteria on the surface by using the method described elsewhere.[31] Briefly, MB7H9 base medium (Difco) was solidified with 0.3% high grade agarose (Sigma) supplemented with 2% glucose. Plates were inoculated into their centre with the cultures (10 μL) after adjusting the OD_{600} to 0.5. Spreading was evaluated after incubation for 5–7 days at 37 °C in a humidified incubator.

SPR studies. These studies were conducted with a Biacore 3000 SPR instrument. A continuous flow of HEPES buffer was maintained over the sensor surface at a flow rate of 10 μl min^{-1}. The carboxyl groups on four flow channels were activated with an injection of a solution containing *N*-ethyl-*N*′-(3-diethylamino-propyl)carbodiimide (EDC) (0.2 M) (70 μL) and *N*-hydroxysuccinimide (NHS) (0.05 M). Arabinomannan compounds in sodium acetate buffer were injected over three different flow channels at a flow rate of 10 μL min^{-1}. The immobilization procedure was completed by an injection of ethanolamine hydrochloride (1 M) (70 μL), followed by a flow of the buffer (100 μL min^{-1}.) to eliminate the physically adsorbed compounds. Cell pellet from of each culture (5 mL) was resuspended in lysis buffer (0.5 mL) containing NaCl (150 mM), 6% Triton X-100, 5% β-mercaptoethanol and Roche Protease Inhibitor Cocktail (1mg mL^{-1}). Lysis was carried out by 5 rounds of sonication for 2 min, each with 2 s pulse at 4 °C. Centrifugation was carried out at 15 000 rpm for 20 min at 4 °C and the lysate was carefully decanted. The bacterial cell lysate containing all soluble proteins (10 mg mL^{-1}) was diluted in the running HEPES buffer and passed over flow cells and binding study was performed. Cell lysates were dissolved in buffer, passed for 60 s for the association phase and buffer alone for 60 s for the dissociation phase. The sensor chip was regenerated between each cycle using a 0.05% SDS solution for 60 s.

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