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Novel synthetic (1 → 6)-α-D-mannodisaccharide substrates support processive mannosylation catalysed by the mycobacterial cell envelope enzyme fraction†

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Three new (1 → 6)-α-D-mannodisaccharides with cyclohexylalkyl or octylsulfonyl function like aglycone were synthesized and screened in the mycobacterial mannosyltransferase assay. 2-Cyclohexylethyl (1 → 6)-α-D-Man2 acted as the best acceptor substrate, whereas the sulfonyl group significantly reduced the ability of the mannodisaccharide to serve as the acceptor. Despite these differences, mass spectrometric analysis confirmed the capability of all synthetic mannodisacharides to accept up to ten additional mannose units, *i.e.* the transfer was not affected by the type of aglycone. The results reported here suggest that the enzyme responsible for the consecutive mannose attachment is the processive α-mannopyranosyltransferase present in the cell-free system of the mycobacterial cell envelope.

Introduction

Mycobacteria represent a large group of microorganisms, with more than 150 species including important human pathogens such as *M. tuberculosis* and *M. leprae* (http://www.bacterio.cict.fr/m/mycobacterium.html). The cell envelopes of these bacteria contain significant amounts of unique lipids and heteropolysaccharides that play critical roles in modulating the host response during infection.[1]

Among them, lipoglycans termed lipoarabinomannan (LAM) and lipomannan (LM) display several immunomodulatory properties through interactions with different receptors of the immune system.[2] Mannan polymers of these molecules are composed of linear chains of α-(1 → 6)-linked mannose units, frequently branched with α-(1 → 2)-mannoses. These are attached to a phosphatidyl-*myo*-inositol anchor through the 6-position of inositol carrying an additional mannose at the 2-position. Moreover, a LAM molecule contains between 50–80 arabinose units organized in distinct structural motifs.[3] A report describing the activity of a cell-free mannosyltransferase (ManT) that could participate in biosynthesis of mycobacterial mannan polymers was published more than two decades ago.[4] However, the first enzyme involved in biosynthesis of LAM/LM mannan chains, mannopyranosyltransferase Rv2181 responsible for the addition of α-(1 → 2) branches, was identified only in 2006.[5] This discovery was soon followed by recognition of the first α-(1 → 6)-mannopyranosyltransferase from *M. tuberculosis*, Rv2174 (MptA), involved in biosynthesis of the distal part of LAM/LM.[6,7] Another α-(1 → 6)-mannopyranosyltransferase, MptB from *Corynebacterium glutamicum*, along with its homolog in *M. tuberculosis* Rv1459c, was proposed to extend phosphatidylinositol dimannoside and thus synthesize the proximal part of LAM/LM.[8] Surprisingly, the *M. smegmatis* strain with a disrupted copy of the Rv1459c homolog did not show any defect in LAM/LM synthesis, so it is likely that the gene is redundant in mycobacteria.[8]

A neoglycolipid acceptor α-D-Man*p*-(1 → 6)-α-D-Man*p*-*O*-C8 (octyl (1 → 6)-α-D-Man2) was used in the cell free assays to confirm the activities of MptA and MptB,[7,8] and exemplified the value of synthetic acceptors for investigation of glycosyltransferases involved in biosynthesis of bacterial cell wall-associated polymers.

The pilot study demonstrating the efficacy of synthetic mannosides as acceptor substrates for mycobacterial ManTs was published in 2001.[9] In the assay with GDP-[^{14}C]Man, synthetic (1 → 6)-α-D-mannodisaccharides with variable aglycone structures (octyl, dec-9-enyl, thiooctyl) and dec-9-enyl (1 → 6)-α-D-Man3 acted as acceptor substrates for ManT. Thorough analysis of the reaction products revealed that each of the active compounds accepted one or two mannose residues attached through α-(1 → 6)-glycosidic linkage. The transfer was sensitive to the presence of amphomycin, which confirmed that the direct mannose donors for these ManTs

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† Electronic supplementary information (ESI) available: Experimental. ^{1}H and ^{13}C NMR spectra of the targeted disaccharides **27–30**. Supplementary Fig. S1–S7. See DOI: 10.1039/c3ra43575j

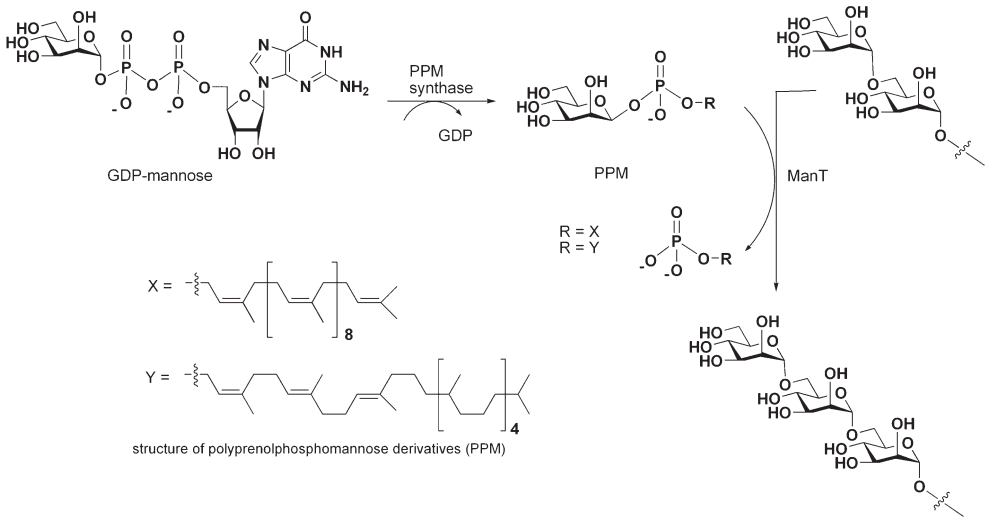


Fig. 1 Schematic illustration of the reaction catalyzed by the polyprenolphosphomannose-dependent α-(1 → 6)-mannosyltransferase (ManT). Polyprenolmonophospomannose synthase (PPM synthase) produces polyprenolphosphomannoses, direct donors for ManT from GDP-Man and polyprenolphosphates.

were polyprenylphosphoryl mannoses formed *in situ* from GDP-[^{14}C]Man (Fig. 1).

The cell-free ManT reaction was further explored with mycobacterial membranes as a source of the enzymatic activities and compounds derived from octyl (1 → 6)-α-D-Man2 by modification at the saccharide unit by deoxygenation, etherification or epimerization.[10–14] In our recent study, we reported a modification achieved by replacement of glycosidic linkage with a triazole linker.[15] Detailed investigation of the reaction products in cell-free ManT assay showed that conjugates consisting of two or three mannose units and octyl aglycone were tolerated by the enzyme; however the effectiveness was reduced in comparison to that observed for the acceptor analogues containing a glycosidic linkage.

We have also previously described the synthesis of mannose glycosides with a variable aglycone moiety: linear C6, C8, C12, C16 and C20, cyclohexylalkyl (alkyl = methyl or ethyl), linear SC6, SC8 and their oxidized forms as SO_2C6, SO_2C8.[16] Examination of these monosaccharide compounds in the mycobacterial ManT assay indicated that glycosides with cyclohexylalkyl aglycone acted as substrates in the ManT reaction with an efficacy similar to that observed for octyl α-D-mannopyranoside. On the other hand, oxidized thiooctyl glycosides, *i.e.* sulfone derivatives exhibited significantly decreased efficiency in the transfer reaction. This study allowed us to identify new aglycone structures since so far only two models having structures other than octyl aglycone have been evaluated.[9]

In the present work we further investigated the effect of the aglycone structure on the acceptor ability of the newly synthesized disaccharide homologs **28–30** since the optimal glycone size for the transferase is disaccharide. The efficiency of compounds **28–30** to act as ManT acceptors was compared with that of octyl (1 → 6)-α-D-Man2 **27**, which can be considered a reference substrate (Fig. 2).

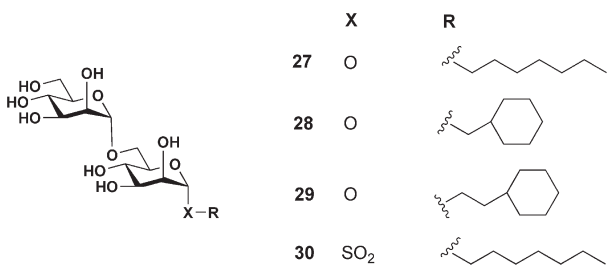


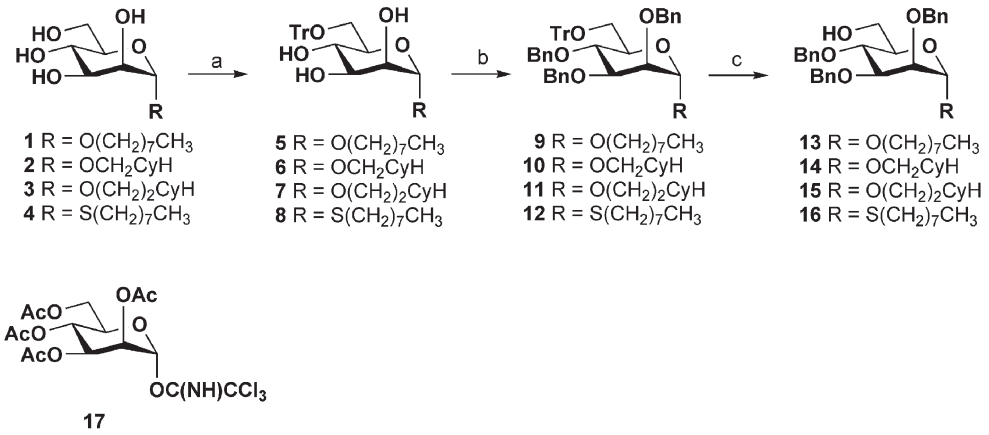
Fig. 2 Structures of the synthesized disaccharide models **27–30**.

Results and discussion

Synthesis

Synthesis of glycosyl acceptors **13–16** was commenced from the corresponding mannose glycosides **1–4**[16] over three steps (Scheme 1). Selective protection of the primary hydroxyl group at C-6 of the saccharide unit by reaction with TrCl was followed by benzylation of the secondary OH groups. Then, removal of the acid labile bulky trityl function by treatment with *p*-TsOH in a solvent mixture of CH_2Cl_2 : MeOH gave the target acceptors. Imidate **17**, chosen as a donor, was synthesized according to a procedure reported recently[17] (Scheme 1).

In the next step, a $BF_3{\cdot}OEt_2$-promoted glycosylation of the prepared acceptors **13–16** with mannopyranosyl trichloroacetimidate **17** provided the required protected disaccharides **18–21** with full α-selectivity (Scheme 2). Disaccharide **21** with a thiooctyl function as aglycone was next transformed into the corresponding sulfone **22** by oxidation with *m*CPBA in 49% yield (over two steps, coupling and oxidation). Subsequent removal of the acetyl followed by benzyl protective groups



Scheme 1 Reagents and conditions: a) TrCl, py, 24 h, 55 °C; b) BnBr, NaH, 16 h, 0 °C to rt; c) *p*-TsOH, CH_2Cl_2 : MeOH, 35 min, rt.

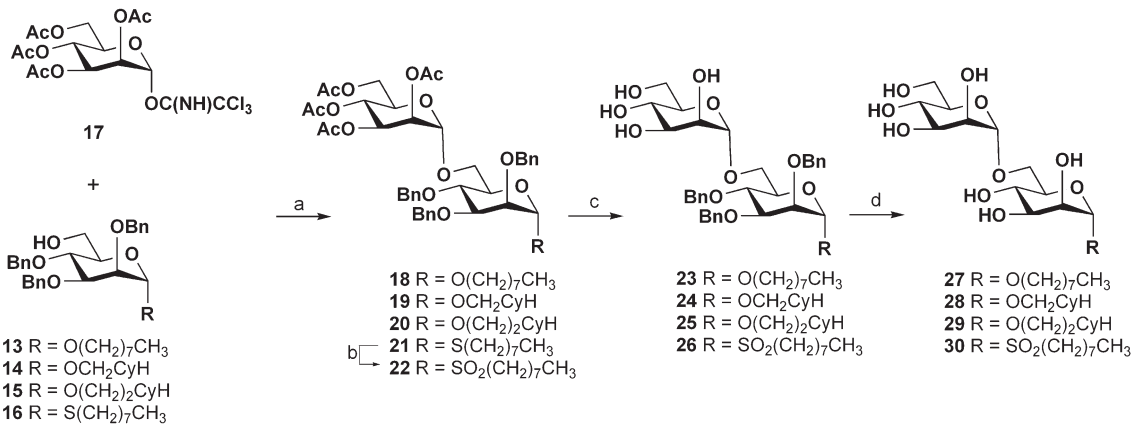
under deprotection conditions (saponification and hydrogenation) provided the desired disaccharides **27–30** in high yields. Evidence for α-stereochemistry of the glycosidic linkages in the target disaccharides **27–30** was confirmed by the $^1J_{C\text{-}1,H\text{-}1}$ heteronucelar coupling constant for the anomeric carbon atoms[18] with values between 168.9 Hz and 170.1 Hz.

Biological evaluation

Once the disaccharides **27–30** were prepared, they were screened for their ability to act as acceptor substrates for the mannosyltransferases present in cell envelope fractions of *Mycobacterium smegmatis* (mc^2155). Each of the synthetic disaccharides was incubated with this enzymatic fraction in the presence of radioactive GDP-[^{14}C]Man which served as an indirect mannose nucleotide donor. TLC analysis of the reaction products followed by autoradiography showed the incorporation of the radioactive label into compounds **27–30**. Comparison of the spot intensity corresponding to the monomannosylated adducts showed that octyl (1 → 6)-α-D-Man2 **27** and 2-cyclohexylethyl (1 → 6)-α-D-Man2 **29** acted as the best acceptor substrates with similar activities; cyclohexylmethyl (1 → 6)-α-D-Man2 **28** was about 20% less active and disaccharide **30** with alkylsulfonyl aglycone showed about 60% decrease of incorporation of the radioactive [^{14}C]Man when compared to the reference disaccharide **27** (Fig. 3).

Interestingly, prolonged exposure of the TLC plate to the autoradiography film revealed several additional products of higher polarity, suggesting further step-wise additions of [^{14}C]Man to the acceptor substrates (data not shown).

To establish the identity of the products, large scale enzymatic reactions with synthetic disaccharides **27–30** and unlabeled GDP-Man were carried out. The reactions were stopped by addition of ethanol, followed by centrifugation and re-extraction of the resulting pellet with the same solvent. The combined dried ethanol extracts were then analyzed by MALDI-MS and MS/MS or with an ESI mass spectrometer coupled to HPLC. These experiments enabled us to validate the presence of oligosaccharide products formed in the enzymatic reactions; products detected clearly confirmed addition of one to ten mannose units to each disaccharide acceptor substrate. A representative example, obtained for the synthetic acceptor **29**, is shown in Fig. 4. The uppermost panel represents the HPLC chromatogram (Fig. 4a) and mass spectra recorded from the peaks are shown in this figure as panels b1–



Scheme 2 Reagents and conditions: a) $BF_3{\cdot}OEt_2$, CH_2Cl_2, 30 min, 0 °C; b) *m*CPBA, CH_2Cl_2, 2 h, rt; c) MeONa, CH_2Cl_2 : MeOH, 16 h, rt; d) 10%Pd/C, H_2, MeOH, 4 h, rt.

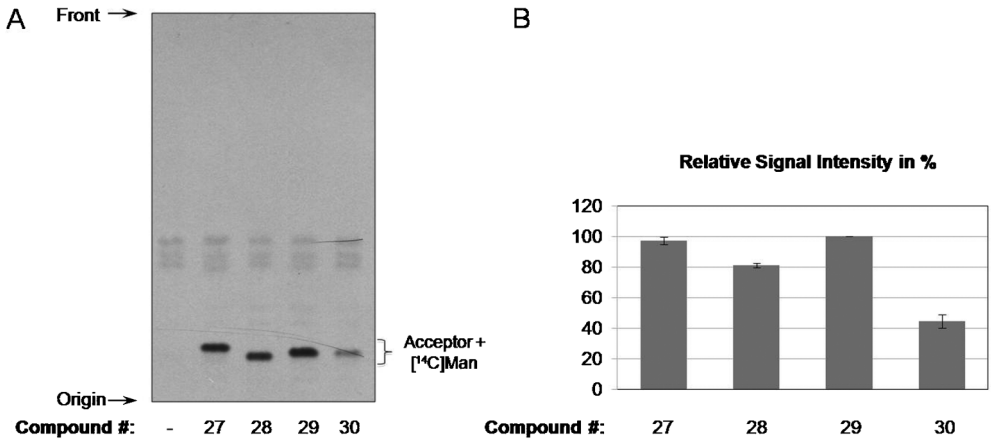


Fig. 3 Analysis of the products of the *in vitro* mannosyl transferase reaction with GDP [^{14}C]Man and synthetic acceptors. Panel A, TLC analysis of the reaction products. The bands were visualized by autoradiography. Panel B, quantification of the reaction products derived from synthetic acceptors corresponding to the addition of the first [^{14}C]Man using IMAGEJ (NIH) software. The values in the graph represent the means of duplicate reactions.

5. The largest chromatographic peak (elution time 39 min) under ESI-MS conditions produced $[M + Na]^+$ ions at *m*/*z* 475 and corresponded to the synthesized disaccharide **29** (Fig. 4, b1). The next chromatographic peak with elution time 38 min (peak 2, in Fig. 4a) gave molecular ions at *m*/*z* 637 and indicated addition of one mannose residue to the parent disaccharide **29** (mass increase of 162 Da; Fig. 4, b2). MS spectra shown in Fig. 4 as b3–5 were recorded from smaller chromatographic peaks 3–5 and provided evidence for additional consecutive mass increases of 162 Da indicating the presence of di-, tri- and tetramannosylated products.

An LC-MS analytical approach was also carried out for the identification of products in the reaction mixtures obtained with the acceptors **28** and **30**. Here again the same phenomenon regarding processive extension of the disaccharide chains was observed (Supplementary Fig. S1 and S2, ESI†). LC-MS analysis confirmed 4-fold procession, as shown for **29**. In the case of **28**, only 3 products of the enzymatic ManT reaction were detected – at *m*/*z* 623.4, 785.5 and 1109.5, corresponding to mono-, di- and tetramannosylated products, respectively (Supplementary data, Fig. S1, b2–4, ESI†). The lack of a trimannosylated product (expected value of *m*/*z* at 947) might be explained by its own consumption in a subsequent ManT reaction resulting in a product with higher mass. Interestingly, in the reaction mixture with **30**, despite reduced efficiency due to the presence of the octylsulfonyl aglycone, the processivity was not negatively affected. The products with increasing masses of 162 Da indicated the ability of the enzyme to extend even this poorer substrate (Supplementary data, Fig. S2, ESI†).

The presence of additional higher mannosylated adducts ($n > 4$) was detected by MALDI-TOF/TOF-MS. Similar results were obtained for the reference compound **27** (Supplementary data, Fig. S3, ESI†) and as well as for new acceptors. The peaks corresponding to these multiple mannosylated products were relatively small and some of them were accompanied with peaks originating from the mycobacterial membrane fraction. Therefore an additional SPE purification was carried out to eliminate unwanted compounds and to obtain a fraction enriched with mannosylated adducts (*e.g.* Supplementary data, Fig. S4, ESI†). Although the products with more than seven additional mannose units ($n = 8–10$) were generally detected as minor peaks, still the ensuing increase in mass of 162 Da provided good evidence for their presence. In the case of a reaction mixture of **30**, it was possible to detect products indicating even 11 additional mannose residues (Fig. S5, ESI†).

For investigation of the glycosidic linkages in products formed by ManT in the enzymatic reactions with non-radioactive GDP-Man, each mannosylated sample was digested enzymatically with jack bean α-mannosidase and then analyzed by MALDI-MS, as described in the Experimental section. Fig. 5 represents MS spectra obtained from the reaction mixture with **28** before and after α-mannosidase treatment. After 1 h of incubation with the enzyme, only peaks corresponding to di- and mono-mannosylated adducts were detected (Fig. 5b). When the reaction mixture was analyzed after 3 h, only the monosaccharide, cyclohexylmethyl α-D-mannopyranoside **2** was detected (*m*/*z* 299, Fig. 5c). Similar results were observed for the products of the enzymatic reactions with acceptors **29** and **30** (Supplementary data, Fig. S6 and S7, ESI†).

The combination of α-mannosidase treatment with MS-analysis confirmed that newly formed linkages in mannosylated products have α-configuration and therefore the synthesized disaccharides **28–30** are appropriate substrates for mycobacterial α-ManT. Several lines of evidence, as reported in our and other studies, demonstrate that the predominant activity of the α-mannosyltransferase in extension of synthetic acceptors in the mycobacterial cell-free system is responsible for α-(1 → 6)-glycosidic bond formation.[9,12,15,16] It was shown that efficiency of ManT catalyzed mannosyl transfer is strongly affected by the length of saccharide unit and the most active acceptor substrates are octyl (1 → 6)-α-D-Man2 and octyl (1 → 6)-α-D-Man3.[12] The former has been extensively used as an acceptor in enzymology studies, *e.g.* during investigation of compartmentalization of lipid biosynthesis in mycobacteria[19]

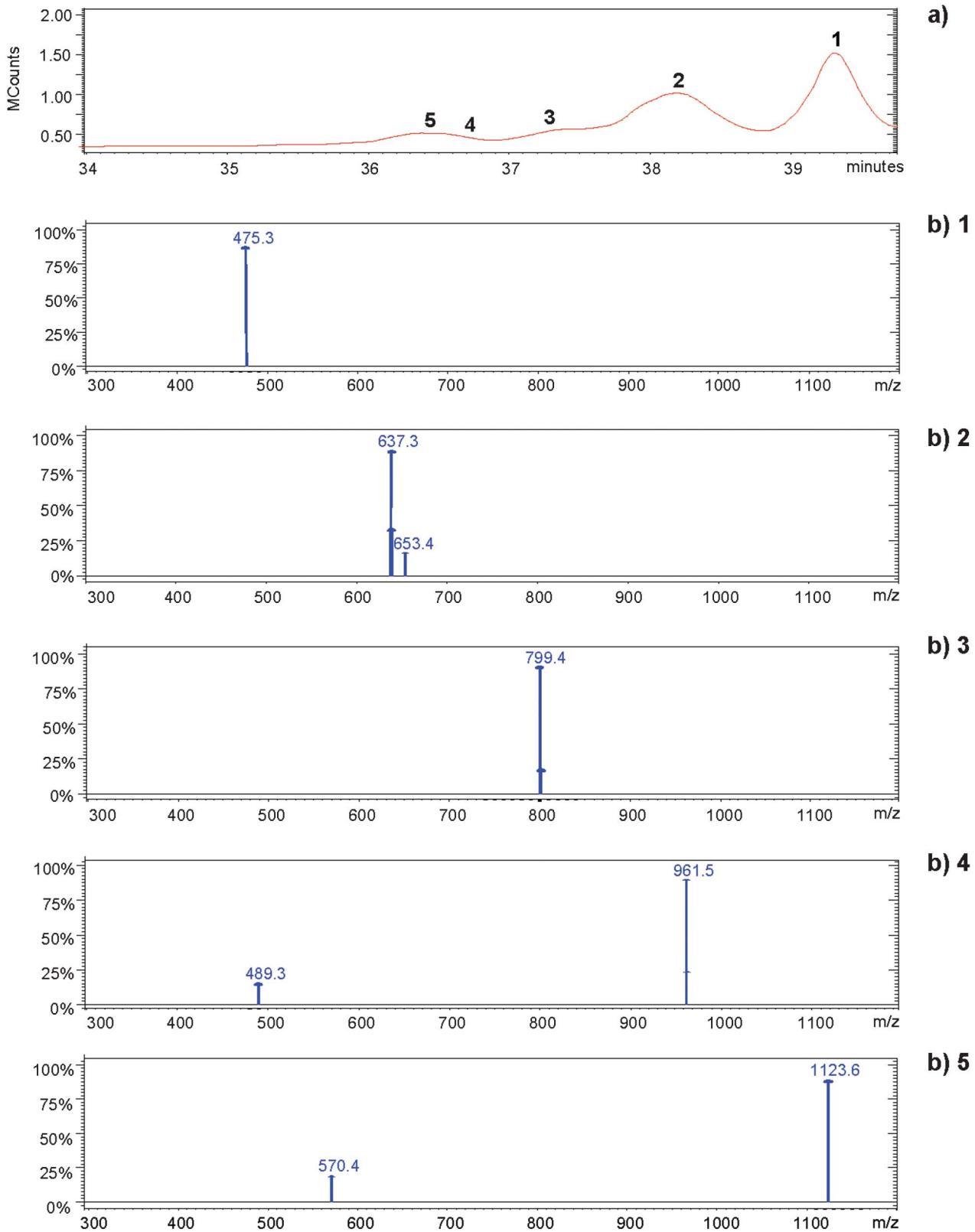


Fig. 4 LC-MS analysis of the enzymatic reaction mixture **29**. Panel (a) HPLC profile. Panel (b) ESI mass spectra obtained from peaks **1–5** in figure (a); all peaks are as $[M + Na]^+$.

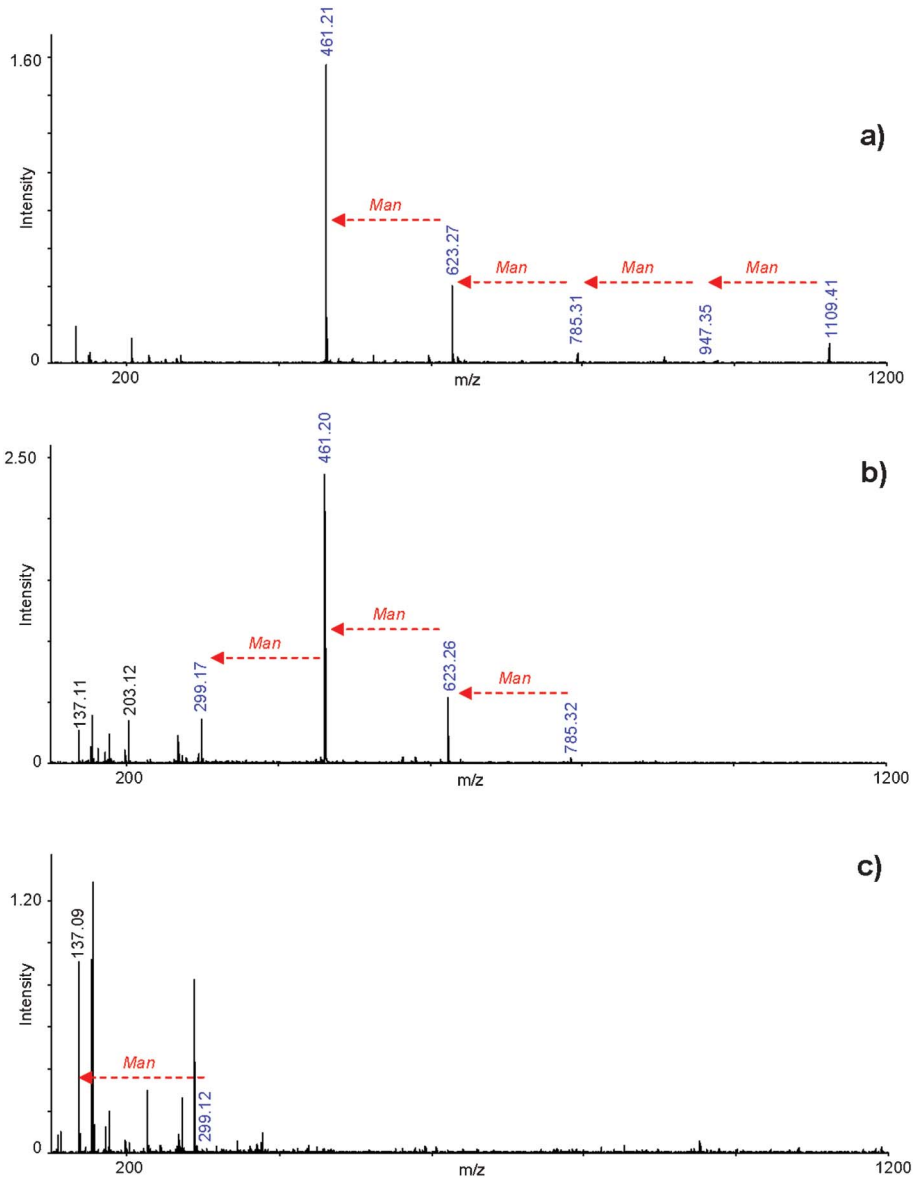


Fig. 5 MALDI-MS spectra recorded from sample **28**: a) before treatment with mannosidase (the presence of up to deca mannosylated products is shown in Fig. S4, ESI†); b) after 1 h; and c) after 3 h incubation with mannosidase. Peaks corresponding to saccharide adducts are in blue color.

or identification of the activities of the mannosyltransferases MptA and MptB.[7,8] Our new model compounds **28–30**, carrying the optimal (1 → 6)-α-D-mannodisaccharide unit, have been prepared with the aim to investigate the influence of the aglycone structure on the ability of the synthetic dimannoside analogs to serve as acceptors in the cell free ManT reaction. Our results showed that the reference compound **27**, as well as the newly synthesized disaccharides **28** and **29** with *O*-glycosidically linked aglycones of different types, but similar in size, were generally good acceptor substrates for ManT. Therefore, they both (preferably **29**) can be utilized as an efficient alternative of a so far commonly used acceptor substrate **27**. Similar data has also been previously reported for disaccharide with slightly longer *O*-linked dec-9-enyl aglycone, which acted as an acceptor substrate comparable with **27**, although in a limited concentration range.[9] Another report dealing with the synthesis and evaluation of disaccharides comprising octyl aglycone and a modified disaccharide unit did not provide better acceptor substrates than the reference compound **27**. While those being etherified or deoxygenated[11,12] were comparable or weaker than **27**, epimerization of the mannosyl unit to either *talo*- or *gluco*-configuration led to a reduction or loss of activity.[14] A substantial decrease in activity has also been observed upon a change of the atom connecting aglycone with mannose unit, *i.e.* upon a replacement of oxygen with sulfur. Octyl thiomannodisaccharide was a weaker acceptor substrate than reference **27** in cell free ManT assay.[9] Oxidation of the sulfur atom resulting in disaccharide **30** carrying alkylsulfonyl aglycone neither recovered nor reduced the acceptor ability in comparison with

thiomannodisaccharide. However, it is worth noting that in contrast to our previous results with monosaccharide derivatives,[16] the presence of the sulfonyl aglycone in the disaccharide acceptor **30** did not completely abolish the reaction, probably due to a longer distance of the site of mannosylation from the aglycone attachment.

Moreover, mass spectrometric analysis of ManT reaction products of compounds **27–30** revealed a series of mannooligosaccharides corresponding to the transfer of up to ten mannosyl residues to the original synthetic substrates. This transfer was independent of the type of mannose attachment to the aglycone. These results suggest that the enzyme responsible for mannose attachment is the processive α-mannopyranosyltransferase. So far, the addition of more than two mannose units was reported only in one study, when using free mannose as the acceptor substrate in the mycobacterial cell free system indicated the formation of oligosaccharides with up to 12 mannoses.[4] However, characterization of the products larger than trisaccharides was based only on the elution profile of the radiolabeled adducts separated by gel filtration chromatography.[4] Although Berg *et al.*[20] proposed the involvement of an α-(1 → 6) processive ManT in LM/LAM biosynthesis, such an enzyme has not been identified so far in mycobacteria. Our results suggest that processive mannosylation of synthetic mannodisaccharide substrates does occur in the cell free system derived from the mycobacterial cell envelope and offers new opportunities in the search for the specific enzyme to catalyse this reaction.

Conclusions

In this work, we have described the synthesis of three new (1 → 6)-α-D-Man2 model compounds **28–30** and their evaluation in the mycobacterial ManT assay. The results showed that these disaccharide models have been recognized by α-mannosyltransferase as acceptor substrates with different efficacies. We observed that the nature of the glycosidic atom connecting the mannose unit to the aglycone had a higher impact on the acceptor substrate ability than the structure of the lipid-like aglycone. Interestingly, none of the new compounds affected the processivity of the enzyme, since mass spectrometric analysis revealed attachment of up to ten additional mannose units to each original synthetic substrate.

Experimental

General methods

All commercially available solvents were distilled, dried and then distilled again immediately before use. Reactions containing sensitive reagents were carried out under an argon atmosphere. TLC was performed on aluminium sheets precoated with a silica gel 60 F_{254} (Merck). Flash column chromatography was conducted on a silica gel 60 (0.040–0.060 mm, Merck) with distilled solvents (hexanes, ethylacetate, dichloromethane, chloroform, methanol). ^{1}H and ^{13}C NMR spectra were recorded at 25 °C on a Bruker AM300, VNMRS 400 MHz Varian or INOVA AV 600 MHz spectrometers. Chemical shifts are referenced to either TMS (δ 0.00, $CDCl_3$ for ^{1}H) or HOD (δ 4.87, CD_3OD for ^{1}H), and to internal $CDCl_3$ (δ 77.23) or CD_3OD (δ 49.15) for ^{13}C. The assignment of resonances in the ^{1}H and ^{13}C NMR spectra was based on data obtained from two-dimensional homonuclear and heteronuclear shift correlation experiments. Optical rotations were measured on a Perkin Elmer 241 polarimeter at 20 °C. The purity (⩾95%) of targeted compounds **27–30** was determined by microanalyses using a Fisons EA-1108 instrument. Unless otherwise stated, mass spectrometry-high-resolution mass spectral (HRMS) and tandem mass spectrometry (MS/MS) analyses were performed on a UltrafleXtreme™ (Bruker) operated in the positive ion mode. 2,5-dihydroxybenzoic acid (DHB, Sigma) was used as a matrix. The aqueous sample solutions (~0.7 μL) were spotted onto a matrix predeposited on the surface of a stainless steel MALDI target and were air-dried. The instrument was calibrated externally over a mass range 100–3000 Da. Individual parent ions of interest were manually selected for the structural analysis.

Chemistry

General procedure for removal of benzyl groups. Synthesis of targeted disaccharides 27–30. Compound **23**, **24**, **25** or **26** (0.12 mmol, 1 eq.) was dissolved in MeOH (5 mL) and 10% Pd/C was added. The solution was stirred under a H_2 atmosphere for 4 h, then the catalyst was filtered off and washed with MeOH (10 mL). The solvent was evaporated and purification of the crude product by column chromatography ($CHCl_3$: MeOH 10 : 1 → 3 : 1) yielded targeted disaccharides **27–30**, which were lyophilized before use in biological tests.

Octyl α-D-mannopyranosyl-(1 → 6)-α-D-mannopyranoside (27). (50.2 mg, 92%); $[\alpha]_D$ + 31 (*c* 0.8, H_2O); lit[11] $[\alpha]_D$ + 27.3 (*c* 0.7, H_2O); ^{1}H NMR (400 MHz, CD_3OD): δ 4.82 (d, 1H, $J_{1',2'}$ = 1.7 Hz, H-1′), 4.71 (d, 1H, $J_{1,2}$ = 1.6 Hz, H-1), 3.91 (ddd, 1H, *J* = 10.7 Hz, *J* = 3.1 Hz, *J* = 2.0 Hz, H-6a), 3.86–3.81 (m, 2H, H-2′, H-6′a), 3.79 (dd, 1H, $J_{2,3}$ = 2.8 Hz, H-2), 3.75–3.61 (m, 9H, H-3, H-4, H-5, H-6b, H-3′, H-4′, H-5′, H-6′b, $OCH_2C_7H_{15}$), 3.41 (dt, 1H, *J* = 6.2 Hz, *J* = 9.6 Hz, $OCH_2C_7H_{15}$), 1.63–1.54 (m, 2H, $OCH_2CH_2C_6H_{13}$), 1.39–1.28 (m, 10H, $O(CH_2)_2(CH_2)_5CH_3$), 0.90 (t, 3H, *J* = 6.8 Hz, $O(CH_2)_7CH_3$). ^{13}C NMR (100 MHz, CD_3OD): δ 101.7 (C-1, $^1J_{C,H}$ = 169.5 Hz), 101.5 (C-1′, $^1J_{C,H}$ = 170.0 Hz), 74.4, 73.2, 73.0, 72.8 (C-3, C-3′, C-5, C-5′), 72.3(2×) (C-2, C-2′), 68.8 (C-4), 68.7(2×) (C-4′, $OCH_2C_7H_{15}$), 67.5 (C-6), 63.0 (C-6′), 33.2, 30.8, 30.7, 30.6, 27.6, 23.9 ($OCH_2(CH_2)_6CH_3$), 14.6 ($O(CH_2)_7CH_3$). HRMS (MALDI): *m*/*z* 477.2336 MNa^+; calcd 477.2312 for $C_{20}H_{38}O_{11}Na$. Anal. Calcd for $C_{20}H_{38}O_{11}$: C, 52.85; H, 8.43. Found: C, 52.74; H, 8.53.

Cyclohexylmethyl α-D-mannopyranosyl-(1 → 6)-α-D-mannopyranoside (28). (50.5 mg, 96%); $[\alpha]_D$ + 37 (*c* 0.7, H_2O); ^{1}H NMR (400 MHz, CD_3OD): δ 4.82 (d, 1H, $J_{1',2'}$ = 1.6 Hz, H-1′), 4.68 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 3.90 (ddd, 1H, *J* = 10.7 Hz, *J* = 3.0 Hz, *J* = 2.1 Hz, H-6a), 3.85–3.81 (m, 2H, H-2′, H-6′a), 3.79 (dd, 1H, $J_{2,3}$ = 2.6 Hz, H-2), 3.75–3.60 (m, 8H, H-3, H-4, H-5, H-6b, H-3′, H-4′, H-5′, H-6′b), 3.51 (dd, 1H, *J* = 6.9 Hz, *J* = 9.4 Hz, $OCH_2C_6H_{11}$), 3.22 (dd, 1H, *J* = 5.9 Hz, *J* = 9.4 Hz, $OCH_2C_6H_{11}$), 1.81–0.96 (m, 11 H, $OCH_2C_6H_{11}$). ^{13}C NMR (100 MHz, CD_3OD): δ 101.8 (C-1, $^1J_{C,H}$ = 169.5 Hz), 101.5 (C-1′, $^1J_{C,H}$ = 169.9 Hz),

74.5, 73.2, 73.0, 73.3, 72.8 (C-3, C-3′, C-5, C-5′, $OCH_2C_6H_{11}$), 72.3(2×) (C-2, C-2′), 68.8(2x) (C-4, C-4′), 67.5 (C-6), 63.0 (C-6′), 39.4, 31.4, 31.2, 27.8, 27.1(2×) ($OCH_2C_6H_{11}$). HRMS (MALDI): *m*/*z* 461.1996 MNa^+; calcd 461.1999 for $C_{19}H_{34}O_{11}Na$. Anal. Calcd for $C_{19}H_{34}O_{11}$: C, 52.05; H, 7.82. Found: C, 51.82; H, 7.97.

2-Cyclohexylethyl α-D-mannopyranosyl-(1 → 6)-α-D-mannopyranoside (29). (51.0 mg, 94%); $[\alpha]_D$ + 39 (*c* 0.7, H_2O); ^1H NMR (400 MHz, CD_3OD): δ 4.82 (d, 1H, $J_{1',2'}$ = 1.6 Hz, H-1′), 4.70 (d, 1H, $J_{1,2}$ = 1.5 Hz, H-1), 3.90 (ddd, 1H, *J* = 10.8 Hz, *J* = 3.3 Hz, *J* = 2.1 Hz, H-6a), 3.86–3.81 (m, 2H, H-2′, H-6′a), 3.78 (dd, 1H, $J_{2,3}$ = 2.6 Hz, H-2), 3.77–3.63 (m, 9H, H-3, H-4, H-5, H-6b, H-3′, H-4′, H-5′, H-6′b, $OCH_2CH_2C_6H_{11}$), 3.43 (dt, 1H, *J* = 5.9 Hz, *J* = 9.8 Hz, $OCH_2C_6H_{11}$), 1.81–0.88 (m, 13H, $OCH_2CH_2C_6H_{11}$). ^{13}C NMR (100 MHz, CD_3OD): δ 101.7 (C-1, $^1J_{C,H}$ = 168.9 Hz), 101.6 (C-1′, $^1J_{C,H}$ = 169.3 Hz), 74.4, 73.3, 73.0, 72.8 (C-3, C-3′, C-5, C-5′), 72.3, 72.2 (C-2, C-2′), 68.8, 68.7 (C-4, C-4′), 67.6 (C-6), 66.5 ($OCH_2CH_2C_6H_{11}$), 63.0 (C-6′), 38.3, 36.0, 34.8, 34.4, 27.8, 27.6, 27.5 ($OCH_2CH_2C_6H_{11}$). HRMS (MALDI): *m*/*z* 475.2185 MNa^+; calcd 475.2155 for $C_{20}H_{36}O_{11}Na$. Anal. Calcd for $C_{20}H_{36}O_{11}$: C, 53.09; H, 8.02. Found: C, 53.32; H, 7.89.

Octyl α-D-mannopyranosyl-(1 → 6)-α-D-mannopyranosyl sulfone (30). (57.3 mg, 95%); $[\alpha]_D$ + 45 (*c* 0.7, H_2O); ^1H NMR (400 MHz, CD_3OD): δ 4.90 (d, 1H, $J_{1,2}$ = 1.4 Hz, H-1), 4.82 (d, 1H, $J_{1',2'}$ = 1.7 Hz, H-1′), 4.50 (dd, $J_{2,3}$ = 3.7 Hz, H-2), 4.27 (ddd, 1H, $J_{4,5}$ = 9.8 Hz, $J_{5,6a}$ = 6.2 Hz, $J_{5,6b}$ = 2.0 Hz, H-5), 3.95 (dd, 1H, $J_{3,4}$ = 9.2 Hz, H-3), 3.88 (dd, 1H, $J_{6a,6b}$ = 11.2 Hz, H-6a), 3.84–3.81 (m, 2H, H-2′, H-6′a), 3.78 (dd, 1H, H-6b), 3.73–3.61 (m, 5H, H-4, H-3′, H-4′, H-5′, H-6′b), 3.25–3.13 (m, 2H, $SO_2CH_2C_7H_{15}$), 1.86–1.78 (m, 2H, $SO_2CH_2CH_2C_6H_{13}$), 1.53–1.46 (m, $SO_2(CH_2)_2CH_2C_5H_{11}$), 1.36–1.29 (m, 8H, $SO_2(CH_2)_3(CH_2)_4CH_3$), 0.91 (t, 3H, *J* = 6.9 Hz, $SO_2(CH_2)_7CH_3$). ^{13}C NMR (100 MHz, CD_3OD): δ 101.6 (C-1′, $^1J_{C,H}$ = 169.5 Hz), 92.9 (C-1, $^1J_{C,H}$ = 170.1 Hz), 78.2 (C-5), 74.6 (C-5′), 73.0, 72.9, 72.2 (C-2′, C-3, C-3′), 68.7 (C-4′), 67.8, 67.7 (C-4, C-6), 66.9 (C-2), 63.0 (C-6′), 51.3 ($SO_2CH_2(CH_2)_6CH_3$), 33.1, 30.4, 30.2, 29.7, 23.8, 22.6 ($SO_2CH_2(CH_2)_6CH_3$), 14.6 ($SO_2CH_2(CH_2)_6CH_3$). HRMS (MALDI): *m*/*z* 525.1985 MNa^+; calcd 525.1982 for $C_{20}H_{38}O_{12}SNa$. Anal. Calcd for $C_{20}H_{38}O_{12}$: C, 47.80; H, 7.62; S, 6.38. Found: C, 47.94; H, 7.50; S, 6.22.

Biological evaluation

***In vitro* mannosyltransferase assay and preparation of the enzyme reaction products for structural characterization.** Synthetic disaccharides **27–30** were tested for their ability to serve as the acceptors for mycobacterial mannosyltransferases in the *in vitro* reaction. The enzymatically active cell envelope (membrane and cell wall) fractions of *Mycobacterium smegmatis* mc^2155, which were grown in the nutrient broth (EM Science) were prepared essentially as described previously.[15,16] The composition of the reaction mixtures was as follows: 1.5 mg membrane protein, 1 mg cell wall protein, 0.05 μCi GDP-[^{14}C]mannose (Amersham, 275 mCi/mmol), 60 μM ATP, DMSO in final concentration of 0.8% (v/v), 4 mM synthetic disaccharide, and buffer A (50 mM MOPS, pH 7.9, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol) in the final volume of 160 μL. In the cold reactions for obtaining material for structural characterization radioactive GDP-[^{14}C]mannose was replaced by 200 μM GDP-mannose and the final volume of the reaction was adjusted with buffer A to 320 μL. These reactions were carried out in triplicate. After 1 h incubation at 37 °C the reactions were stopped by an addition of 1 mL, or 2 mL of 96% ethanol, respectively. The radioactive reaction products were obtained by n-butanol/water partitioning, as described before.[15,16] The butanol extracts were dried under nitrogen and resuspended in n-butanol. 10% of these extracts were analyzed by TLC on aluminum-coated silica 60 F_{254} plate (Merck) developed in $CHCl_3$/MeOH/conc NH_4OH/H_2O (65 : 25 : 0.5 : 4; by vol.). The TLC plates were exposed to X-ray film (Kodak Bio-Max MR) and the reaction products arising from the synthetic acceptors were quantified by IMAGEJ (NIH) software.

Ethanol extracts from the non-radioactive reactions of **27–30** were clarified by centrifugation and the resulting pellets were re-extracted with 2 mL 96% ethanol. Ethanol extracts were combined and dried under the stream of N_2 at 37 °C.

LC-MS analysis of the products formed in non-radioactive enzymatic reactions. On-line LC-MS experiments were performed using a HPLC system (Varian, ProStar). Eluent A was 0.01 M formic acid in water and eluent B consisted of 0.01 M formic acid in 100% acetonitrile. The proportion of B was maintained at 10% between 5–10 min and then linearly increased to 80% over 70 min. The reaction mixtures were separated at a flow rate of 0.4 mL min^{-1} on a reversed-phase analytical column (Vydac 218-TP54 C18, 300-Å, Grace, Hesperia, USA). The column effluent was introduced directly into the mass spectrometer equipped with an electrospray ionization source (Varian 500 LC-MS). The nebulizing gas pressure was 50 psi and drying gas pressure 39 psi at 350 °C. Mass spectra were recorded in the positive mode with a scan rate of 3 s/scan.

Linkage determination. To confirm the α-linkages of mannose units in all enzymatic products, ethanol extracts from the whole enzymatic reaction mixtures were first partially purified by SPE on Starta-XC cartridges (30 mg/1 ml, Phenomenex, Torrance, CA) with deionized water. All collected fractions were evaporated and the volume was adjusted with deionized water to 50 μL and then analyzed by MALDI-TOF/TOF-MS. The fractions enriched with enzymatic adducts were subjected to α-mannosidase (Jack beans, Sigma) treatment at 37 °C for 1–20 h according to the protocol supplied by manufacturer. After the incubation, 1 μL of the digested mixture was spotted on the MALDI target and analyzed under the same conditions as the original non-digested reaction mixtures.

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