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Synthetic Mannosides Act as Acceptors for Mycobacterial α1-6 Mannosyltransferase

Jillian R. Brown,[a,*] Robert A. Field,[b] Adam Barker,[a] Mark Guy,[c] Ravinder Grewal,[a] Kay-Hooi Khoo,[d] Patrick J. Brennan,[a] Gurdyal S. Besra[c,†] and Delphi Chatterjee[a,†]

[a]*Department of Microbiology, Mycobacterial Research Laboratories, Colorado State University, Fort Collins, CO, 80523, USA*
[b]*School of Chemistry, University of St. Andrews, Purdie Building, St. Andrews, Fife KY16 9ST, UK*
[c]*Department of Microbiology and Immunology, University of Newcastle upon Tyne, The Medical School, Framlington Place, Newcastle upon Tyne, NE2 4HH, UK*
[d]*Institute of Biological Chemistry, Academica Sinica, Taipei, Taiwan, Republic of China*

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Abstract—A series of synthetic mannosides was screened in a cell-free system for their ability to act as acceptor substrates for mycobacterial mannosyltransferases. Evaluation of these compounds demonstrated the incorporation of [^{14}C]Man from GDP-[^{14}C]Man into a radiolabeled organic-soluble fraction and analysis by thin layer chromatography and autoradiography revealed the formation of two radiolabeled products. Each synthetic acceptor was capable of accepting one or two mannose residues, resulting in a major and a minor mannosylated product. Both products from each acceptor were isolated and their mass was confirmed by fast-atom bombardment–mass spectrometry (FABMS). Characterization of each mannosylated product by *exo*-glycosidase digestion, acetolysis and linkage analysis by gas chromatography–mass spectrometry of partially per-*O*-methylated alditols, revealed only α1-6-linked products. In addition, the antibiotic amphomycin selectively inhibited the formation of mannosylated products suggesting polyprenolmonophosphate-mannose ($C_{35/50}$-P-Man) was the immediate mannose donor in all mannosylation reactions observed. The ability of synthetic disaccharides to act as acceptor substrates in this system, is most likely due to the action of a mycobacterial polyprenol-P-Man:mannan α1-6 mannosyltransferase involved in the biosynthesis of linear α1-6-linked lipomannan.

Introduction

Mycobacterial diseases, such as tuberculosis and leprosy, remain serious human health concerns. One critical feature that contributes to the problematic pathogenicity of mycobacteria is the unique and intricate structure of the mycobacterial cell wall, which results in low permeability to most chemotherapeutic agents and thus promotes resistance. The cell wall structure, which is made up of polysaccharides, proteins and lipids, has been shown[1–4] to contain two major polysaccharide components, arabinogalactan (AG) and lipoarabinomannan (LAM). Both AG and LAM contain approximately 70 arabinosyl residues and 30 hexosyl residues (galactosyl in AG and mannosyl in LAM). The synthesis of these cell wall components requires the concerted action of a large number of glycosyltransferases.[5,6] Biological studies have implicated LAM as an important cell-surface molecule involved in host-pathogen interactions[5] and agents that interfere with the biogenesis of either AG or LAM are expected to have serious consequences for cell pathogenicity. Indeed, the biological importance of the mycobacterial lipoglycans, LAM, lipomannan (LM) and the phosphatidylinositol mannosides (PIMs), has led to a preliminary investigation into their biosynthesis.[4] Briefly, LAM and LM originate from a phosphatidylinositol (PI) core which is elaborated to give Ac_1PIM_2, linear α1-6-linked LM, mature LM and finally LAM[7,8] (Fig. 1). Three families of mannosyltransferases have been suggested to be involved in the biosynthesis of linear α1-6-linked LM. The first family of enzymes catalyzes the transfer of Man residues from GDP-Man to PI and other short PIM intermediates.[8] A second family catalyzes the transfer of Man residues from GDP-Man to a variety of polyprenol monophosphates, while a third

*Corresponding author at current address: Department of Cellular and Molecular Medicine, Glycobiology Research and Training Center, University of California, San Diego, La Jolla, CA, 92093-0687, USA. Tel.: +1-858-822-1102; fax: +1-858-534-5611; e-mail: jibrown@ucsd.edu
†Both authors shared responsibility for this work.

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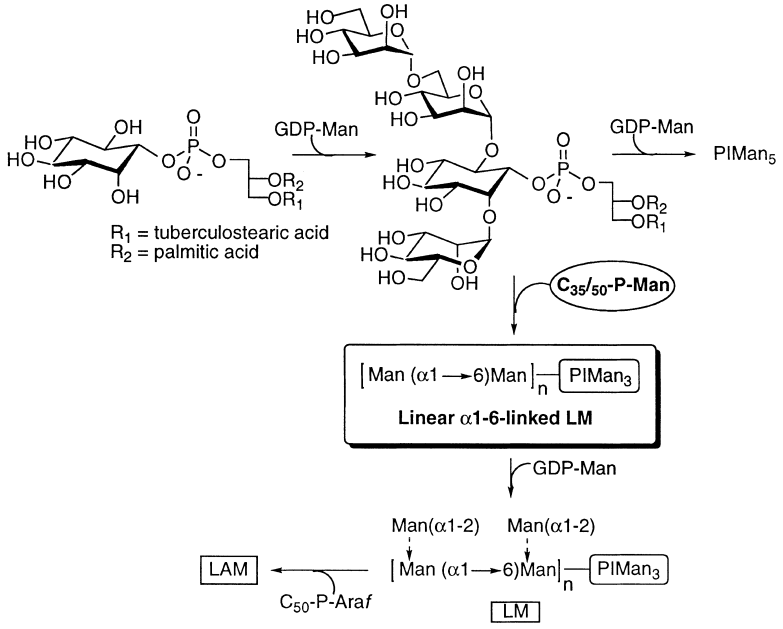

Figure 1. Postulated routes for the biosynthesis of mycobacterial PIMs, linear α1-6-linked LM, native LM and LAM.

family catalyzes the transfer of Man residues from polyprenol-monophosphate-mannose to PIM intermediates during the assembly of linear α1-6-linked LM.

In order to better understand the biosynthesis of the mycobacterial cell wall, cell-free assay systems have previously been described, by ourselves and others, for arabinosyltransferases[9,10] and galactosyltransferases.[11] In an attempt to establish an assay for the mycobacterial α1-6 mannnosyltransferases as well as to probe the acceptor/donor substrate specificities of these activities, we have prepared and tested a series of synthetic mannosides with variable aglycones (Fig. 2). Previously, the thiooctyl and octyl mannosides described herein have been shown to act as acceptors in a trypanosomal cell-free system.[12,13] In this paper we demonstrate that the mycobacterial cell-free system transfers up to two mannose residues from the polyprenol-P-mannose to four different synthetic mannoside acceptors. The mycobacterial polyprenol-P-Man: mannan α1-6 mannosyltransferase involved in the biosynthesis of linear α1-6-linked LM is most likely responsible for catalyzing these transformations.

Results

Synthesis of thiooctyl, octyl and decenyl mannopyranosides

Octyl 6-*O*-α-D-mannopyranosyl-1-thio-α-D-mannopyranoside [Manα1-6Manα1-S-C_8, **1**], octyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside [Manα1-6Manα1-O-C_8, **2**] and octyl 3-*O*-α-D-mannopyranosyl-α-D-mannopyranoside [Manα1-3Manα1-O-C_8, **4**] were prepared as reported previously[12,14] [where C_8 denotes the alkyl chain -$(CH_2)_7CH_3$]. Dec-9-enyl 6-*O*-α-D-mannopyranosyl- α-D-mannopyranoside [Manα1-6Manα1-O-C_{10}, **3**] was prepared essentially as described previously for compound **2** [where C_{10} denotes the alkenyl chain -$(CH_2)_8CH{=}CH_2$]. Briefly, compound **3** was prepared by selective glycosylation of dec-9-enyl 2,3,4-tri-*O*-benzoyl-α-D-mannopyranoside[15] with benzobromomannose[16] using mercury cyanide as a promoter[17] in 55% yield. Dec-9-enyl 2,3,4-tri-*O*-benzoyl-6-*O*-(2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl)-α-D-mannopyranoside was deprotected using 2 M ammonia in methanol in 90% yield (Fig. 3). Octa-*O*-acetyl-α-D-isomaltose was prepared by reaction of α1-6-isomaltose with acetic anhydride in pyridine. The crude product was converted into the peracetylated thioglycoside intermediate, octyl 2,3,4-tri-*O*-acetyl-6-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-1-thio-α-D-glucopyranoside using the coupling procedure previously described by Ferrier and Furneux[28] in 38% yield and the intermediate was then deprotected using 2 M ammonia in methanol to give octyl 6-*O*-α-D-glucopyranosyl-1-thio-α-D-glucopyranoside [Glcα1-6Glcα1-S-C_8, **5**] in 86% yield. Dec-9-enyl 6-*O*-α-D-mannopyranosyl-6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside [Manα1-6Manα1-6Manα1-O-C_{10}, **6**] was prepared by selective 6′-*O*-*tert*-butyldimethylsilylation of dec-9-enyl 6-*O*-α-D-mannopyranosyl - α - D - mannopyranoside as reported previously[18] followed by per-*O*-acetylation. Desilylation of the 6-*tert*-butyldimethylsilyl protecting group using acetic acid/water (80:20) followed by deacetylation (sodium methoxide/methanol) and desalting (Dowex AG-50X8 eluted with methanol) gave the glycosyl acceptor, dec-9-enyl 2,3,4-tri-*O*-acetyl-6-*O*-(2,3,4-tri-*O*-acetyl-α-D-mannopyranoside)-α-D-mannopyranoside in three steps with 75% overall yield. Glycosylation of the primary alcohol of dec-9-enyl 2,3,4-tri-*O*-acetyl-6-*O*-(2,3,4-tri-*O*-acetyl-α-D-mannopyranoside)-α-D-mannopyranoside with commercially available acetochloromannose using silver triflate as a promoter[28] followed by deprotection using sodium methoxide in methanol yielded the target trimannoside **6** (Fig. 3).

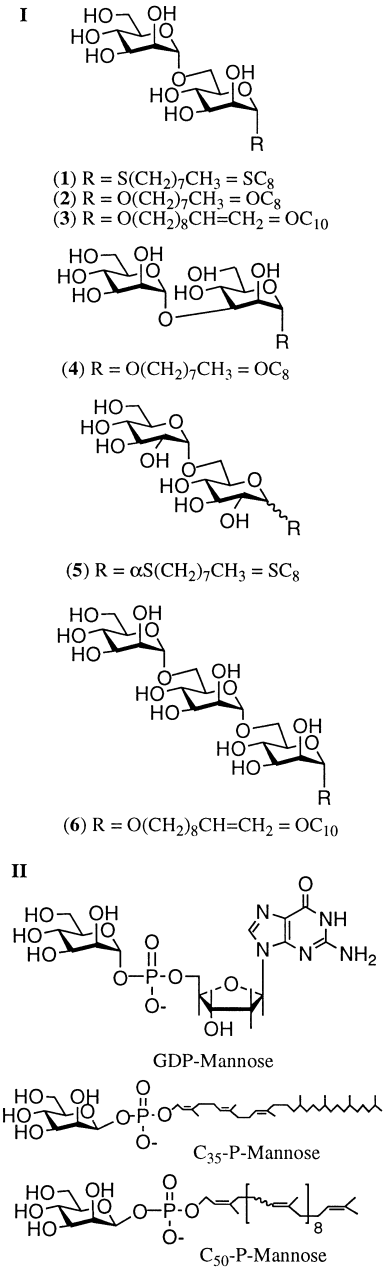

Figure 2. I. Structures of the six synthetic glycosides screened for their ability to act as potential acceptor substrates for mycobacterial mannosyltransferases and **II**. structures of possible mannose donors utilized in these mannosyltransferase assays.

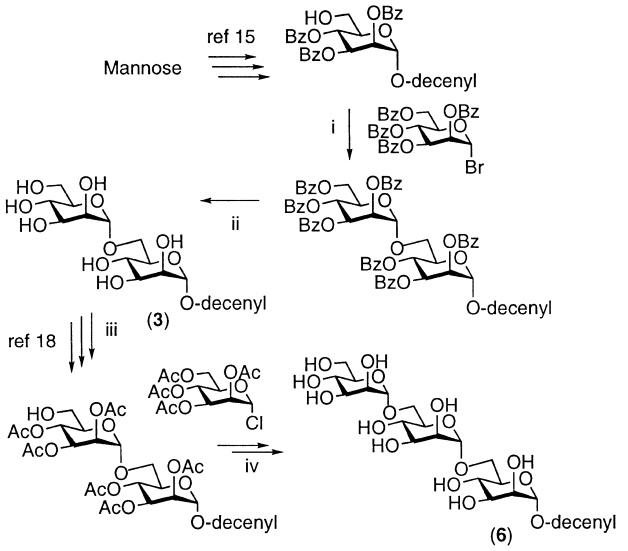

Figure 3. Synthesis of *O*-decenyl mannosides (**3**) and (**6**).

Biological studies

Synthetic mannosides act as mannosyltransferase acceptor substrates in a mycobacterial cell-free system

A series of six synthetic glycosides (**1**–**6**) was screened for their abilities to act as acceptor substrates for the mannosyltransferases present in washed *Mycobacterium smegmatis* mc^{2}155 membranes (see Fig. 2). GDP-[^{14}C]Man was used as the indirect sugar nucleotide donor in incubations with mycobacterial membranes and the aforementioned synthetic compounds at various concentrations. The [^{14}C]mannosylated products were recovered by solvent extraction, partitioned between water and butan-1-ol and the butan-1-ol phases analyzed by HPTLC. Under these conditions the turnover of endogenous phosphatidylinositol-mannoside (PIM) intermediates indicated that α-mannosyltransferases in the membrane preparation were active (Fig. 4, lane 1 Ctrl). In this assay the α1-3-linked compound **4** did not act as a mannose acceptor, while the α1-6-linked glucose compound **5** showed negligible acceptor activity (data not shown). In contrast, significant radiolabeled product bands were identified for α1-6-linked dimannosides and the trimannoside, **1**, **2**, **3**, and **6**, respectively. The major and minor [^{14}C]mannosylated products generated by each of the four acceptor substrates **1**, **2**, **3**, and **6** are shown in Figure 4 Man_2SC_8, Man_2OC_8, Man_2OC_{10}, and Man_3OC_{10}, respectively. For the dimannosides and the trimannoside acceptor substrates, these labeled products were judged to be tri- and tetra-saccharide, and tetra- and penta-saccharide, respectively, based on their HPTLC mobilities and their masses as measured by FAB-mass spectrometry confirmed that these were the saccharide products formed (Table 1). The amount of radioactivity associated with each [^{14}C]mannosylated product was determined by scintillation counting of the excised HPTLC bands. The [^{14}C]mannosylation of each of the four active acceptors is of similar magnitude to the mannosylation of endogenous PIM intermediates, suggesting that all four are good acceptors for the mycobacterial α-mannosyltransferase(s). In addition to the major and minor radiolabeled mannosylated product bands there are other minor radiolabeled bands seen on HPTLC, which are due to the synthesis of endogenous PIM intermediates similar to those seen in the control incubation. For each synthetic acceptor, the recovered counts increased with acceptor substrate concentration up to a maximum 2 mM, 2 mM, 0.5 mM, and 3 mM for **1**, **2**, **3**, and **6**, respectively (Fig. 5). Beyond these concentrations a decrease in the incorporation of [^{14}C]Man into the radiolabeled products was observed, which is most likely due to the detergent-like properties of these neoglycolipids.

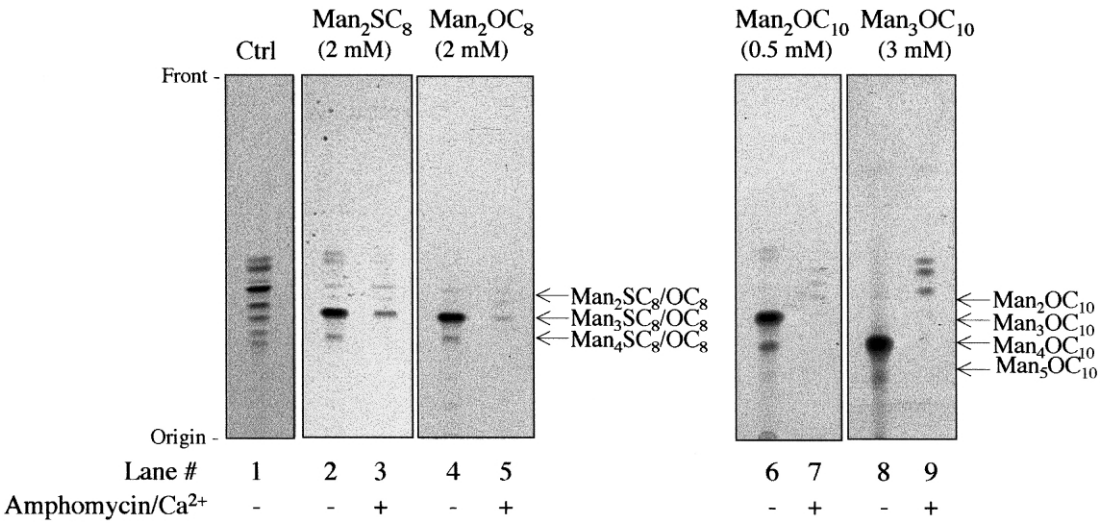

Figure 4. Enzymatic product formation and inhibition of the [^{14}C]Man transfer to each acceptor substrate in the mycobacterial cell-free system. Synthetic mannosides were incubated with GDP-[^{14}C]Man and washed *M. smegmatis* membranes. The glycolipid enzymatic products were extracted and analyzed by HPTLC using solvent system A and fluorography. In the absence of exogenous acceptor, there are endogenous phosphatidylinositol intermediates (lane 1, Ctrl) indicating that the mycobacterial enzymes are active in this crude enzyme preparation. The [^{14}C]mannosylated products for Manα1-6Manα1-S-C_8 (Man_2SC_8), Manα1-6Manα1-O-C_8 (Man_2OC_8), Manα1-6Manα1-O-C_{10} (Man_2OC_{10}), and Manα1-6Manα1-6Manα1-O-C_{10} (Man_3OC_{10}) at optimal concentrations, in the absence (−) or presence (+) of amphomycin and Ca^{2+}. The position of non-radioactive standards as judged by HPTLC mobilities and mass are indicated on the right hand side of the chromatograms.

Table 1. Glycosyl linkage analysis of all mannosylated products formed as a result of the transfer of Man residues to each of the four active acceptors

Synthetic mannoside	Mannosylated products[a]	Linkage of Man transferred[b]		Ratio of Man residues[c]	
		Type	Position	t-Man	6-Man
Manα1-6Manα1-S-C_8	Man-(Manα1-6Manα-1-S-C_8)	α	1,6	1	2
	Man-Man-(Manα1-6Manα-1-S-C_8),	α	1,6	1	3
Manα1-6Manα1-O-C_8	Man-(Manα1-6Manα-1-O-C_8)	α	1,6	1	2
	Man-Man-(Manα1-6Manα-1-O-C_8),	α	1,6	1	3
Manα1-6Manα1-O-C_{10}	Man-(Manα1-6Manα-1-O-C_{10})	α	1,6	1	2
	Man-Man-(Manα1-6Manα-1-O-C_{10})	α	1,6	1	3
Manα1-6Manα1-6Manα1-O-C_{10}	Man-(Manα1-6Manα1-6Manα-1-O-C_{10})	α	1,6	1	3
	Man-Man-(Manα1-6 Manα1-6Manα-1-O-C_{10})	α	1,6	1	4

[a]FABMS confirmed [$M + Na^+$] molecular ions (m/z) for all mannosylated products formed using the cell-free system.
[b]The nature of each glycosidic linkage present in each mannosylated product was determined by (i) exoglycosidase digests, (ii) acetolysis, and (iii) gas chromatography–mass spectrometry (GC–MS) analysis on the partially *O*-methylated alditol acetates.
[c]The ratio of terminal Man (t-Man) to 6-linked Man (6-Man) for each mannosylated product was determined by GC and GC–MS.

Rates of [^{14}C]Man transfer to each of the four active mannosides

In all assays, two [^{14}C]Man radiolabeled bands, one major and one minor, were consistently formed with each of the four active acceptors **1**, **2**, **3**, and **6** (e.g., see Fig. 4). The efficiencies of the different acceptors at their optimal concentrations, relative to the rate of the first mannose transferred using **2** at 2 mM (100%, i.e., ~0.6 pmol/mg protein/min), were comparable for dimannosides **1** and **3** tested in the system (67%, i.e., ~0.4 pmol/mg protein/min). Despite a substantially higher (3 mM) acceptor concentration of trimannoside **6**, the efficiency of the first mannose transferred was significantly lower (45%, i.e., ~0.27 pmol/mg protein/min, see Fig. 5). By comparing all acceptors at 0.5 mM, we can conclude that **1** and **6** have the ability to act as mannose acceptors with a lower efficiency than **2** and **3**. At higher concentrations of acceptor (2 mM), dimannoside **2** works most efficiently. In contrast, dimannoside **3** acts as an acceptor efficiently at lower concentrations (0.5 mM), although this compound contains a slightly longer lipid-like aglycone than either **1** and **2**, which results in significant reduction in the transferase activity at higher concentrations (see Fig. 5). The rate of the second [^{14}C]Man residue transferred to each acceptor was also measured and these data were approximately 10–15% of the rate of the first [^{14}C]Man transferred. The reduction in the efficiency of mannose transfer is presumably due to a much lower acceptor concentration during the second transfer reaction.

Trimannoside **6** was chemically synthesized as an extended linear acceptor substrate in an attempt to detect α1-2 mannosyltransferase activity (see Fig. 1). The optimal acceptor concentration for the **6** was considerably higher (3 mM) than for **1**, **2**, or **3**, and thus indicates that the

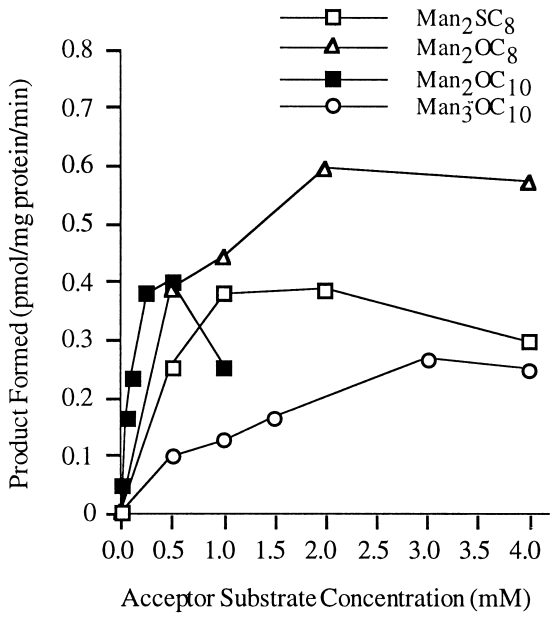

Figure 5. [^{14}C]Man transferred to each of the four acceptor substrates in the mycobacterial cell-free system. Incubations contained washed *M. smegmatis* membranes, GDP-[^{14}C]Man and synthetic acceptors over a range of acceptor concentrations (as indicated). The radioactivity associated with each major [^{14}C]mannosylated product (pmol/mg protein/min) for each synthetic acceptor was determined by scintillation counting of the excised HPTLC bands. The values in these graphs are the mean of at least two determinations.

trimannoside **6** was a poorer acceptor substrate for the mycobacterial α1-6 mannosyltransferase when tested in the cell-free system. Despite a higher concentration, **7** produced both a major (tetrasaccharide) and minor (pentasaccharide) radiolabeled product, a similar result to all of the other assays, however still no α1-2 mannosyltransferase activity was detected.[8]

Enzymatic characterization of the [^{14}C] mannosylated products derived from the synthetic mannosides

The glycosidic linkages present in each of the [^{14}C]mannosylated products were characterized by *exo*-glycosidase digestions and acetolysis. In each case, digestion using jack bean α-mannosidase resulted in the quantitative removal of [^{14}C]Man residues from each acceptor, as well as the loss of the labeled bands upon analysis by HPTLC (data not shown). These results confirm that the newly formed glycosidic linkages present in each enzymatic product were α-configured and these mannosides were therefore acceptor substrates for mycobacterial α-mannosyltransferase(s).

Digestion of each [^{14}C]mannosylated product using the Manα1-2Man-specific *Aspergillus phoenicis* α-mannosidase was carried out in order to determine the nature of glycosidic linkage(s) formed between the transferred [^{14}C]Man residues and each acceptor. This particular digest enabled the relative proportions of α1-2, α1-6 and α1-3/4-linked [^{14}C]Man to be determined. In all cases, digestion did not remove any [^{14}C]Man residues attached to any of the acceptors (data not shown). These results confirm the absence of any α1-2-linked [^{14}C]Man and demonstrated that none of the mannosides evaluated could act as acceptor substrates for the α1-2 mannosyltransferase activity present in the mycobacterial membranes.

Three dimannosides (**1**, **2**, and **4**) were used as standards for acetolysis, showing quantitative cleavage of Manα1-6Man glycosidic linkages and only partial cleavage of Manα1-3Man, as expected. The results also showed that the glycosidic linkage to the agylcone was cleaved in this procedure forming free Man from Manα1-6Manα1-S-C_8 and Manα1-6Manα1-*O*-C_8, and Manα1-3Man from Manα1-3Manα1-*O*-C_8. Acetolysis therefore confirms that when a free [^{14}C]Man residue is released during this procedure, an α1-6-linkage exists between the [^{14}C]Man residue and the acceptor. In contrast, the release of a radiolabeled disaccharide confirms an α1-3/4 linkage. In all cases, only free [^{14}C]Man was liberated consistent with only α1-6-linked [^{14}C]Man (data not shown). From these data, it is most likely the same enzyme activity is operating on each acceptor substrate twice, resulting consistently in the formation of major and minor α1-6-linked mannosylated products.

Linkage analysis using gas chromatography–mass spectrometry

In order to provide sufficient quantities of 'cold' mannosylated products for complete linkage analysis, large scale cell-free assays using unlabeled GDP-Man were performed. Such mannosylated products were isolated by preparative HPTLC as described in Experimental. A sample of each purified major and minor mannosylated product was analyzed by fast-atom bombardment–mass spectrometry (FABMS) where the [$M + Na^+$] molecular ions confirmed addition of one or two hexose units to each acceptor substrate (Table 1). Representative data for the major mannosylated product derived from **2** (i.e., Manα1-6Manα1-6Manα1-OC_8) is shown in (Fig. 6(A)), confirming the addition of one hexose residue with an increase in m/z 162 over the mass of the parent dimannoside.

To further characterize the glycosyl linkages formed between the transferred Man residues and each of the four active acceptors, each mannosylated product was methylated and alditol acetates were generated as described in Experimental. These alditol acetates were subsequently analyzed by gas chromatography (GC) and gas chromatography–mass spectrometry (GC–MS), giving quantitative measurements of the ratio of terminal Man to 6-linked-Man present in each mannosylated product. The results revealed that for the all the synthetic acceptor substrates tested there was consistently the introduction of one 6-linked Man followed subsequently by a second 6-linked Man residue. These data are summarized in Table 1 and confirm the data from the *exo*-glycosidase digests and acetolysis, in that there was a consistent increase in only 6-linked mannosylated products to all four acceptors. GC-MS data clearly confirmed that the glycosidic linkages formed in each of the cell-free assays were indeed α1-6-configured. These data indicate that all four active acceptors presumably utilize only one enzyme activity present in the washed

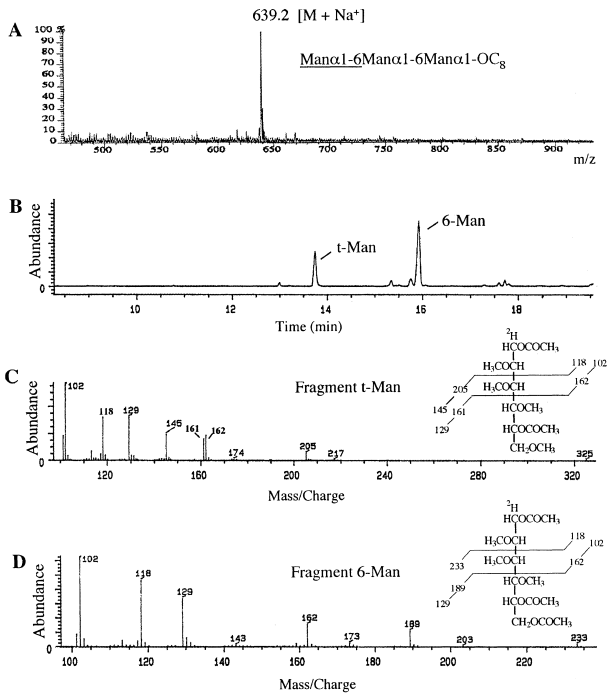

Figure 6. GC–MS profile of the alditol acetates derived from the *O*-methylated oligomannosyl alditols prepared for the major mannosylated product of Manα1-6Manα-1-O-C_8, i.e., Manα1-6Manα1-6Manα-1-O-C_8. (**A**) FABMS spectra $[M+Na^+]$ molecular ion at m/z 639.2 corresponding to Manα1-6Manα1-6Manα-1-O-C_8) (**B**) The total ion chromatograph generated for Manα1-6Manα1-6Manα-1-O-C_8, showing a ratio of ~1:2; terminal Mannose (t-Man, R_T 13.5) to 6-linked Mannose (6-Man, R_T 15.8) residues (**C**) Mass spectrum of the major diagnostic fragment ions for t-Man; and (**D**) Mass spectrum of the major diagnostic fragment ions for 6-linked Mannose.

mycobacterial membranes, most likely the mycobacterial polyprenol-P-Man: mannan α1-6 mannosyltransferase activity.

The nature of the α1-6 mannosyltransferase activities

The mycobacterial cell-free system contains two mannosyl phospholipids, namely C_{35}-P-Man and C_{50}-P-Man[19] that have the ability to act as the donor of mannose to exogenous acceptors.[20,21] A crude mixture of polyprenol-P-Man (C_{35} and C_{50}-P-Man) has previously been shown to be the direct donor of mannose in mannosylation reactions involved in LM and LAM biosynthesis.[8] In order to assess whether the mannosyltransferase responsible for the [^{14}C]mannosylation of acceptor substrates **1**, **2**, **3**, and **6**, was GDP-Man or C_{35}/C_{50}-P-Man-dependent, enzymatic assays were performed in the presence and absence of amphomycin and calcium. In the presence of Ca^{2+}, this antibiotic is known to specifically inhibit a variety of translocase enzymes by chelating with polyprenol monophosphates, thus inhibiting the transfer of mannose residues from GDP-[^{14}C]Man to polyprenyl-P carriers.[8,22–25] Significantly, preincubation of the cell-free system with amphomycin and Ca^{2+} substantially abrogated the formation of C_{35}/C_{50}-P-Man and [^{14}C]mannosylated products (Fig. 4, lanes 3, 5, 7, and 9). Therefore, the α1-6 mannosyltransferase activity observed for all four acceptors appears to be C_{35}/C_{50}-P-Man-dependent, and not GDP-Man-dependent.

Discussion

Synthetic glycosides can be utilized as acceptor substrate analogues for assaying a variety of glycosyltransferases.[10,11,26,27] and the most commonly used aglycones in synthetic glycoside preparations are fluorogenic (e.g., 4-methyl-umbelliferyl), chromogenic (e.g., *para*-nitrophenol) and hydrophobic (e.g., $(CH_2)_7CH_3$) moieties, since they impart properties which enable convenient product purification and characterization. The data presented here represent an extension of the use of synthetic acceptor substrates for specific glycosyltransferases involved in mycobacterial biosynthetic pathways. The present report describes the establishment of a cell-free system that utilizes synthetic mannosides as acceptors in vitro to assay for the mycobacterial α1-6 mannosyltransferase involved in the biosynthesis of linear α1-6-linked LM.

In each transfer assay, two mannose residues were added sequentially to all four active synthetic mannosides with comparable amounts of enzymatic products being formed. The enzyme activities were shown to be polyprenol-P-Man dependent rather than GDP-Man dependent. *exo*-Glycosidase digestion and linkage analysis of partially per-*O*-methylated alditols by GC–MS confirmed the dimannosides and trimannoside acted as acceptor substrates for only the mycobacterial α1-6 mannosyltransferase. Therefore, the synthetic acceptors **1**, **2**, **3**, and **6**, appear to be recognized by only one specific enzyme activity, most likely the mycobacterial polyprenol-P-Man: mannan α1-6 mannosyltransferase. This is in contrast to the corresponding trypanosome cell-free system, where only one mannose residue was added to each of the aforementioned synthetic acceptor substrates, and multiple enzyme activities were recognized by three of the same acceptors.[12]

In order to ascertain whether an extended linear α1-6-linked structure would be required for α1-2 branching, trimannoside acceptor **6** was chemically synthesized. Surprisingly, only α1-6 mannosyltransferase activity, with no evidence of any α1-2 branching mannosyltransferase activity, was detected. Despite showing reasonable α1-6 mannosyltransferase activity, this compound is elongated no more efficiently than any of the smaller, simpler dimannosides. A comparable result whereby larger glycans were not significantly better acceptor substrates was observed by Ayers and colleagues in their mycobacterial arabinosyltranferase cell-free assays.[10] In addition, this trisaccharide acceptor substrate is active only at a much higher optimum concentration.

Finally, the synthetic acceptor substrates **1**, **2**, **3**, and **6**, might be used to aid in purification of the mycobacterial α1-6 mannosyltransferase. In particular, Manα1-6Manα1-*O*-C_{10}, with the 9-dec-1-ene aglycone moiety can be attached through the aglycone to a polymer matrix, forming a potential affinity column. In addition, all of these synthetic acceptor substrates will provide invaluable information towards mapping the substrate specificity of the individual mycobacterial biosynthetic enzymes once they are purified. Based on these observations, this study

is a good starting point for further modification of these mannose-based disaccharides in the design of specific inhibitors of LM biosynthesis. An important implication is that small inhibitor analogues could be utilized as potential lead compounds in the development of novel chemotherapeutic agents for the treatment of mycobacterial infections.

Experimental

General methods

All synthetic and associated analytical procedures were carried out using established methods, as described previously.[29] Where appropriate, deprotected products were dissolved in water, washed with diethyl ether, and purified by gel filtration on a Bio-Gel P4 column. NMR spectra were recorded on a Varian Inova 300 (^{1}H, 300 MHz; ^{13}C, 75.4 MHz). Electrospray-mass spectrometry data were recorded on a Micromass Quattro-single quadrapole mass spectrometer (Micromass, U.K.). Thio- and Octyl- dimannosides were chemically synthesized as previously described.[12] All compounds that were subjected to biological testing gave spectral and analytical data consistent with their proposed structures. GDP-[^{14}C]Man (251 mCi/mmol) was purchased from DuPont NEN. Aluminum-backed silica gel 60 high-performance thin-layer chromatography plates (Art. 5547) were obtained from Merck. Amphomycin (calcium salt) was a gift from C. J. Waechter, University of Kentucky, KY). Jack bean α-mannosidase (JBAM) and *Aspergillus phoenicis* α-mannosidase (APAM) were obtained from Oxford GlycoSystems. Manα1-6Man, Manα1-3Man and Manα1-2Man disaccharides were obtained from Dextra-Laboratories. Acetochloromannose was obtained from Toronto Research Chemical Inc and isomaltose was obtained from Sigma Chemical Company. All solvents and general reagents were from BDH-Merck, Aldrich or Sigma Chemical Company.

Octyl 6-*O*-α-D-glucopyranosyl-1-thio-α-D-glucopyranoside (5). To an ice-cold solution of isomaltose (Glcα1-6Glc) (51 mg, 0.149 mmol) in pyridine (3 mL) was added acetic anhydride (1 mL), and the solution was stirred overnight at room temperature; thin layer chromatography (TLC) (hexane/EtOAc, 1/1) then showed the reaction to be complete. The mixture was evaporated to dryness and acetic acid and pyridine were removed by co-evaporation with toluene. A solution of the resulting oil in dichloromethane (40 mL) was subjected to a standard work up to give the per-*O*-acetylated isomatose (37 mg, 95%), which was converted directly into the thioglycoside using the coupling procedure described by Ferrier and Furneaux.[28] To an ice-cold solution of isomaltose octaacetate (37 mg, 0.055 mmol) in anhydrous dichloromethane (5 mL) was added 1-octanethiol (0.025 mL, 2 equiv) and boron trifluoride etherate (0.08 mL, 10 equiv) and the solution was stirred overnight; TLC (light petroleum/EtOAc, 1/1) then showed the reaction to be complete. Dichloromethane (15 mL) was added and the resulting solution was subjected to a standard work up. Flash-column chromatography of the residue (gradient elution, light petroleum/EtOAc. 4/1 to 1/1) yielded α-isomer, octyl 2,3,4-tri-*O*-acetyl-6-*O*-(2,3,4,6-tetra-*O*-acetyl-α-D-glucopyranosyl)-1-thio-α-D-glucopyranoside (16 mg, 38%); δ_H ($CDCl_3$) 0.8–1.6 (15H, 3×m, octyl *H*), 1.8–2.2 (21H, 4×s, CH_3CO), 2.6 (2H, m, –SCH_2), 3.5 (1H, dd, $J_{5,6a}$ = 2.6 and $J_{6a,6b}$ = 11.3 Hz, H-6a/6a′), 3.75 (1H, dd, $J_{5,6b}$ = 5.1 and $J_{6a,6b}$ = 11.3 Hz, H6b/b′), 5.1 (1H, dd, $J_{1,2}$ = 5.2 Hz, H-1), 5.6 (1H, dd, $J_{1,2}$ = 4.1 Hz, H-1′); δ_C ($CDCl_3$) 14.0, 20.6, 22.5, 28.5, 28.8, 29.1, 29.7, 31.7, 61.7, 66.2, 67.2, 68.2, 69.2, 69.9, 70.4, 70.6, 70.8, 81.1 (C-1), 95.5 (C-1′), 169.5–169.8, α/β mixture (5 mg, 12%) and β-isomer (13 mg, 32%). All three fractions were recovered as immobile oils, however, the α/β mixture and the β-isomer were not used in any biological assays. Sodium methoxide (4.6 M, 0.01 mL) was added to a solution of octyl 2,3,4-tri-*O*-acetyl-α-D-glucopyranosyl)-1 - thio - α - D - glucopyranoside (16 mg, 0.02 mmol) in methanol (5 mL). The reaction mixture was stirred at rt for 6 h, whereupon Dowex AG50X8 (H^+) ion-exchange was added and stirring was continued for a further 30 min. The resin was removed by filtration and the filtrate was evaporated to dryness. The residue was dissolved in water and the aqueous solution was freeze-dried to give the title compound (**5**) (8.5 mg, 86%) as and amorphous solid; δ_H ($CDCl_3$) 0.8–1.6 (15H, 3×m, octyl *H*), 2.7 (2H, m, -SCH_2), 4.1 (1H, m, H-5/5′), 5.3 (1H, d, $J_{1,2}$ = 5 Hz, H-1/1′); δ_C ($CDCl_3$) 14.9, 24.1, 24.7, 30.4, 30.8, 31.1, 31.5, 33.4, 63.0, 67.8, 72.1, 72.2, 72.8, 73.5, 73.9, 74.1, 75.6, 76.1, 87.6, (C-1; J_{C-H} = 168 Hz), 101.6 (C-1′, J_{C-H} = 165 Hz). ES–MS: calcd for [$C_{20}H_{38}O_{10}S$]: *m*/*z* 470.2. Found $[M-1]^-$: *m*/*z* 469.1 and $[M+Cl]^-$: *m*/*z* 505.0.

Dec-9-enyl 6-*O*-α-D-mannopyranosyl-6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside (6). Disaccharide (**3**) was synthesized as described by Nikolaev and co-workers for the corresponding dec-9-enyl synthetic oligomers.[15] To an ice-cold solution of dec-9-enyl 6-*O*-α-D-mannopyranosyl - 6 - *O* - α - D - mannopyranoside (**3**) (100 mg, 0.208 mmol) in anhydrous pyridine (2 mL) was added *tert* butyldimethylsilyl chloride (60 mg, 2.5 equiv) and the solution was stirred in the cold for 8 h; TLC ($CHCl_3$/MeOH, 10/2) then showed the reaction to be complete. The reaction mixture was cooled in an ice-bath and acetic anhydride was added (0.5 mL) and the reaction was stirred overnight at rt; TLC (hexane/EtOAc, 3/2) then showed the reaction to be complete. The reaction was evaporated to dryness and co-evaporated three times with methanol and toluene to give dec-9-enyl 6′-*O*-*tert*-butyldimethylsilyl 6-*O*-α-D-mannopyranosyl-α-D-mannopyranoside which was used directly in the next step. Acetic anhydride and water mixture (80/20, 3 mL) was added to the reaction mixture and stirred overnight at rt for 3 days; TLC (hexane/EtOAc, 3/2) then showed the reaction to be complete and yield dec-9-enyl 2,3,4-tri-*O*-acetyl-6-*O*-(2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl) - α - D - mannopyranoside (3 steps, 120 mg, 75%); δ_H ($CDCl_3$) 1.3 (10H, m, 5×CH_2), 1.8 (2H, m, CH_2), 1.9, 2.0, 2.1 (9H, 3×s, 3×Ac), 2.4 (2H, m CH_2), 3.4 (2H, m, OCH_2CH_2), 4.7 (1H, m, H1/1′), 4.8 (1H, m, H1/1′), 4.9 (1H, d, CH=CH_2) 5.0 (1H, d, CH=CH_2), 5.8 (1H, m, CH_2CH=CH_2); δ_C ($CDCl_3$) 26.1–33.7 (7×CH_2), 61.3, 66.4, 66.7 (2), 68.4 (OCH_2CH_2), 68.7, 69.1, 69.3, 69.5, 69.7, 70.8, 97.2 (C-1/1′, J_{C-H} = 169 Hz), 97.7 (C-1/1′,

J_{C-H} = 169 Hz), 114.1 (CH=CH_2), 139.2 (CH=CH_2), 167.7–170.8 (carbonyl *C*). Silver triflate[29] (14 mg, 0.054 mmol) was added to a cooled (−35 °C) solution of dec-9-enyl 2,3,4-tri-*O*-acetyl-6-*O*-(2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside (20 mg, 0.027 mmol) and acetochloromannose (20 mg, 0.054 mmol) in anhydrous dichloromethane (1 mL) containing crushed 4 Å molecular sieves. After 2 h, the reaction was quenched with collidine (0.005 mL), filtered through Celite and subjected to a standard work up to give dec-9-enyl 2,3,4-tri-*O*-acetyl-6-*O*-(2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl)-6-*O*-(2,3,4-tri-*O*-acetyl-α-D-mannopyranosyl)-α-D-mannopyranoside which was used directly in the next step. A solution of the reaction mixture in methanol (1 mL) was added sodium methoxide (4.6 M, 0.02 mL). The reaction was stirred overnight at rt, whereupon Dowex AG50X8 (H^+) ion-exchange resin was added and the mixture was stirred for a further 30 min. The resin was removed by filtration and washed with methanol and the filtrate was evaporated to dryness. The residue was dissolved in water and the aqueous solution was freeze-dried. The final deprotected trimannoside (**6**) was purified by reverse-phase (Sep-pak, C18, Waters Associates) chromatography as previously described by Palcic and colleagues[30] to give the title compound (**6**) (2 steps, 7.3 mg, 41%) as a white solid; δ_H (D_2O) 1.2–2.1 (14H, m, 7×CH_2), 3.7 (2H, m, OCH_2CH$_2$), 4.7 (3H, m, H1/1′/1″), 4.8 (1H, d, CH=CH_2) 4.9 (1H, d, CH=CH_2), 5.8 (1H, m, CH_2CH=CH_2). The configuration of each of the glycosidic linkages was confirmed to be α-linked by exhaustive digestion with jack bean α-mannosidase. FABMS: calcd for [$C_{28}H_{50}O_{16}$]: *m*/*z* 642.3. Found $[M+1]^+$: *m*/*z* 643.3 and $[M+Na]^+$: *m*/*z* 665.3.

Preparation of mycobacterial membranes and cell-free system. *M. smegmatis* mc^{2}155 cells were grown in Bacto Nutrient Broth (Difco Labs, Detroit, MI) to mid log-phase (~24 h) and harvested. The cells were washed and resuspended at 4 °C in 50 mM MOPS buffer, pH 7.9, containing 5 mM β-mercaptoethanol, 10 mM $MgCl_2$ (Buffer A), 150 μg of DNAse (type IV, Sigma), 250 μg of RNase (microsomal nuclease, Sigma) and subjected to six passes though a French Press Cell (Aminco, Silver Spring, MD) at 10,000 psi. The lysed cells were centrifuged initially at 600×*g* to remove cell debris and then at 25,000×*g* for 20 min at 4 °C to remove cell wall and finally membranes were obtained by centrifugation of the supernatant at 100,000×*g* for 1 h at 4 °C. Membranes were resuspended in Buffer A in 50 μL aliquots and frozen at −20 °C at a known protein concentration.

Synthetic dimannosides and trimannoside (over a range of acceptor concentrations) were incubated with 1 μCi of GDP-[^{14}C]Man in buffer A. The reactions were initiated by the addition of 50 μL of washed membranes (~10 mg/mL protein) in 160 μL total volume. For experiments with amphomycin, membranes were pre-incubated with and without 2 mg/mL amphomycin and/or 10 mM $CaCl_2$ (per assay) for 15 min on ice prior to starting reactions. After incubation at 37 °C for 1 h, 1067 μL of chloroform/methanol (1/1, v/v) was added to terminated the reactions and lipids were extracted at 4 °C for 16 h. The supernatants were dried under a stream of nitrogen, redissolved in 1 mL ethanol/water (1/1, v/v) and applied to 1 mL Whatman strong anion exchange (SAX) cartridge. Radiolabeled products were eluted from the cartridges with ethanol (3 mL) and dried under a stream of nitrogen. The radiolabeled products were resuspended in 3 mL of water saturated with butan-1-ol and extracted once more with 3 mL of butan-1-ol saturated with water. The combined butan-1-ol phases were washed three times with 3 mL of water saturated with butan-1-ol. Aliquots of the combined butan-1-ol and/or aqueous phase(s) were analyzed by HPTLC.

Generation of the mannosylated and [^{14}C]mannosylated products for structural characterization. Large scale mannosyltransferase reactions were performed to generate sufficient material for linkage analysis. These reactions were performed in 10×360 μL final volume containing variable amounts of buffer A, 2 mM Manα1-6Manα1-S-C_8 (**1**), 2 mM Manα1-6Manα1-*O*-C_8 (**2**), 0.5 mM Manα1-6Manα1-*O*-C_{10} (**3**), and 3 mM Manα1-6Manα1-6Manα1-*O*-C_{10} (**6**), 2 mM GDP-Man and 100 μL of mycobacterial membranes (10 mg/mL protein). Radioactive tracer reactions were also performed in 1×360 μL final volume under exactly the same conditions as the above reactions except 50 μL GDP-[^{14}C]Man (1 μCi) was used instead of 2 mM GDP-Man. After reactions were incubated at 37 °C for 16 h, the reactions were stopped by the addition of 2.4 mL of chloroform/methanol (1/1, v/v) and extracted at 4 °C for 16 h. The lipid extracts were dried under N_2 and partitioned between butan-1-ol and water saturated butan-1-ol (using 3 mL volumes). The butan-1-ol phases from all 10× cold reactions were applied on an HPTLC plate (20×20 cm), alongside radioactive tracer lanes (approx. 3,000 cpm), and developed in solvent system A. Immediately after the plate was developed the radioactive tracer products (tri-, tetra- and penta-saccharide products) were detected using a BIOSCAN-system 200 imaging scanner. The regions of the HPTLC plate corresponding to the major cold α-mannosylated products, namely, Man-(Manα1-6Manα-1-S-C_8/-O-C_8/-O-C_{10}), Man-Man-(Manα1-6Manα-1-S-C_8/-O-C_8/-O-C_{10}), Man-(Manα1-6Manα1-6Manα-1-O-C_{10}) and Man-Man-(Manα1-6Manα1-6Manα-1-O-C_{10}), were excised and extracted twice using 3 mL of butan-1-ol and the butan-1-ol phase back extracted 3 times using water saturated butan-1-ol. The extracts containing the aforementioned cold mannosylated products were analyzed (i) by fast-atom bombardment mass spectrometry and (ii) by linkage analysis. The radiolabeled tracer products were also excised and extracted twice using 3 mL of butan-1-ol and the butan-1-ol phase back extracted three times using water saturated butan-1-ol. Approximately 3,000 cpm from the butan-1-ol phase of each mixture, namely; [^{14}C]Man-(Manα1-6Manα-1-S-C_8/-O-C_8/-O-C_{10}), [^{14}C] Man-[^{14}C]Man-(Manα1-6Manα-1-O-C_8/-O-C_8/-O-C_{10}), [^{14}C]Man-(Manα1-6Manα1-6Manα-1-O-C_{10}), and [^{14}C] Man-[^{14}C]Man-(Manα1-6Manα1-6Manα-1-*O*-C_{10}) were dried and used in exoglycosidase digests and acetolysis experiments.

High-performance, thin-layer chromatography (HPTLC). Samples were applied to silica gel-60 aluminum-backed HPTLC plates (Merck). Unless otherwise stated, the

plates were developed for 10 cm, with solvent system A: one development with chloroform/methanol/1 M ammonium acetate/13 M ammonia/water (180/140/9/9/23, v/v) or with solvent system B: one development with propan-1-ol/acetone/water (5/4/1, v/v) followed by one development with butan-1-ol/acetone/water (5/3.5/1.5, v/v). The HPTLC plates were scanned with a BIOSCAN-system 200 imaging scanner with Autochanger 3000 and subsequently exposed to Kodak XAR-5 film at −70 °C for fluorography. The lanes containing nonradioactive compounds (5–10 μg) were cut out after development of the HPTLC plate, sprayed with α-naphthol reagent[31] and heated at 110 °C for 5 min.

Preparation of partially per-*O*-methylated oligomannosyl alditols for GC-MS analysis. Samples of Manα1-6Manα-1-S-C_8/-O-C_8/-O-C_{10} (standards) as well as Man-(Manα1-6Manα-1-S-C_8/-O-C_8/-O-C_{10}, Man-Man-(Manα1-6Manα-1-S-C_8/O-C_8/-O-C_{10}, Man-(Manα1-6Manα1-6Manα-1-O-C_{10}) and Man-Man-(Manα1-6Manα1-6Manα-1-O-C_{10}) were per-*O*-methylated in screw-capped glass tubes by the addition of a sodium hydroxide-dimethyl sulfoxide slurry (1 mL) according to the procedure previously described.[32] The derivatized oligosaccharides were extracted into chloroform (1 mL) and subsequently dried under a stream of nitrogen. Partially per-*O*-methylated oligomannosyl alditols were prepared for GC-MS analysis as previously described[33] for the purpose of determining sugar sequences and linkage patterns. In brief, all the above per-*O*-methylated samples were hydrolyzed with 250 μL of 2 M trifluoroacetic acid for 2 h at 120 °C, reduced with 100 μL of 10 mg/mL sodium borodeuteride solution ($NaBD_4$/2 M NH_4OH) at rt for 2 h and then acetylated with 100 μL acetic anhydride at 100 °C for 1 h.

FABMS analysis

FABMS in the positive-ion mode was performed on a Fisons VG Autospec equipped with liquid secondary ion monitor (LSIMS) using Cesium ion gas operated at 25 kV. Spectra were computer processed. The matrix was thioglycerol and derivatized oligosaccharides were dissolved in methanol prior to loading on the target. Samples were analyzed and spectra recorded as described.[34,35] Quantitation of all samples analyzed was performed in relation to the standards, namely, Manα1-6Manα-1-S-C_8/-O-C_8/-O-C_{10} and Manα1-6Manα1-6Manα-1-O-C_{10}.

GC–MS analysis

GC-mass spectrometry data were recorded on a Hewlett Packard Gas Chromatograph 5890 connected to Hewlett Packard 5790B Mass Detection. The partially methylated alditol acetates were dissolved in chloroform prior to injection on BPX5 (0.25 microns) SGE column (J&W Scientific) at 100 °C. The temperature was then increased to 125 °C over 30 min and held for 1 min before increasing to 180 °C for 5 min.

exo-Glycosidase digestions and partial acetolysis

Enzymatic reaction products were extracted and analyzed as described.[12,13] In brief, the [^{14}C]mannosylated products were dried and redissolved in 0.1 M sodium acetate buffer, pH 5, containing 0.1% (w/v) sodium taurodeoxycholate and incubated at 37 °C for 16 h with and without 1 U of jack bean α-mannosidase (30 μL final volume) (Oxford GlycoSystems).

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References

1. Daffe, M.; Draper, P. *Adv. Microb. Physiol.* **1998**, *39*, 131.
2. Chatterjee, D.; Khoo, K.-H. *Glycobiology* **1998**, *8*, 113.
3. Lee, R. E.; Brennan, P. J.; Besra, G. S. In *Current Topics in Microbiology and Immunology, Tuberculosis*; Shinnick, T. M., Ed.; Springer-Verlag: Heidelberg, 1996; Vol. 215, p 1.
4. Besra, G. S.; Brennan, P. *Biochem. Soc. Trans.* **1997**, *25*, 845.
5. Besra, G. S.; Chatterjee, D. In *Tuberculosis: Pathogenesis, Protection and Control*; Bloom, B. R., Ed.; American Society for Microbiology: Washington, DC, 1994; p 285.
6. Xin, Y.; Lee, R. E.; Scherman, M. S.; Khoo, K.-H.; Brennan, P. J.; Besra, G. S.; McNeil, M. *Biochem. Biophys. Acta* **1997**, *1335*, 231.
7. Chatterjee, D.; Hunter, S. W.; McNeil, M.; Brennan, P. J. *J. Biol. Chem.* **1992**, *267*, 6228.
8. Besra, G. S.; Moorehouse, C. B.; Rittner, C. M.; Waechter, C. J.; Brennan, P. J. *J. Biol. Chem.* **1997**, *272*, 18460.
9. Lee, R. E.; Brennan, P. J.; Besra, G. S. *Glycobiology* **1997**, *7*, 1121.
10. Ayers, J. D.; Lowary, T. L.; Moorehouse, C. B.; Besra, G. S. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 437.
11. Pathak, A. K.; Besra, G. S.; Crick, D.; Maddry, J. A.; Moorehouse, C. B.; Suling, W. J.; Reynolds, R. C. *Bioorg. Med. Chem.* **1999**, *7*, 2407.
12. Brown, J. R.; Güther, M. L. S.; Field, R. A.; Ferguson, M. A. J. *Glycobiology* **1997**, *7*, 549.
13. Brown, J. R.; Smith, T. K.; Ferguson, M. A. J.; Field, R. A. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2051.
14. Pingel, S.; Field, R. A.; Güther, M. L. S.; Duszenko, M.; Ferguson, M. A. J. *Biochem. J.* **1995**, *309*, 877.
15. Nikolaev, A. V.; Rutherford, T. J.; Ferguson, M. A. J.; Brimacombe, J. S. *J. Chem. Soc., Perkin Trans. 1* **1995**, 1977.
16. Ness, R. K.; Fletcher, H. G., Jr.; Hudson, C. S. *J. Am. Chem. Soc.* **1950**, *72*, 2200.
17. Lemieux, R. U.; Bundle, D. R.; Baker, D. A. *J. Am. Chem. Soc.* **1975**, *97*, 4076.
18. Corey, E. J.; Venkateswarlu, A. *J. Am. Chem. Soc.* **1972**, *94*, 6190.
19. Schultz, J.; Elbein, A. D. *Arch. Biochem. Biophys.* **1974**, *160*, 311.
20. Adamany, A. M.; Spiro, R. G. *J. Biol. Chem.* **1975**, *250*, 830.
21. Yokoyama, K.; Ballou, C. E. *J. Biol. Chem.* **1989**, *264*, 21621.
22. Tanaka, H.; Iwai, Y.; Oiwa, R.; Shinohara, S.; Shimizu, S.; Oka, T.; Omura, S. *Biochem. Biophys. Acta* **1977**, *497*, 633.
23. Tanaka, H.; Oiwa, R.; Matsukura, S.; Omura, S. *Biochem. Biophys. Res. Commun.* **1979**, *86*, 902.
24. Kang, M. S.; Spencer, J. P.; Elbein, A. D. *J. Biol. Chem.* **1978**, *253*, 8860.

25. Banerjee, D. K.; Scher, M. G.; Waechter, C. J. *Biochemistry* **1981**, *20*, 1561.
26. Brown, G. M.; Millar, A. R.; Masterson, C.; Brimacombe, J. S.; Nikolaev, A. V.; Ferguson, M. A. J. *Eur. J. Biochem.* **1996**, *242*, 410.
27. Palcic, M. M.; Heerze, L. D.; Pierce, M.; Hindsgaul, O. *Glycoconj. J.* **1988**, *5*, 49.
28. Ferrier, R. J.; Furneaux, R. H. *Methods Carbohydr. Chem.* **1980**, *8*, 251.
29. Field, R. A.; Otter, A.; Fu, W.; Hindsgaul, O. *Carbohydr. Res.* **1995**, *276*, 347.
30. Palcic, M. M.; Heerze, L. D.; Srivastava, O. P.; Hinsdgaul, O. *J. Biol. Chem.* **1989**, *264*, 17174.
31. Siakotos, A. N.; Rouser, G. *J. Am. Oil Chem. Soc.* **1965**, *42*, 913.
32. Dell, A. *Methods Enzymol.* **1990**, *193*, 647.
33. Albersheim, P.; Nevins, D. J.; English, P. D.; Karr, A. *Carbohydr. Res.* **1967**, *5*, 340.
34. Chatterjee, D.; Khoo, K.-H.; McNeil, M.; Dell, A.; Morris, H. R.; Brennan, P. J. *Glycobiology* **1993**, *3*, 497.
35. Dell, A. *Adv. Carbohydr. Chem. Biochem.* **1987**, *45*, 19.