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# Exploring the Substrate Specificity of a Mycobacterial Polyprenol Monophosphomannose-Dependent α-(1→6)-Mannosyltransferase

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*A series of synthetic α-(1→6)-linked octyl mannopyranoside oligomers was evaluated as potential acceptors of a polyprenol monophosphomannose-dependent α-(1→6)-mannosyltransferase that is involved in the biosynthesis of the mannan core of mycobacterial lipoarabinomannan. Initial evaluation demonstrated that the enzyme recognizes di-, tri- and tetramannosides (**5**, **6** and **7**) as substrates with different activities. While the highest mannosyltransferase activities were observed when the di- and trisaccharide were used as substrates, diminished enzymatic activity was seen with the tetramannoside. As octyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranoside (**5**) appears to be the minimum structural element required for mannosyltransferase catalysis, a panel of methoxy and deoxy disaccharide analogues (**8**–**21**) were used to probe the substrate specificity of the enzyme further. In terms of the steric requirements at the active site, the enzyme does not recognize either C2′- and C2-methoxy analogues as substrates, a result that suggests that the α-(1→2)-mannopyranosyl branches, which are present in the mannan core of LAM must be added on a larger α-(1→6)-oligomannan intermediate. In contrast, the presence of a methoxy functionality at the C3′, C3, C4′ and C4 positions are somewhat tolerated by the enzyme, although diminished enzyme activities were observed with the C4′- and C4-methoxy analogues. Moreover, the 2′- and 4-hydroxyl groups appear not to be critical for substrate binding at the active site, as both 2′- and 4-deoxy analogues are substrates for the enzyme. In contrast, replacement of the hydroxyl groups at other positions essentially abolished enzymatic activity. Further kinetic characterization of the enzyme by using the effective acceptor substrates gave apparent $K_M$ values ranging from 111 to 437 μM, which are within two-fold higher or lower than that for the parent dimannoside (**5**). Although the $K_M$ values indicate that the enzyme binds those acceptors with comparable affinities, the C4′-methoxy analogue (**12**) turns over more slowly than the others, as indicated by the apparent $V_{max}$ values.*

## Introduction

Tuberculosis (TB), which is caused by the bacterium *Mycobacterium tuberculosis*, is a disease that kills nearly three million people worldwide each year.[1–3] TB has been the subject of increasing recent concern due not only to difficulties in treating the disease in individuals with compromised immune systems (e.g., those with HIV/AIDS[4]) but also due to the emergence of *M. tuberculosis* strains that are resistant to one or more of front-line anti-TB drugs.[5] In particular, extremely-drug resistant TB (XDR-TB) has been the subject of substantial recent media coverage[6] stemming in part from an incident[6c] in which an individual, who was thought to be infected with an extreme drug-resistant strain travelled between Europe and North America, potentially infecting large numbers of people. While it was subsequently shown that this individual did not have XDR-TB, but the rather less serious multi-drug resistant TB,[6d] this incident underscored the contagious nature of the disease and the need for new anti-TB agents.

Successful treatment of TB and other mycobacterial diseases requires a protracted drug regimen that involves multiple antibiotics.[7] Such intensive treatments are made necessary by the unusual structure of the mycobacterial cell wall, which both protects the organism from the immune system of the host, and also serves as a formidable barrier to the passage of therapeutic agents.[8] The two major entities of the mycobacterial cell wall are the mycolyl-arabinogalactan (mAG) complex and lipoarabinomannan (LAM).[8] The largest structural component of the cell wall is the mAG, which serves as a permeability barrier to the passage of antibiotics.[8] LAM is a major antigenic component of the cell wall and a number of recent studies have clearly shown that this glycoconjugate is an important modulator of the immune response that arises from mycobacterial infections.[9]

LAM contains four major structural features (**1**, Scheme 1): a phosphatidylinositol (PI) anchor, a core mannan chain, an arabinan domain and terminal capping motifs, which are found at the non-reducing end of the molecule. The polysaccharide, together with its truncated analogues, lipomannan (LM) and the phosphatidyl-*myo*-inositol mannosides (PIMs), constitute the major lipoglycans of the mycobacterial cell wall.[8,9] In LAM, the PI anchor is non-covalently attached to the plasma membrane

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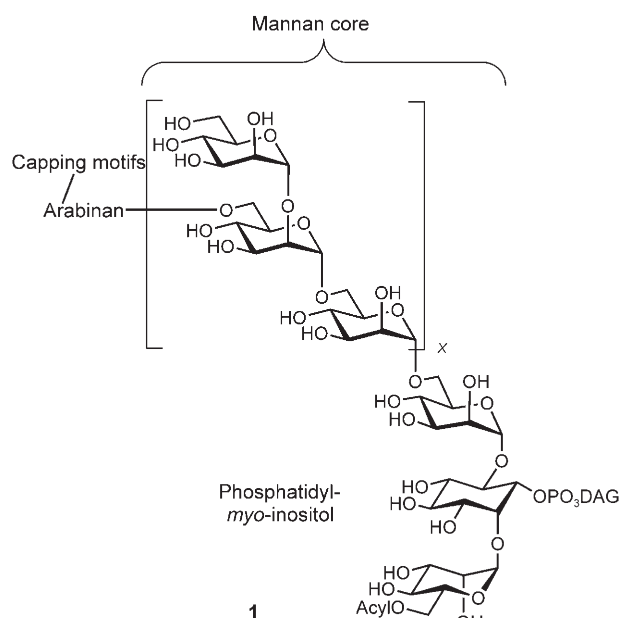


**Scheme 1.** Structure of LAM in which the mannan core is highlighted; DAG = diacylglycerol.

and the attached polysaccharide extends to the exterior of the cell wall complex. The mannan core is a polymer of ~20–25 mannopyranose residues that are linked α-(1→6), and this linear structure is further elaborated by additional α-mannopyranose units on approximately half of these residues. In species such as *M. tuberculosis*, *M. leprae*, *M. kansasii* and *M. smegmatis*[9] these side-chains are attached to O-2 of the α-(1→6)-linked mannopyranose residues, while in *M. chelonae* they are attached to O-3.[10] The arabinan domain of LAM is a highly branched motif that is composed of α-(1→5)-, α-(1→3)-, and β-(1→2)-linked arabinofuranose residues, the exact structure and complexity of which depends on the identity of the mycobacterial species.[11–13] The nature of the LAM capping motifs are also species-specific and these groups include short oligomannopyranosides (*M. tuberculosis, M. leprae, M. bovis* and *M. avium*),[14] inositol phosphate motifs (*M. smegmatis*)[15] and 5-thiomethyl-xylofuranose residues (*M. tuberculosis*).[16]

A model for the biosynthesis of mycobacterial LAM was proposed ten years ago by Brennan and co-workers (Scheme 2),[17] and since that time many of the steps in this process have been supported by biochemical and genetic evidence.[18–26] Of particular relevance to the work that is described here is that the biosynthesis of the mannan core has received significant scrutiny, and current evidence points to a process in which a number of acyltransferases[19] and mannosyltransferases (ManT's),[20,23–26] and act in concert by adding single monosaccharide residues and acyl groups to PI; this leads initially to AcPIM2 and then to AcPIM4, which serves as a key branch point between PIM and LM/LAM biosynthesis. From AcPIM4, the α-(1→6)-linked backbone of the mannan core is assembled, and the α-(1→2)-linked mannopyranosyl side-chains are introduced.

Whether the α-(1→2)-linked residues are introduced after, or simultaneously with the formation of the α-(1→6)-linked backbone is unknown. However, in the initial model that was suggested by Brennan and co-workers, the α-(1→2)-branching was proposed to follow the complete synthesis of the α-(1→6)-linked mannan.[17]

The donor substrates for these ManTs are either GDP-mannose (GDP-Man, **2**) or polyprenolphosphomannose (PPM, **3**), which is synthesized from **2** by the enzyme polyprenol monophosphomannose synthase.[22] The ManTs that are involved in the initial biosynthetic steps that lead to AcPIM2 and AcPIM4, including PimA, PimB and PimC (only found in *M. tuberculosis* CDC1551), which use GDP-Man as the donor species, have received the most attention.[20] Among the achievements in this area is the recent report of a crystal structure of PimA in complex with GDP-Man.[27] In contrast, the enzymes that use PPM as the donor species have been less well studied, but three PPM-dependent α-(1→2)-ManT's, which are involved in LM/LAM core branching,[24] arabinan motif capping,[25] and AcPIM6 biosynthesis[26] have been identified. To date, the PPM-dependent α-(1→6)-ManT that is involved in LM/LAM assembly has remained elusive, but a cell-free assay for its activity has been developed and has been used to screen the potential substrates and inhibitors of the enzyme.[22,28–34] It is, at this point, unclear whether a single ManT is responsible for the installation of all the α-(1→6)-linked residues of the core mannan. Indeed, very recently, through genetic knockout and complementation studies, Brennan and coworkers have provided evidence that suggests that more than one α-(1→6)-ManT might be involved in the full-length LM/LAM biosynthesis.[35]

Given the important roles of LM and LAM in the progression of mycobacterial disease, a better understanding of their biosynthesis is of interest. Our efforts in this area have been focused on probing the substrate specificity of the PPM-dependent ManT that are responsible for the synthesis of the α-(1→6)-linked mannan core of LM and LAM.[33,34] The specificity of this enzyme remains poorly understood, and knowledge of the steric and hydrogen-bonding requirements in the active site of the enzyme would facilitate the design of potent and specific inhibitors. Herein, a panel of octyl mannopyranoside analogues (Scheme 3) were screened against this PPM-dependent mannosyltransferase. A homologous series of mono- through tetrasaccharides (**4**–**7**) was synthesized and screened to probe the effect of acceptor length on activity. In addition, a panel of methoxy and deoxy analogues (**8**–**21**) of the known[31] disaccharide substrate α-D-Man*p*-(1→6)-α-D-Man*p*-$O(CH_2)_7CH_3$ (**5**), were evaluated to explore the acceptor specificity of the enzyme further. Singly modified oligosaccharide analogues such as **8**–**21** have been of great utility in probing carbohydrate–protein interactions,[36] and these studies have provided compounds that are not only useful biochemical tools but have also led to the identification of potent glycosyltransferase inhibitors. In the present case, compounds with the latter activity are potential lead compounds for new classes of anti-mycobacterial agents.

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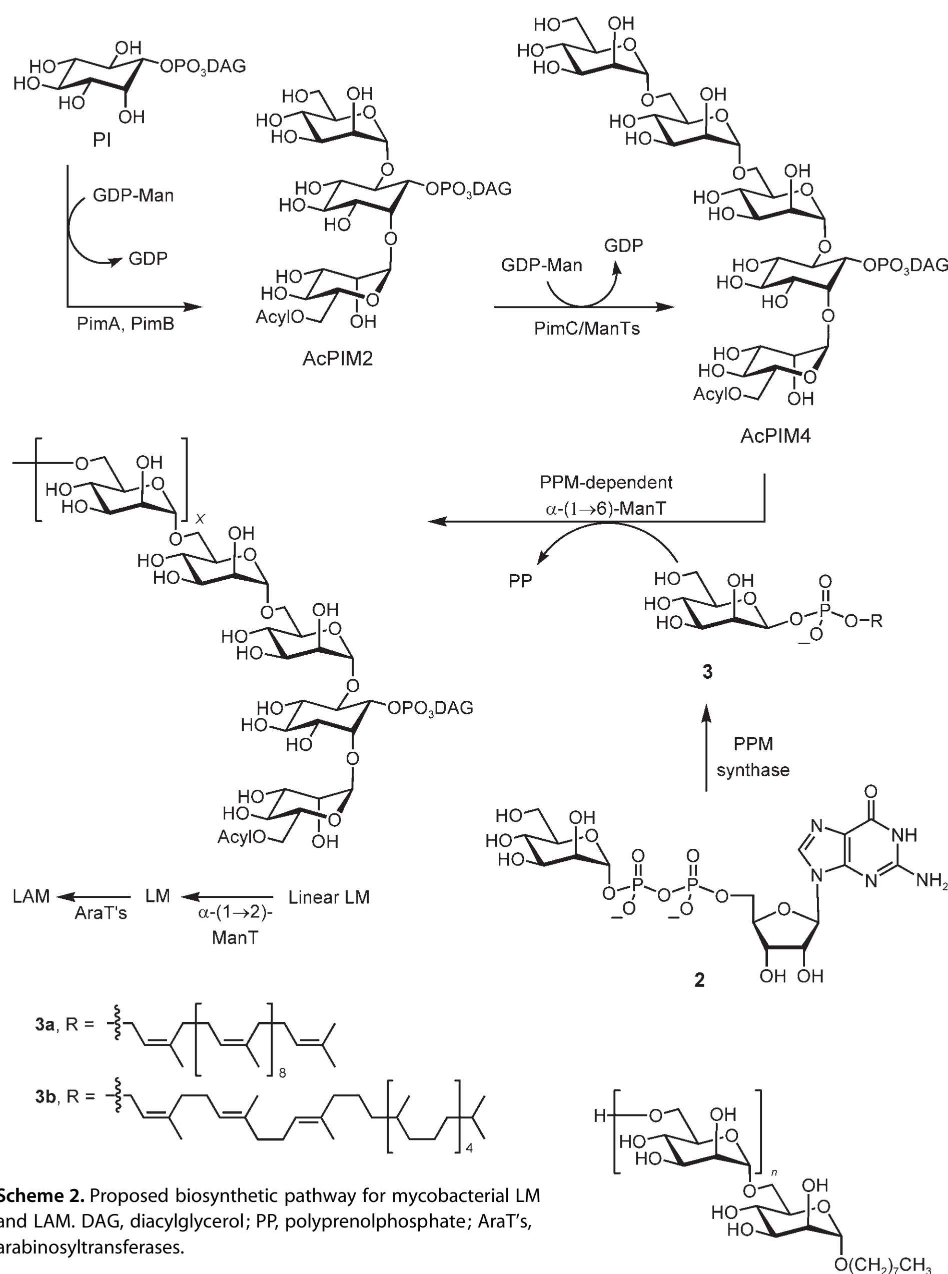


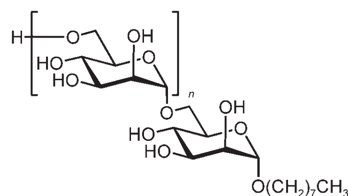

**Scheme 2.** Proposed biosynthetic pathway for mycobacterial LM and LAM. DAG, diacylglycerol; PP, polyprenolphosphate; AraT's, arabinosyltransferases.

## Results and Discussion

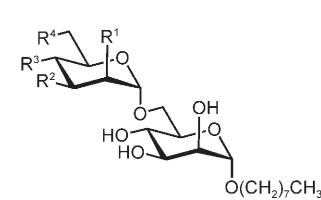
The activity of a PPM-dependent α-(1→6)-mannosyltransferase in a membrane preparation from *M. smegmatis* was first demonstrated by Yokoyama and Ballou[28] and a cell-free assay was later developed by Brown et al.[31] Both studies showed that the ManT utilizes β-D-mannopyranosyl phosphodecaprenol (**3**), which is synthesized in situ from **2** and decaprenol phosphate, as the donor and catalyzes the α-(1→6) mannosylation of oligomannopyranoside acceptors. In particular, the latter studies demonstrated that octyl dimannopyranoside **5** (Scheme 3) is a good acceptor for the enzyme.[31] The hydrophobic nature of the octyl agylcone allows convenient product isolation and characterization after the assays,[38] and a small panel of analogues of **5** was recently screened as potential substrates and inhibitors of the enzyme.[33,34] In this paper, a larger series of synthetic octyl mannopyranoside acceptors was tested as substrates for ManT under the established assay conditions to probe the specificity of the enzyme further.[28–34]

### Synthesis of octyl mannopyranosides 5–7

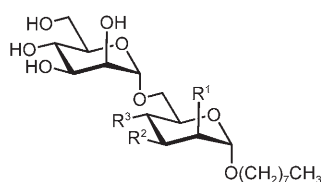
Considering that AcPIM4, which is the proposed initial substrate for the PPM-dependent α-(1→6)-ManT consists of a linear α-(1→6)-trimannopyranoside, oligomannosides that are longer than two residues might be better acceptors. Indeed, the early studies of Yokoyama and Ballou demonstrated that longer methyl oligomannosides could act as substrates for this α-(1→6)-ManT with improved $K_M$ values.[28] To determine the effect of the acceptor length of the octyl glycoside counterparts on ManT catalysis, oligosaccharides **5**–**7** were synthesized from the



**4**, $n$ = 0
**5**, $n$ = 1
**6**, $n$ = 2
**7**, $n$ = 3



**8**, $R^1 = OCH_3$, $R^2$ = OH, $R^3$ = OH, $R^4$ = OH
**9**, $R^1$ = H, $R^2$ = OH, $R^3$ = OH, $R^4$ = OH
**10**, $R^1$ = OH, $R^2 = OCH_3$, $R^3$ = OH, $R^4$ = OH
**11**, $R^1$ = OH, $R^2$ = H, $R^3$ = OH, $R^4$ = OH
**12**, $R^1$ = OH, $R^2$ = OH, $R^3 = OCH_3$, $R^4$ = OH
**13**, $R^1$ = OH, $R^2$ = OH, $R^3$ = H, $R^4$ = OH
**14**, $R^1$ = OH, $R^2$ = OH, $R^3$ = OH, $R^4 = OCH_3$
**15**, $R^1$ = OH, $R^2$ = OH, $R^3$ = OH, $R^4$ = H



**16**, $R^1 = OCH_3$, $R^2$ = OH, $R^3$ = OH
**17**, $R^1$ = H, $R^2$ = OH, $R^3$ = OH
**18**, $R^1$ = OH, $R^2 = OCH_3$, $R^3$ = OH
**19**, $R^1$ = OH, $R^2$ = H, $R^3$ = OH
**20**, $R^1$ = OH, $R^2$ = OH, $R^3 = OCH_3$
**21**, $R^1$ = OH, $R^2$ = OH, $R^3$ = H

**Scheme 3.** Synthetic acceptor analogues that were used as probes of the PPM-dependent ManT that is involved in the synthesis of the α-(1→6)-linked mannan core of LM and LAM.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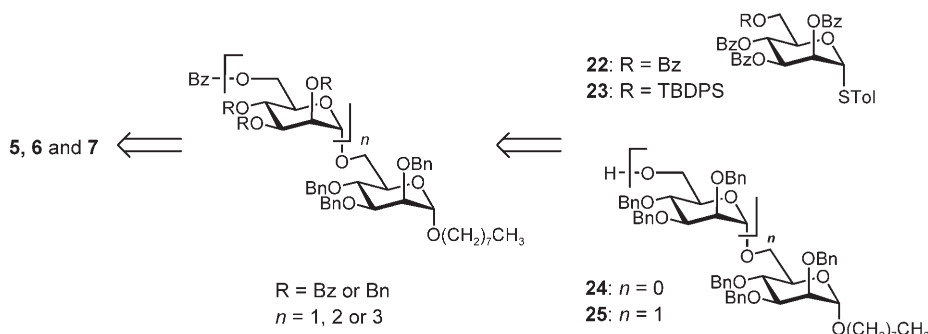
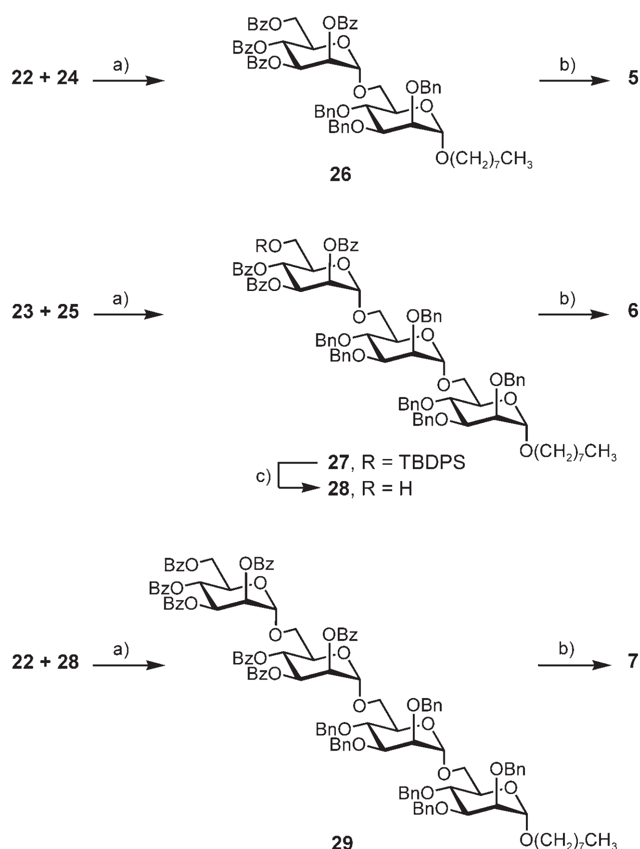
known thioglycosides **22** and **23**[37,39] and acceptors **24** and **25**[33,37] as shown in Scheme 4 by using the overall general strategy that was developed by Watt and Williams.[39]

As illustrated in Scheme 5, the coupling of thioglycoside **22** with alcohol **24** by using *N*-iodosuccinimide–trimethylsilyl tri-




**Scheme 4.** Retrosynthetic analysis of **5**, **6** and **7**.




**Scheme 5.** Reagents and conditions: a) NIS, TMSOTf, 4 Å MS, $CH_2Cl_2$, 0 °C, 96 % for **26**, 98 % for **27**, 90 % for **29**; b) *i.* $NaOCH_3$, MeOH or MeOH/$CH_2Cl_2$ (8:1); *ii.* $H_2$, 20 % $Pd(OH)_2$, MeOH; over two steps: 87 % for **5**, 95 % for **6**, 62 % for **7**; c) HF·pyridine/pyridine/THF (1:2:5), 90 %.

flate (NIS–TMSOTf) activation[40] afforded the corresponding disaccharide **26** in 96 % yield. Subsequent debenzoylation of **26** by using sodium methoxide followed by hydrogenolysis afforded the desired disaccharide **5** in 87 % over two steps.[33] Under the same glycosylation conditions, the coupling of donor **23** and acceptor **25** afforded trisaccharide **27** in excellent yield (98 %). To avoid possible acyl migration and debenzoylation that can occur by using tetra-*n*-butylammonium fluoride, intermediate **27** was desilyated by using hydrogen fluoride in pyridine to give **28** in 90 % yield.[41] Subsequent removal of the benzoyl and benzyl protecting groups under standard conditions afforded the target trimannoside **6** in 95 % overall yield.[39] With alcohol **28** in hand, coupling with thioglycoside **22** under NIS–TMSOTf activation provided the corresponding protected tetrasaccharide **29** in good yield (90 %). Final deprotection furnished the desired oligomannoside **7** in 62 % yield over two steps.[39] In the glycosylation reactions described above, the α-stereochemistry of the glycosidic linkages was confirmed by the one-bond $^1J_{C-1,H-1}$ heteronuclear coupling constants for the anomeric carbon atoms.[42] For all products, this value was between 167 and 174 Hz, which clearly indicates the α-stereochemistry.

### Optimal length of oligomannopyranosides for ManT catalysis

To determine the optimal length of oligomannopyranosides for ManT catalysis, oligosaccharides **4**–**7** were screened as potential substrates for the enzyme in a cell-free assay by using $^3$H-labeled GDP-mannose (**2**, Scheme 2), which is converted to PPM (**3**, Scheme 2), by the membrane fraction.[31] The results are summarized in Table 1. At 2 mм concentration, disaccharide **5** and trisaccharide **6** were the best acceptor substrates for

**Table 1.** Comparison of the ManT activities by using potential acceptors **4**–**7**.

| Acceptor | Relative activity [%][a] | Mass of oligosaccharide product[b] | | Glycosidic linkage formed by enzymatic reaction[c] |
|---|---|---|---|---|
| | | calcd | found | |
| **4** | 8 ± 2 | 477.5 | 477.5 | n.d.[d] |
| **5** | 100 ± 2 | 639.3 | 639.5 | α-(1→6) |
| **6** | 108 ± 4 | 801.4 | 801.7 | α-(1→6) |
| **7** | 54 ± 10 | 963.9 | 963.9 | n.d. |

[a] Relative activities were measured at 2.0 mм acceptor concentration with 0.2 μCi of [$^3$H] GDP-Man and are expressed with respect to disaccharide **5**. 100 % activity corresponds to 0.36 pmol mg$^{-1}$ min$^{-1}$. [b] The enzymatic products were isolated from large-scale incubations and their masses were determined by MALDI mass spectrometry. The calculated and found values correspond to the sodium adducts. [c] The structure of the enzymatic products were elucidated by $^1$H NMR spectroscopy. The chemical shifts of the anomeric protons were shown to be identical with those of the authentic tri- and tetrasaccharides obtained by chemical synthesis. [d] Not determined.

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the enzyme, and showed roughly comparable activities. On the other hand, monosaccharide **4** and tetrasaccharide **7** were relatively poor substrates for the ManT; they possessed only 8 and 54% activity relative to **5**, respectively. Our findings are consistent with the previous report by Yokoyama and Ballou,[28] in which methyl α-D-mannopyranoside was shown to act as an acceptor with much lower efficiency than larger oligomers.

The lower efficiency of tetramannoside **7** to act as an acceptor substrate may result from the presence of an endogenous α-(1→6)-*endo*-mannosidase, which catalyzes the removal of trisaccharide units from the nonreducing end of the pentasaccharide product, as was previously observed.[28] The simultaneous synthesis and degradation of a pentasaccharide product from **7**, by ManT and mannosidase activities, respectively, would be expected to lead to the lower apparent acceptor efficiency of the tetrasaccharide. It is also possible that the tetrasaccharide substrate is degraded by this α-(1→6)-*endo*-mannosidase to afford a trisaccharide that lacks an octyl group (and which would therefore not be detected in the assay) and octyl α-D-mannopyranoside, **4**, which is a very poor substrate for the ManT. In the mass spectrum of the product that was obtained from the incubation mixtures with **7** (below), a peak that corresponds to **4** was detected, which supports this hypothesis. In addition, treatment of the tetrasaccharide under the assay conditions, both with and without the donor substrate, followed by TLC analysis revealed the formation of mono-, di-, and trisaccharides (see Figure S1 in the Supporting Information). To date, only limited information about the specificity this α-(1→6)-*endo*-mannosidase is available,[28] and no structural information on this protein, or to the best of our knowledge any other *endo*-mannosidase has been reported. This dearth of information makes it difficult to make a more definitive statement about the interference of this glycosidase with the assay. Nevertheless, based on these results, it is possible to conclude that the disaccharide unit appears to be the minimal-length acceptor that is required for ManT catalysis, and that significant increases in activity are not observed by increasing the size of the acceptor to a trisaccharide.

### Milligram-scale incubations and product characterizations

In addition to the PPM-dependent α-(1→6)-mannosyltransferase, the crude membrane extract of *M. smegmatis* that was used in these assays also contained α-(1→2)-ManTs. For example, recent studies have identified three PPM-dependent α-(1→2)-ManTs that are involved in LM branching, the capping of the arabinan domain, and the biosynthesis of AcPim6.[24–26] In addition, the earlier study by Yokoyama and Ballou detected trace amounts of products that arise from α-(1→2)-ManT activity.[28] To confirm that the observed addition of radiolabeled mannose to **4**–**7** arose from α-(1→6)-ManT activity, and not from α-(1→2)-ManT activity, more detailed structural characterization of the products was required. Therefore, in addition to the radiochemical assays described above, milligram-scale enzymatic incubations of **4**–**7** with unlabelled GDP-Man and the membrane fraction were carried out. After the incubations, the enzymatic products were purified by using a $C_{18}$ SepPak cartridge,[38] and then were analyzed by MALDI mass spectrometry. As shown in Table 1, these analyses confirmed that a single mannopyranose unit was transferred to each of the acceptor substrates that were examined. In addition, TLC analysis (data not shown) of the products clearly showed that the $R_f$ values of the enzymatic products were identical to those of the authentic samples that were obtained by chemical synthesis (above). Finally, the enzymatic products from the incubations of acceptors **5** and **6** were purified by preparative TLC and the structures of the products were analyzed by $^1$H NMR spectroscopy. Based on the mass spectrometry results, the product of the reaction with disaccharide **5** would be **6**, and trisaccharide **6** would yield tetrasaccharide **7**. Comparison of the anomeric region of the spectrum of the product that was obtained from the incubation of **5** with the membrane preparation and GDP-Man revealed excellent agreement with the spectrum of an authentic synthetic **6**. Similarly, the spectrum of the product that was obtained for the reaction with **6** was a match with an authentic sample of **7** (Supporting Information). As further support for structure, the product that resulted from tetrasaccahride **7**, a pentasaccharide, was treated with an α-(1→2)-specific mannosidase,[26,43] and, as determined by TLC, no cleavage of the polysaccharide was observed (see Figure S2 in the Supporting Information).

Given the proposed processive nature of this ManT, it would be expected that a homologous series of products would be observed in these incubations. In an earlier study,[31] by monitoring the transfer of $^{14}$C-labelled mannose to the acceptor, disaccharide **5** was shown to give predominantly trisaccharide **6** and trace amounts of tetrasaccharide **7**. In our hands, the larger oligomers were not observed from TLC analyses. This is likely due to the poor sensitivity of this detection method compared the radiochemical method that was used earlier,[31] as well as the existence of an extremely small amount of the longer oligosaccharides compared to the products that result from the addition of a single mannose residue. Further complicating the situation is the presence of the aforementioned (1→6)-*endo*-mannosidase, which would preferentially degrade the longer oligomers. In the MALDI-MS analyses, although longer oligosaccharides were observed (e.g., disaccharide **5** going to trisaccharide **6** and tetrasaccharide **7** and similar observations when using **4** and **6** as the substrates), the peak intensities were comparable to background noise. While the lack of the longer oligomers could be the result of the cleavage of the products by the *endo*-mannosidase, it could also be that these oligosaccharides are generally poor substrates compared to those that are present in the natural system, which would include the AcPIM4 core (Scheme 2). The relative importance of these issues is impossible to resolve in the absence of a pure ManT that is free from any *endo*-mannosidase activity.

### Kinetic characterization of 5–7

Acceptors **5**, **6** and **7** were sufficiently active to allow further kinetic characterization, and representative examples of the kinetic experiments are shown in Figure 1. First, to determine the quantity of the membrane preparation that is needed for

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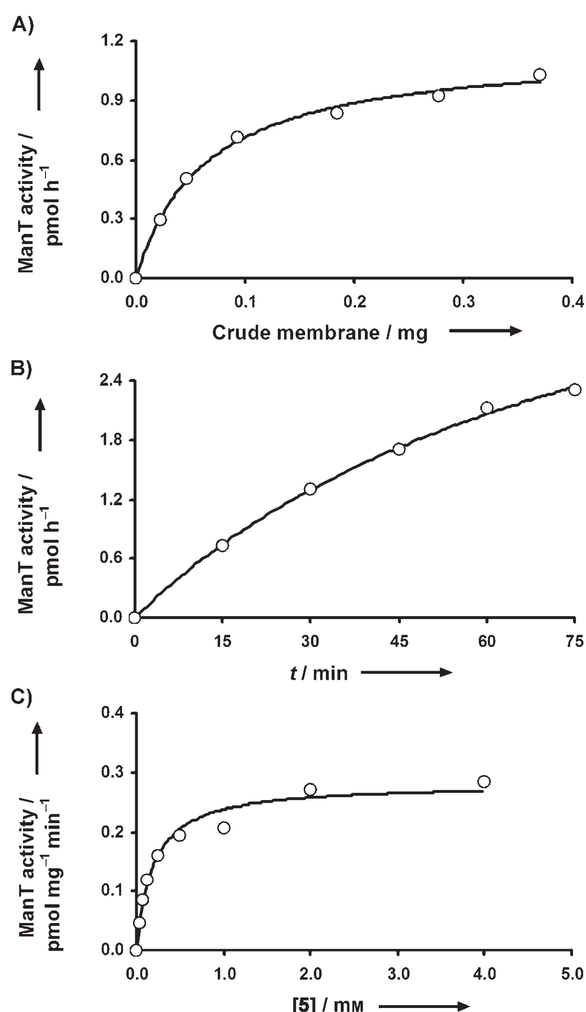


**Figure 1.** Representative graphs for ManT kinetics with acceptor **5**. A) Incorporation of radiolabeled [$^3$H]-mannose into product relative to protein amount. The activity of the enzyme was determined by using different protein amounts. All other reaction conditions were identical to those described in the cell-free assay as described in the experimental section. B) Incorporation of radiolabeled [$^3$H]-mannose into **5** as a function of time. An acceptor concentration of 4 mM was used and the assays were terminated at the indicated time points. C) Incorporation of radiolabeled [$^3$H]-mannose into **5** as a function of acceptor concentration. Assays were performed at different substrate concentrations (0.03, 0.06, 0.13, 0.25, 0.5, 1, 2, and 4 mM). Control experiments without the addition of acceptor were also performed in parallel. The data obtained were subjected to nonlinear regression analysis by using GraphPad Prism 4.0.

the assays, we studied product formation from **5** as a function of the mass of the membrane fraction. As shown in Figure 1 A, product formation was proportional to the amount of cell membrane preparation that was added to the assays up to ~100 ug, at which point, the product formation reached a plateau. Also, with acceptor **5**, the incorporation of the radiolabeled mannose was linear with time up to 60 min (Figure 1 B). This linear correlation between the incorporation of radiolabeled product and time was also observed in the subsequent kinetic studies by using other acceptor substrates. Finally, the reaction rate with **5** reached a plateau at acceptor concentrations above 2 mM. The Michaelis–Menten plot for **5** is given in Figure 1 C and those for **6** and **7** can be found in the Supporting Information. As shown in Table 2, the apparent $K_M$ values for these compounds were in the range of 147 to 234 μM. The

**Table 2.** Kinetic parameters for oligosaccharide substrates of ManT[a]

| Analogue | $K_M$ [μM] | $V_{max}$ [pmol mg$^{-1}$ min$^{-1}$] |
|---|---|---|
| **5** | 188 ± 33 | 0.28 ± 0.013 |
| **6** | 234 ± 50 | 0.25 ± 0.015 |
| **7** | 147 ± 27 | 0.13 ± 0.001 |
| **10** | 201 ± 39 | 0.16 ± 0.010 |
| **12** | 437 ± 104 | 0.072 ± 0.010 |
| **18** | 216 ± 31 | 0.22 ± 0.010 |
| **20** | 272 ± 56 | 0.16 ± 0.010 |
| **9** | 307 ± 49 | 0.23 ± 0.010 |
| **21** | 111 ± 25 | 0.23 ± 0.012 |

[a] Kinetic parameters were determined by using a range of acceptor concentrations (0.03 to 4.0 mM) by nonlinear regression analysis of the Michaelis–Menten equation with the GraphPad Prism 4.0 program.

$V_{max}$ values for **5** and **6** were very similar, 0.28 and 0.25 pmol mg$^{-1}$ min$^{-1}$, respectively. On the other hand, the $V_{max}$ value for tetrasaccharide **7** was reduced by 2.2-fold (0.13 pmol mg$^{-1}$ min$^{-1}$), compared to that of disaccharide **5**. However, as discussed above, it is plausible that the apparent slower turnover of acceptor **7** might be due to the simultaneous degradation of the radiolabeled product and the acceptor substrate, which consequently affects the apparent kinetic parameters of **7**.

### Acceptor specificity of mannosyltransferase by using analogues of disaccharide 5

Because the above-presented data demonstrated that the octyl dimannoside **5** appeared to be the minimal acceptor required for ManT catalysis, and that the trisaccharide did not lead to substantially better activity, a panel of disaccharide analogues **8**–**21** were screened to explore the enzyme substrate specificity further. In **8**–**21**, one of the hydroxyl groups of the parent disaccharide has either been replaced with a methoxy group, or deoxygenated. The synthesis of this panel of disaccharides has been described previously[37] and with them in hand, their ability to act as acceptor substrates for the ManT were compared with the parent compound **5** (Figure 2). Among the disaccharides synthesized, only **8** and **9**, which are the C-2′-methoxy and C-2′-deoxy analogues of **5**, respectively, had been tested against the ManT enzyme previously.[33] Our findings here are consistent with that previous report, which showed that the C-2′-deoxy disaccharide **9** but not **8** served as a substrate for the ManT. Interestingly, the 2-methoxy analogue **16** was also inactive, and the 2-deoxy analogue **17** was also a poor substrate for ManT with a relative mannosylation rate of 11 %. The results with the methoxy analogues **8** and **16** demonstrate that the enzyme is not tolerant of bulky substituents at the C-2′ and C-2 positions. These results have implications in the overall pathway for LAM biosynthesis because they suggest that all (or at least more than two) of the α-(1→6)-linked

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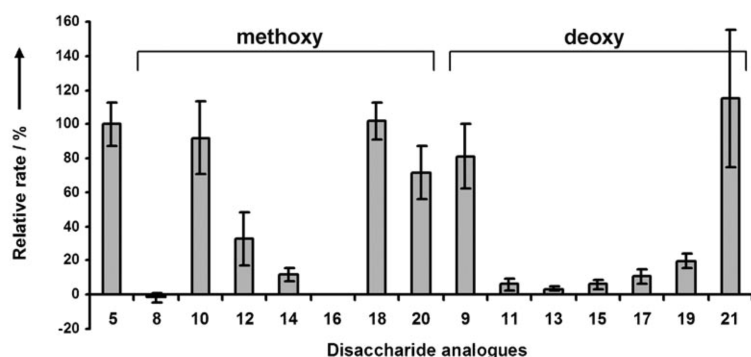



**Figure 2.** Acceptor specificity of the mycobacterial mannosyltransferase with disaccharide **5** and derivatives. Each acceptor was tested at 2 mM and was incubated with 0.05 μCi of [$^3$H] GDP-Man under the assay conditions as described in the experimental section. Relative activities for each acceptor are expressed as a percentage of the incorporation of [$^3$H]-mannose into the parent compound **5**. 100% activity corresponds to 2.67 pmol mg$^{-1}$ h$^{-1}$ α-(1→6)-ManT activity.

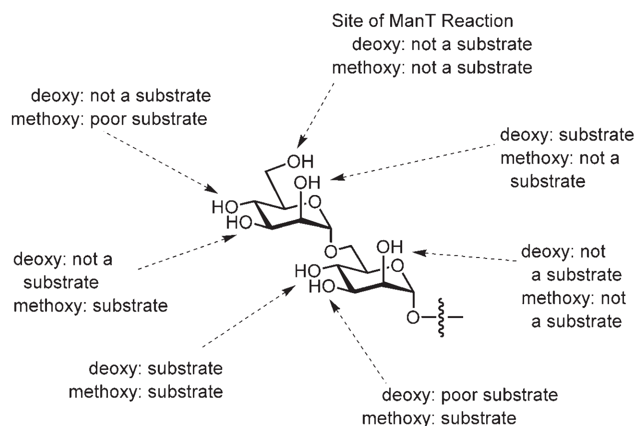
residues must be assembled before the α-(1→2)-mannopyranosyl branch points are attached. If the relatively small methyl group shuts down the α-(1→6)-ManT activity, a much larger monosaccharide residue would be expected to do the same. While this hypothesis should be studied with longer oligomers, these results support the original model for the introduction of these branching residues into the polymer.[17] In addition, the data that were obtained with the deoxy analogues **9** and **17** show that while the C2′ hydroxyl group does not appear to form a critical hydrogen bond with the enzyme, the interaction between the C-2 hydroxyl group and the protein appears to be essential for activity.

Although both the methoxy analogues at C2′ and C2 (**8** and **16**) are not substrates for the enzyme, methylation of O-3′/O-3 and O-4′/O-4 of disaccharide **5** resulted in compounds (**10**, **12**, **18** and **20**) that are accepted by the ManT, but a substantial decrease in activity is observed for the 4′-methoxy analogue, **12**. In terms of the polar interactions with the enzyme, the hydroxyl groups at C-3′, C-4′, C-2 and C-3 of **5** seem to be essential for recognition because deoxygenation of any of them produces compounds (**11**, **13**, **17** and **19**) that are essentially inactive. Similar to the 2′-deoxy derivative **9**, deoxygenation at C-4, which leads to compound **21** does not influence recognition by the enzyme.

Finally, although lacking a reactive C-6′ hydroxyl group, small amounts of [$^3$H] mannosylated products are detected when the **6**′ methoxy and deoxy analogues, **14** and **15**, respectively, are screened as acceptor substrates. These unexpected observations could be explained by the presence in the crude cell membrane preparation of an α-(1→2)-ManT, as was previously noted by Yokoyama and Ballou.[28] While the NMR spectra of the products that were obtained from the incubations of **5** and **6** demonstrated the formation of α-(1→6)-linkages, it is possible that some α-(1→2)-ManT activity is present in levels that cannot be detected by a relatively insensitive technique such as NMR spectroscopy. While it is expected that this membrane fraction contains the ManT that is responsible for the addition of the α-(1→2)-linked branches in the core mannan, if one assumes that the enzyme normally recognizes a longer mannan substrate, it is conceivable that disaccharide **5** is only a very weak substrate for the α-(1→2)-ManT. Therefore, this activity can only be detected when using compounds (e.g., **14** and **15**) that cannot act as α-(1→6)-ManT substrates.

Unfortunately, the small turnover that is observed for these compounds precluded the isolation and characterization of the products. However, to test if the introduction of α-(1→2)-linked mannopyranose residues into **14** and **15** was responsible for the observed radioactivity transfer, the products of the reactions were treated with an α-(1→2)-specific mannosidase and then the amount of radioactivity quantitated again (see Table S1 in the Supporting Information). This treatment led, in the case of **14**, to no appreciable decrease in radioactivity and, in the case of **15**, only a marginal reduction; this suggests that the formation of (1→2)-linked mannopyranosyl linkages was not leading, at least exclusively, to the apparent substrate activity of these analogues. We remain unsure as to the origin of radioactivity transferred to **14** and **15** but other possibilities include the ability of these compounds to serve as substrates for another mannosyltransferase in the membrane fraction (e.g., the α-(1→4)-ManT that is involved in the biosynthesis of 3-*O*-methyl-mannose polysaccharides[44]), or the cleavage of these substrates by an endogenous mannosidase to a monosaccharide that is then a substrate for the ManT. We note, however, that the latter possibility is problematic given the data that is provided in Table 1 and earlier studies[28] that demonstrate that monosaccharides are poor substrates for the enzyme. The possibility that **14** and **15** were contaminated with small amounts of the parent disaccharide, **5**, was ruled out by the MS and NMR spectroscopic data that was obtained for these compounds.[37]

A graphical summary of the results of screening disaccharides **8**–**21** against the enzyme is presented in Scheme 6.




**Scheme 6.** Summary of the substrate specificity of the ManT. "Not a substrate" refers to a relative ManT activity ≤ 12% of that for the natural substrate **5**; "poor substrate" refers to a relative ManT activity ≤33% of that for the natural substrate **5**.

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**Kinetic parameters of selected disaccharide analogues**

Among the disaccharide analogues that were screened, compounds **9**, **10**, **12**, **18**, **20**, and **21** were the most efficient acceptor substrates for the ManT, with relative activities $>20\%$ of that for the natural substrates **5**. Full kinetic characterization of these six compounds was carried out, and their kinetic constants are listed in Table 2; the Michaelis–Menten plots for these disaccharide analogues can be found in the Supporting Information. The kinetic constants for these analogues were generally similar to those that were observed with the parent compound **5**. For example, compounds **9**, **10**, **18**, **20**, and **21** had $K_M$ values that ranged from 111–307 μM and $V_{max}$ values of 0.16–0.23 $pmol\,mg^{-1}\,min^{-1}$, which are comparable to the values that were obtained for **5** ($K_M = 188$ μM and $V_{max} = 0.28\,pmol\,mg^{-1}\,min^{-1}$). The only exception was the 4′-methoxy analogue **12**, which has a $K_M$ (437 μM) that is ~2.3-fold higher than that of **5**, and is also significantly larger than the other disaccharide analogues that were evaluated (Table 2). In addition, **12** has a much smaller $V_{max}$ ($0.072\,pmol\,mg^{-1}\,min^{-1}$), which indicates that this acceptor turns over significantly more slowly than the other derivatives. It is possible that the steric bulk of the methyl group hinders mannosylation of the 6′-OH group by the enzyme, as has been observed previously in a substrate for N-acetylglucosaminyltransferase-V (GlcNAcT-V).[45] In this earlier example, the glycosylation of a mannose 6-OH group in a trisaccharide substrate for GlcNAcT-V, was inhibited by methylation at O-4 of the mannose residue undergoing glycosylation. Apparently, the C-4′ hydroxyl group of the dimannoside acceptor not only plays an important role in enzyme catalysis, but also in substrate recognition because deoxygenation at this position resulted in no enzymatic activity.

## Conclusions

In this paper, we report studies on the substrate specificity of a PPM-dependent α-(1→6)-mannosyltransferase that is involved in the mycobacterial LAM biosynthesis. Screening of a homologous series of octyl glycoside oligomers that range in size from monosacccharides to tetrasaccharides, revealed that, in agreement with earlier studies,[28,31] a disaccharide motif is the minimum epitope that is recognized by the enzyme and that significant increases in activity were not gained by moving to larger substrates. Through the subsequent analysis of a panel of monomethoxy and monodeoxy analogues of disaccharide substrate **5**, key interactions with the protein were identified (Figure 2 and Scheme 6). Among the most important findings is that methylation of the hydroxyl groups at C-2 of either mannopyranose residue in **5** leads to complete loss of activity. These results suggest that the attachment of the α-(1→2)-mannopyranosyl branches in the mannan core of LM/LAM must occur after a larger α-(1→6)-linked mannan is assembled. Further support for this hypothesis came from an experiment in which treatment of the pentasaccharide that resulted from tetrasaccharide **7** with an α-(1→2) specific mannosidase did not leave to cleavage of the product as detected by TLC. Another important conclusion is that the enzyme appears to form critical hydrogen-bonding interactions with a number of the hydroxyl groups on the substrate as deoxygenation leads, in all but two cases (C-2′ and C-4), to essential total loss of activity. We are currently using the results of this study to design and synthesize additional compounds that are anticipated to act as more potent inhibitors of this ManT.

## Experimental Section

**General methods for chemical synthesis**: All reagents used were purchased from commercial sources and were used without further purification unless noted. The solvents that were used in reactions were purified by successive passage through columns of alumina and copper under nitrogen. Unless indicated otherwise, all reactions were performed at room temperature and under a positive pressure of argon. The reactions were monitored by analytical TLC on silica gel 60-$F_{254}$ (0.25 mm, Silicycle, Quebec City, Canada) and spots were detected under UV light or by charring with acidified anisaldehyde solution in ethanol. Organic solvents were evaporated under reduced pressure at $<40\,°C$. Products were purified by column chromatography by using silica gel (40–60 μm) or Sep-Pak $C_{18}$ reversed-phase cartridges (Waters, Milford, MA, USA). Before use, the cartridges were prewashed with MeOH (10 mL) followed by $H_2O$ (10 mL). Optical rotations were measured at $22\pm2\,°C$ and are in units of degrees·mL/(g·dm). $^1$H NMR spectra were recorded at 500 or 600 MHz, and chemical shifts are referenced to either TMS ($\delta=0.0$ ppm, $CDCl_3$), or HOD ($\delta=4.78$ ppm, $D_2O$ and $CD_3OD$). $^{13}$C NMR spectra were recorded at 100 or 125 MHz and chemical shifts are referenced to internal $CDCl_3$ ($\delta=77.23$ ppm, $CDCl_3$), or $CD_3OD$ ($\delta=48.9$ ppm, $CD_3OD$). Assignments of NMR spectra were made based on two-dimensional ($^1$H-$^1$H COSY and HMQC) experiments. All $^1$H and $^{13}$C NMR spectra of synthesized compounds can be found in the Supporting Information. The stereochemistry at the anomeric centers of the pyranose rings were proven by measuring the $^1J_{C1\text{-}H1}$.[42] Electrospray mass spectra were recorded on samples that were suspended in mixtures of THF with MeOH and added NaCl.

**Synthesis of acceptors substrates**: The deoxy and methoxy analogues **8–21** were synthesized as described elsewhere.[37] The synthesis of octyl mannosides **5–7** were synthesized as described below from the known thioglycosides **22** and **23**,[37,39] and octyl glycosides **24** and **25**.[33,37]

**Octyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranoside (5)**: Disaccharide **26** (359 mg, 0.31 mmol) was dissolved in MeOH (25 mL) and $NaOCH_3$ (135 mg) was added. After 2 h, the solution was neutralized with acetic acid and the debenzoylated intermediate was purified by chromatography ($CH_2Cl_2$/MeOH, 10:1, $R_f=0.32$). The resulting colorless oil (200 mg, 0.28 mmol) was subsequently dissolved in MeOH (15 mL) and 20% $Pd(OH)_2$ (50 mg) was added. The mixture was stirred overnight under a $H_2$ atmosphere, and the catalyst was separated by filtration through a short pad of Celite. The filtrate was concentrated to give **5** (124 mg, 87% over two steps) as a foam. $R_f=0.15$ ($CH_2Cl_2$/MeOH, 4:1); $^1$H NMR (600 MHz, $CD_3OD$): $\delta=4.81$ (d, $J=1.8$ Hz, 1H; H-1′), 4.70 (d, $J=1.8$ Hz, 1H; H-1), 3.89 (ddd, $J=10.8$, 3.3, 2.0 Hz, 1H; H-6a), 3.84 (dd, $J=3.6$, 1.8 Hz, 1H; H-2′), 3.82 (dd, $J=11.4$, 1.8 Hz, 1H; H-6a′), 3.78 (dd, $J=3.0$, 1.8 Hz, 1H; H-2), 3.60–3.74 (m, 9H; H-3, H-4, H-5, H-6b, H-3′, H-4′, H-5′, H-6b′, octyl $OCH_2$), 3.40 (dt, $J=9.0$, 6.6 Hz, octyl $OCH_2$), 1.52–1.64 (m, 2H; octyl $OCH_2CH_2$), 1.24–1.44 (m, 10H; octyl $CH_2$), 0.89 ppm (t, $J=6.9$ Hz, 3H; octyl $CH_3$); $^{13}$C NMR (125 MHz, $CD_3OD$) $\delta_C$ 101.6 (C-1/C-1′), 101.5 (C-1/C-1′), 74.3, 73.1, 72.9, 72.7 (4C, C-3, C-3′, C-5, C-5′),

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72.2 (C-2/C-2′), 72.1 (C-2/C-2′), 68.7 (C-4), 68.6 (octyl O$CH_2$), 68.6 (C-4′), 67.4 (C-6), 62.9 (C-6′), 33.0 (octyl $CH_2$), 30.7 (octyl $CH_2$), 30.5 (octyl $CH_2$), 30.4 (octyl $CH_2$), 27.4 (octyl $CH_2$), 23.7 (octyl $CH_2$), 14.4 ppm (octyl $CH_3$); HRMS (ESI) calcd for $C_{20}H_{38}O_{11}$: 477.2306 [M+Na]; found: 477.2306.

**Octyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→6)-α-D-mannopyranoside (6)**: Trisaccharide **26** (73 mg, 0.050 mmol) was dissolved in MeOH/$CH_2Cl_2$ (8:1; 6 mL) and $NaOCH_3$ (32 mg) was added. After stirring overnight, the solution was neutralized with AcOH and the crude product was purified by chromatography ($CH_2Cl_2$/MeOH, 15:1) to give the partially deprotected trisaccharide as a colorless oil ($CH_2Cl_2$/MeOH, 15:1, $R_f$ = 0.36). The partially deprotected compound was then dissolved in MeOH (4 mL) and 20% $Pd(OH)_2$ (23 mg) was added. The mixture was stirred overnight under a $H_2$ atmosphere and the catalyst was separated by filtration through a short pad of Celite. The filtrate was concentrated and the residue was dissolved in $H_2O$ (1 mL) and loaded onto a prewashed Sep-pak $C_{18}$ reversed-phase cartridge. The column was washed with $H_2O$ (10 mL) and the desired product was eluted with MeOH (8 mL), concentrated, and lyophilized to give **6** (23 mg, 95%) as a foam. $R_f$ = 0.43 (ethyl acetate/MeOH/$H_2O$, 7:2:1); $^1H$ NMR (500 MHz, $CD_3OD$): δ = 4.85 (d, J = 1.5 Hz, 1H; H-1′), 4.77 (d, J = 2.0 Hz, 1H; H-1′′), 4.72 (d, J = 2.0 Hz, 1H; H-1), 3.56–3.88 (m, 19H; H-2, H-3, H-4, H-5, H-6a, H-6b, H-2′, H-3′, H-4′, H-5′, H-6a′, H-6b′, H-2′′, H-3′′, H-4′′, H-5′′, H-6a′′, H-6b′′, octyl O$CH_2$), 3.40 (dt, J = 10.0, 6.3 Hz, 1H; octyl O$CH_2$), 1.53–1.62 (m, 2H; octyl O$CH_2CH_2$), 1.25–1.42 (m, 10H; octyl $CH_2$), 0.90 ppm (t, J = 7.0 Hz, 3H; octyl $CH_3$); $^{13}C$ NMR (125 MHz, $CD_3OD$): δ = 101.5 (C-1), 101.1 (C-1′′), 100.9 (C-1′), 74.5, 73.1, 72.9, 72.9, 72.5, 72.3, 72.3, 72.1, 72.0, 68.7, 68.7, 68.7 (12 C, C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2′′, C-3′′, C-4′′, C-5′′), 68.6 (octyl O$CH_2$), 67.4 (C-6/C-6′), 67.2 (C-6/C-6′), 63.0 (C-6′′), 33.0 (octyl $CH_2$), 30.6 (octyl $CH_2$), 30.5 (octyl $CH_2$), 30.4 (octyl $CH_2$), 27.5 (octyl $CH_2$), 23.7 (octyl $CH_2$), 14.4 (octyl $CH_3$); HRMS (ESI) calcd for $C_{26}H_{48}O_{16}$: 639.2835 [M+Na]; found: 639.2835.

**Octyl α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→6)-α-D-mannopyranosyl-(1→6)-α-D-mannopyranoside (7)**: Tetrasaccharide **27** (78 mg, 0.038 mmol) was dissolved in MeOH/$CH_2Cl_2$ (8:1; 9 mL) and $NaOCH_3$ (43 mg) was added. After overnight stirring, the solution was neutralized with AcOH and the crude product was purified by chromatography to give the partially deprotected tetrasaccharide as pale-yellow oil ($CH_2Cl_2$/MeOH, $R_f$ = 0.30). The partially deprotected compound was dissolved in MeOH (6 mL) and 20% $Pd(OH)_2$ (25 mg) was added. The mixture was stirred overnight under a $H_2$ atmosphere and the catalyst was separated by filtration through a short pad of Celite. The filtrate was concentrated and the residue was dissolved in $H_2O$ (1 mL) and loaded on a prewashed Sep-pak $C_{18}$ reversed-phase cartridge. The column was washed with $H_2O$ (10 mL) and the desired product was eluted with MeOH (8 mL), concentrated, and lyophilized to give **7** (18 mg, 62%) as a foam. $R_f$ = 0.27 (ethyl acetate/MeOH/$H_2O$, 7:2:1); $^1H$ NMR (600 MHz, $D_2O$): δ = 4.91 (d, J = 1.8 Hz, 1H; H-1′), 4.90 (brs, 1H; H-1′′), 4.88 (brs, 1H; H-1′′′), 4.84 (brs, 1H; H-1), 3.64–3.99 (m, 25H; H-2, H-3, H-4, H-5, H-6a, H-6b, H-2′, H-3′, H-4′, H-5′, H-6a′, H-6b′, H-2′′, H-3′′, H-4′′, H-5′′, H-6a′′, H-6b′′, H-2′′′, H-3′′′, H-4′′′, H-5′′′, H-6a′′′, H-6b′′′, octyl O$CH_2$), 3.52 (dt, J = 9.6, 6.0 Hz, 1H; octyl O$CH_2$), 1.54–1.66 (m, 2H; octyl O$CH_2CH_2$), 1.23–1.42 (m, 10H; octyl $CH_2$), 0.88 ppm (t, J = 6.9 Hz, 3H; octyl $CH_3$); $^{13}C$ NMR (125 MHz, $D_2O$): δ = 100.8, 100.5, 100.2, 100.2 (4 C, C-1, C-1′, C-1′′, C-1′′′), 73.6, 71.9, 71.8, 71.8, 71.7, 71.7, 71.6, 71.5, 71.5, 71.1, 70.9, 70.9, 70.8, 67.6, 67.5, 67.5 (16 C, C-2, C-3, C-4, C-5, C-2′, C-3′, C-4′, C-5′, C-2′′, C-3′′, C-4′′, C-5′′, C-2′′′, C-3′′′, C-4′′′, C-5′′′), 68.8 (octyl O$CH_2$), 66.5, 66.5, 66.4 (3 C, C-6, C-6′, C-6′′), 61.8 (C-6′′′), 32.2 (octyl $CH_2$), 29.6 (octyl $CH_2$), 29.6 (octyl $CH_2$), 29.5 (octyl $CH_2$), 26.5 (octyl $CH_2$), 23.1 (octyl $CH_2$), 14.5 ppm (octyl $CH_3$); HRMS (ESI) calcd for $C_{32}H_{58}O_{21}$: 801.3363 [M+Na]; found: 801.3363.

**Octyl 2,3,4,6-tetra-O-benzoyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-α-D-mannopyranoside (26)**: Thioglycoside **22**[37] (620 mg, 0.89 mmol), alcohol **24**[33] (369 mg, 0.66 mmol), and powdered 4 Å molecular sieves (625 mg) were dried overnight under vacuum with $P_2O_5$. Dry $CH_2Cl_2$ (25 mL) was added and the mixture was cooled to 0 °C before the addition of N-iodosuccinimide (230 mg, 0.99 mmol) and TMSOTf (44 mg, 0.20 mmol). The mixture was stirred for 1 h at 0 °C then neutralized with triethylamine, and filtered through Celite and concentrated. The crude residue was purified by chromatography (hexane/ethyl acetate, 4:1) to give **26** (724 mg, 96%) as a yellow oil. $R_f$ = 0.34 (hexane/ethyl acetate, 4:1); $[\alpha]_D$ = +5.7 (c 3.0, $CHCl_3$); $^1H$ NMR (500 MHz, $CDCl_3$): δ = 8.11–8.15 (m, 2H; Ar), 8.05–8.09 (m, 2H; Ar), 7.91–7.94 (m, 2H; Ar), 7.81–7.85 (m, 2H; Ar), 7.49–7.62 (m, 3H; Ar), 7.20–7.45 (m, 24H; Ar), 6.12 (dd, J = 10.0, 10.0 Hz, 1H; H-4′), 5.95 (dd, J = 10.0, 3.0 Hz, 1H; H-3′), 5.77 (dd, J = 3.0, 1.8 Hz, 1H; H-2′), 5.22 (d, J = 1.8 Hz, 1H; H-1′), 5.04 (d, J = 11.5 Hz, 1H; Ph$CH_2$), 4.84 (d, J = 1.0 Hz, 1H; H-1), 4.78 (d, J = 12.5 Hz, 1H; Ph$CH_2$), 4.73 (d, J = 11.5 Hz, 1H; Ph$CH_2$), 4.69 (d, J = 11.0 Hz, 1H; Ph$CH_2$), 4.62–4.69 (m, Ph$CH_2$, 3H; H-6a′), 4.53 (ddd, J = 10.0, 3.5, 3.5 Hz, 1H; H-5′), 4.45 (dd, J = 12.5, 3.5 Hz, 1H; H-6b′), 4.00 (dd, J = 12.0, 6.0 Hz, 1H; H-6a), 3.94–3.99 (m, 4H; H-3, H-4, H-5, H-6b), 3.82 (brs, 1H; H-2), 3.77 (dt, J = 9.5, 6.5 Hz, 1H; octyl O$CH_2$), 3.42 (dt, J = 9.5, 6.5 Hz, 1H; octyl O$CH_2$), 1.55–1.65 (m, 2H; octyl O$CH_2CH_2$), 1.20–1.42 (m, 10H; octyl $CH_2$), 0.85 ppm (t, J = 7.0 Hz, 3H; octyl $CH_3$); $^{13}C$ NMR (125 MHz, $CDCl_3$): δ = 166.2 (C=O), 165.4 (C=O), 165.2 (C=O), 165.2 (C=O), 138.5 (Ar), 138.5 (Ar), 133.3 (Ar), 133.0 (Ar), 132.9 (Ar), 130.0 (Ar), 129.9 (Ar), 129.8 (Ar), 129.8 (Ar), 129.7 (Ar), 129.5 (Ar), 129.3 (Ar), 129.2 (Ar), 128.5 (Ar), 128.4 (Ar), 128.4 (Ar), 128.4 (Ar), 128.3 (Ar), 128.2 (Ar), 127.9 (Ar), 127.9 (Ar), 127.7 (Ar), 127.7 (Ar), 127.5 (Ar), 97.8 (C-1′, $^1J_{C,H}$ = 172.9 Hz), 97.6 (C-1, $^1J_{C,H}$ = 167.0 Hz), 80.5 (C-3), 75.1 (Ph$CH_2$), 74.9 (C-2/H-4), 74.9 (C-2/H-4), 72.7 (Ph$CH_2$), 72.1 (Ph$CH_2$), 71.4 (C-5), 70.5 (C-2′), 70.0 (C-3′), 68.7 (C-5′), 67.8 (octyl O$CH_2$), 67.1 (C-6), 67.1 (C-4′), 62.8 (C-6′), 31.8 (octyl $CH_2$), 29.5 (octyl $CH_2$), 29.5 (octyl $CH_2$), 29.3 (octyl $CH_2$), 26.2 (octyl $CH_2$), 22.7 (octyl $CH_2$), 14.1 ppm (octyl $CH_3$); HRMS (ESI) calcd for $C_{69}H_{72}O_{15}$: 1163.4763 [M+Na]; found: 1163.4763.

**Octyl 2,3,4-tri-O-benzoyl-6-O-(tert-butyldiphenylsilyl)-α-D-mannopyranosyl-(1→6)- 2,3,4-tri-O-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-O-benzyl-α-D-mannopyranoside (27)**: Thioglycoside **23**[39] (125 mg, 0.15 mmol), alcohol **25**[37] (121 mg, 0.12 mmol), and powdered 4 Å molecular sieves (100 mg) were dried overnight under vacuum with $P_2O_5$. Dry $CH_2Cl_2$ (4 mL) was added and the mixture was cooled to 0 °C before the addition of N-iodosuccinimide (43 mg, 0.18 mmol) and TMSOTf (7 μL, 0.036 mmol). The mixture was stirred for 1 h at 0 °C and neutralized with triethylamine, filtered through a short pad of Celite, and concentrated. The crude residue was purified by chromatography (hexane/ethyl acetate, 4:1) to give **27** (204 mg, 98%) as a pale-yellow oil. $R_f$ = 0.36 (hexane/ethyl acetate, 4:1); $[\alpha]_D$ = −7.2 (c 0.8, $CHCl_3$); $^1H$ NMR (500 MHz, $CDCl_3$): δ = 8.11–8.15 (m, 2H; Ar), 7.80–7.88 (m, 4H; Ar), 7.70–7.74 (m, 2H; Ar), 7.10–7.60 (m, 47H; Ar), 6.16 (dd, J = 10.0, 10.0 Hz, 1H; H-4′′), 5.87 (dd, J = 10.0, 3.5 Hz, 1H; H-3′′), 5.76 (dd, J = 3.5, 2.0 Hz, 1H; H-2′′), 5.17 (d, J = 2.0 Hz, 1H; H-1′′), 5.14 (d, J = 1.5 Hz, 1H; H-1′), 5.04 (d, J = 12.0 Hz, 1H; Ph$CH_2$), 4.91 (d, J = 12.0 Hz, 1H; Ph$CH_2$), 4.80 (d, J = 1.5 Hz, 1H; H-1), 4.65–4.77 (m, 7H; Ph$CH_2$), 4.57 (d, J = 12.0 Hz, 1H; Ph$CH_2$), 4.52 (d, J = 11.5 Hz, 1H; Ph$CH_2$), 4.49 (d, J = 12.0 Hz, 1H; Ph$CH_2$), 4.11 (ddd, J = 10.0, 2.5, 2.5 Hz, 1H; H-5′′), 3.98–4.06 (m, 3H; H-4, H-4′, H-6a′), 3.90–3.96 (m, 3H; H-3, H-2′, H-3′), 3.87 (dd, J = 11.3, 4.8 Hz, 1H; H-6a), 3.70–3.81

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(m, 7H; H-2, H-5, H-6b, H-5′, H-6b′, H-6a′′, H-6b′′), 3.59 (dt, $J$=9.5, 6.5 Hz, 1H; octyl OC$H_2$), 3.29 (dt, $J$=9.5, 6.5 Hz, 1H; octyl OC$H_2$), 1.41–1.49 (m, 2H; octyl OCH$_2$C$H_2$), 1.18–1.30 (m, 10H; octyl C$H_2$), 1.05 (s, 9H; *tert*-butyl), 0.86 ppm (t, $J$=7.3 Hz, 3H; octyl C$H_3$); $^{13}$C NMR (100 MHz, CDCl$_3$): $\delta$=165.1 (C=O), 165.1 (C=O), 165.1 (C=O), 138.7 (Ar), 138.6 (Ar), 138.5 (Ar), 138.3 (Ar), 138.2 (Ar), 135.7 (Ar), 135.7 (Ar), 135.4 (Ar), 133.1 (Ar), 133.0 (Ar), 132.9 (Ar), 132.8 (Ar), 132.7 (Ar), 130.0 (Ar), 129.9 (Ar), 129.7 (Ar), 129.6 (Ar), 129.6 (Ar), 129.4 (Ar), 129.4 (Ar), 129.3 (Ar), 128.4 (Ar), 128.3 (Ar), 128.2 (Ar), 128.2 (Ar), 128.2 (Ar), 128.2 (Ar), 128.1 (Ar), 128.1 (Ar), 127.8 (Ar), 127.7 (Ar), 127.6 (Ar), 127.5 (Ar), 127.5 (Ar), 127.5 (Ar), 127.4 (Ar), 127.4 (Ar), 127.4 (Ar), 127.4 (Ar), 127.2 (Ar), 127.1 (Ar), 98.0 (C-1′, $^1J_{C,H}$=171.4 Hz), 97.9 (C-1′′, $^1J_{C,H}$=174.3 Hz), 97.7 (C-1, $^1J_{C,H}$=167.0 Hz), 80.4 (C-3), 79.5 (C-3′), 75.0 (C-2), 74.9 (PhCH$_2$), 74.8 (PhCH$_2$), 74.7 (C-2′), 74.5 (C-4), 74.5 (C-4′), 72.6 (PhCH$_2$), 72.3 (PhCH$_2$), 72.1 (PhCH$_2$), 71.7 (C-5′), 71.3 (C-5), 71.2 (PhCH$_2$), 71.1 (C-5′′), 70.6 (C-2′′), 70.6 (C-3′′), 67.5 (octyl OCH$_2$), 66.6 (C-4′′), 66.6 (C-6), 65.9 (C-6′), 62.3 (C-6′′), 31.7 (octyl CH$_2$), 29.3 (octyl CH$_2$), 29.3 (octyl CH$_2$), 29.1 (octyl CH$_2$), 26.6 (C(CH$_3$)), 26.1 (octyl CH$_2$), 22.5 (octyl CH$_2$), 19.1 (C(CH$_3$)), 14.0 ppm (octyl CH$_3$); HRMS (ESI) calcd for $C_{105}H_{114}O_{19}Si$: 1729.7616 [$M$+Na]; found: 1729.7620.

**Octyl 2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-benzyl-α-D-mannopyranoside (28)**: Trisaccharide **27** (229 mg, 0.13 mmol) was stirred in a solution of 70% HF·pyridine/pyridine/THF (3.2 mL, ratio of 1:2:5). Upon completion, the mixture was diluted with ethyl acetate, washed twice with 0.5 M HCl, sat. aq. CuSO$_4$, and dried (MgSO$_4$). The solvent was evaporated and the crude residue was purified by chromatography (hexane/ethyl acetate, 3:1) to give 28 (178 mg, 90%) as a colorless oil. $R_f$=0.18 (hexane/ethyl acetate, 3:1); $[\alpha]_D$=−3.6 ($c$ 2.8, CHCl$_3$); $^1$H NMR (500 MHz, CDCl$_3$): $\delta$=8.09–8.13 (m, 2H; Ar), 7.93–7.98 (m, 2H; Ar), 7.78–7.82 (m, 2H; Ar), 7.59–7.64 (m, 1H; Ar), 7.47–7.54 (m, 3H; Ar), 7.15–7.44 (m, 35H; Ar), 6.00 (dd, $J$=10.3, 3.5 Hz, 1H; H-3′′), 5.77 (dd, $J$=10.3, 10.3 Hz, 1H; H-4′′), 5.75 (dd, $J$=3.5, 1.5 Hz, 1H; H-2′′), 5.18 (d, $J$=1.5 Hz, 1H; H-1′′), 5.13 (d, $J$=1.0 Hz, 1H; H-1′), 5.09 (d, $J$=11.5 Hz, 1H; PhC$H_2$), 4.93 (d, $J$=11.5 Hz, 1H; PhC$H_2$), 4.82 (d, $J$=1.5 Hz, 1H; H-1), 4.67–4.75 (m, 5H; PhC$H_2$), 4.64 (s, 2H; PhC$H_2$), 4.59 (d, $J$=12.0 Hz, 1H; PhC$H_2$), 4.53 (d, $J$=11.5 Hz, 1H; PhC$H_2$), 4.50 (d, $J$=12.0 Hz, 1H; PhC$H_2$), 4.05 (dd, $J$=9.5, 9.5 Hz, 1H; H-4′), 3.95–4.04 (m, 5H; H-4, H-6a/6a′, H-2′, H-3′, H-5′′), 3.93 (dd, $J$=9.8, 3.3 Hz, 1H; H-3), 3.88 (dd, $J$=11.5, 5.0 Hz, 1H; H-6a/6a′), 3.79–3.84 (m, 2H; H-2, H-5′), 3.66–3.78 (m, 4H; H-5, H-6b, H-6b′, H-6a′′), 3.55–3.64 (m, 2H; H-6b′′, octyl OC$H_2$), 3.33 (dt, $J$=10.0, 6.8 Hz, 1H; octyl OC$H_2$), 2.57 (dd, $J$=8.0, 6.0 Hz, 1H; O$H$), 1.45–1.54 (m, 2H; octyl OCH$_2$C$H_2$), 1.20–1.33 (m, 10H; octyl C$H_2$), 0.88 ppm (t, $J$=7.0 Hz, 3H; octyl C$H_3$); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$=166.5 (C=O), 165.2 (C=O), 165.2 (C=O), 138.8 (Ar), 138.7 (Ar), 138.6 (Ar), 138.6 (Ar), 138.4 (Ar), 138.3 (Ar), 133.5 (Ar), 133.3 (Ar), 133.0 (Ar), 130.0 (Ar), 129.9 (Ar), 129.7 (Ar), 129.6 (Ar), 129.4 (Ar), 128.9 (Ar), 128.6 (Ar), 128.5 (Ar), 128.4 (Ar), 128.3 (Ar), 128.3 (Ar), 128.3 (Ar), 128.3 (Ar), 128.2 (Ar), 128.2 (Ar), 127.9 (Ar), 127.8 (Ar), 127.7 (Ar), 127.7 (Ar), 127.6 (Ar), 127.5 (Ar), 127.5 (Ar), 127.5 (Ar), 127.4 (Ar), 127.3 (Ar), 98.2 (C-1′), 97.9 (C-1′′), 97.8 (C-1), 80.5 (C-3′), 79.5 (C-3), 75.1 (C-2), 75.0 (PhCH$_2$), 74.9 (PhCH$_2$), 74.8 (C-2′), 74.6 (C-4/C-4′), 74.5 (C-4/C-4′), 72.8 (PhCH$_2$), 72.5 (PhCH$_2$), 72.2 (PhCH$_2$), 71.7 (C-5), 71.3 (PhCH$_2$), 71.2 (C-5′), 70.8 (C-2′′), 70.6 (C-5′′), 69.6 (C-3′′), 67.6 (octyl OCH$_2$), 67.6 (C-4′′), 67.0 (C-6), 66.2 (C-6′), 61.2 (C-6′′), 31.8 (octyl CH$_2$), 29.4 (octyl CH$_2$), 29.4 (octyl CH$_2$), 29.2 (octyl CH$_2$), 26.2 (octyl CH$_2$), 22.7 (octyl CH$_2$), 14.1 ppm (octyl CH$_3$); HRMS (ESI) calcd for $C_{89}H_{96}O_{19}$: 1491.6438 [$M$+Na]; found: 1491.6437.

**Octyl 2,3,4,6-tetra-*O*-benzoyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-benzoyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-benzyl-α-D-mannopyranosyl-(1→6)-2,3,4-tri-*O*-benzyl-α-D-mannopyranoside (29)**: Thioglycoside **22**[37] (45 mg, 0.064 mmol), alcohol **28** (86 mg, 0.050 mmol), and powdered 4 Å molecular sieves (50 mg) were dried overnight under vacuum with P$_2$O$_5$. Dry CH$_2$Cl$_2$ (2 mL) was added and the mixture was cooled to 0 °C before the addition of *N*-iodosuccinimide (19 mg, 0.080 mmol) and TMSOTf (3 μL, 0.016 mmol). The mixture was stirred at 0 °C for 30 min and neutralized with triethylamine, before being filtered through a short of Celite and concentrated. The crude residue was purified by chromatography (hexane/ethyl acetate, 3:1) to give 29 (93 mg, 90%) as a colorless oil. $R_f$=0.21 (hexane/ethyl acetate, 3:1); $[\alpha]_D$=+12.5 ($c$ 1.2, CHCl$_3$); $^1$H NMR (600 MHz, CDCl$_3$): $\delta$=8.20–8.22 (m, 2H; Ar), 7.99–8.06 (m, 6H; Ar), 7.93–7.96 (m, 2H; Ar), 7.84–7.88 (m, 4H; Ar), 7.13–7.60 (m, 51H; Ar), 6.18 (dd, $J$=10.2, 10.2 Hz, 1H; H-4′′′), 6.11 (dd, $J$=10.2, 10.2 Hz, 1H; H-4′′), 6.06 (dd, $J$=10.2, 3.3 Hz, 1H; H-3′′), 5.96 (dd, $J$=10.2, 3.0 Hz, 1H; H-3′′′), 5.88 (dd, $J$=3.3, 1.8 Hz, 1H; H-2′′), 5.84 (dd, $J$=3.0, 1.8 Hz, 1H; H-2′′′), 5.20 (d, $J$=1.8 Hz, 1H; H-1′′′), 5.17 (brs, 1H; H-1′), 5.13 (d, $J$=1.8 Hz, 1H; H-1′′), 5.09 (d, $J$=11.4 Hz, 1H; PhC$H_2$), 4.92 (d, $J$=11.4 Hz, 1H; PhC$H_2$), 4.82 (d, $J$=1.8 Hz, 1H; H-1), 4.67–4.75 (m, 5H; PhC$H_2$), 4.64 (s, 2H; PhC$H_2$), 4.58 (d, $J$=12.0 Hz, 1H; PhC$H_2$), 4.54 (d, $J$=11.4 Hz, 1H; PhC$H_2$), 4.50 (d, $J$=11.4 Hz, 1H; PhC$H_2$), 4.38 (dd, $J$=12.0, 2.4 Hz, 1H; H-6a′′′), 4.27–4.32 (m, 2H; H-5′′, H-5′′′), 4.20 (dd, $J$=12.0, 4.2 Hz, 1H; H-6b′′′), 4.12 (dd, $J$=9.6, 9.6 Hz, 1H; H-4′), 4.00–4.05 (m, 2H; H-4, H-6a′), 3.91–3.96 (m, 5H; H-3, H-6a, H-2′, H-3′, H-6a′′), 3.85 (ddd, 1H; $J$=9.6, 4.8, 1.5 Hz, H-5′), 3.75–3.80 (m, 4H; H-2, H-5, H-6b, H-6b′), 3.66 (dd, $J$=11.4, 2.1 Hz, 1H; H-6b′′), 3.60 (dt, $J$=9.6, 6.6 Hz, 1H; octyl OC$H_2$), 3.31 (dt, $J$=9.6, 6.6 Hz, 1H; octyl OC$H_2$), 1.42–1.50 (m, 2H; octyl OCH$_2$C$H_2$), 1.18–1.30 (m, 10H; octyl C$H_2$), 0.86 ppm (t, 3H; $J$=7.2 Hz, octyl C$H_3$); $^{13}$C NMR (125 MHz, CDCl$_3$): $\delta$=166.0 (C=O), 165.5 (C=O), 165.4 (C=O), 165.3 (C=O), 165.3 (C=O), 165.1 (C=O), 165.1 (C=O), 138.9 (Ar), 138.7 (Ar), 138.6 (Ar), 138.4 (Ar), 138.3 (Ar), 133.3 (Ar), 133.2 (Ar), 132.9 (Ar), 132.9 (Ar), 132.8 (Ar), 130.1 (Ar), 129.9 (Ar), 129.9 (Ar), 129.7 (Ar), 129.7 (Ar), 129.6 (Ar), 129.5 (Ar), 129.4 (Ar), 129.4 (Ar), 129.2 (Ar), 129.2 (Ar), 128.8 (Ar), 128.5 (Ar), 128.5 (Ar), 128.4 (Ar), 128.4 (Ar), 128.3 (Ar), 128.3 (Ar), 128.3 (Ar), 128.3 (Ar), 128.2 (Ar), 127.9 (Ar), 127.9 (Ar), 127.8 (Ar), 127.7 (Ar), 127.6 (Ar), 127.6 (Ar), 127.6 (Ar), 127.5 (Ar), 127.5 (Ar), 127.4 (Ar), 127.3 (Ar), 98.3 (C-1′′′, $^1J_{C,H}$=174.0 Hz), 98.2 (C-1′, $^1J_{C,H}$=169.5 Hz), 98.1 (C-1′′, $^1J_{C,H}$=172.3 Hz), 97.8 (C-1, $^1J_{C,H}$=167.0 Hz), 80.5 (C-3), 79.6 (C-3′), 75.1 (C-2), 75.0 (PhCH$_2$), 74.9 (PhCH$_2$), 74.7 (C-2′), 74.5 (C-4), 74.5 (C-4′), 72.7 (PhCH$_2$), 72.4 (PhCH$_2$), 72.2 (PhCH$_2$), 71.8 (C-5), 71.5 (C-5′), 71.3 (PhCH$_2$), 70.4, 70.3, 70.3, 70.2 (4C, C-2′′, C-3′′, C-2′′′, C-3′′′), 69.2 (C-5′′), 68.8 (C-5′′′), 67.6 (octyl OCH$_2$), 67.0 (C-6/C-6′), 66.9 (C-4′′/C-4′′′), 66.6 (C-4′′/C-4′′′), 66.3 (C-6/C-6′), 66.1 (C-6′′), 62.4 (C-6′′′), 31.8 (octyl CH$_2$), 29.4 (octyl CH$_2$), 29.4 (octyl CH$_2$), 29.2 (octyl CH$_2$), 26.2 (octyl CH$_2$), 22.6 (octyl CH$_2$), 14.1 ppm (octyl CH$_3$); ESI calcd for $C_{123}H_{122}O_{28}$: 2071.3 [$M$+Na]; found 2070.8.

**Bacterial strains and growth conditions**: *M. smegmatis* mc$^2$155 was a generous gift from Professor William R. Jacobs, Jr. at the Albert Einstein College of Medicine. The bacteria were grown at 37 °C in Luria Bertoni (LB) medium (100 mL) that contained 0.05% Tween 80 to an $A_{600\,nm}$ of <1.0 (~2 days from a frozen bacterial stock). The liquid cultures (50 mL) were then transferred to 2×1 L of fresh media and cultured further for 24 h at 37 °C. Cells were harvested by centrifugation, washed with phosphate buffered saline (PBS) and stored at −20 °C until use.

**Preparation of membrane fractions from *M. smegmatis*:** The *M. smegmatis* cell pellet (~10 g wet weight) was washed and resuspended in 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS;

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100 mL) buffer (adjusted to pH 7.9 with KOH) that contained 5 mM β-mercaptoethanol and 10 mM $MgCl_2$ and was supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche) at 4 °C. The cells were subjected to two passes through a French Press cell (Thermo Spectronic) at 20000 psi. The cell lysate was centrifuged at 600 *g* for 15 min and then at 27000 *g* for 20 min. The resulting supernatant was centrifuged at 100000 *g* for 60 min. The supernatant was carefully removed and the membrane pellets were gently resuspended in 50 mM MOPS buffer (1 mL), pH 7.9, that contained 5 mM β-mercaptoethanol and 10 mM $MgCl_2$. Protein concentrations were determined by the BCA™ Protein Assay (Pierce) by using bovine serum albumin as the standard.

**Radiochemical activity assays**: The ManT enzyme activity was determined by using the previously established cell-free system.[31] Unless indicated otherwise, the synthetic acceptor analogues at a concentration of 2.0 mM were incubated with 0.20 µCi of guanosine diphosphate mannose, [mannose-2-$^3$H] (American Radiolabeled Chemicals, Inc., 20 Ci mmol$^{-1}$) in 50 mM MOPS buffer, pH 7.9, containing 1 mM ATP, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 0.25 mM decaprenol phosphate (in 0.25% CHAPS) (Larodan Fine Chemicals, Malmö, Sweden) and membrane fraction (92.5 µg of protein) in a total volume of 80 µL. All assays were performed in duplicate and control assays without acceptor were also performed in parallel to correct for the presence of endogenous acceptor. The enzymatic activities were determined by using SepPak radiochemical $C_{18}$ assays.[38] Briefly, after incubation at 37 °C for 1 h, the reactions were stopped by adding $CHCl_3$/MeOH (120 µL, 2:1, *v*/*v*) and the mixtures were centrifuged. The supernatants were recovered and further diluted with $H_2O$ before loading onto SepPak $C_{18}$ cartridges (Waters). The unreacted donor was removed by washing the cartridges with $H_2O$ (50 mL) and the radiolabeled products were eluted with MeOH (4.0 mL). The isolated products in the eluants were quantified by liquid scintillation counting on a Beckman LS6500 Scintillation Counter by using Ecolite cocktail (10 mL). For kinetic analysis, the ManT activities were determined by using a range of acceptor concentrations (0.03 to 4.0 mM). All other reaction conditions were identical to the cell-free assay described above. Assays were performed under the conditions in which the formations of radiolabeled products were linear for both time and protein concentration. The kinetic parameters $K_M$ and $V_{max}$ were obtained by nonlinear regression analysis by using the Michaelis–Menten equation with the GraphPad Prism 4.0 program (GraphPad Software, San Diego, CA).

**Product characterizations from milligram-scale incubations**: Large-scale ManT reactions were performed for the structural characterization by using acceptor substrates **4**–**7**. A typical reaction that contained 50 mM MOPS buffer, pH 7.9, 1 mM ATP, 10 mM $MgCl_2$, 5 mM β-mercaptoethanol, 2 mM acceptor, 2 mM GDP-mannose and the *M. smegmatis* membrane preparation was incubated at 37 °C with gentle rotation for 5 days. The reaction mixture was loaded directly on the $C_{18}$ reversed-phase cartridge and the unreacted donor was washed away with $H_2O$ (50 mL) and the product was eluted subsequently with MeOH (4 mL). The solvent was evaporated and the residue was redissolved in $H_2O$ (50 µL). The conversion of the acceptor substrate to the enzymatic product could be visualized by thin layer chromatography (TLC) on SIL G-25 silica gel plates (Macherey–Nagel) after developing with the following solvent systems: A) ethyl acetate/MeOH/$H_2O$ (17:2:1) gave $R_f$ values of 0.5 and 0.23 for mono- and dimannoside, respectively; B) ethyl acetate/MeOH/$H_2O$ (17:2:1), developed three times to give $R_f$ values of 0.38 and 0.13 for the di- and trimannosides, respectively; ethyl acetate/MeOH/$H_2O$ (7:21) and developed twice to give $R_f$ values of 0.35 and 0.22 for the tetra- and pentamannosides, respectively. Visualization of compounds was achieved by heating the TLC plates after dipping them in a solution of 3% anisaldehyde in sulfuric acid. In addition, the mass of the product was characterized by MALDI mass spectrometry on a Voyager Elite time-of-flight spectrometer on sample suspended in 2,5-dihydroxy benzoic acid, by using the delayed-extraction mode and positive-ion detection.

For characterization of products by using $^1$H NMR spectroscopy, the reaction products were purified by preparative TLC. The area that corresponds to the product on the TLC plate was scraped and dissolved in $H_2O$. The resulting solution was stirred for 15 min and centrifuged. The solution was filtered through a 0.2 µm nylon membrane filter (Pall Life Sciences, Ann Arbour, MI, USA) before being applied to the SepPak $C_{18}$ cartridge. After washing the column with $H_2O$, the product was eluted with MeOH (4 mL). The solvent was evaporated and the product was lyophilized overnight and dissolved in $D_2O$. One-dimensional $^1$H NMR spectra were recorded on a Varian i600 instrument.

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