Epimeric and amino disaccharide analogs as probes of an α-(1→6)-mannosyltransferase involved in mycobacterial lipoarabinomannan biosynthesis†

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Mycobacterial lipoarabinomannan (LAM) is an important, immunologically active glycan found in the cell wall of mycobacteria, including the human pathogen *Mycobacterium tuberculosis*. At the core of LAM is a mannan domain comprised of α-(1→6)-linked-mannopyranose (Man*p*) residues. Previously, we and others have demonstrated that α-Man*p*-(1→6)-α-Man*p* disaccharides (*e.g.*, Man*p*-(1→6)-α-Man*p*Octyl, **1**) are the minimum acceptor substrates for enzymes involved in the assembly of the LAM mannan core. We report here the synthesis five epimeric and three amino analogs of **1**, and their subsequent biochemical evaluation against an α-(1→6)-ManT activity present in a membrane preparation from *M. smegmatis*. Changing the *manno*- configuration of either residue of **1** to *talo*- or *gluco*- led to a reduction or loss of activity, thus confirming earlier work showing that the C-2 and C-4 hydroxyl groups of each monosaccharide were important for enzymatic recognition. Characterization of the products formed from these analogs was done using a combination of mass spectrometry and glycosidase digestion, and full substrate kinetics were also performed. The analogs in which the acceptor hydroxyl group had been replaced with an amino group were, as expected, not substrates for the enzyme, but were weak inhibitors.

Introduction

Lipoarabinomannan (LAM) is an important cell wall constituent in mycobacteria, including those that cause the human diseases tuberculosis (*Mycobacterium tuberculosis*) and leprosy (*M. leprae*).[1] This glycoconjugate, which contains both arabinofuranose (Ara*f*) and mannopyranose (Man*p*) residues, has been implicated in a host of biochemical and immunological processes associated with mycobacterial infection.[2,3] There has consequently been significant interest not only in obtaining a molecular-level understanding of its immunomodulatory activity, but also in elucidating the biosynthetic pathway by which LAM is assembled.[2–6]

All mycobacteria, and a number of related organisms, produce LAMs, the structure of which varies from species to species.[2,3] In mycobacterial LAM (Fig. 1), the molecule is built upon an acylated phosphatidyl inositol anchor from which extends a chain of Man*p* residues connected *via* α-(1→6) linkages. Approximately half of these core mannan residues are further elaborated by single α-(1→2)-linked Man*p* branching units, and this structure serves as a scaffold to which an arabinan domain containing α-(1→2)-, α-(1→3)- and α-(1→5)-linked Ara*f* residues is bound.[1–3] Depending on the species, the arabinan can be modified at its periphery by a number of capping groups such as short α-(1→2)-linked Man*p* oligosaccharides,[2,3,7] 5-deoxy-5-thiomethyl-xylofuranose residues[8–11] or inositol phosphate moieties.[12]

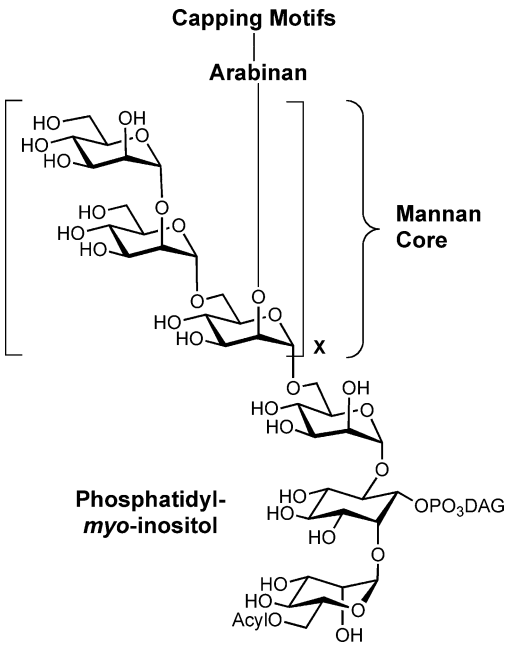

Fig. 1 Composite structure of mycobacterial LAM; DAG = diacylglycerol.

The role of each structural domain on the immunomodulatory function of LAM remains relatively poorly understood. For example, although previous studies suggested the capping motifs were important for virulence,[2,3,7] more recent studies have suggested this is not the case.[13] Other recent investigations have demonstrated that the ability of these glycans to bind to the toll-like receptor 2 (TLR-2) is influenced by mannan chain length[14] and acylation

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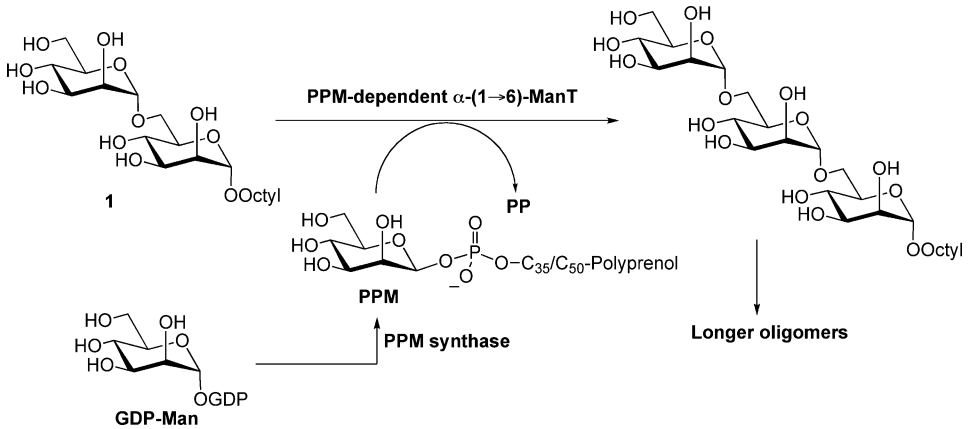


Fig. 2 Reported assay for measuring PPM-dependent α-(1→6)-ManT activity in mycobacterial membrane preparations. PPM = polyprenolphoshomannose; GDP = guanosine disphosphate.

state.[15] Similarly, the recognition of LAM fragments by anti-LAM antibodies has also been studied recently.[16–18]

The model proposed more than a decade ago by Brennan and coworkers[19] for the biosynthesis of LAM has, for the most part, been borne out through genetic and biochemical investigations carried out since that time.[4,5] Of particular relevance to the work reported in this paper are a family of polyprenolphosphomannose (PPM)-dependent mannosyltransferases (ManTs) that are involved in the assembly of the mannan domain of this glycan. Earlier studies reported the use of a membrane preparation from *M. smegmatis* to assay for the α-(1→6)-ManT activity required for the synthesis of the LAM mannan core.[20,21] This assay, in which the donor substrate for the enzyme, is generated *in situ* from GDP-mannose (Fig. 2), has been used to screen potential substrates and inhibitors of this enzymatic activity,[22,23] and it has been demonstrated that α-Man*p*-(1→6)-α-Man*p* disaccharides (*e.g.*, **1**) are the minimum acceptor substrates.[21,24] It is currently unknown how many enzymes are involved in the assembly of the mannan core. Although earlier investigations in this area assumed, without substantial experimental support, that a single enzyme was involved, recent work with mycobacterial gene knock-out strains,[25] has provided evidence that more than one α-(1→6)-ManT is required.

In previous papers, we reported the preparation[26] and biochemical evaluation[24] of a panel of monomethoxy and monodeoxy analogs of disaccharide acceptor **1** (Fig. 3) as potential substrates and inhibitors for this α-(1→6)-ManT activity. Among the important findings from these investigations were that the enzyme appears to form critical hydrogen bonding interactions with a number of hydroxyl groups on the substrate because deoxygenation leads, in all but two cases (C-2′ and C-4), to essentially a total loss of activity. To extend our understanding of the substrate specificity of this enzymatic activity further, we describe here the synthesis five epimeric (**3**, **5**, **7–9**) and three amino (**2**, **4** and **6**) analogs of **1** (Fig. 3), and their subsequent biochemical evaluation against the α-(1→6)-ManT activity present in the previously mentioned *M. smegmatis* membrane preparation.

The epimers were selected as targets to determine if the *manno*-configuration was absolutely required by the enzyme, or if disaccharides with rings in the *gluco*- (**3**, **7**, **9**) or *talo*- (**5**, **8**, **9**) configuration were also recognized. The results obtained from these analogs would provide additional insight into the important steric and

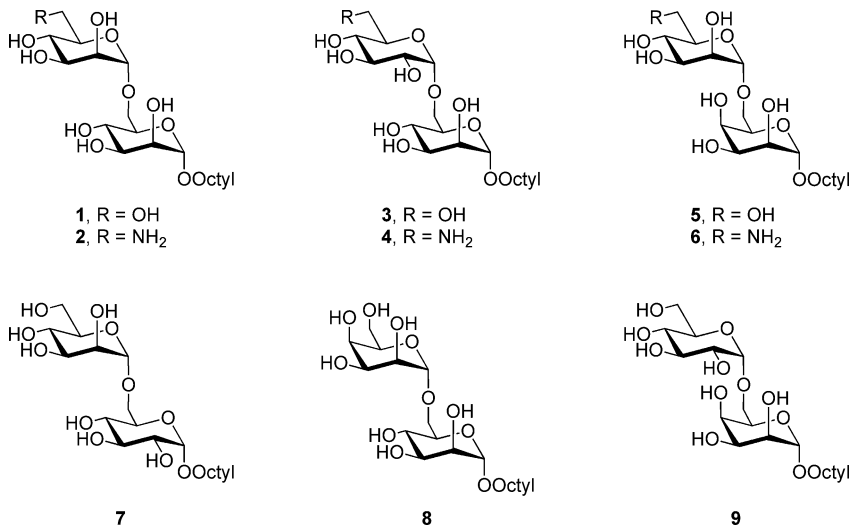
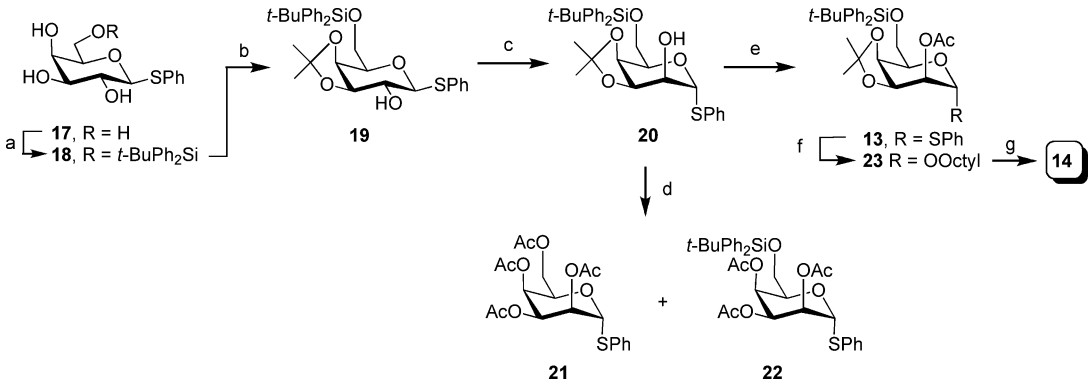


Fig. 3 Synthetic disaccharide targets **2–9**.



Scheme 1 *Reagents and conditions*: a) *t*-$BuPh_2SiCl$, imidazole, DMF, 45 °C, 94%; b) 2,2-dimethoxypropane, *p*TsOH, acetone, quant.; c) i: oxalyl chloride, DMSO, −78 °C; then alcohol **19**, warm to −60 °C; then Et_3N, warm to rt.; ii: $NaBH_4$, MeOH, 62%; d) i: 80% aq. AcOH, 50 °C; ii: Ac_2O, DMAP, CH_2Cl_2, pyridine, 65% for **21** and 26% for **22**; e) Ac_2O, CH_2Cl_2, pyridine (1 : 1), 89%; f) octanol, NIS, TMSOTf, 4 Å MS, CH_2Cl_2, 0 °C, 89%; g) TBAF, THF, 74%.

hydrogen bonding interactions in the active site of these proteins. In addition, the amino analogs were synthesized and evaluated as potential inhibitors. We envisioned that the amino groups of **2**, **4** and **6** would be protonated at physiological pH, and the resulting ammonium derivatives would serve as inhibitors *via* an ionic interaction with a negatively charged residue in the enzyme active site.[27,28] It has been shown previously that octyl oligosaccharides are efficient substrates for glycosyltransferases from mycobacteria and other organisms.[21,27–30] Moreover, the attachment of this hydrophobic group to an oligosaccharide allows for convenient product isolation using reversed-phase chromatography.[30] Thus, the target compounds **2–9** were synthesized as octyl glycosides.

Results and discussion

Preparation of monosaccharide building blocks

We envisioned that disaccharides **2–9** could be assembled from monosaccharide precursors **10–16** (Fig. 4). Previously reported methods were used to prepare octyl glycoside **10**[31] and **16**,[32] as well as thioglycosides **11**[33] and **12**.[26] The other building blocks, talopyranosides **13** and **14**, and glucopyranoside **15**, were synthesized as outlined below.

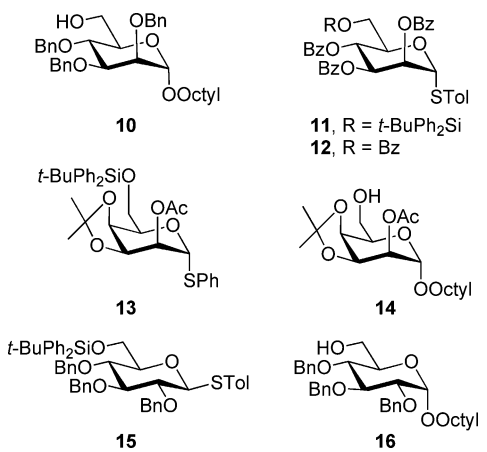


Fig. 4 Monosaccharide building blocks used for the synthesis of **2–9**.

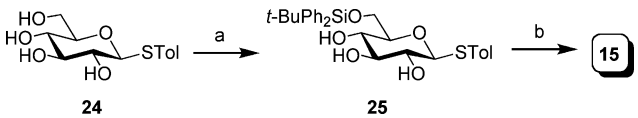
Thioglycoside **13** and octyl glycoside **14** were synthesized from the known thioglycoside **17**[34] as illustrated in Scheme 1. First, reaction of **17** with *tert*-butylchlorodiphenylsilane and imidazole provided silyl ether **18** in 94% yield. Subsequent reaction of **18** with 2,2-dimethoxypropane in the presence of a catalytic amount of *p*-TsOH gave a quantitative yield of alcohol **19**.

Oxidation of the C-2 hydroxyl group under Swern conditions and subsequent reduction with sodium borohydride gave an anomeric mixture of thioglycoside **20** (62% yield of a 6 : 1 α/β mixture of isomers, which could be separated by chromatography). In the ^1H NMR spectrum of the major isomer, the H-1 resonance was shifted to 5.33 ppm, which is in good agreement with the α-configuration at the anomeric center. Presumably, the basic conditions of the oxidation reaction resulted in the anomerization of the ketone product, as has been reported previously.[35] However, the $J_{1,2}$ (7.4 Hz) of the product obtained after borohydride reduction was larger than would be expected for thioglycosides with either the α-*galacto*- or α-*talo*-configuration.

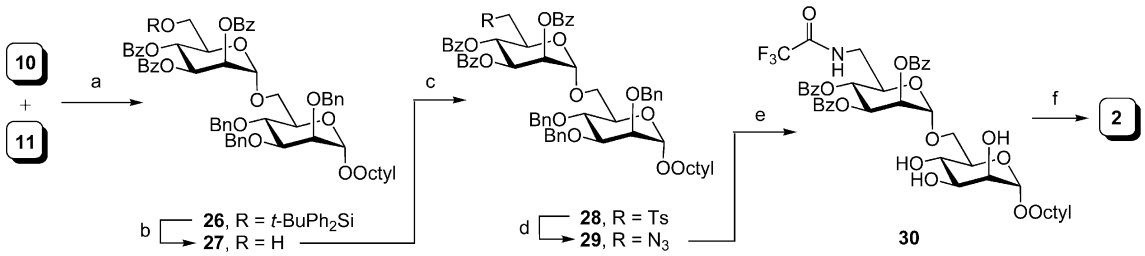
We postulated that the larger than expected coupling constant value was due to the distortion of the chair conformation of the pyranose ring by the isopropylidene protecting group. Therefore, to confirm the stereochemistry at C-2, **20** was hydrolyzed with 80% aqueous AcOH and then reacted with acetic anhydride in the presence of pyridine and DMAP to afford a mixture of **21** and **22**. The $J_{1,2}$ and $J_{2,3}$ of **22** (1.3 and 3.7 Hz, respectively) are consistent with the α-*talo*-configuration, in turn confirming the structure of **20** as that shown in Scheme 1.

Having determined that the oxidation–reduction sequence had provided the correct product, **20** was treated with acetic anhydride thus providing an 89% yield of **13**. Glycosidation of **13** with *n*-octanol gave octyl talopyranoside **23** in 89% yield. Finally, treatment of **33** with tetra-*n*-butylammonium fluoride provided a 74% yield of the expected alcohol **14**.

As illustrated in Scheme 2, thioglycoside **15**, was obtained from the known thioglucoside **24**.[36] Reaction of **24** with



Scheme 2 *Reagents and conditions*: a) *t*-$BuPh_2SiCl$, imidazole, DMF, 45 °C; b) NaH, BnBr, DMF, 95% two steps.



Scheme 3 *Reagents and conditions*: a) NIS, TMSOTf, 4 Å MS, CH_2Cl_2, 0 °C, 85%; b) HF·pyridine–pyridine–THF (1 : 2 : 20), 66%; c) TsCl, pyridine, 91%; d) NaN_3, DMF, reflux, 98%; e) i: $Pd(OH)_2$–C, pyridine, H_2; ii: $(CF_3CO)_2O$, pyridine, 0 °C to rt.; iii: $Pd(OH)_2$–C, MeOH, H_2, 68% over three steps; f) NaOMe, MeOH, 61%.

tert-butylchlorodiphenylsilane and imidazole provided silyl ether intermediate **25**. Without purification, the remaining hydroxyl groups were protected as benzyl ethers upon treatment with sodium hydride and benzyl bromide to afford **15** in 95% yield over the two steps.

Synthesis of disaccharides

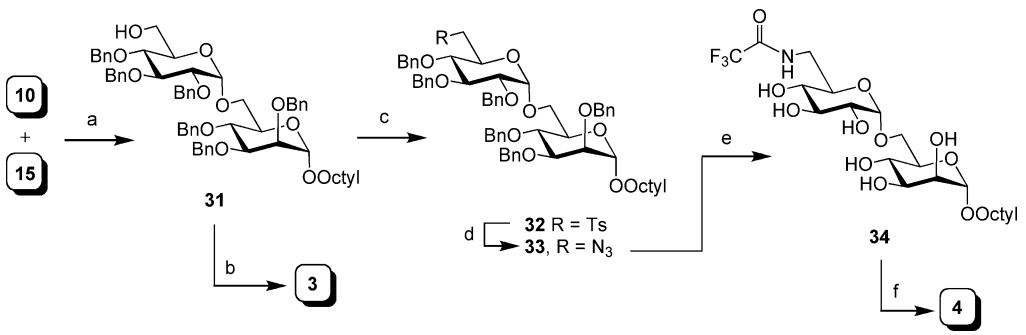
As illustrated in Scheme 3, the synthesis of amino disaccharide **2** was began with the NIS–TMSOTf activated[37] glycosylation of thioglycoside **11**[33] and alcohol **10**,[31] which afforded disaccharide **26** in 85% yield. For this reaction, and all other glycosylations described in this paper using mannopyranoside and talopyranoside donors, the α-stereochemistry of the glycosidic linkages was confirmed by measurement of the one-bond heteronuclear coupling constant for the anomeric carbon atom ($^1J_{C\text{-}1,H\text{-}1}$). In all cases, this value was between 167 and 176 Hz, clearly indicating the α-stereochemistry.[38]

Next, disaccharide **26** was desilyated using hydrogen fluoride in pyridine[39] to give **27** in 66% yield. The resulting primary alcohol in **27** was then tosylated with tosyl chloride in pyridine (91%); treatment of the product, **28**, with sodium azide gave azidodisaccharide **29** in 98% yield. That the substitution had occurred was confirmed from the ^{1}H NMR spectrum of **29**, in which the protons on C-6′ resonated between 3.38 and 3.48 ppm, as would be expected for hydrogens adjacent to an azido functionality. In addition, in the ^{13}C NMR spectrum, a resonance at 51.2 ppm could be assigned to C-6′.

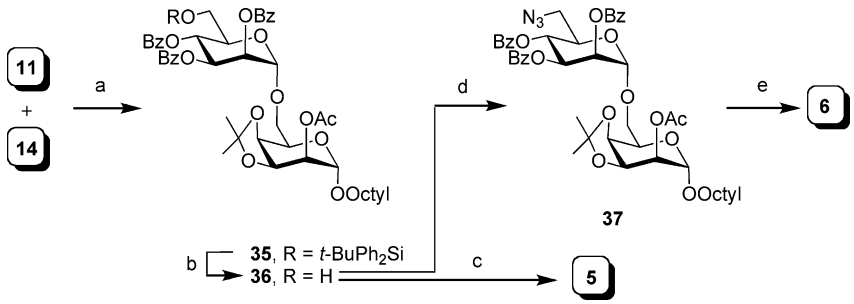
Similar to an earlier observation on related disaccharides,[40] simultaneous reduction of the azide and deprotection of the benzyl groups by treatment of **29** with hydrogen and a palladium catalyst failed. Thin layer chromatographic analysis of the reaction mixture showed several products, presumably a series of partially-debenzylated derivatives of **29**. No further reduction was observed, even after prolonged reaction times. Instead, the azido group of **29** was reduced to the amine by hydrogenation over $Pd(OH)_2$–C in pyridine, and the product was immediately converted to its *N*-trifluoroacetamide using trifluoroacetic anhydride in pyridine.[40] Subsequent hydrogenolysis of the benzyl groups using $Pd(OH)_2$–C catalyst was achieved without incident and provided triol **30** in 68% yield over three steps. Final deprotection of **30** using sodium methoxide in methanol furnished a 61% yield of the desired amino disaccharide **2**.

As illustrated in Scheme 4, the synthesis of disaccharides **3** and **4** were achieved by first coupling thioglycoside **15** with alcohol **10**,[31] under NIS–TMSOTf activation. This reaction afforded an inseparable diastereoisomeric mixture (~5 : 1 α : β ratio) of products. Subsequent desilylation using hydrogen fluoride followed by chromatographic purification gave the α-linked disaccharide **31** in 50% yield over the two steps. Final deprotection of the benzyl ethers afforded **3** in 82% yield.

The amino disaccharide **4** was synthesized *via* a route analogous to **2**, starting with **31**. Thus, the primary hydroxyl group in **31** was first tosylated to provide **32**, which upon heating in reflux in DMF with sodium azide gave azidosugar **33** in 76% yield over the two steps. As was seen for compound **29**, the primary azido functionality in **33** was evident in the ^{13}C NMR spectrum, which showed a resonance for C-6′ at 51.2 ppm. The azido group of **33** was then converted to the corresponding *N*-trifluoroacetamide derivative in two steps (azide reduction and trifluoroacetylation of



Scheme 4 *Reagents and conditions*: a) i: NIS, TMSOTf, 4 Å MS, CH_2Cl_2, 0 °C, 5 : 1 of α : β isomers; ii: HF·pyridine–pyridine–THF (1 : 2 : 20), 50%; b) $Pd(OH)_2$–C, MeOH, H_2, 82%; c) TsCl, pyridine, 89%; d) NaN_3, DMF, reflux, 85%; e) i: $Pd(OH)_2$–C, pyridine, H_2; ii: $(CF_3CO)_2O$, pyridine, 0 °C to rt.; iii: $Pd(OH)_2$–C, MeOH, H_2, 72% over three steps; f) NaOMe, MeOH, 79%.

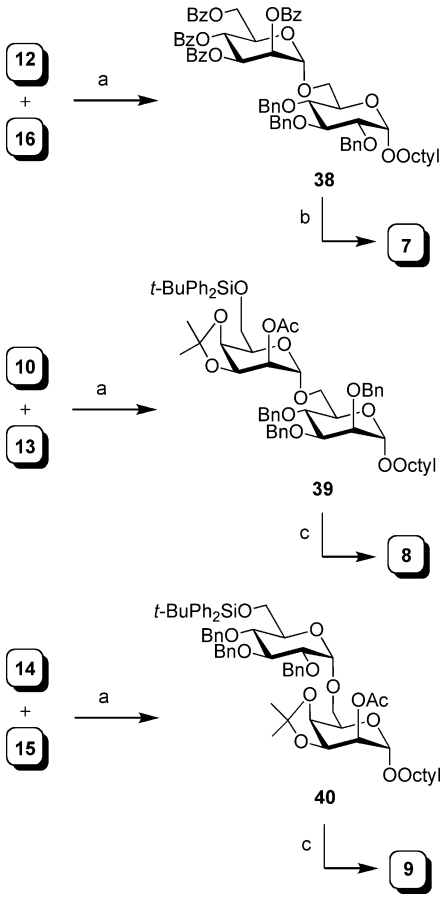


Scheme 5 *Reagents and conditions*: a) NIS, TMSOTf, 4 Å MS, CH_2Cl_2, 0 °C, 90%; b) HF·pyridine–pyridine–THF (1 : 2 : 20), 77%; c) i: 80% aq. AcOH, 50 °C; ii: NaOMe, MeOH, 91%; d) i: TsCl, pyridine, 91%; ii: NaN_3, DMF, reflux, 84%; e) i: 80% aq. AcOH, 50 °C; ii: NaOMe, MeOH; iii: $Pd(OH)_2$–C, MeOH, H_2, 86% over three steps.

the product amine). Without purification, the crude intermediate was hydrogenated to provide **34** in 72% yield over three steps. Final deprotection by treatment with sodium methoxide furnished a 79% yield of the target analog **4**.

The synthesis of **4** and **5**, starting from octyl talopyranoside **14**, is shown in Scheme 5. Coupling of this acceptor with thioglycoside **11**[33] under NIS–TMSOTf activation afforded the corresponding protected disaccharide **35** in 90% yield. Deprotection of the silyl ether proceeded under standard conditions to give a 77% yield of **36**. Cleavage of the isopropylidene acetal and treatment with sodium methoxide in methanol gave **5** in 91% overall yield. To prepare **6**, alcohol **36** was converted to azido disaccharide **37** *via* the two step sequence (91% and 84% yields, respectively) described for the preparation of **29**. Reduction of azide **37**, following removal of the isopropylidene acetal and acyl protecting groups, provided aminosugar **6** in 86% yield.

The preparation of disaccharides **7–9** is illustrated in Scheme 6. Glycosylation of alcohols **10**,[31] **14**, **16**[32] with thioglycosides **12**,[26] **13**, **15** using NIS–TMSOTf activation, gave disaccharides **38**, **39**, and **40** in excellent yields (92%, 81% and 88%, respectively). Debenzoylation and hydrogenolysis of **38** provided the desired compound **7** (84% yield). To obtain **8** and **9**, disaccharides **39** and **40** were subjected to a four-step sequence (desilylation, deacetylation, acetal cleavage and hydrogenolysis) to give the expected products in overall yields of 45% and 62%, respectively. NMR analysis of the final compounds suggested that the modified oligosaccharides have a conformation similar to the parent compounds. For example, the chemical shifts and coupling constants were consistent with all of the pyranose rings adopting a $^4C_1$ conformation.



Scheme 6 *Reagents and conditions*: a) NIS, TMSOTf, 4 Å MS, CH_2Cl_2, 0 °C, 92% for **38**, 81% for **39**, 88% for **40** (5 : 1 of α : β isomers); b) i: NaOMe, MeOH; ii: $Pd(OH)_2$–C, MeOH, H_2, 84%; c) i: TBAF, THF; ii: NaOMe, MeOH; iii: 80% aq. AcOH, 50 °C; iv: $Pd(OH)_2$–C, MeOH, H_2, 45% for **8**; 62% for **9**.

Screening analogs as substrates and inhibitors

Once synthesized, **2–9** were screened as potential substrates and inhibitors of the PPM-dependent α-(1→6)-ManT activity, present in the *M. smegmatis* membrane preparation.[20,21] Each analog was incubated with [^{3}H]-labeled GDP-mannose (Fig. 2) and the membrane extracts, the radiolabeled products were recovered by solvent extraction and reversed-phase chromatography, and the radioactivity was measured by scintillation counting.

The ability of **2–9** to act as acceptor substrates for ManT were compared with the parent compound **1** and the results are summarized in Table 1. The initial screening revealed that analogs **3** (α-Glc*p*-(1→6)-α-Man*p*) and **5** (α-Man*p*-(1→6)-α-Tal*p*) are moderate substrates of ManT with 41% and 57% activity, relative to **1** (α-Man*p*-(1→6)-α-Man*p*). Our previous data suggested that the hydroxyl groups at C-2′ and C-4 of **1** were not crucial for substrate–enzyme interaction, as the replacement with hydrogen had no dramatic effect on ManT catalysis.[24] However, it appears that epimerization at either position, leading to analog **3** or **5**, significantly affects the efficiency to serve as a substrate. This finding is further indicated by the apparent kinetic parameters

Table 1 Summary of ManT activities using analogs **1–9**

Analog	Relative activity[a] (%)	Apparent K_M [b]/mM	Apparent V_{max} [b]/ pmol mg min^{-1}	Remaining ManT activity[c] (%)	Mass of oligosaccharide product[d]
1	100	0.09 ± 0.01	0.37 ± 0.005	—	639.2, 801.3
2	4	–[e]	—	21	—
3	41	1.80 ± 0.71	0.34 ± 0.071	—	639.1, 801.1
4	0	—	—	9	—
5	57	1.20 ± 0.24	0.40 ± 0.031	—	639.2, 801.2
6	<1	—	—	30	—
7	10	3.04 ± 0.69	0.10 ± 0.014	—	639.3, 801.3, 963.3
8	<1	—	—	—	—
9	10	2.27 ± 0.12	0.11 ± 0.003	—	639.3, 801.3

[a] Relative activities were measured at 2.0 mM acceptor concentration with 0.2 μCi of [^{3}H]GDP-Man and are expressed with respect to disaccharide **1**. 100% activity corresponds to 29.8 pmol mg^{-1} h^{-1}. [b] Kinetic parameters were determined using a range of acceptor concentrations by nonlinear regression analysis of the Michaelis–Menten equation with the GraphPad Prism 4.0 program. [c] Compounds were screened at a concentration of 2.0 mM with **1** as the substrate at 0.2 mM. [d] The enzymatic products were isolated from larger-scale incubations and their masses were determined by MALDI mass spectrometry. The found values correspond to the sodium adducts, which were in good agreement with the calculated values. [e] Not determined.

shown in Table 1. These acceptor analogs, have K_M values of 1.8 mM (**3**) and 1.2 mM (**5**), which are 13- and 20-fold larger, respectively, than that of **1** (0.09 mM). The V_{max} values of **3** or **5** are comparable to that of the parent compound.

The lower relative activity of **3** and **5** compared to **1** appears to be due to the steric requirements of the ManT. For both compounds, the hydroxyl group is placed in the opposite orientation in the parent compound (changed from axial to equatorial in **3**, or *vice versa* in **5**). This change appears to lead to a potential negative steric interaction with amino acid residues in the active site thus resulting in an unfavorable enzyme–substrate interaction and a higher K_M. These observations suggest that while the enzyme preferentially recognizes disaccharides in which both residues are in the *manno*-configuration, it will also accept analogs of different stereochemical configurations, but with lower affinity.

This finding is further supported by the results obtained from analogs **7–9**, in which α-Tal*p*-(1→6)-α-Man*p* analog **8** is not a substrate, and both the α-Man*p*-(1→6)-α-Glc*p* **7** and α-Glc*p*-(1→6)-α-Tal*p* analogs **9** are poor substrates with only 10% relative activity compared to **1**. Consistent with the results for **3** and **5**, subsequent kinetic analysis provided larger apparent K_M values for **7** and **9**, 3.04 mM and 2.27 mM, respectively. However, the V_{max} values for these substrates was only marginally less than the native substrate, **1**. The poor substrate activity of **7**, and the lack of activity of **8**, is consistent with our previous work,[24] which indicated that the C-4′ and C-2 hydroxyl groups are essential for ManT catalysis. In **3** and **5**, the orientations of the C-4′ and C-2 hydroxyl groups were the same as in the parent compound, while the stereochemistry at one other centre was inverted (C-2′ in **3** and C-4 in **5**), and these compounds were weak substrates for the enzyme. However, evaluation of an analog in which the stereochemistry at both C-2′ and C-4 were inverted, the α-Glc*p*-(1→6)-α-Tal*p* isomer **9**, showed even weaker ManT substrate activity.

In summary, the change of the configuration of the disaccharide motif, regardless of whether the change was at the reducing or non-reducing end, did not enhance but rather reduced the ManT activity. This finding indicates that the enzyme requires a *manno*-configuration of both rings for optimal activity.

Characterization of enzymatic products by mass spectrometry

In addition to the radiochemical assays, milligram-scale enzymatic incubations of **1**, **3**, **5**, **7** and **9** with unlabelled GDP-Man and the membrane fraction were carried out to determine the structure of the oligosaccharide products that were produced from these analogs. After the incubations, the enzymatic products were purified and analyzed by MALDI mass spectrometry. As shown in Fig. S1, these reactions all resulted in the formation of the corresponding trisaccharides and tetrasaccharides as the major and minor products, respectively. Contrary to the proposed processive nature of this enzyme,[20] a homologous series of products was not observed. The absence of these larger oligosaccharides is probably due to much lower acceptor concentrations of the resulting tri- and tetrasaccharide products, resulting in slower rates of the subsequent elongation reactions. It is also possible that the larger enzymatic products (*e.g.*, tetra- and pentasaccharide) are degraded by an α-(1→6)-*endo*-mannosidase present in the membrane preparation.[24]

Characterization of enzymatic products by glycosidase digestion

In addition to the PPM-dependent α-(1→6)-mannosyltransferases, the crude membrane extract of *M. smegmatis* used in these assays also contains α-(1→2)-ManT's involved in mannan core branching and the capping of the arabinan domain.[25,41–43] To confirm that the observed addition of radiolabeled mannose to **3**, **5**, **7** and **9** arose from α-(1→6)-ManT activity, the radiochemical assays were repeated on a larger scale with [^{3}H]-labelled GDP-Man. After the reaction, the [^{3}H]Man-labeled enzymatic products were purified, divided evenly and digested with glycosidases.

As illustrated in Fig. 5, there is no significant difference in the radioactivities between the control experiments and the samples that have undergone treatment with the α-Man-(1→2)-α-Man-specific *Aspergillus saitoi* α-(1→2)-mannosidase (AS). These results demonstrate that none of the mannosylated products contained an α-Man-(1→2)-α-Man linkage. On the other hand, digestions of the radiolabeled products using α-mannosidases from jack bean (JB, α-(1→2,3,6)-specific) and *Xanthomonas manihotis* (XM, unbranched α-(1→6)-specific) removed essentially

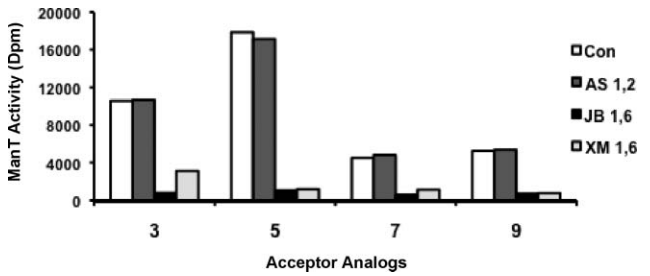


Fig. 5 Mannosidase digestion of products formed from **3**, **5**, **7** and **9**. Each acceptor at 2 mM was incubated with [^{3}H] GDP-Man under the assay conditions as described in the experimental section. The radiolabeled enzymatic products were divided evenly after purification and treated with *exo*-mannosidases including *Aspergillus saitoi* α-(1→2)-mannosidase (AS), jack bean α-(1→2,3,6)-mannosidase (JB) and *Xanthomonas manihotis* α-(1→6)-mannosidase (XM). The mannosidase-digested samples were purified using C_{18} reversed-phase column and the radioactivities were compared with the controls (without any mannosidase treatment).

all the [^{3}H]-labeled mannose units. The combination of these mannosidases enabled the relative proportions of the α-(1→6)- and α-(1→3/4)-linkages to be determined. These results further suggested that the observed mannosylations, in all cases except **3**, resulted exclusively from the action of an α-(1→6)-specific ManT in the membrane preparation.

In the case of **3**, the small difference of the residual radioactivity between the initial product and that treated with mannosidases from jack bean and *X. manihotis* suggests that a small amount of the enzymatic products from **3** might contain α-(1→3/4)-linkages. Besra and co-workers have recently demonstrated that in *M. tuberculosis* an enzyme, MgtA is able to utilize Glc*p*AGroAc$_2$ (1,2-di-*O*-C_{16}/$C_{18:1}$-α-D-glucopyranosyluronic acid-(1→3)-glycerol as a substrate to form α-Man*p*-(1→4)-Glc*p*AGroAc$_2$ (Fig. 6).[44]

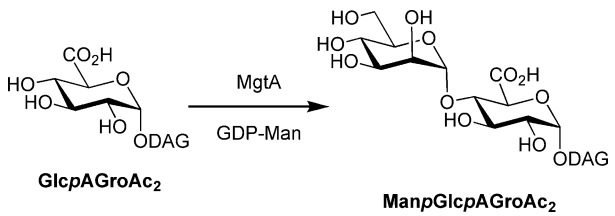


Fig. 6 Reaction catalyzed by MgtA. DAG = diacylglycerol.

It is therefore plausible to speculate that MgtA has a relaxed substrate specificity, which allows it to recognize both disaccharides such as **3** as well as GlcAGroAc$_2$. With regard to this proposal, it is important to note that the non-reducing residue in **3**, is a Glc*p* residue and this may be a reasonable substrate for this newly discovered mannosyltransferase activity. It is also important to consider that an MgtA-like enzymatic activity has not, to date, been identified in *M. smegmatis*, the source of the membrane fraction used in these investigations. Although disaccharide **9** also carries a Glc*p* residue at its non-reducing terminus, no α-(1→3/4)-linkage was detected from the *exo*-mannosidase treatments. Unfortunately, we were unable to carry out any further investigation due to the small turnover of **3**, and the negligible amount of the observed α-(1→3/4)-linked products obtained in the assay.

Inhibition effects of amino analogs

As expected, analogs **2**, **4** and **6**, which lack a hydroxyl group at C-6′, are not substrates of ManT; all compounds show relative activities below 5% (Table 1). These amino derivatives were next tested as inhibitors against ManT using **1** as the substrate. In these studies, analogs **2**, **4** and **6** were screened at a concentration of 2.0 mM with the parent compound **1** at 0.2 mM. As shown in Table 1, disaccharides **2**, **4** and **6** inhibit the mannosylation of **1** by 79, 91 and 70%, respectively. These compounds are thus very weak inhibitors (at 10-fold excess of **1**) and not comparable to other aminosugar-containing acceptor analogs, which have been demonstrated to be very potent glycosyltransferase inhibitors.[28] To examine if the inhibition was proportional to an increase in inhibitor concentration, we studied the mannosylation of **1** (fixed concentration of 0.2 mM) by the α-(1→6)-ManT in the presence of these amino analogs at concentrations up to 4 mM. In all cases, 50% inhibition was observed at about 1 mM under these conditions, as shown in Fig. 7.

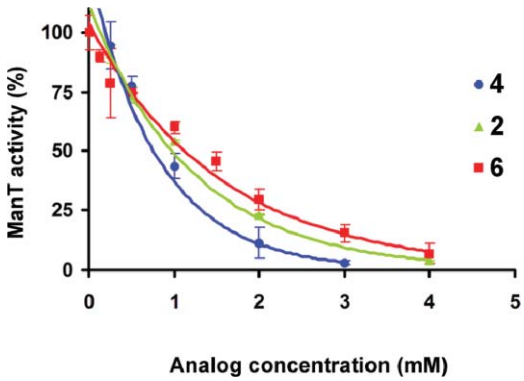


Fig. 7 Inhibition effects of amino analogs **2**, **4** and **6** on ManT activity. The activities were determined using aminosugar analog concentrations up to 4.0 mM with **1** as the acceptor substrate at 0.2 mM. All other reaction conditions were identical to the cell-free assay as described in the experimental section.

Based on the data shown in Table 1, the substrate preference for ManT, based on the relative activity of the epimeric substrate analogs, appears to follow the order of α-Man*p*-(1→6)-α-Man*p* **1** (100%) > α-Man*p*-(1→6)-α-Tal*p* **5** (57%) > α-Glc*p*-(1→6)-α-Man*p* **3** (41%). We were therefore surprised to see that the 6-amino-α-Glc*p*-(1→6)-α-Man*p* analog **4**, rather than 6-amino-α-Man*p*-(1→6)-α-Man*p* analog **2** is a comparably better inhibitor. One explanation for this result is that these amino analogs may not be competing with **1** in the active site, but rather act in a noncompetitive or uncompetitive fashion. However, given that these analogs are at best modest inhibitors of α-(1→6)-mannosylation, we chose not to further probe the mechanism by which they inhibit the enzyme.

Conclusions

In this paper we have described the synthesis of a panel of epimeric and amino analogs of disaccharide **1** and their evaluation as potential substrates and inhibitors of a PPM-dependent α-(1→6)-ManT involved in LAM/LM biosynthesis. The results presented here are in agreement with our previous findings that the hydroxyl

groups at C-4′ and C-2 positions in **1** are important for both the polar interactions and steric requirements. In particular, a change of the *manno*- configuration of the parent substrate **1**, at either the reducing or non-reducing end, to the *gluco*- or *talo*-configuration, results in poor turnover by the ManT, with the most pronounced loss of activity being observed for a compound in which the stereochemistry at C-4′ and C-2 are both inverted. Analogs **2**, **4** and **6**, in which the OH group at C-6′ has been substituted with an amino group, are weak inhibitors against ManT. Although the observed inhibition may result from an ionic interaction of the protonated amino groups of **2**, **4** and **6** with a negatively charged residue in the enzyme active site, the substrate specificity trends observed in the epimeric substrates are different from those seen with the aminosugar analogs. This suggests that the mode of inhibition may not be competitive. Regardless of the mode of action, the α-Man*p*-(1→6)-α-Man*p* (**2**), α-Glc*p*-(1→6)-α-Man*p* (**4**), and α-Man*p*-(1→6)-α-Tal*p* (**6**) analogs all show 50% inhibition at a concentration of ~1 mM. A summary of these results in presented graphically in Fig. 8.

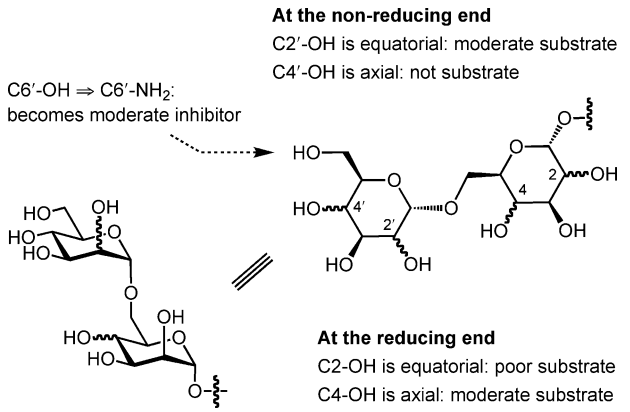


Fig. 8 Graphical summary of activity of analogs **1–9** with α-(1→6)-ManT.

Experimental section

General methods for chemical synthesis

All reagents used were purchased from commercial sources and were used without further purification unless noted. Solvents 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 (rt) and under a positive pressure of argon. The reactions were monitored by analytical TLC on silica gel 60-F_{254} (0.25 mm, Silicycle) 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 chromatography using silica gel (40–60 μM), Iatrobeads (Iatron Laboratories, Tokyo) or SepPak C_{18} reversed-phase cartridges (Waters). Before use, the SepPak cartridges were prewashed with 10 mL of MeOH followed by 10 mL of H_2O. The crude residue in water was loaded onto the prewashed column. After washing the column with water (10 mL), the desired product was eluted with MeOH (4 mL). Optical rotations were measured at 22 ± 2 °C and are in units of degrees mL/(g dm). ^{1}H NMR spectra were recorded at 400, 500 or 600 MHz, and chemical shifts are referenced to either TMS (0.0, $CDCl_3$), or HOD (4.78, D_2O and CD_3OD). ^{13}C NMR spectra were recorded at 100 or 125 MHz and chemical shifts are referenced to internal $CDCl_3$ (77.23, $CDCl_3$), or CD_3OD (48.9, CD_3OD). Assignments of NMR spectra were made based on two-dimensional (^{1}H–^{1}H COSY and HMQC) experiments. The stereochemistry at the anomeric centres of the mannopyranose and talopyranose rings were proven by measuring the $^1J_{C1\text{-}H1}$.[7] ^{19}F spectra were recorded at 376 MHz, and chemical shifts were referenced to external $CFCl_3$. Samples were prepared by the cast film method and infrared spectra were measured on a FT-IR spectrometer. Electrospray mass spectra were recorded on samples suspended in mixtures of THF with MeOH and added NaCl.

Octyl 6-amino-6-deoxy-α-D-mannopyranosyl-(1→6)-α-D-mannopyranoside (2). Disaccharide **30** (17 mg, 0.020 mmol) was dissolved in MeOH (4 mL) and 1 M NaOMe (1 mL) was added. After stirring overnight, the solution was neutralized with Amberlite 120 resin (H^+ form), filtered and concentrated. The crude was dissolved in satd aq $NaHCO_3$ (5 mL) and extracted with EtOAc (5 mL). The aqueous layer was purified by SepPak C_{18} reversed-phase column to give **2** (6 mg, 61%), after lyophilization, as white solid; $[\alpha]_D$ = +75.0 (*c* 0.3, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD) δ_H 4.78 (d, 1H, *J* = 1.7 Hz, H-1′), 4.69 (d, 1H, *J* = 1.7 Hz, H-1), 3.87–3.93 (m, 1H, H-5′), 3.83 (dd, 1H, *J* = 3.5, 1.7 Hz, H-2′), 3.76 (dd, 1H, *J* = 2.9, 1.7 Hz, H-2), 3.58–3.74 (m, 6H, H-3′, H-4, H-5, H-6a, H-6b, octyl OC*H*$_2$), 3.57 (dd, 1H, *J* = 7.2, 2.9 Hz, H-3), 3.50 (dd, 1H, *J* = 9.5, 9.5 Hz, H-4′), 3.39 (dt, 1H, *J* = 9.5, 6.3 Hz, octyl OC*H*$_2$), 2.97 (dd, 1H, *J* = 13.4, 2.7 Hz, H-6a′), 2.74 (dd, 1H, *J* = 13.4, 7.2 Hz, H-6b′), 1.52–1.64 (m, 2H, octyl OCH$_2$C*H*$_2$), 1.22–1.44 (m, 10H, octyl C*H*$_2$), 0.89 (t, 3H, *J* = 6.9 Hz, octyl C*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD) δ_C 101.7 (C-1′), 101.4 (C-1), 73.1 (2C, C-5′, C-5), 72.9 (C-3), 72.5 (C-3′), 72.2 (C-2′/C-2), 72.1 (C-2′/C-2), 70.1 (C-4′), 68.7 (octyl O*C*H$_2$), 68.6 (C-4), 67.4 (C-6), 43.2 (C-6′), 33.0 (octyl *C*H$_2$), 30.6 (octyl *C*H$_2$), 30.5 (octyl *C*H$_2$), 30.4 (octyl *C*H$_2$), 27.4 (octyl *C*H$_2$), 23.7 (octyl *C*H$_2$), 14.4 (octyl *C*H$_3$). HRMS (ESI) calcd. for (M + H) $C_{20}H_{40}NO_{10}$: 454.2647. Found: 454.2645.

Octyl α-D-glucopyranosyl-(1→6)-α-D-mannopyranoside (3). Disaccharide **31** (37 mg, 0.036 mmol) was dissolved in CH_3OH (4 mL) and 20% $Pd(OH)_2$–C (15 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 crude product was purified by chromatography on Iatrobeads (4 : 1 CH_2Cl_2–CH_3OH) to give **3** (14 mg, 82%) as clear glass. R_f 0.54 (4 : 1 CH_2Cl_2–MeOH); $[\alpha]_D$ = +99.1 (*c* 0.3, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD) δ_H 4.82 (d, 1H, *J* = 3.7 Hz, H-1′), 4.70 (d, 1H, *J* = 1.7 Hz, H-1), 4.00 (dd, 1H, *J* = 10.6, 4.0 Hz, H-6a), 3.61–3.82 (m, 9H, H-3′, H-5′, H-6a′, H-6b′, H-2, H-3, H-4, H-5, octyl OC*H*$_2$), 3.60 (dd, 1H, *J* = 10.6, 2.3 Hz, H-6b), 3.39 (dt, 1H, *J* = 9.6, 6.3 Hz, octyl OC*H*$_2$), 3.35 (dd, 1H, *J* = 9.6, 3.7 Hz, H-2′), 3.30 (overlap with residual CD_2HOD peak, 1H, H-4′), 1.52–1.62 (m, 2H, octyl OCH$_2$C*H*$_2$), 1.23–1.42 (m, 10H, octyl C*H*$_2$), 0.89 (t, 3H, *J* = 7.0 Hz, octyl C*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD) δ_C 101.8 (C-1), 100.0 (C-1′), 74.3 (C-5′/C-5), 73.9 (C-2′), 73.5 (C-5′/C-5), 72.8 (C-3′, C-3), 72.3 (C-2), 71.8 (C-4′), 68.8 (octyl O*C*H$_2$), 68.4 (C-4), 67.3 (C-6), 62.6 (C-6′), 33.0 (octyl *C*H$_2$), 30.6(3) (octyl *C*H$_2$), 30.5(6) (octyl *C*H$_2$), 30.4 (octyl *C*H$_2$), 27.4

(octyl CH_2), 23.7 (octyl CH_2), 14.4 (octyl CH_3). HRMS (ESI) calcd. for (M + Na) $C_{20}H_{38}O_{11}$: 477.2306. Found: 477.2305.

Octyl 6-amino-6-deoxy-α-D-glucopyranosyl-(1→6)-α-D-mannopyranoside (4). Disaccharide **34** (61 mg, 0.062 mmol) was dissolved in MeOH (4.5 mL) and 1 M NaOMe (1 mL) was added. After stirring overnight, the solution was neutralized with Amberlite 120 resin (H^+ form), filtered and concentrated. The crude was dissolved in satd aq $NaHCO_3$ (5 mL) and extracted with EtOAc (5 mL). The aqueous layer was purified by SepPak C_{18} reversed-phase column to give **4** (14 mg, 79%), after lyophilization, as white solid; $[\alpha]_D = +149.2$ (*c* 0.1, CH_3OH); ^1H NMR (500 MHz, CD_3OD) δ_H 4.81 (d, 1H, $J = 3.8$ Hz, H-1′), 4.71 (d, 1H, $J = 1.8$ Hz, H-1), 4.01 (dd, 1H, $J = 10.5$, 3.9 Hz, H-6a), 3.77 (dd, 1H, $J = 9.6$, 9.6 Hz, H-4), 3.77 (dd, 1H, $J = 3.2$, 1.8 Hz, H-2), 3.58–3.74 (m, 6H, H-3′, H-5′, H-3, H-5, H-6b, octyl OCH_2), 3.40 (dt, 1H, $J = 9.6$, 6.3 Hz, octyl OCH_2), 3.35 (dd, 1H, $J = 9.7$, 3.8 Hz, H-2′), 3.13 (dd, 1H, $J = 9.8$, 8.9 Hz, H-4′), 3.00 (dd, 1H, $J = 13.4$, 2.8 Hz, H-6a′), 2.68 (dd, 1H, $J = 13.4$, 7.7 Hz, H-6b′), 1.52–1.64 (m, 2H, octyl OCH_2CH_2), 1.23–1.42 (m, 10H, octyl CH_2), 0.88 (t, 3H, $J = 7.0$ Hz, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ_C 101.9 (C-1), 99.8 (C-1′), 75.3 (C-5′/C-5), 73.9 (C-5′/C-5), 73.6 (C-2′), 73.5 (C-4′), 72.9, 72.8 (C-3′, C-3), 72.2 (C-2), 68.9 (octyl OCH_2), 68.2 (C-4), 67.1 (C-6), 43.8 (C-6′), 33.0 (octyl CH_2), 30.6(3) (octyl CH_2), 30.5(5) (octyl CH_2), 30.4 (octyl CH_2), 27.4 (octyl CH_2), 23.7 (octyl CH_2), 14.4 (octyl CH_3). HRMS (ESI) calcd. for (M + H) $C_{20}H_{40}NO_{10}$: 454.2647. Found: 454.2649.

Octyl α-D-mannopyranosyl-(1→6)-α-D-talopyranoside (5). Protected disaccharide **36** (84 mg, 0.077 mmol) was dissolved in THF (5 mL) and AcOH (12 μL, 0.46 mmol) and 1.0 M tetra-*n*-butylammonium fluoride in THF (0.15 mL, 0.15 mmol) were added. After stirring overnight, the mixture was filtered through a short pad of silica gel and concentrated. The crude product was redissolved in 4 : 1 AcOH–H_2O (5 mL) and heated at 50 °C for 3 h. The mixture was diluted with EtOAc (20 mL), washed with satd aq $NaHCO_3$ (5 mL), dried (Na_2SO_4), and concentrated. The resulting product was subsequently dissolved in MeOH (5 mL) and NaOMe (54 mg) was added. After 2 h, the solution was neutralized with AcOH and, following concentration, the crude product was purified by chromatography on Iatrobeads (4 : 1 CH_2Cl_2–CH_3OH) to give **5** (21 mg, 91% over three steps) as clear glass. R_f 0.54 (4 : 1 CH_2Cl_2–MeOH); $[\alpha]_D = +78.2$ (*c* 0.4, CH_3OH); ^1H NMR (600 MHz, CD_3OD) δ_H 4.80 (br s, 1H, H-1), 4.79 (d, 1H, $J = 1.8$ Hz, H-1′), 3.60–3.74 (m, 2H, H-5, H-6a), 3.82 (dd, 1H, $J = 11.8$, 2.3 Hz, H-6a′), 3.80 (dd, 1H, $J = 3.3$, 1.8 Hz, H-2′), 3.58–3.74 (m, 9H, H-3′, H-4′, H-5′, H-6b′, H-2, H-3, H-4, H-6b, octyl OCH_2), 3.43 (dt, 1H, $J = 9.7$, 6.3 Hz, octyl OCH_2), 1.52–1.62 (m, 2H, octyl OCH_2CH_2), 1.24–1.41 (m, 10H, octyl CH_2), 0.89 (t, 3H, $J = 7.0$ Hz, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ_C 102.2 (C-1), 101.6 (C-1′), 74.6 (C-5′), 72.7, 72.6 (C-3′, C-3), 72.2 (C-2), 71.7 (C-2′), 71.0 (C-5), 68.9 (octyl OCH_2), 68.5 (C-4′), 67.9 (C-6), 67.2 (C-4), 62.9 (C-6′), 33.0 (octyl CH_2), 30.6 (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 (octyl CH_3). HRMS (ESI) calcd. for (M + Na) $C_{20}H_{38}O_{11}$: 477.2306. Found: 477.2307.

Octyl 6-amino-6-deoxy-α-D-mannopyranosyl-(1→6)-α-D-talopyranoside (6). Disaccharide **37** (61 mg, 0.062 mmol) was dissolved in 4 : 1 AcOH–H_2O (5 mL) and heated at 50 °C for 6 h. The mixture was cooled, diluted with EtOAc (20 mL), washed with satd aq $NaHCO_3$ (5 mL), dried (Na_2SO_4), and concentrated. The crude product was subsequently dissolved in MeOH (4 mL) and 1 M NaOMe (1 mL) was added. After stirring overnight, the solution was neutralized with Amberlite 120 resin (H^+ form), filtered and concentrated. The azidosugar intermediate was dissolved in CH_3OH (8 mL) and 20% $Pd(OH)_2$–C (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 eluant was concentrated, redissolved in satd aq $NaHCO_3$ (5 mL) and extracted with EtOAc (5 mL). The aqueous layer was purified by on a SepPak C_{18} reversed-phase cartridge to give **6** (24 mg, 86%), after lyophilization, as white solid; $[\alpha]_D = +100.0$ (*c* 0.2, CH_3OH); ^1H NMR (500 MHz, CD_3OD) δ_H 4.81 (br s, 1H, H-1), 4.79 (d, 1H, $J = 1.6$ Hz, H-1′), 3.86–3.94 (m, 2H, H-5, H-6a), 3.80 (dd, 1H, $J = 3.3$, 1.8 Hz, H-2′), 3.77–3.81 (m, 1H, H-2), 3.58–3.75 (m, 6H, H-3′, H-5′, H-3, H-4, H-6b, octyl OCH_2), 3.52 (dd, 1H, $J = 9.5$, 9.5 Hz, H-4′), 3.42 (dt, 1H, $J = 9.6$, 6.3 Hz, octyl OCH_2), 3.05 (dd, 1H, $J = 13.5$, 2.9 Hz, H-6a′), 2.83 (dd, 1H, $J = 13.5$, 7.0 Hz, H-6b′), 1.52–1.64 (m, 2H, octyl OCH_2CH_2), 1.23–1.42 (m, 10H, octyl CH_2), 0.90 (t, 3H, $J = 6.9$ Hz, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ_C 102.2 (C-1), 101.6 (C-1′), 73.7 (C-5′), 72.6, 72.5 (C-3′, C-3), 72.1 (C-2′), 71.8 (C-2), 71.1 (C-5), 69.8 (C-4′), 68.9 (octyl OCH_2), 68.2 (C-6), 67.2 (C-4), 43.4 (C-6′), 33.0 (octyl CH_2), 30.6 (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 (octyl CH_3). HRMS (ESI) calcd. for (M + H) $C_{20}H_{40}NO_{10}$: 454.2647. Found: 454.2649.

Octyl α-D-mannopyranosyl-(1→6)-α-D-glucopyranoside (7). Disaccharide **38** (45 mg, 0.041 mmol) was dissolved in MeOH (6 mL) and NaOMe (32 mg) was added. After stirring overnight, the solution was neutralized with acetic acid, concentrated, and the residue was purified by chromatography (10 : 1 CH_2Cl_2–CH_3OH). The pure fractions were collected, concentrated, and redissolved in CH_3OH (8 mL) and 20% $Pd(OH)_2$–C (30 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 **7** (16 mg, 84%) as clear glass. R_f 0.36 (4 : 1 CH_2Cl_2–MeOH); $[\alpha]_D = +95.7$ (*c* 0.5, CH_3OH); ^1H NMR (600 MHz, CD_3OD) δ_H 4.80 (d, 1H, $J = 1.7$ Hz, H-1′), 4.74 (d, 1H, $J = 3.9$ Hz, H-1), 3.89 (dd, 1H, $J = 11.1$, 5.3 Hz, H-6a), 3.78–3.84 (m, 2H, H-2′, H-6a′), 3.65–3.74 (m, 5H, H-3′, H-6b′, H-5, H-6b, octyl OCH_2), 3.58–3.65 (m, 3H, H-4′, H-5′, H-3), 3.43 (dt, 1H, $J = 9.6$, 6.3 Hz, octyl OCH_2), 3.37 (dd, 1H, $J = 9.7$, 3.9 Hz, H-2), 3.33 (dd, 1H, $J = 10.0$, 9.0 Hz, H-4), 1.56–1.69 (m, 2H, octyl OCH_2CH_2), 1.24–1.46 (m, 10H, octyl CH_2), 0.89 (t, 3H, $J = 6.6$ Hz, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ_C 101.5 (C-1′), 100.2 (C-1), 75.3 (C-3), 74.4 (C-2), 73.6, 72.7, 72.2, 72.1 (4*C*, C-2′, C-3′, C-5′, C-5), 71.7 (C-4), 69.3 (octyl OCH_2), 68.6 (C-4′), 67.1 (C-6), 62.9 (C-6′), 33.0 (octyl CH_2), 30.7 (octyl CH_2), 30.6 (octyl CH_2), 30.4 (octyl CH_2), 27.4 (octyl CH_2), 23.7 (octyl CH_2), 14.4 (octyl CH_3). HRMS (ESI) calcd. for (M + Na) $C_{20}H_{38}O_{11}$: 477.2306. Found: 477.2305.

Octyl α-D-talopyranosyl-(1→6)-α-D-mannopyranoside (8). Disaccharide **39** (39 mg, 0.037 mmol) was dissolved in THF (3 mL) and 1.0 M tetra-*n*-butylammonium fluoride in THF (0.15 mL, 0.15 mmol) was added and the solution was stirred at rt overnight. The reaction mixture was diluted with CH_2Cl_2

(15 mL), washed with H_2O (5 mL), and the organic phase was dried (Na_2SO_4) and concentrated. The resulting crude product was redissolved in MeOH (4 mL) and NaOMe was added (22 mg). The reaction mixture was stirred for 3 h and neutralized with AcOH. The solution was then diluted with CH_2Cl_2 (15 mL), washed with H_2O (5 mL), and the organic phase was concentrated. The partially deprotected intermediate was dissolved and stirred in 4 : 1 HOAc–H_2O (5 mL) at 50 °C overnight. The mixture was diluted with CH_2Cl_2 (15 mL), washed with satd aq $NaHCO_3$ (5 mL × 2), and the organic phase was dried (Na_2SO_4) and concentrated. The resulting crude product was redissolved in CH_3OH (8 mL) and 20% $Pd(OH)_2$–C (15 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 purified by chromatography on Iatrobeads (4 : 1 CH_2Cl_2–MeOH) to give **8** (8 mg, 45%) as clear glass. R_f 0.38 (4 : 1 CH_2Cl_2–MeOH); $[\alpha]_D$ = +83.5 (*c* 0.3, CH_3OH); ^{1}H NMR (600 MHz, CD_3OD) δ_H 4.90 (d, 1H, *J* = 1.5 Hz, H-1′), 4.70 (d, 1H, *J* = 1.7 Hz, H-1), 3.80–3.93 (m, 3H, H-3′, H-4′, H-6a), 3.77 (dd, 1H, *J* = 3.2, 1.7 Hz, H-2), 3.71–3.77 (m, 5H, H-2′, H-5′, H-6a′, H-6b′, H-6b), 3.60–3.69 (m, 4H, H-3, H-4, H-5, octyl OC*H*$_2$), 3.40 (dt, 1H, *J* = 9.6, 6.4 Hz, octyl OC*H*$_2$), 1.52–1.62 (m, 2H, octyl OCH$_2$C*H*$_2$), 1.24–1.42 (m, 10H, octyl C*H*$_2$), 0.90 (t, 3H, *J* = 7.2 Hz, octyl C*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD) δ_C 102.0 (C-1′), 101.7 (C-1), 73.1, 72.8, 72.5, 72.2 (5C, C-2′, C-3′, C-2, C-3, C-4), 71.8 (C-4′), 68.6 (octyl O*C*H$_2$), 68.6 (C-5), 67.5 (C-6), 67.3 (C-5′), 62.9 (C-6′), 33.0 (octyl *C*H$_2$), 30.6 (octyl *C*H$_2$), 30.5 (octyl *C*H$_2$), 30.4 (octyl *C*H$_2$), 27.4 (octyl *C*H$_2$), 23.7 (octyl *C*H$_2$), 14.4 (octyl *C*H$_3$). HRMS (ESI) calcd. for (M + Na) $C_{20}H_{38}O_{11}$: 477.2306. Found: 477.2303.

Octyl α-D-glucopyranosyl-(1→6)-α-D-talopyranoside (9). Disaccharide **40** (85 mg, 0.081 mmol) was dissolved in THF (7 mL) and 1.0 M tetra-*n*-butylammonium fluoride in THF (0.32 mL, 0.32 mmol) was added and the solution was stirred at rt overnight. The reaction mixture was diluted with CH_2Cl_2 (15 mL), washed with H_2O (5 mL), and the organic phase was dried (Na_2SO_4) and concentrated. The resulting crude product was redissolved in MeOH (8 mL) and NaOMe was added (43 mg). The solution was stirred for 2 h and neutralized with AcOH. The mixture was diluted with CH_2Cl_2 (15 mL), washed with H_2O (5 mL), and the organic phase was concentrated. The partially deprotected intermediate was dissolved and stirred in 4 : 1 HOAc–H_2O (5 mL) at 50 °C overnight. The mixture was diluted with CH_2Cl_2 (15 mL), washed with sat. aq. $NaHCO_3$ (5 mL x 2), and the organic phase was dried (Na_2SO_4) and concentrated. The resulting crude product was redissolved in CH_3OH (8 mL) and 20% $Pd(OH)_2$–C (20 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 purified by chromatography on Iatrobeads (4 : 1 CH_2Cl_2–MeOH) to give **9** (23 mg, 62%) as clear glass. R_f 0.33 (4 : 1 CH_2Cl_2–MeOH); $[\alpha]_D$ = +102.9 (*c* 1.3, CH_3OH); ^{1}H NMR (500 MHz, CD_3OD) δ_H 4.82 (d, 1H, *J* = 3.7 Hz, H-1′), 4.80 (d, 1H, *J* = 1.5 Hz, H-1), 3.93 (dd, 1H, *J* = 6.3, 6.3 Hz, H-5), 3.84–3.90 (m, 2H, H-4, H-6a), 3.79 (dd, 1H, *J* = 11.9, 2.4 Hz, H-6a′), 3.66–3.76 (m, 5H, H-6b′, H-2, H-3, H-6b, octyl OC*H*$_2$), 3.59–3.66 (m, 2H, H-3′, H-5′), 3.41 (dt, 1H, *J* = 9.7, 6.4 Hz, octyl OC*H*$_2$), 3.40 (dd, 1H, *J* = 9.8, 3.7 Hz, H-2′), 3.31 (dd, 1H, *J* = 9.8, 8.9 Hz, H-4′), 1.52–1.62 (m, 2H, octyl OCH$_2$C*H*$_2$), 1.24–1.42 (m, 10H, octyl C*H*$_2$), 0.89 (t, 3H, *J* = 7.2 Hz, octyl C*H*$_3$); ^{13}C NMR (125 MHz, CD_3OD) δ_C 102.3 (C-1), 100.2 (C-1′), 75.2 (C-3′), 73.8 (C-5′), 73.6 (C-2′), 72.7 (C-2), 71.7 (C-4′), 71.4 (C-3), 70.8 (C-5), 69.0 (octyl O*C*H$_2$), 68.0 (C-6), 67.1 (C-4), 62.6 (C-6′), 33.0 (octyl *C*H$_2$), 30.6 (octyl *C*H$_2$), 30.4 (2C, octyl *C*H$_2$), 27.4 (octyl *C*H$_2$), 23.7 (octyl *C*H$_2$), 14.5 (octyl *C*H$_3$). HRMS (ESI) calcd. for (M + Na) $C_{20}H_{38}O_{11}$: 477.2306. Found: 477.2299.

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 100 mL of Luria Bertoni (LB) broth medium containing 0.05% Tween 80 to an A_{600nm} of < 1.0 (~two days from a frozen bacterial stock). 50 mL liquid cultures 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 100 mL of 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS) buffer (adjusted to pH 7.9 with KOH) containing 5 mM β-mercaptoethanol and 10 mM $MgCl_2$ supplemented with Complete Protease Inhibitor Cocktail Tablets (Roche) at 4 °C. The cells were subjected to two passes through a Thermo Spectronic French Pressure Cell Press at 20 000 psi. The cell lysate was centrifuged at 600 × *g* for 15 min and then at 27 000 × *g* for 20 min. The resulting supernatant was centrifuged at 100 000 × *g* for 60 min. The supernatant was carefully removed and the membrane pellets were gently resuspended in 1 mL of 50 mM MOPS buffer, pH 7.9, containing 5 mM β-mercaptoethanol and 10 mM $MgCl_2$. Protein concentration (31.6 mg mL^{-1}) was determined by the BCA(tm) Protein Assay (Pierce) using bovine serum albumin as the standard.

Radiochemical activity assays

The ManT enzyme activity was determined using the previously established cell-free system.[21] Unless indicated otherwise, the synthetic acceptor analogs at a concentration of 2.0 mM were incubated with 0.20 μCi of guanosine diphosphate mannopyranose (GDP-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, and membrane fraction (94.8 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 using radiochemical SepPak C_{18} assays.[30] Briefly, after incubation at 37 °C for 1 h, the reactions were stopped by adding 100 μL of $CHCl_3$–MeOH (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 using 10 mL of Ecolite cocktail. For kinetic analysis, the ManT activities were determined using a range of acceptor concentrations. All other reaction conditions were identical to the cell-free assay as 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 using the Michaelis–Menten equation with the GraphPad Prism 4.0 program (GraphPad Software, San Diego, CA).

Product characterizations from milligram-scale incubations

Larger-scale ManT reactions were performed for the structural characterization using acceptor substrates **3**, **5**, **7** and **9**. A typical reaction containing 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 (3.2 mg) in a total volume of 0.5 mL, was incubated at 37 °C with gentle rotation for 2 days. The reaction was stopped by adding equal volume of $CHCl_3$–MeOH (2 : 1, v/v) and the mixtures were centrifuged. The supernatant was recovered and further diluted with H_2O before loading onto 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 was analyzed by MALDI mass spectrometry on a Voyager Elite time-of-flight spectrometer on sample suspended in 2,5-dihydroxy benzoic acid, using the delayed-extraction mode and positive-ion detection.

Glycosidic linkage analysis by *exo*-mannosidases

Larger-scale ManT reactions (160 μL) were performed using acceptor substrates **3**, **5**, **7** and **9**. A typical reaction 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 (189.6 μg) in a total volume of 160 μL. The radioactive enzymatic products were purified using SepPak C_{18} cartridges. After removal of methanol, the residues were redissolved in 200 μL H_2O. Ten microliters of the suspension were digested with *exo*-mannosidases, *Aspergillus saitoi* α-(1→2)-mannosidase (AS) (Glyko), jack bean α-(1→2,3,6)-mannosidase (JB) (Glyko) and *Xanthomonas manihotis* α-(1→6)-mannosidase (XM) (New England Biolabs), according to the manufacturers procedures. After incubation for one day, the reaction mixtures were purified using a C_{18} reversed-phase cartridge as described above.[30]

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