University of Alberta

Studies of a Mycobacterial α-(1→4)-Mannosyltransferase involved in 3-*O*-methyl-mannose Polysaccharide Biosynthesis

by

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Dedicated to All My Friends and Family!

Abstract

Mycobacteria produce a number of unique carbohydrates, the majority of which are found in the cell wall of the organism. In addition to these extracellular glycans, mycobacteria also produce intracellular glycans. One class of them are the 3-*O*-methy-lmannose polysaccharides (MMPs), whose physiological functions and biosynthesis have not been fully established.

An α-(1→4)-mannosyltransferase (ManT), one of the enzymes involved in MMP biosynthesis, incorporates mannose *via* an unusual α-(1→4)-linkage. However, to date, there is only one report studying this uncommon glycosyltransferase. In this thesis, we sought to expand upon this study to fully characterize the activity, specificity, and identity of ManT.

We hypothesized that access to synthetic MMP fragments would lead to a greater understanding of the substrate specificity of ManT. When using synthetic MMP analogs to probe this enzyme, an unexpected activity for ManT was discovered. This enzyme was previously reported to recognize only substrates having a terminal methylated residue. However, we demonstrated that ManT also recognizes substrates without a methyl group on this terminal mannose residue, and with even better affinity. This finding was supported through careful structural elucidation of the isolated enzymatic products with various techniques including glycosidase digestion, Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) and Nuclear Magnetic Resonance (NMR) spectroscopy, and a comparison of the kinetic parameters of two representative tetrasaccharide substrates.

To further our understanding of MMP biosynthesis we sought to obtain pure ManT enzyme. Our first attempt to purify ManT using synthetic MMP-functionalized affinity chromatography was not successful. Therefore, a bioinformatic approach was used. Following this approach, we identified a putative ManT gene from *M. smegmatis*, and expressed this gene recombinantly in *E. coli*. The activity of the gene product was found to be identical to the native ManT from *M. smegmatis*. This will facilitate further studies of MMP biosynthesis.

In the course of synthesizing MMP analogs for these studies, we developed a four-step methodology to quickly install several methyl groups directly on oligosaccharides. The key reaction involves *n*-Bu_2SnCl_2-mediated simultaneous activation of multiple *cis*-diols. By tuning protecting groups on the substrates, we were able to functionalize multiple *cis*-diols in a consistent and highly regioselective manner.

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$[\alpha]_D$	specific rotation (sodium D line)
3-*O*-Me-Man	3-*O*-methyl-mannose
3-*O*-MT	3-*O*-methyltransferase
6-*O*-Me-Glc	6-*O*-methyl-glucose
6-*O*-MT	6-*O*-methyltransferase
Å	Angstrom
Ac	Acetyl
AcT	Acyltransferase
ADP	adenosine diphosphate
All	Allyl
AMMP	Acetylated MMP
app	apparent
Ar	aromatic
ATCC	American Type Culture Collection
BLAST	basic local alignment search tool
Bn	Benzyl
Boc	*tert*-butoxycarbonyl
Bu	Butyl
Bz	Benzoyl
CAZy	Carbohydrate-Active enZymes

Cb	4-chlorobenzyl
coenzyme A	CoA
COSY	COrrelation SpectroscopY
dba	dibenzylideneacetone
DBU	diazabicyclo[5.4.0]undec-7-ene
DHB	2,5-dihydroxylbenzoic acid
DIPEA	*N*,*N*-Diisopropylethylamine
DMAP	*N*,*N*-Dimethyl-4-amminopyridine
DMF	*N*,*N*-Dimethyl formamide
Dol	dolichol
dpm	decay per minute
EDCI	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EGTA	Ethylene Glycol Tetraacetic Acid
ESI	Electrospray ionization
Et	Ethyl
FAS	fatty acid synthase
GDP-mannose	guanosine diphosphate mannose
GG	glucosylglycerate
GlcT	glucosyltransferase
GpgP	glucosyl-3-phosphoglycerate phosphatase
GpgS	glucosyl-3-phosphoglycerate synthetase
GPI	glycosylphosphatidylinositol
GT	glycosyltransferase

His	hexahistidine
HMBC	Heteronuclear Multiple Bond Coherence
HMQC	Heteronuclear Multiple Quantum Coherence
HSQC	Heteronuclear Single Quantum Coherence
Im	imidazole
IMAC	Immobilized Metal ion Affinity Chromatography
IPTG	isopropyl β-D-thiogalactopyranoside
IR	Infrared
k_{cat}	Apparent unimolecular rate constant
K_d	Dissociation constant
K_m	Michaelis constant
LAM	lipoarabinomannan
LB	Luria-Bertani
MALDI	Matrix assisted laser desorption ionization
ManT	α-(1→4)-mannosyltransferase
Me	Methyl
MGLP	6-*O*-methyl-glucose lipopolysaccharide
MGP	6-*O*-methyl-glucose polysaccharide
MMP	3-*O*-methyl-mannose polysaccharide
MS	Mass spectrometry
MSH	Mycothiol
MTs	mannosyltransferases
NAC	no acceptor control

NBS	*N*-bromosuccinimide
NCBI	National Center for Biotechnology Information
NHS	*N*-hydroxylsuccinimide
NIS	*N*-iodosuccinimide
NMO	*N*-Methylmorpholine *N*-oxide
NMR	Nuclear magnetic resonance
Ns	*p*-nitrobenzenesulfonyl
NTA	nitrilotriacetic acid
Octyl	Octanyl
OD_{600}	Optical density at 600 nanometer
OMT	3-*O*-methyltransferase
OS	Oligosaccharide
OTf	Trifluoromethanesulfonyl
P	phosphate
Ph	phenyl
Piv	Pivaloyl
PMB	*p*-methoxybenzyl
PMPS	polymethylated polysaccharide
ppm	parts per million
Py	pyridine
R_f	Retention factor
rManT	recombinant ManT
rpm	rounds per minute

SAH	*S*-Adenosyl-L-homocysteine
SAM	*S*-adenosylmethionine
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SM	starting material
TAPS	*N*-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid
TBS	*tert*-Butyldimethylsilyl
TES	Triethylsilyl
THF	Tetrahydrofuran
TIPS	Triisopropylsilyl
TLC	thin layer chromatography
TMS	Trimethylsilyl
TOCSY	Total Correlation SpectroscopY
TOF	Time Of Flight
Tol	*p*-Tolyl
Tr	triphenylmethyl or trityl
Tris	Tris(hydroxymethyl)aminomethane
Ts	*p*-toluenesulfonyl
UDP	uridine diphosphate
V_{max}	maximum velocity

Chapter 1: Introduction

1.1 Mycobacterial intracellular polysaccharides

Mycobacteria, both pathogenic (*e.g., Mycobacterium tuberculosis*) and nonpathogenic (*e.g*., *Mycobacterium smegmatis*), are rich in carbohydrates, of which the majority are anchored extracellularly on the cell wall.[1-2] The resulting multi-layer cell envelope is directly associated with mycobacterial pathogenesis, and hence is a primary target of drug development for the treatment of tuberculosis.[3-5]

In addition to the above mentioned extracellular carbohydrates, mycobacteria also produce a smaller number of intracellular glycoconjugates, including glycogen,[6] trehalose,[7] mycothiol[8] and a group of polymethylated polysaccharides (PMPSs).[9] These intracellular glycans have received much less attention compared with the extracellular cell wall glycans, as they appear not to be directly associated with pathogenesis. For the most part, their physiological functions are still not fully understood. Here, we briefly summarize reports on mycobacterial intracellular glycoconjugates, with a focus on their structures, distributions and functions.

1.1.1 Glycogen structure, distribution and function

Glycogens are branched glucose polymers composed of approximately 90% α-(1$\rightarrow$4)-linked glucopyranose residues as the core structure, with the remaining residues in branched α-(1$\rightarrow$6)-linkages (Figure 1-1).[10-11] These polymers can contain hundreds to thousands of glucose units, but have no defined structures and molecular weight. Their sizes and degrees of branching vary in different

organisms. Glycogens are commonly found in animals and fungi as an energy source.[12] Interestingly, structurally similar glycogens are also present in bacteria.[11]

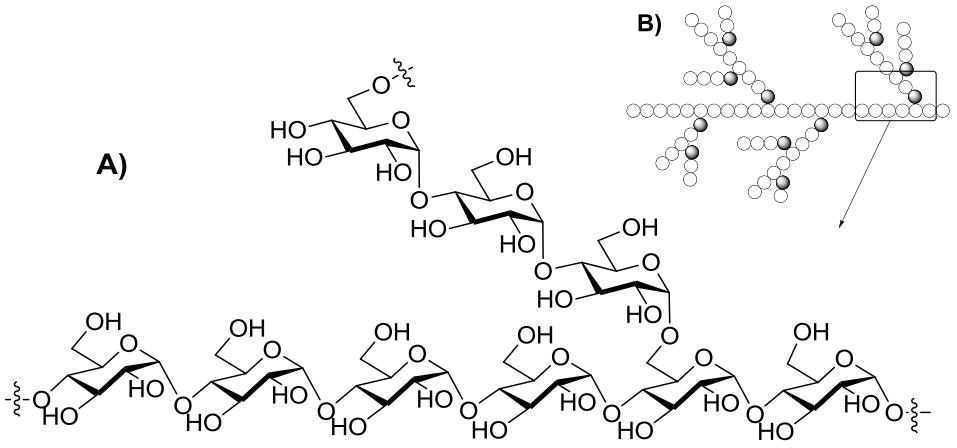


Figure 1-1. Structures of mycobacterial glycogen.

A) Structural component of glycogen; B) Schematic representation of glycogen. White circles are α-(1→4)-linked glucose residues and shaded circles are α-(1→6)-linked glucose residues. The structure in the box is shown in A.

The first mycobacterial glycogen was identified from *M. phlei* in 1961.[13] Since then, glycogen production has also been discovered in other mycobacterial species, including pathogenic *M. tuberculosis* and non-pathegenic *M. smegmatis*.[14] The major difference between mammalian and bacterial glycogens is that mammalian glycogens are attached to proteins in the form of glycoproteins, while bacterial glycogens are not and, therefore, exist in a free state in the cytoplasm.[15] Intracellular glycogen appears as particles in the cytoplasm with sizes ranging from 20 to 150 nm in diameter.[10,14] Similar to glycogen from animals and fungi, the function of mycobacterial glycogen is also as an energy reserve.[6]

1.1.2 Trehalose structure, distribution and function

Extracellular trehalose-containing lipids are important structural components of the mycobacterial cell wall.[16] In addition, free trehalose and trehalose-based oligosaccharides are also found in the cytoplasm of mycobacteria, including *M. tuberculosis* and *M. smegmatis* (Figure 1-2).[7] In one particular case, sulfated trehalose (trehalose-2-sulfate) has been observed in the cytoplasmic fraction of both *M. tuberculosis* and *M. smegmatis*.[17] These trehalose-based small molecules are proposed to be biosynthetic precursors of the related trehalose-containing glycolipids in the cell envelope.[17]

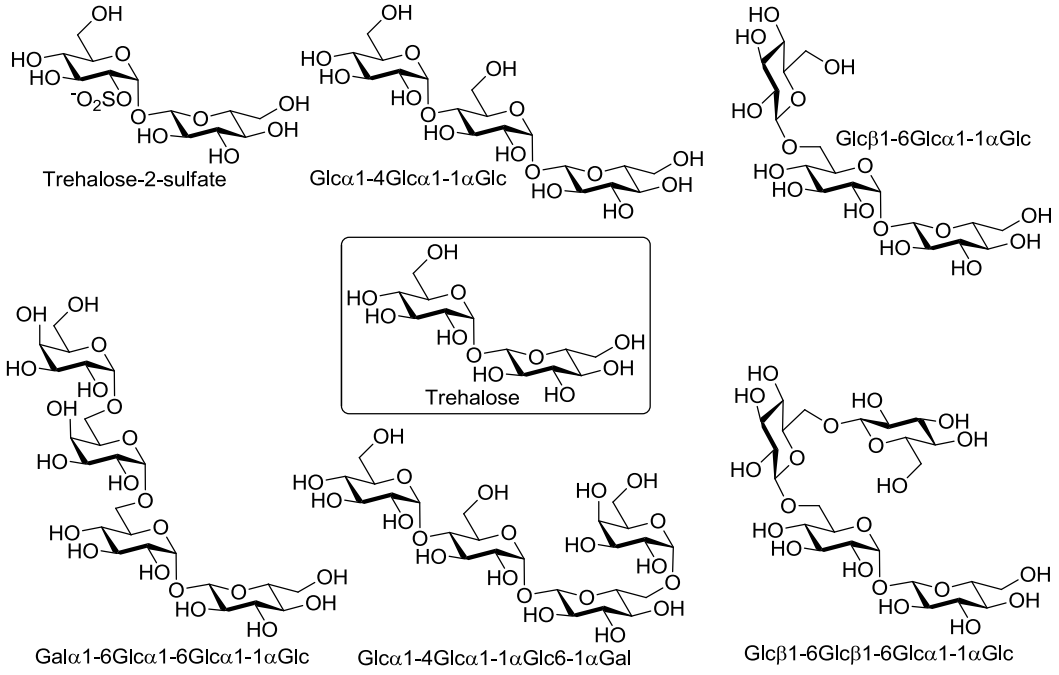


Figure 1-2. Intracelluar trehalose-based oligosaccharides in mycobacteria.

Differing from most other bacteria, which only use one trehalose biosynthetic pathway, mycobacteria possess three possible routes to this species and synthesize more complicated trehalose-based molecules.[18] This suggests a

distinct role for trehalose in mycobacteria. Most bacteria only use free trehalose as an energy source or protectant from osmotic stress;[16] however, the exact function of free trehalose in mycobacteria is still not fully understood.[16] Some evidence suggests that trehalose-based molecules play multifunctional roles, in energy storage, as structural components, as well as a stress protectant.[6,19]

1.1.3 Mycothiol structure, distribution and function

Mycothiol (MSH) is a small molecule composed of L-cysteine, D-glucosamine and 1D-*myo*-inositol (Figure 1-3).[8,20] This intracellular glycothiol was first identified from *Streptomyces* sp. strain AJ 9463 in 1994, and later also isolated from *Mycobacteria bovis*.[21-22] A survey of distribution of MSH in nature shows that a broad spectrum of microorganisms produce MSH, with predominance in the streptomyces and mycobacteria.[23] Six species of mycobacteria have been identified to produce MSH, including *M. tuberculosis*, *M. bovis*, *M. smegmatis*, *M. avium*, *M. chelonae* and *M. fortuitum*.

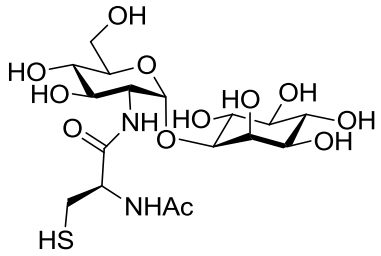


Figure 1-3. Structure of mycobacterial mycothiol (MSH).

MSH production plays an important role in regulating mycobacterial intracellular oxidative stress by removal of foreign oxidants such as H_2O_2 and formaldehyde.[8,24] The mechanism of MSH-dependent detoxification involves

reaction of the thiol group of MSH with an oxidative agent.[25] Because the MSH biosynthetic pathway is absent in humans and other mammals, inhibitors for MSH biosynthesis might provide an alternative solution for the treatment of tuberculosis.[25]

1.1.4 PMPSs structure, distribution and function

PMPSs are another group of glycans located in the cytoplasm of mycobacteria. Two classes of these methylated polysaccharides have been identified, first from *M. phlei*. One class is the 6-*O*-methyl-glucose lipopolysaccharides (MGLPs)[26] and the other is 3-*O*-methyl-mannose polysaccharides (MMPs) (Figure 1-4).[27]

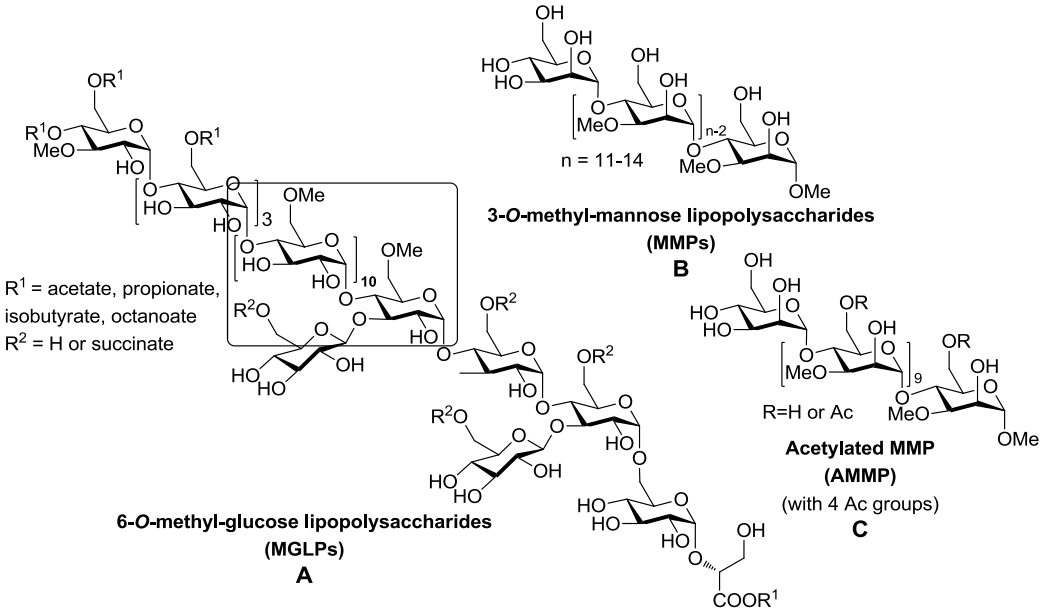


Figure 1-4. Structure of mycobacterial PMPSs.

A). The major MGLP from *M. bovis* BCG (core structure is highlighted in the box); B). MMPs from *M. smegmatis*; C). Structure of AMMPs from *Streptomyces griseus* (four of the *O*-6 positions are acetylated).

These two classes of polysaccharides have been discovered not only in mycobacteria, but also in other bacterial species (Table 1-1). In mycobacteria, MMPs are produced only by fast growing species, while MGLPs are found in nearly all mycobacterial species, as well as in other bacteria, such as Nocardia.[28] In addition to these two polysaccharides, another polysaccharide, structurally related to MMPs, has also been identified in *Streptomyces griseus*,[29-30] which produces partially acetylated forms of MMPs (AMMP) (Figure 1-4, Table 1-1).[31]

Table 1-1. Distribution of PMPSs in bacterial species.

Bacteria	Growth rate	Species	MMPs	MGLPs	Reference
Mycobacteria	fast	*M. phlei*	+	+	26
		M. smegmatis	+	+	32
		M. parafortuitum	+	+	33
		M. cuneatum	+	+	33
		M. petrophilum	+	+	33
		M. chitea	+	+	33
		M. vaccae	+	N.D.	34
	slow	*M. tuberculosis*	–	+	28,35
		M. bovis	N.D.	+	36
		M. leprae	N.D.	+	37
		M. xenopi	N.D.	+	38
Stretomyces	N/A	*S.griseus*	+	N.D.	29-30
Nocardia	N/A	*N. otitidis-caviarurn*	N.D.	+	39
		N.brasiliensis	N.D.	+	39
		N. farcinica	N.D.	+	39
		N. kirovani	N.D.	+	39

+: detected; –: not detected; N.D.: not determined; N/A: Not applicable.

MMPs exist as a mixture of four homologs containing 11–14 α-(1→4)-linked mannopyranose residues with molecular weights ranging in 2000–2500.[32] Each monosaccharide is methylated at the O-3 position, except for the terminal residue at the non-reducing end, which is unsubstituted. The reducing end is blocked with a methyl aglycon (Figure 1-4). AMMP resembles the smallest MMP

with 11 mannose residues, and contains an additional four acetyl groups at the O-6 position of some of the mannose residues (Figure 1-4).[30-31]

Compared with the two mannose-containing PMPSs, MGLPs are more structurally complex. The reported structures of MGLPs have been revised three times since their first discovery, with the final revision being reported in 1998.[36,40-41] MGLPs vary in the total numbers of glucose units and methyl groups present, as well as in the number and nature of acyl group modifications. As a representative, the major component of MGLPs from *M. bovis BCG* is composed of up to 20 glucose units with an average molecular weight of 3500 (Figure 1-4).[36] Of these glucose units, 17 are connected through consecutive α-(1→4)-linkages as the core structure, one in an α-(1→6)-linkage, and the remaining two glucose units are branched off the main chain via β-(1→3)-linkages.

Similar to MMPs, MGLPs are also highly methylated. For example, MGLP from *M. bovis BCG* contains 12 methyl groups in total. Eleven are present at the O-6 positions of backbone glucose units (highlight in box, Figure 1-4) and the final one at the O-3 position of the non-reducing end residue. In contrast to MMPs, the aglycon of MGLPs is a glyceric acid substituted at O-2. MGLPs also differ from MMPs in that, in addition to methyl groups, MGLPs contain a number of acyl groups, including acetates, propionates, isobutyrates, octanoates and succinates. These are located mainly on the O-6 position of unmethylated monosaccharides; however, their exact locations are difficult to determine.[42-43] Another component of MGLPs from the *M. bovis BCG* strain is a branched 2-acetamido-2,6-dideoxy-β-glucopyranose residue instead of a β-(1→3)-glucose at

the first branch point near the aglycon.[36] MGLPs from *M. phlei* and *M. smegmatis* have the same backbone structure but vary by 0–3 succinyl groups.[41,43] Conversely, the major MGLPs from *M. xenopi* lack the tetrasaccharide moiety at the non-reducing end.[38]

A striking property associated with PMPSs is that, *in vitro*, both polysaccharides form stable complexes with long-chain fatty acids and palmityol-coenzyme A (CoA) derivatives (K_d ~ 0.1 μM).[44-45] Nuclear magnetic resonance (NMR) spectroscopy studies have shown that upon titration with lipids, the random coiled MMPs undergo substantial conformational change to adopt a helical conformation, with all the methyl groups pointing inward to create a hydrophobic channel.[46-48] The lipids are postulated to insert into these channels to form a complex with the MMP (Figure 1-5).

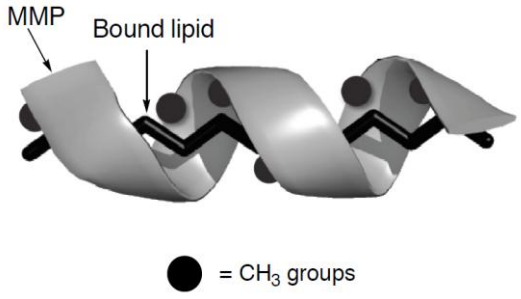


Figure 1-5. Schematic representation of MMP–lipid complex.

In contrast to MMPs, which require an external lipid to drive the conformational change from a random coil to an organized helix, MGLPs are postulated to exist in a helical conformation in nature due to the presence of the additional acetate, propionate, isobutyrate, octanoate and succinate groups.[9] Fluorometric titration studies indicate the complexation of PMPSs with lipids is

thermodynamically favored due to a significant increase in entropy during complexation.[49] If it is assumed that the formed complex is spherical, its size is calculated to have a diameter of 23–26 Å, based on a model of a MGLP–stearic acid complex.[49] More recently, synthetic analogs of both MMPs and MGLPs were found to bind with fatty acids with equal efficiency as natural PMPSs.[50-51]

The tight binding observed for PMPSs with lipids *in vitro* suggests a possible *in vivo* biological role for these polysaccharides as storage vehicles for lipids in mycobacteria.[45] Because the intracellular concentration of PMPSs in *M. smegmatis* (1 mM) is much higher than the concentration of long-chain acyl-CoAs (0.3 mM), all the cytoplasmic fatty acids could theoretically complex with PMPSs. One benefit of this complexation would be to protect acyl-CoA from degradation by thioesterases.[52] In this regard, PMPSs have been shown to influence the activity of enzymes involved in mycobacterial fatty acid biosynthesis.[53] They are proposed to facilitate the release of the long-chain lipid acyl-CoA product from fatty acid synthase I (FAS-I), hence reactivating the enzyme to continuously synthesize more acyl-CoA.[53]

Despite the evidence suggesting a role for PMPSs in fatty acid metabolism, this hypothesis is still questionable. In mutant strains of *M. smegmatis* and *M. tuberculosis* whose abilities to synthesize PMPSs were impaired, the levels of fatty acids were not altered dramatically.[28,54] Thus, an alternative role for PMPSs in maintaining membrane stability during hyperthermal conditions was recently proposed.[55] In this work, it was suggested that PMPSs modify the content of outer membrane mycolic acids, which, in turn, alters the structure of the cell envelope.

More details regarding the possible physiological roles of PMPSs have been recently reviewed by both Brennan and Empadinhas.[9,55]

To date, a complete understanding of the physiological roles of PMPSs is still not known. Furthermore, as not all mycobacteria produce PMPSs, they are likely not essential for mycobacterial survival. Gaining further insight into the biological role of PMPSs will require a full understanding of their biosynthesis. This information will enable the production of mutants lacking PMPS, which will provide further information about their function, *in vivo*. Therefore, next, we will introduce what is known regarding the biosynthesis of MGLPs and MMPs, including genetic information.[9]

1.2 6-*O*-methyl-glucose polysaccharides (MGLPs)

Because MGLPs are produced by pathogenic *M. tuberculosis* while MMPs are not, the biosynthesis of MGLPs has received much more attention. As a result, more is known about the biosynthesis of MGLPs compared to MMPs. The initial studies into MGLP biosynthesis were conducted mainly in the Ballou laboratory.[42,56-59] Recently, with the aid of genetic tools, Jackson and Empadinha independently explored further details about the biosynthetic pathway of MGLPs.[28,56-57,59-62]

1.2.1 Enzymes involved in MGLP biosynthesis

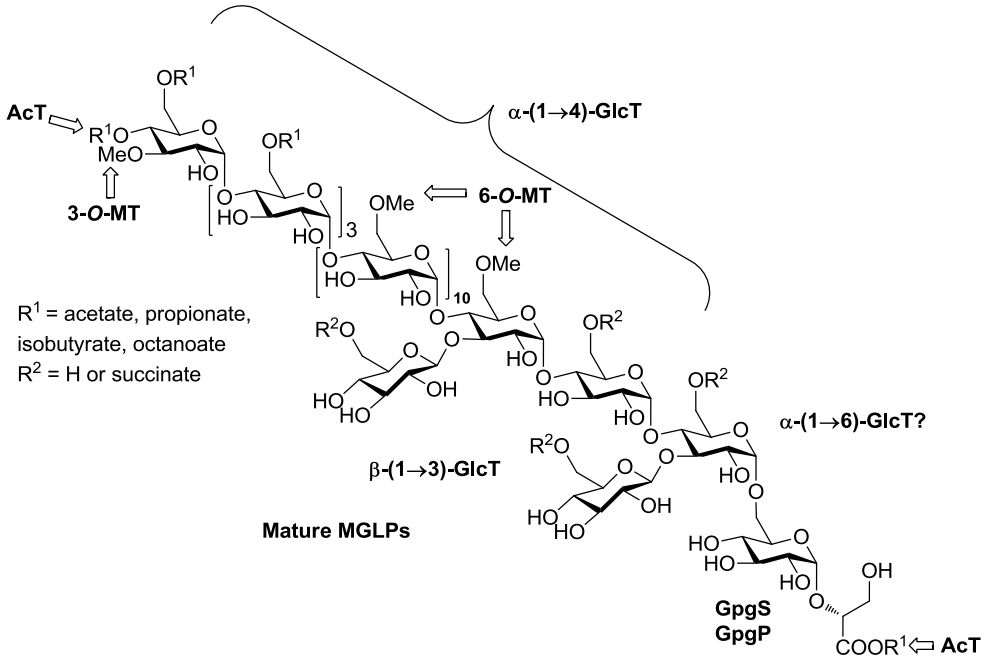


Figure 1-6. Enzymes involved in MGLP biosynthesis.

3-*O*-MT: 3-*O*-methyltransferase; 6-*O*-MT: 6-*O*-methyltransferase; AcT: acyltransferase; GlcT: glucosyltransferase; GpgS: glucosyl-3-phosphoglycerate synthetase; GpgP: glucosyl-3-phosphoglycerate phosphatase.

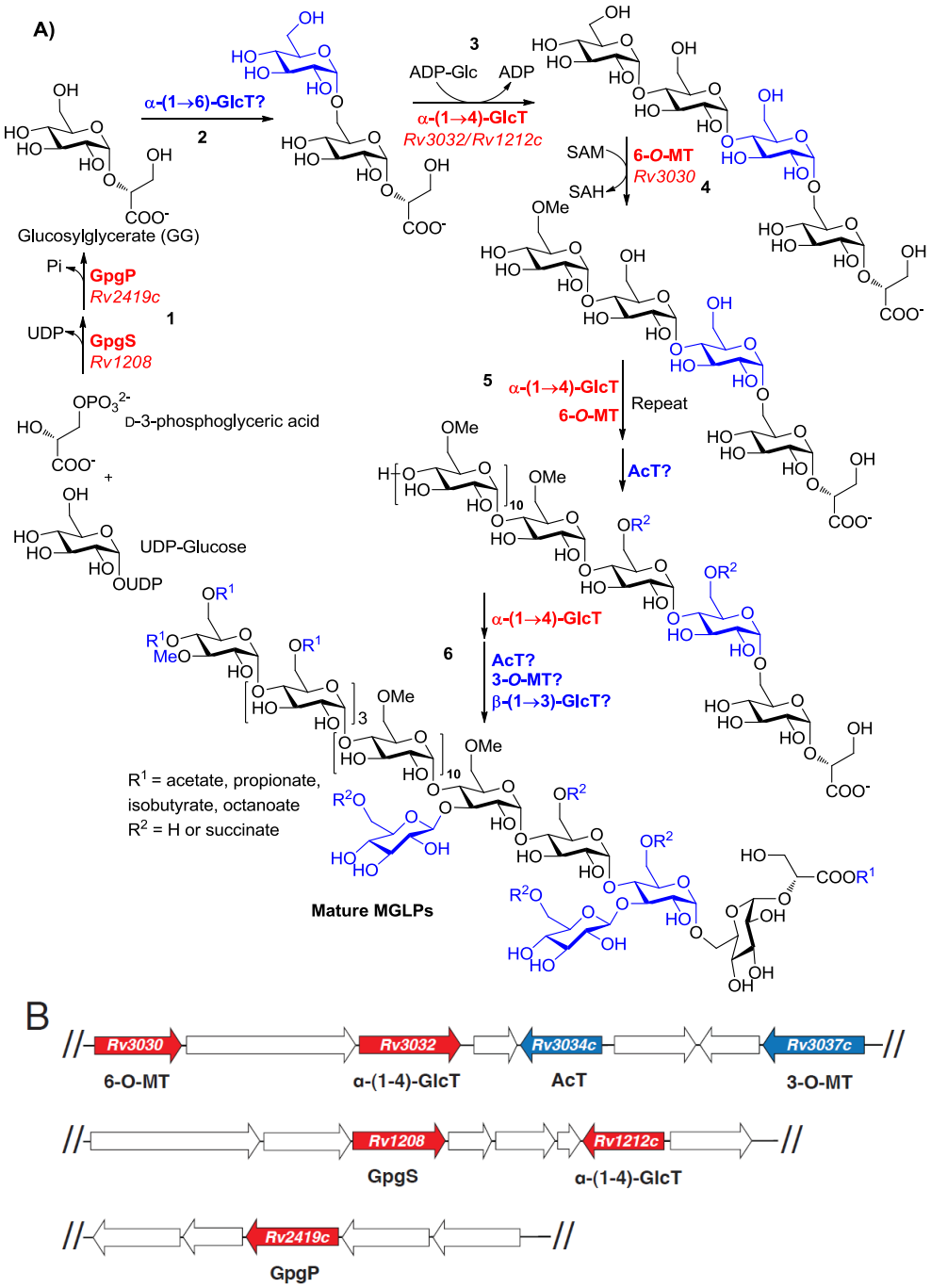
The complex structure of MGLPs suggests a number of enzymes, including glycosyltransferases, methyltransferases and different acyltransferases, would be required for their biosynthesis. In fact, several of these enzymes have now been identified including a 6-*O*-methyltransferase (6-*O*-MT),[28,56-57] some acyltransferases (AcTs)[59], and an α-(1→4)-glucosyltransferase (α-(1→4)-GlcT) enzyme.[28] A glucosyl-3-phosphoglycerate synthase (GpgS)[60-61] and a glucosyl-3-phosphoglycerate phosphatase (GpgP),[62] both involved in assembly of the glyceric acid moiety in the early stage of MGLP biosynthesis, have also been

identified. However, information about the remaining α-(1→6)-GlcT and β-(1→3)-GlcT are still missing (Figure 1-6).

A detailed description of the role these enzymes play in MGLP biosynthesis has been recently reviewed[9,55] and so will not be discussed here in detail. The genes responsible for MGLP biosynthesis are not found in a single gene cluster, but are instead distributed throughout the mycobacterial genome.[9,55] In *M. tuberculosis* H37Rv, for example, at least two gene clusters,[9] and as many as five,[55] have been proposed to be involved in MGLP biosynthesis. The interest in MGLP biosynthesis stems, at least in part, from their proposed role in lipid metabolism,[45,53] and because they are the sole polymethylated polysaccharides (PMPSs) found in slow growing pathogenic species of mycobacteria.[28]

1.2.2 Proposed biosynthetic pathway

As shown in Scheme 1-1, MGLP biosynthesis starts with a glucosylglycerate (GG) unit at the reducing end.[63] There are two known pathways for the biosynthesis of GG.[64] However, mycobacteria appear to use only the two-step pathway (step 1), through the action of a glucosyl-3-phosphoglycerate synthetase (GpgS, encoded by *Rv1208* gene in *M. tuberculosis* H37Rv)[60-61,65] and a recently reported glucosyl-3-phosphoglycerate phosphatase (GpgP, *Rv2419c*).[62] Interestingly, the synthetase and phosphatase are not encoded in a single gene cluster and share little similarity to enzymes serving the same function in other organisms.[55]



Scheme 1-1. Proposed MGLP biosynthesis pathway (A) and discovered genes (B).

3-*O*-MT: 3-*O*-methyltransferase; 6-*O*-MT: 6-*O*-methyltransferase; AcT: acyltransferase; GlcT: glucosyltransferase; GpgS: glucosyl-3-phosphoglycerate synthetase; GpgP: glucosyl-3-phosphoglycerate phosphatase. UDP: uridine diphosphate; ADP: adenosine diphosphate; SAM: *S*-adenosylmethionine; SAH: *S*-adenosyl-L-homocysteine. Enzymes with genes identified are in red and putative enzymes yet to be identified are in blue.

A yet unknown glucosyltransferase (α-(1→6)-GlcT) is proposed to add the first α-(1→6)-linked glucose residue to GG (step 2).[63] The elongation of MGLPs is then proposed to involve the sequential addition of α-(1→4)-glucose residues and 6-*O*-methyl groups by a key glycosyltransferase (α-(1→4)-GlcT) and methyltransferase (6-*O*-MT) encoded by the *Rv3032* and *Rv3030* genes of *M. tuberculosis* H37Rv, respectively (step 3 and 4).[28,63] It has also been found that the glucose residues can be introduced by another α-(1→4)-GlcT (encoded by *Rv1212c*) involved in glycogen and capsular glucan biosynthesis (step 3).[10] Thus, there appears to be redundant mechanisms available for construction of the α-(1→4)-glucose core of MGLPs. The ability of the glycogen α-(1→4)-GlcT, which normally recognizes unmethylated glucose substrates, to function in MGLP biosynthesis also suggests that elongation of the α-(1→4)-Glc core may not strictly involve alternating methylation and glucose transfer reactions. Also, Empadinha and coworkers pointed out trehalose is likely involved in MGLP biosynthesis.[55]

During the stepwise elongation (step 5), acylation was suggested to proceed together with methylation, as membrane-associated acyltransferase enzymes (AcT) recognize the partially acylated substrate preferentially.[59] Hence, the elongated intermediate may already carry some acyl groups before the tetrasaccharide motif at the non-reducing end is installed (step 6). The remaining acyl groups and 3-*O*-methyl group are installed by a combination of AcTs and an unknown 3-*O*-methyltransferase (step 6).

The glycosyltransferase(s) responsible for adding the two β-(1→3)-glucose branches onto MGLPs have yet to be identified. Thus, there are still a number of questions regarding MGLP biosynthesis that remain to be answered. In particular, the mechanism controlling the length of MGLP is still not known.[9,55]

1.3 3-*O*-methyl-mannose polysaccharides (MMPs)

In contrast to MGLPs, MMPs are not produced by pathogenic *M. tuberculosis*, and, hence, do not appear to be directly associated with pathogenesis. Therefore, their appeal to scientists has been less than for the MGLPs. Only a few studies have been reported since their discovery in 1971[27] and no genetic information about MMP biosynthesis is yet available.

1.3.1 Enzymes involved in MMP biosynthesis

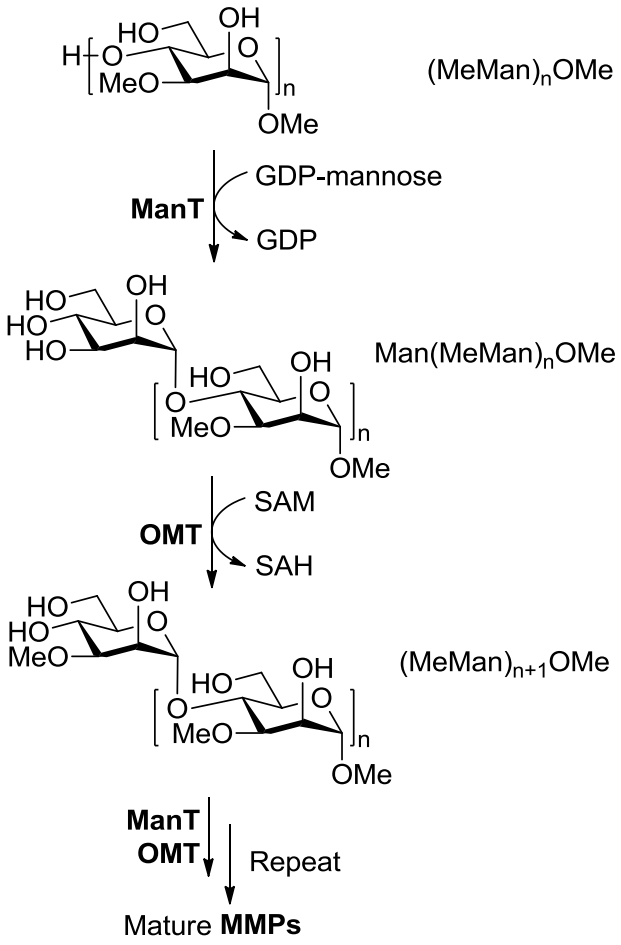
While genetic information about MGLP biosynthesis has been continuously revealed over the past few years, exploration of the biosynthetic pathway of MMPs has not progressed. Prior to the start of my thesis, the only information regarding MMP biosynthesis came from studies by Ballou, which were published in 1984.[33,66] Based on the structural features of MMPs, which are only composed of mannose residues and methyl groups, it was proposed there were two enzymes involved in MMP biosynthesis, a glycosyltransferase and a methyltransferase. Indeed, using cell-free extracts of *M. smegmatis*, Ballou and coworkers were able to detect activities corresponding to an α-(1→4)-mannosyltransferase (ManT),[33] which transfers mannose from guanosine

diphosphate mannose (GDP-mannose) to the O-4 position of mannose units, and a 3-*O*-methyltransferase (OMT)[66], which transfers methyl groups from *S*-adenosylmethionine (SAM) to the O-3 position of the mannose units.

The acceptor specificities of both enzymes are more complicated. According to the previous studies, OMT recognized all the MMP fragments ending with a free mannose (*i.e.,* Man-(MeMan)$_{1\text{-}10}$-OMe), while ManT recognized oligomers ending with a 3-*O*-methyl-mannose moiety (*i.e.*, MeMan-(MeMan)$_{4\text{-}9}$-OMe). However, mono- to trisaccharide MMPs were reported not to be recognized by ManT. The observation that the activity of ManT is detected in membrane fractions while OMT is from the cytoplasmic fraction suggests that ManT is a membrane-associated protein, while OMT is a cytoplasmic protein.

1.3.2 Proposed biosynthetic pathway

Based on the substrate specificities of both ManT and OMT, Ballou and coworkers proposed a model for MMP biosynthesis,[66] in which elongation occurs via alternating mannosylation and methylation reactions (Scheme 1-2). ManT was proposed to recognize the terminal 3-*O*-methyl-mannose residue of the MMP precursor and transfer a mannose residue from GDP-mannose to the O-4 position of the terminal methylated mannose residue. Then, OMT transfers a methyl group from SAM to the O-3 position of the newly introduced mannose residue at the non-reducing terminus. Chain elongation proceeds sequentially through alternating mannosylation and methylation until the polymer reaches 11–14 monosaccharide residues in length.



Scheme 1-2. Proposed biosynthetic pathway for MMP elongation.

GDP: guanosine diphosphate; SAM: *S*-adenosylmethionine; SAH: *S*-Adenosyl-L-homocysteine; ManT: α-(1→4)-mannosyltransferase; OMT: 3-*O*-methyltransferase.

This model is based on the structures of MMP oligomers isolated from *M. smegmatis*.[67] These oligomers, which are terminated with either a free mannose or 3-*O*-methyl-mannose residue at the non-reducing end, were found to be substrates for OMT and ManT, respectively. Therefore, they are possible precursors in MMP biosynthesis. However, it remains unclear whether only two enzymes (*i.e.*, a single ManT and a single OMT) assemble the entire MMP, or whether different ManTs and OMTs are involved at different stages of polymer assembly.

1.3.3 Unsolved problems

1.3.3.1 Initiation of MMP biosynthesis

Although a model of MMP biosynthesis has been established, this model does not explain how the biosynthesis is initiated. ManT was shown to only recognize MMP substrates that are a tetrasaccharide (Man-$(MeMan)_3$-OMe) or larger, suggesting that the tetrasaccharide might be the initial precursor of ManT. However, the question of where, and how, this precursor is assembled remains unanswered. It seems that there might be enzymes, in addition to the proposed ManT and OMT that are involved in MMP biosynthesis. Therefore, further studies to identify and explore these enzymes are necessary.

1.3.3.2 Termination of MMP elongation

Mature MMPs are a mixture of homologous polymers with lengths never longer than 14 sugar residues. This raises the question about how termination occurs during MMP biosynthesis. One possible explanation is attributed to the methylation enzyme OMT. The non-reducing terminal residues of all mature MMPs are not methylated, and thus, OMT was postulated to control the length of MMPs.[66] This enzyme was found to show greater activity with short MMP intermediates, compared to longer ones. As the length of the MMP fragment increases, their affinities with OMT decreases gradually. When used as a OMT substrate, the undecasaccharide (Man-$(MeMan)_{10}$-OMe) was reported to have only 27% of the activity observed for the tetrasaccharide substrate (Man-$(MeMan)_3$-OMe), and the corresponding K_m value determined for the longer

substrate is significantly greater than for the tetrasacharide.[66] Therefore, the longer substrates may no longer be methylated by OMT as MMPs reach certain lengths. As a consequence, ManT, which was thought to recognize substrates having terminal methyl groups, would no longer recognize these substrates.

An alternative proposal is that termination of MMP assembly is regulated by palmitoyl-CoA synthesis in the cytoplasm. Ballou and coworkers added palmitoyl-CoA during the methylation reaction catalyzed by OMT, and found that the activity with long-chain substrate (Man-$(MeMan)_{12}$-OMe) was inhibited by 50% percent, while activity with a shorter pentasaccharide (Man-$(MeMan)_4$-OMe) was not disturbed. Lipids from cell extracts of *M. smegmatis* had a similar effect as palmitoyl-CoA.[66] As tight binding between mature MMPs and palmitoyl-CoA has been observed *in vitro*,[44] it is possible that during MMP biosynthesis the longer MMPs are selectively captured by palmitoyl-CoA, thus, preventing them from serving as substrates for OMT. As mentioned earlier, fatty acid synthesis seems to be regulated by MMPs; therefore, it is reasonable to assume MMP length is also controlled by fatty acid synthesis. Answers to these questions would require knock out studies of the genes encoding both ManT and OMT, which remain to be identified.

1.4 Other molecules containing 6-*O*-Me-Glc and 3-*O*-Me-Man

1.4.1 Other naturally occurring molecules containing 6-*O*-Me-Glc

Glucose is the most abundant carbohydrate in nature, and various modifications on glucose have been observed. Interestingly, O-6 methylation is

rare.[68] Natural products containing a 6-*O*-methyl-glucose (6-*O*-Me-Glc) moiety are occasionally found in plants but rarely in bacteria (Figure 1-7). In one example, a polysaccharide extracted from the leaves of *Catharanthus rosea* has a 6-*O*-Me-Glc moiety in the repeating hexasaccharide unit.[69] A pentasaccharide from flowers, Granatumoside, represents another example.[70] This moiety is also present in some triterpene saponins from plants,[71-72] as well as cyanobacteria (Figure 1-7).[73] This uncommon methylation modification was summarized in a recent review where it was stated that the role of the methyl groups in these structures is still not clear.[68]

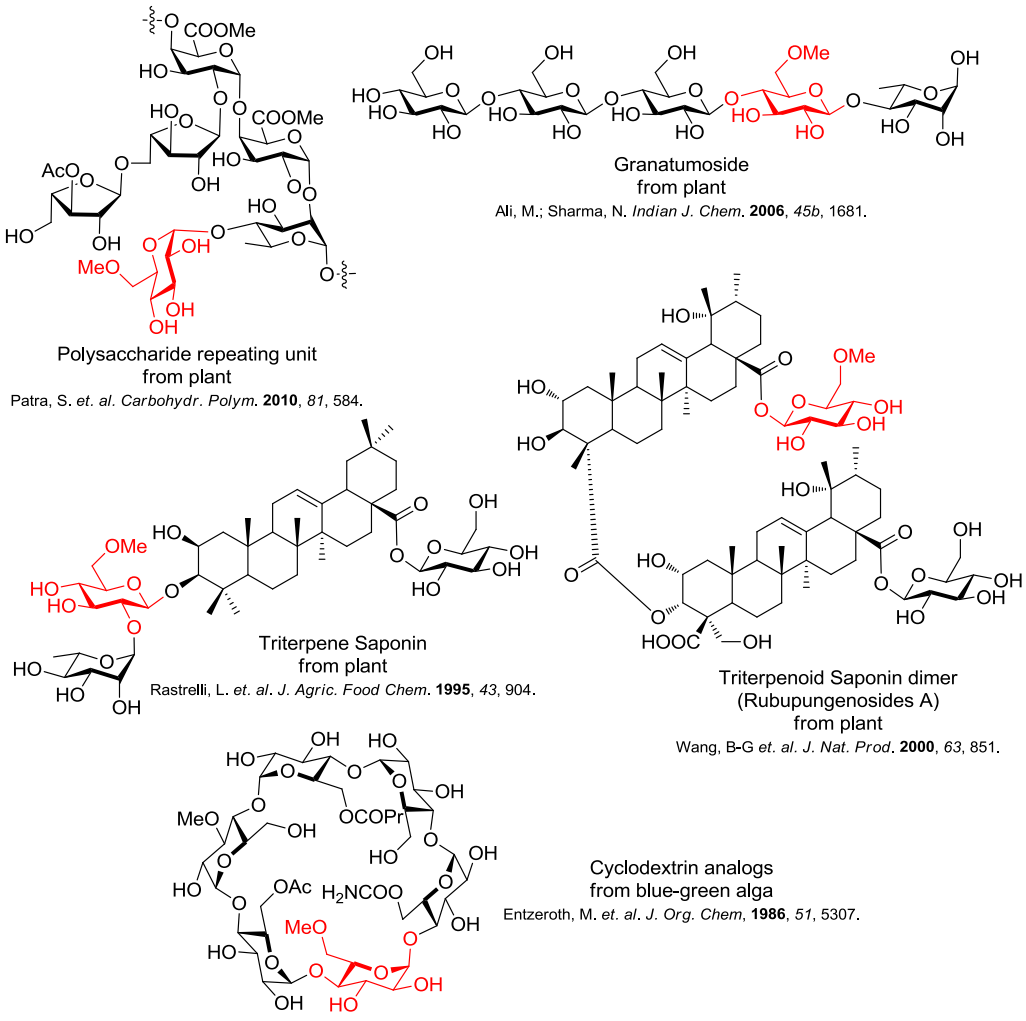
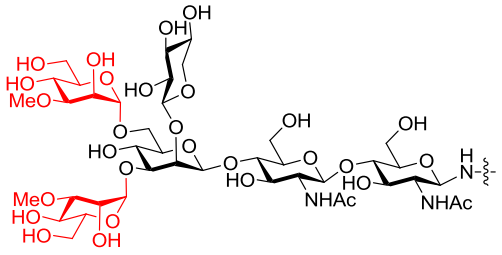


Figure 1-7. Examples of natural products containing a 6-*O*-Me-Glc moiety.

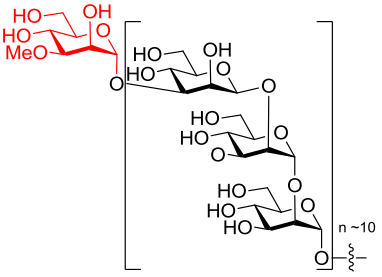
1.4.2 Other naturally occurring molecules containing 3-*O*-Me-Man

Mannose residues also exist widely in nature, in both eukaryotes and prokaryotes.[74-75] However, methylation of the 3-hydroxyl group is not common.[68] Interestingly, in addition to the aforementioned MMPs, 3-*O*-methyl-mannose (3-*O*-Me-Man) moieties are also found in other natural occurring glycans, including those from animals, fungi and bacteria. Summarised in Table 1-2 are a few examples of such glycans.

Table 1-2. Examples and distribution of natural products containing a 3-*O*-Me-Man moiety.



N-glycan from snail *L. stagnalis*
Van Kuik, J. A. *et. al*. *Eur. J. Biochem.* **1986**, *160*, 621.



O-antigen of *Klebsiella* and *E. coli*
Jansson, P-E, *et. al. Carbohydr. Res.* **1985**, *149*, 59.

Kingdom	Species	Location of 3-*O*-Me-Man	Reference
Animals	Snail: *Lymnaea stagnalis*	N-glycan	76
	Snail: *Biomphalaria glabrata*		77
	Snail: *Rapana thomasiana*		78
	Snail: *Arianta arbustorum*		79
	Snail: *Achatina fulica*		79
	Snail: *Planorbarius corneus*		79
	Gastropod: *Arion lusitanicus*		79-80
	Snail: *Arianta arbustorum*	O-glycan	79
	Snail: *Achatina fulica*		79
	Snail: *Planorbarius corneus*		79
	Gastropod: *Arion lusitanicus*		79
Fungi	*Mucor rouxii*	N-glycan	81
	Coccidioides immitis	Cell wall polysaccharide	82
Bacteria	Proteobacteria: *Escherichia coli*	O-antigen	83
	Proteobacteria: *Klebsiella*		83-84
	Proteobacteria: *Rhodopseudomonas viridis*		85
	Cyanobacteria: *Anacystis nidulans*		86

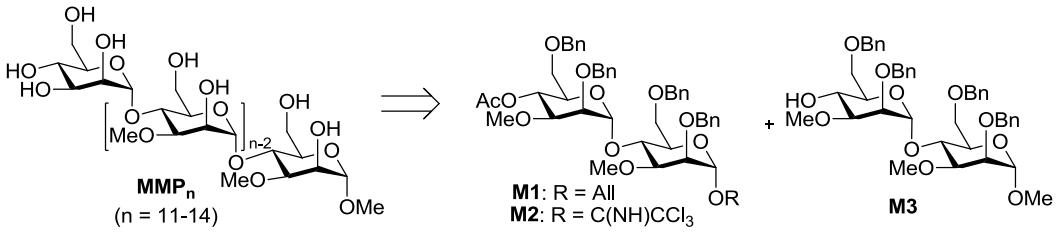
This moiety is frequently found in snails as a constituent of their *N*-glycans and *O*-glycans.[76-80] It also occasionally appears in cell wall components and N-glycans of fungi,[81-82] as well as in O-specific antigens of some bacterial species.[*83-84*] Notably, this moiety is resistant to mannosidase digestion,[81] and appears to frequently occupy the non-reducing end terminus of glycans. Therefore, O-methylation seems to play an important role in controlling the length of glycans[86] and in protecting the glycans from degradation.

1.5 Chemical synthesis of MGLPs and MMPs and their analogs

1.5.1 Synthesis of MMPs and analogs

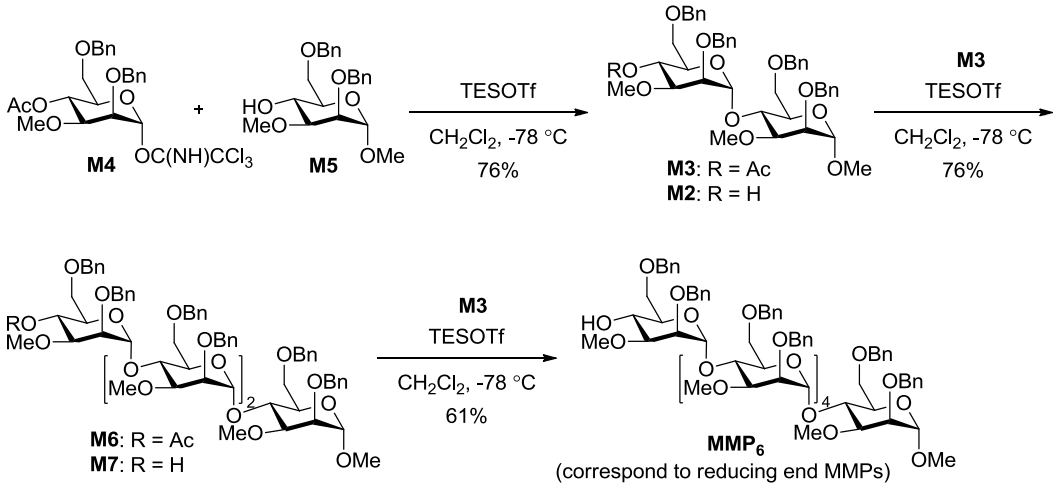
1.5.1.1 Liao's convergent synthesis of $MMP_{2,4,6,8}$

In addition to biochemical studies of MMPs, there have also been a number of efforts towards the chemical synthesis of MMPs and their analogs. The first chemical synthesis of MMPs was reported by Liao and coworkers in 1996. They accomplished construction of a disaccharide (MMP_2), tetrasaccharide (MMP_4), and hexasaccharide (MMP_6), which correspond to the reducing terminus fragments of MMPs. In their synthesis, the MMP analogs were assembled in an iterative manner from two disaccharide building blocks **M1** and **M3** (Scheme 1-3). The orthogonal acetyl and allyl groups were used as temporary protecting groups of **M1** at the O-4 and anomeric positions, respectively. For glycosylation, the allyl group of **M1** was replaced to produce the trichloroacetimidate **M2**[87] as a glycosyl donor. The 3-*O*-methyl groups in both building blocks were installed *via* dibutyltin oxide-mediated regioselective methylation.[88]



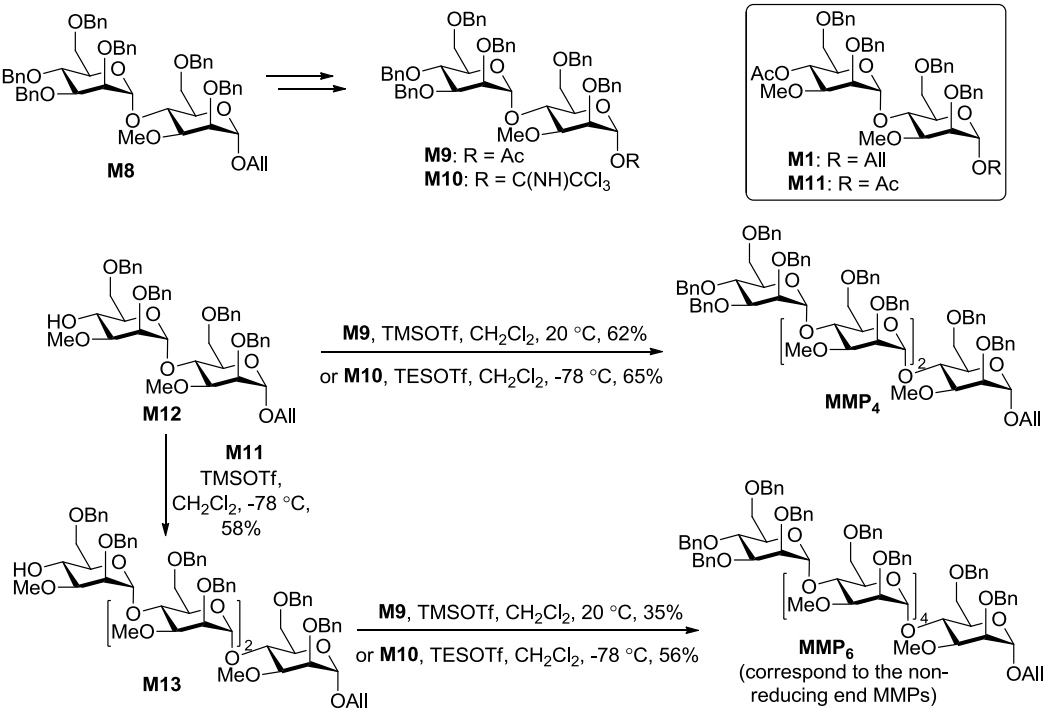
Scheme 1-3. Retrosynthetic analysis of MMPs by Liao's group.

Disaccharide building block **M3** was synthesized from imidate donor **M4** and acceptor **M5**. Convergent coupling between disaccharides **M3** and imidate **M2** generated a tetrasaccharide **M6**. Removal of the acetyl group liberated a new hydroxyl group at O-4, which was further elongated with imidate **M2** to give MMP_6 corresponding to the reducing end of MMPs (Scheme 1-4). All the glycosylation reactions with imidate donors proceeded in good yields (61–76%). In all cases only the α-isomers were isolated. However, formation of β adducts was not mentioned.



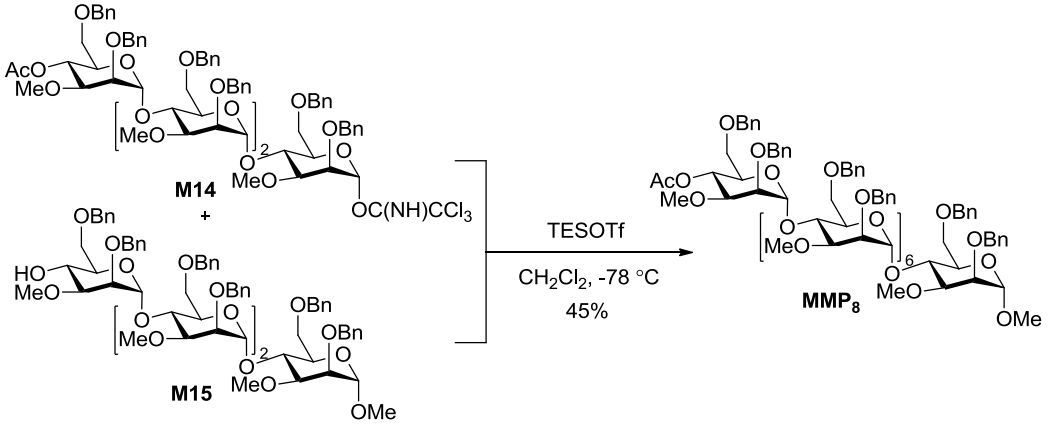
Scheme 1-4. Liao's convergent synthesis of MMP_6 corresponding to reducing end of MMPs.

A year later, the same group reported the synthesis of a hexasaccharide MMP_6 corresponding to the non-reducing terminus of MMPs.[89] In addition to using a trichloroacetimidate donor, they also employed acetates as glycosyl donors. A new building block, **M8**, which could be converted to the corresponding acetate donor **M9** and imidate donor **M10**, was used as the termination unit in the synthesis of elongated MMPs. Coupling of either donor with disaccharide **M12** or tetrasaccharide **M13** produced the corresponding tetrasaccharide MMP_4 and hexasaccharide MMP_6, respectively. Both the imidate donor and acetate donor were equally effective when reacting with disaccharide acceptor **M12** to produce MMP_4. However, when the larger acceptor **M13** was involved, glycosylation using acetate **M9** (35%) turned out to be less efficient than the imidate **M10** (56%) (Scheme 1-5).



Scheme 1-5. Liao's synthesis of MMP_4 and MMP_6 corresponding to the non-reducing end of MMPs.

Using their established method, Liao and coworkers extended their protocol to assemble an octasaccharide MMP_8.[90] Convergent coupling of a tetrasaccharide imidate donor **M14** and a tetrasaccharide acceptor **M15** produced an octasaccharide MMP_8 in moderate yield (52%) (Scheme 1-6). Comparing with analogs of MMPs with shorter length, the coupling efficiency drops as the length of substrates increases. The synthesis of MMP_8 required 20 steps in total from two monosaccharides, allyl α-D-mannopyranoside and methyl 3-*O*-methyl-α-D-mannopyranoside, in a 2.3% overall yield.

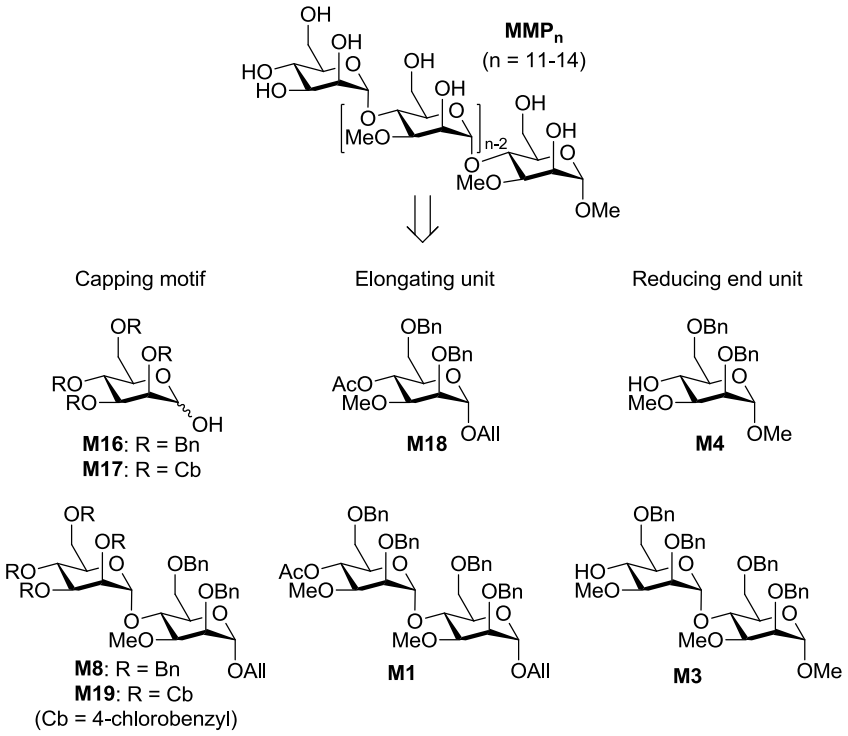


Scheme 1-6. Liao's convergent synthesis of octasaccharide MMP_8.

1.5.1.2 Hirooka's dehydrative glycosylation synthesis of $MMP_{2,3,4}$

In 2002, Hirooka and coworkers developed a direct dehydrative glycosylation approach to construct MMPs.[91] They assembled MMPs using three types of building blocks (Scheme 1-7): a non-reducing end capping motif (**M8**, **M16**–**M19**), a middle elongating unit (**M1**, **M18**) and a reducing end unit (**M3**, **M4**). Similar to Liao's strategy, orthogonal acetyl and allyl groups were used as temporary protecting groups in the elongation units **M1** and **M18**. Using their

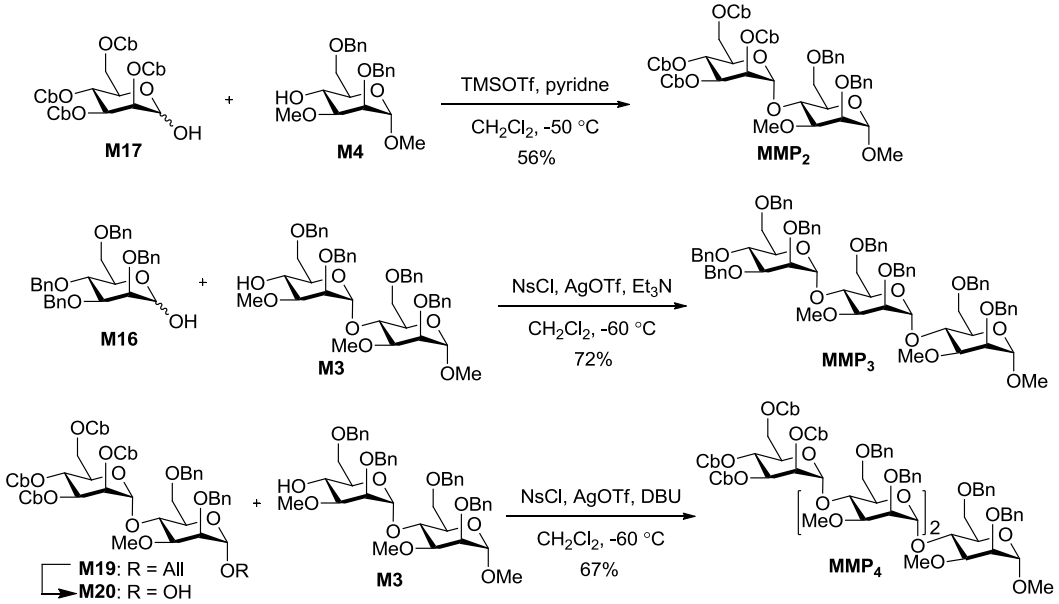
approach, MMPs of different lengths could be assembled by changing the combinations of building blocks, *e.g.*, the trisaccharide MMP_3 was obtained from the reaction of monosaccharide **M16** and disaccharide **M3**, or disaccharide **M8** and monosaccharide **M4**. The coupling of these building blocks was achieved using the activating system of *p*-nitrobenzenesulfonate chloride (NsCl), silver trifluromethanesulfonate (AgOTf) and a base (triethylamine (Et_3N) or diazabicyclo[5.4.0]undec-7-ene (DBU)).[92-94] Combinations of trimethylsilyl trifluromethanesulfonate (TMSOTf) and pyridine were also used as an alternative activating system.[95]



Scheme 1-7. Dehydrative glycosylation strategy towards MMPs.

The disaccharide MMP_2 was assembled in 56% yield from **M17** and **M4**, under dehydrative glycosylation conditions with TMSOTf and pyridine.[95] The

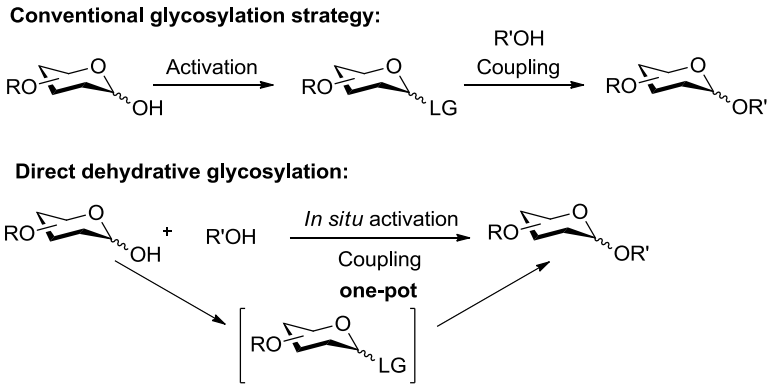
alternative activation system, using NsCl, AgOTf and Et_3N, gave the trisaccharide MMP_3 with relatively higher coupling efficiency (72% yield from **M16** and **M3**). A tetrasaccharide MMP_4 was assembled, using a convergent strategy, from disaccharides **M19** and **M20**. Activation was achieved using NsCl, AgOTf and DBU with 67% coupling efficiency (Scheme 1-8). Regarding the stereoselectivity of the glycosylation, the authors did not comment on formation of the β isomers during the reactions.



Scheme 1-8. Synthesis of MMP_2, MMP_3 and MMP_4 using dehydrative glycosylation.

Conventional glycosylation methods generally require two steps. First, activation of the anomeric hydroxyl group gave a donor bearing a latent leaving group. Then, the isolated donor is coupled with an acceptor to produce the glycosylation product (Scheme 1-9). On the other hand, in a dehydrative glycosylation approach,[96] the anomeric hydroxyl group is activated *in situ* to

generate a reactive intermediate, which is trapped by a nucleophilic acceptor to give a glycosylation product in one-pot.[97]



Scheme 1-9. Conventional indirect glycosylation and direct dehydrative glycosylation.

Although attractive, the dehydrative glycosylation method suffers from certain limitations. One major side reaction is the self-coupling of the 1-hydroxyl glycoside. Thus, formation of byproducts such as **M20** is often unavoidable (Figure 1-8). Minimizing this problem requires careful tuning of the activation conditions. Hirooka and coworkers found the byproduct **M20** was not observed when NsCl, AgOTf and Et_3N was replaced with TMSOTf and pyridine as the activator. Other byproducts observed in this dehydrative glycosylation are **M21** (8%) and **M22** (21% when reacting with **A1**), resulting from activation of accepter **M4** and **M3** by NsCl (Figure 1-8).

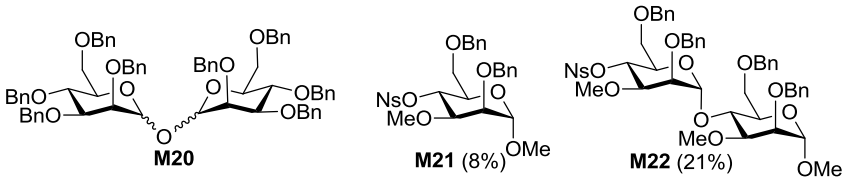
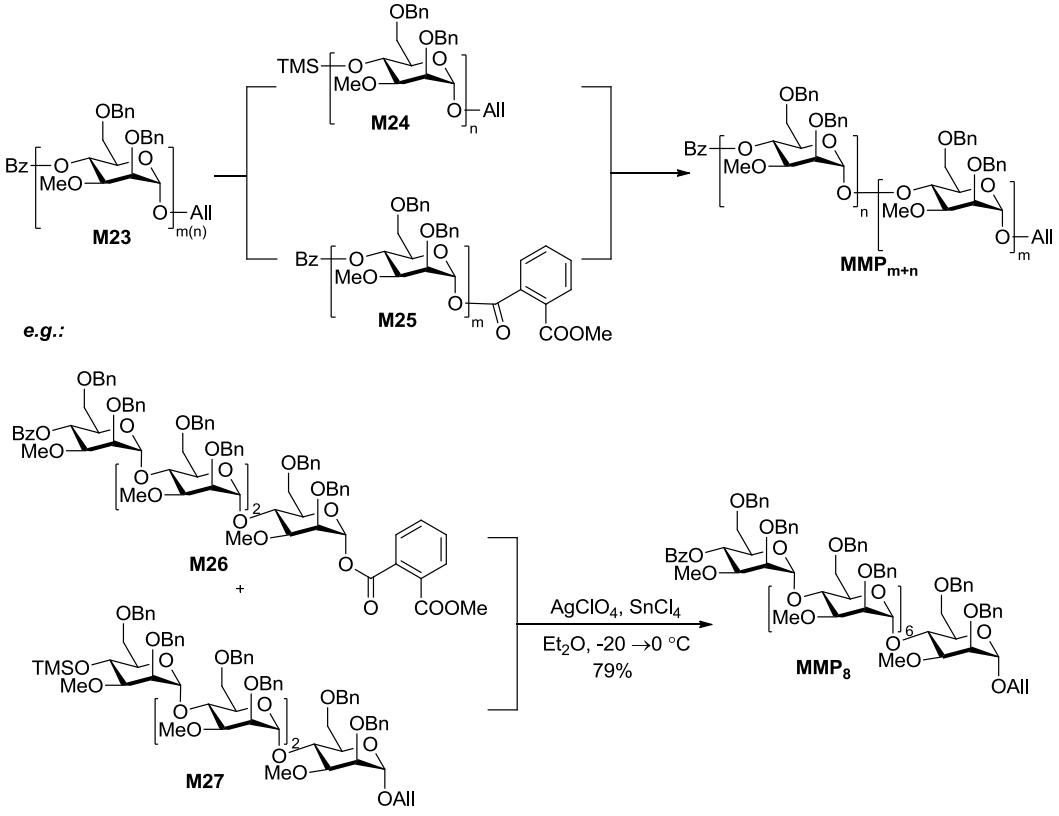


Figure 1-8. Byproducts of dehydrative glycosylation of **M20**, **M21** and **M22**.

1.5.1.3 Kishi's two generations of glycosylation towards $MMP_{2n\ (n=1\text{-}10)}$

More recently, in 2007, Kishi and coworkers developed a highly convergent strategy using Mukaiyama glycosylation to produce MMP analogs up to MMP_{20}.[98] The synthesis started with a single building block, monosaccharide **M23**, with orthogonal benzoyl and allyl protecting groups. This building block was converted to an acceptor **M24** by replacing the benzoyl ester with a TMS ether in two steps. Alternatively, the allyl group of **M23** was converted into phthalate ester donor **M25** in two steps (Scheme 1-10).



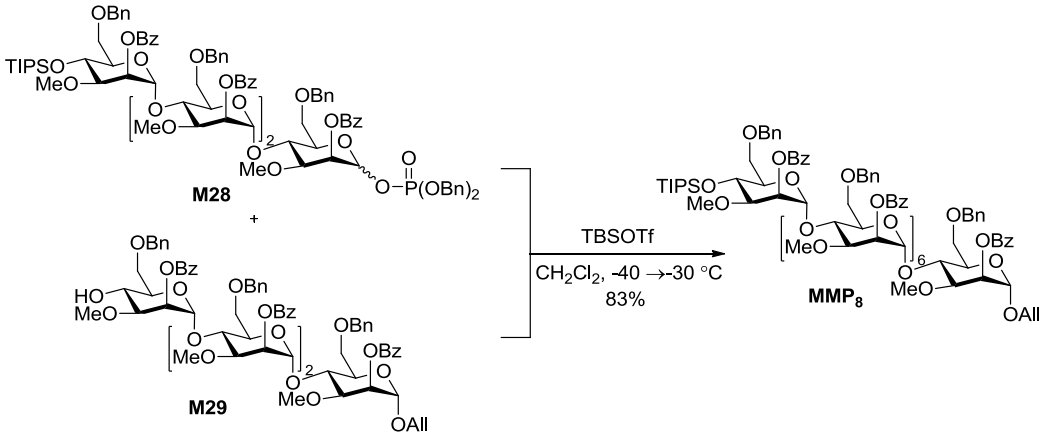
Scheme 1-10. Kishi's glycosylation strategy and construction of MMP_8.

Glycosylation of **M24** with **M25** was promoted by $SnCl_3ClO_4$ generated *in situ* from $SnCl_4$ and $AgClO_4$.[99-100] Each glycosylation cycle only took five steps

from the communal building block **M23**: two steps for each for the synthesis of the donor and acceptor, as well as the glycosylation reaction. This convergent method produced octasaccharide MMP_8 in only three cycles from monosaccharide **M23**. This product was obtained in an overall yield of 4.2% over 18 steps from the D-mannose (Scheme 1-10). The Mukaiyama glycosylations proceeded in high yield (74–79%) and stereoselectivity (α/β > 20:1~50:1). The excellent α-selectivity was attributed to stereospecific anomerization of the β isomer under the glycosylation conditions.[101] Compared with Liao's iterative method, which used 20 steps to prepare MMP_8, Kishi's strategy is more efficient for constructing larger analogs of MMPs. In fact, up to a 16-mer (MMP_{16}) was synthesized using this method.

Despite the advantages, higher yield and selectivity of the above mentioned Mukaiyama glycosylation, the reactions are not suitable for scale up. Also, the produced unstable β isomers are often susceptible to random cleavage, resulting in contamination of the glycosylation products, which makes purification challenging. Thus, Kishi and coworkers improved their method by modifying the protecting groups of the building blocks.[102] Instead of using phthalate ester donors, anomeric phosphates (*e.g.*, **M28**) bearing a benzoyl group at O-2 were employed. Glycosylation with phosphate donors in presence of TBSOTf proved to be more efficient than the phthalate donors. The glycosylation product could be converted into the corresponding donor in two steps, or to an acceptor in one step. An 83% yield of octasaccharide MMP_8 was obtained from tetrasaccharide donor **M28** and acceptor **M29** (Scheme 1-11). Furthermore, oligosaccharides of sizes up to a 20-

mer (MMP_{20}) were successfully synthesized without a loss in glycosylation efficiency. In Kishi's second generation of MMPs synthesis, no scrambled products were formed during glycosylation and high stereoselectivity (α/β > 20:1) was achieved by neighboring group participation of the benzoyl group at O-2.[103]



Scheme 1-11. Kishi's modified glycosylation towards MMP_8 using phosphate as donor.

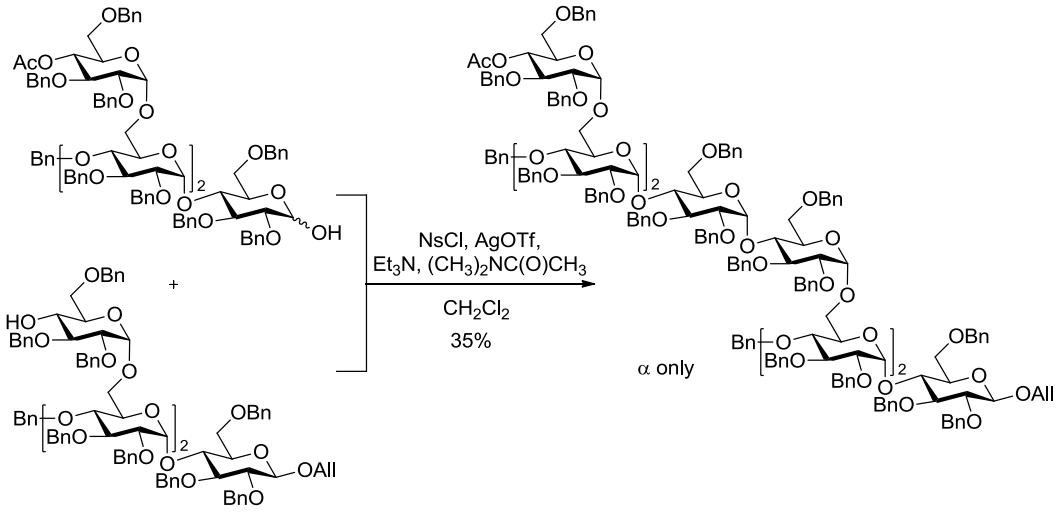
Thus, to date, three individual groups have completed chemical syntheses of MMPs. Five different donors were employed in the glycosylations, including Liao's imidates and acetates, Hirooka's free anomeric alcohols and Kishi's phthalates and phosphates. Of the four methods, Kishi's glycosylation with phosphate donors was superior in not only shortening the reaction steps, but also in giving the highest yield and stereoselectivity.

1.5.2 Synthesis of MGLPs and analogs

1.5.2.1 Kishi's Mukaiyama glycosylation towards $MGP_{12,14,16,20}$

Inspired by the successful construction of MMPs analogs, Kishi's group also initiated the chemical synthesis of MGLP analogs.[104] Previously, α-(1→4)-

linked glucose oligosaccharides have been assembled using a dehydrative strategy employing a combination of NsCl, AgOTf, Et_3N and *N*,*N*-dimethylacetamide.[105] These dehydrative glycosylation conditions gave the desired α-isomer exclusively; however, with only moderate yield (Scheme 1-12).



Scheme 1-12. Assembly of α-(1→4)-linked glucose oligosaccharides via dehydrate glycosylation.

Due to the complexity of the natural MGLPs, Kishi's synthesis only focused on the core structure MGPs (6-*O*-methyl-glucose polysaccharides), which are composed of repeating α-(1→4)-linked 6-*O*-methyl-glucopyranoside units (Figure 1-9).

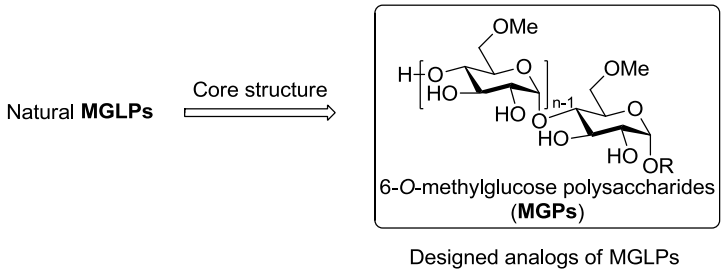
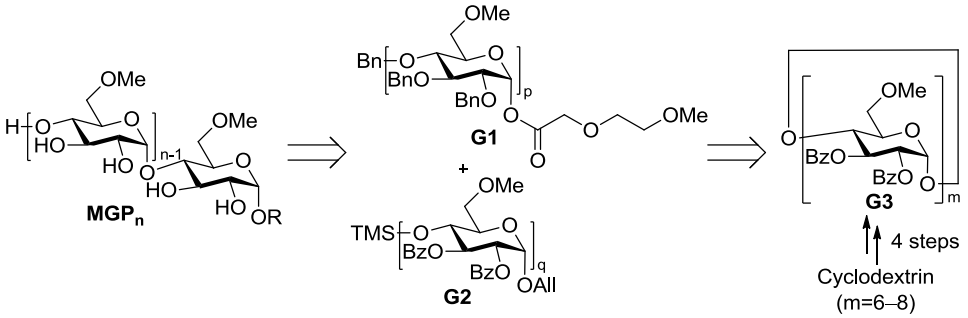


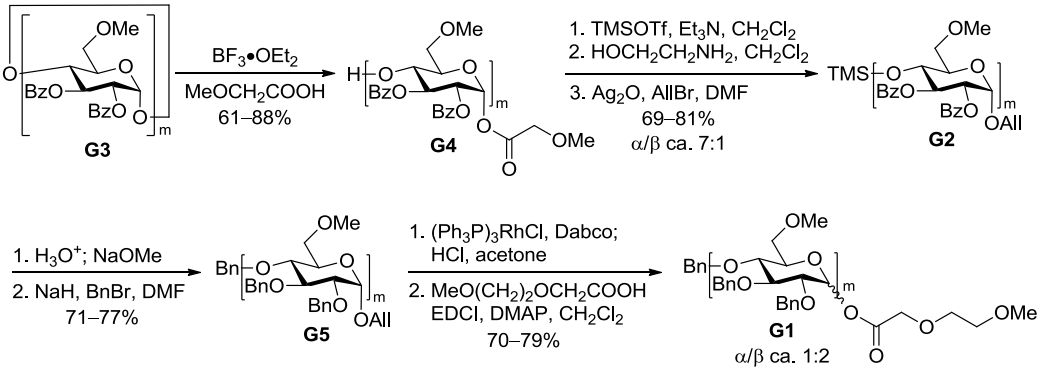
Figure 1-9. Core structures of natural MGLPs and designed analogs.

To minimize manipulations of the stereoselectivity of the newly formed glycosidic bonds, Kishi and coworkers used readily available natural cyclodextrins as the starting materials.[106] Because cyclodextrins are natural-occurring cyclic oligosaccharides composed of α-(1→4)-linked glucose, Kishi's group used them as a template for MGLP synthesis. In their approach both glycosylation donor **G1** and acceptor **G2** were derived from the same intermediate, the modified cyclodextrins **G3**, which was in turn assembled from corresponding α-, β- and γ- cyclodextrins in four steps (Scheme 1-13).



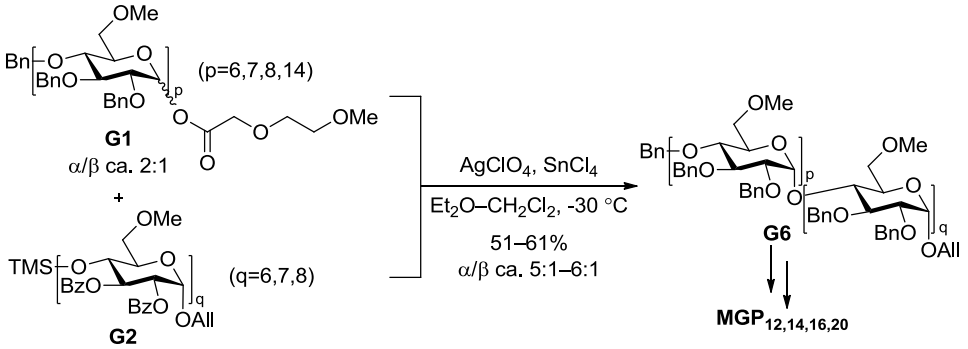
Scheme 1-13. Retrosynthetic analysis of MGPs by Kishi's group.

The key reaction was the selective hydrolysis of the cyclic cyclodextrins **G3** to give the linear product. Over cleavage of glycosidic bonds was minimized when hydrolysis was promoted in the presence of $BF_3{\cdot}OEt_2$. The newly formed anomeric hydroxyl group was simultaneously protected as an ester to give **G4** in an overall yield of 61–88%. The adduct **G4** was then converted to the corresponding TMS-protected acceptors **G2** in three steps and additional four steps produced donors **G1** as an anomeric mixture (Scheme 1-14).



Scheme 1-14. Synthesis of building blocks for MGP synthesis.

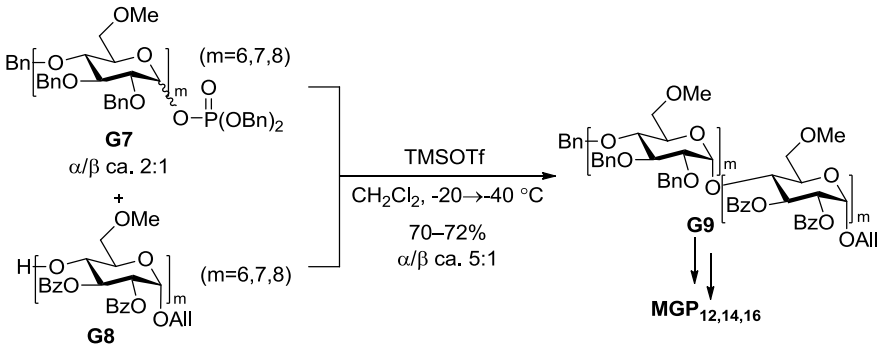
Coupling of donor **G1** and acceptor **G2** was attempted using the same Mukaiyama glycosylation conditions employed for the synthesis of MMPs. However, in this case, a catalytic amount of Lewis acid was not enough to effectively mediate the glycosylation. Instead, at least one equivalent of $SnCl_3ClO_4$ was required. The reaction was found to not be influenced by the stereochemistry of the donor, as glycosylation reactions using donors as anomeric mixture (α/β 2:1) gave glycosylation products in a 5:1α/β ratio. This strategy was used to prepare up to a 20-mer MGLP analog in an average yield of 51–61% (Scheme 1-15).



Scheme 1-15. Kishi's Mukaiyama glycosylation towards $MGP_{12,14,16,20}$.

1.5.2.2 Kishi's modified glycosylation towards $MGP_{12,14,16}$

Although Kishi's first generation MGLP synthesis is highly convergent, it is not suitable for scale-up synthesis. Therefore, they later optimized the synthetic route. In the modified approach, phosphate donor **G7** was used instead and glycosylation was promoted with TMSOTf.[107] The new glycosylation reaction produced the desired products in 70–72% yield and with an α/β ratio of 5:1 (Scheme 1-16).



Scheme 1-16. Kishi's modified glycosylation towards $MGP_{12,14,16}$ using phosphate as donor.

1.6 Overview of thesis research

In this thesis, I will focus on the biosynthesis of 3-*O*-methyl-mannose polysaccharides (MMPs), one of the two classes of polymethylated polysaccharides produced by mycobacteria.[27] In the past three decades, although advances in the chemical synthesis of MMP analogs has been achieved, no attempts have been made to verify the biosynthetic pathway that was proposed in 1984.[66]

Of particular interest to this thesis is the α-(1→4)-mannosyltransferase (ManT) involved in MMP biosynthesis. This glycosyltransferase introduces a

mannose residue *via* an uncommon α-(1→4)-linkage. To date, the enzyme has only been evaluated using a crude extract of mycobacteria and no genetic information for this enzyme is available yet. Therefore, more detailed studies are required to fully characterize this enzyme.

In Chapter 2, I chemically synthesized ten analogs of MMPs and used them to probe the substrate specificity of ManT, which led to the identification of an unexpected activity. It was previously reported ManT recognizes substrates that have terminal methylated residues.[33] However, I demonstrated it also recognizes substrates lacking this terminal methyl group, and with even better affinity.

The results in Chapter 2 suggest the presence of methyl groups on the substrates is not essential for the activity of ManT. To verify this hypothesis, in Chapter 3, I evaluated a tetrasaccharide with no methyl group substitution as substrate of ManT. This compound was indeed turned over by ManT, however, with significantly lower activity when compared with tetrasaccharides that are partially methylated. Comparing the kinetic parameters indicated methyl groups play different roles when present at different positions of substrate.

In the course of synthesizing the aforementioned MMP analogs, I also developed a four-step methodology to quickly install methyl groups directly on oligosaccharides. This method is detailed in Chapter 4 and using it enabled me to access MMP-based ligands for affinity purification of ManT in Chapter 5.

In Chapter 5, to further explore the activity and specificity of ManT, I endeavoured to gain access to the pure ManT enzyme. My first attempt to purify

ManT using synthetic affinity columns containing immobilized MMP ligands was not successful. Therefore, we instead used an alternative bioinformatic approach. Following this approach, a putative ManT gene from *M. smegmatis* was identified and expressed in *E. coli*. The activity of this gene product proved identical to the ManT from *M. smegmatis*. Hence, the gene encoding the ManT from *M. smegmatis* was successfully identified and will facilitate future studies of MMP biosynthesis.

1.7 Bibliography

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Chapter 2: Revisiting the specificity of a mycobacterial α-(1→4)-mannosyltransferase[1]

[1] A version of this chapter has been published:
Xia, L.; Zheng, R. B.; Lowary, T. L. *ChemBioChem* **2012**, *13*, 1139-1151.

2.1 Introduction

Methylmannose polysaccharides (MMPs) are one of two unique methylated glycans found in the cytoplasm of some mycobacterial species.[1] Isolated from *Mycobacterium phlei* independently by Ballou and Bloch,[2-3] their structures were first reported in 1971 and later revised.[2,4] MMPs are a mixture of four homologs containing 11–14 α-(1→4)-linked mannopyranose residues. Each monosaccharide is methylated at the O-3 position except the terminal non-reducing end residue, which is unsubstituted. The reducing end is blocked with a methyl aglycone (Figure 2-1, left).[5]

MMPs bind strongly to lipids (e.g., palmitoyl-CoA), with reported affinities up to 10^7 M^{-1}.[6] NMR investigations[6] suggest that MMPs bind lipids in a hydrophobic channel formed when the glycan adopts a helical conformation in which the methyl groups face the interior (Figure 2-1, right). This observation led to the proposal that these glycans are involved in mycobacterial lipid storage and biosynthesis.[5] However, clear support for a role of MMPs in mycobacterial lipid metabolism is still lacking.

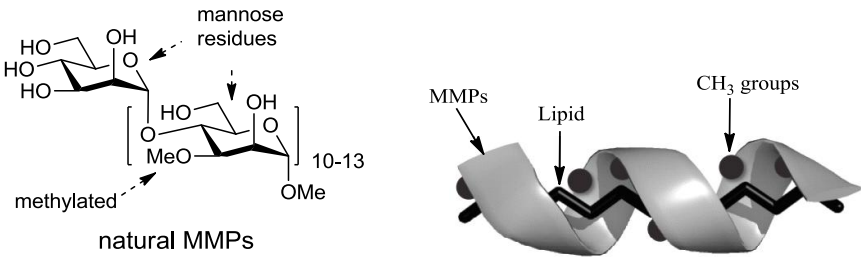
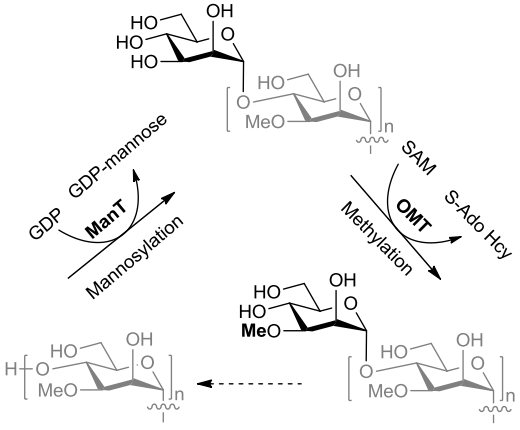


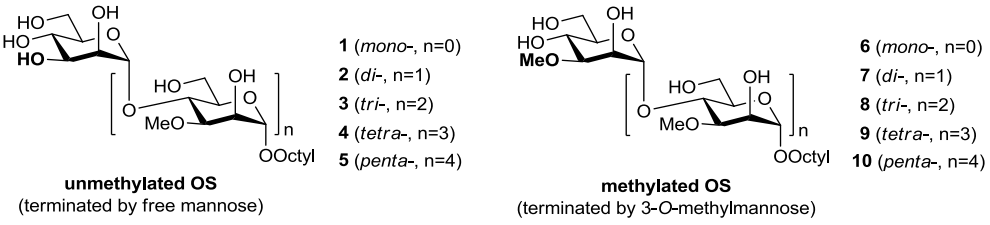
Figure 2-1. Structure of natural MMPs (left) and model of MMP-lipid complex (right).

A pathway for MMP biosynthesis was proposed in 1984 by Ballou and Weisman, based on structural information from isolated MMP precursors[7] and the measurement of α-(1→4)-mannosyltransferase (ManT)[8] and 3-*O*-methyltransferase (OMT)[9] activities in membrane fractions from *Mycobacterium smegmatis.* It was proposed that MMP elongation occurs via alternating mannosylation and methylation reactions (Scheme 2-1).[9] First, the ManT recognizes the terminal 3-*O*-methyl-mannose residue and catalyzes the transfer of mannose from guanosine diphosphate mannose (GDP-mannose) to the O-4 position of the terminal methylated mannose residue. Second, OMT catalyzes the transfer of a methyl group from S-adenosylmethionine (SAM) to O-3 of the unsubstituted mannose residue at the non-reducing terminus. It was proposed that alternating mannosylation and methylation continues until the polymer reaches 11–14 monosaccharide residues in length. It is unknown how the chain elongation is truncated, but it has been suggested that upon reaching an appropriate length the molecule bind to cellular lipids and hence is no longer a substrate for the biosynthetic enzymes.[9]



Scheme 2-1. Previously proposed biosynthetic pathway for MMP elongation.

Despite the logic of the proposed pathway, when I started this project, direct evidence supporting it using well-characterized substrates was absent, and none of the genes encoding for the putative ManT or OMT had been identified.[1] As part of a program focused on characterizing these enzymes, I undertook studies to probe the activity of the ManT, which makes use of a series of synthetic oligosaccharides (**1**–**10**, Scheme 2-2) as the acceptor substrates and a mycobacterial membrane preparation as the source of the ManT enzyme. These investigations provide new insight into the specificity of the ManT and call into question the previously proposed biosynthetic model.



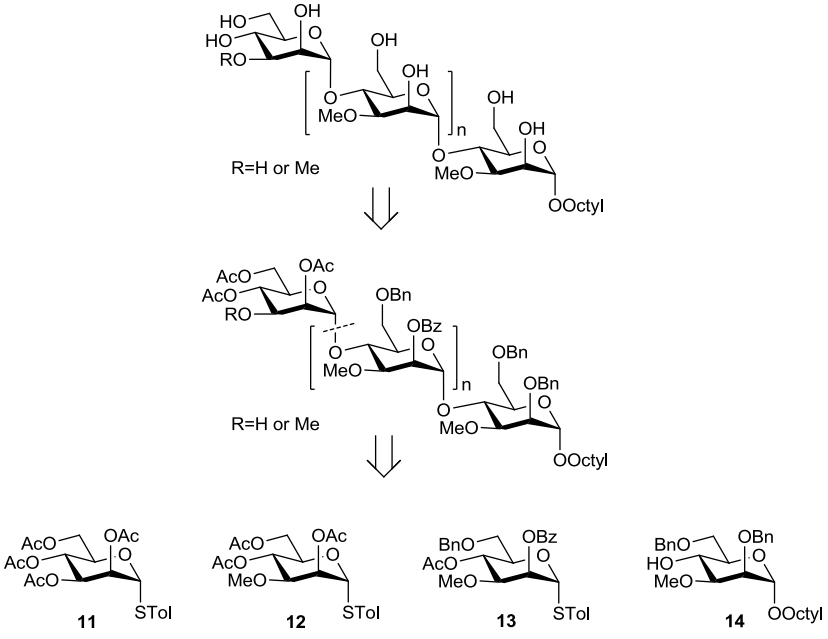
Scheme 2-2. Designed synthetic analogs of MMP fragments (OS is short for oligosaccharides).

2.2 Synthesis of analogs of MMPs

Two series of MMP fragments, ranging in size from mono- to pentasaccharides, were targeted for synthesis. In one series (unmethylated oligosaccharides (OS), **1**–**5**), the "non-reducing" end of the molecule is terminated with a mannose residue without a methyl group. Based upon the proposed biosynthetic model, these compounds were designed as potential acceptors for the OMT, but should not be used by ManT. The other series of oligosaccharides (methylated OS, **6**–**10**) were truncated at the "non-reducing" terminus with a 3-*O*-methylated mannose residue. These were designed as substrates for ManT. To

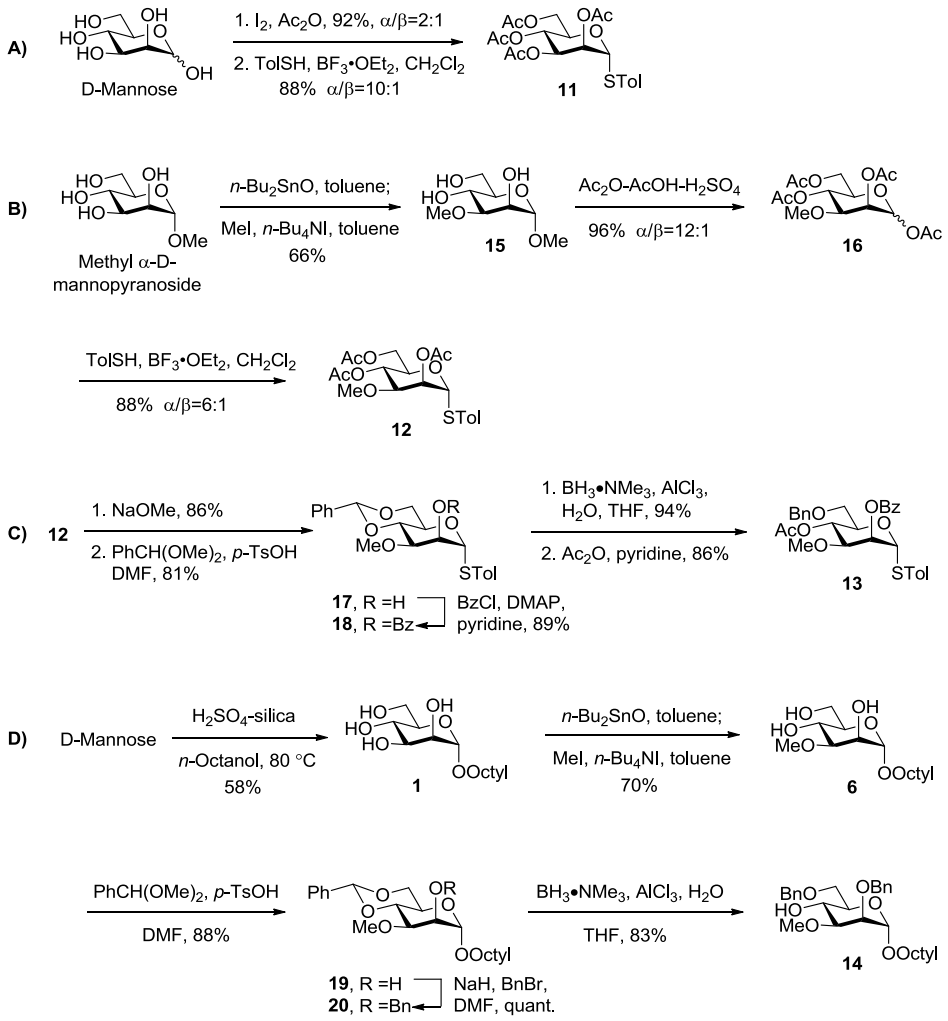
simplify the enzyme assay, the methyl aglycon in natural MMP was substituted with an *n*-octyl chain in the synthetic MMP fragments so that the enzymatic products could be easily purified by C_{18} column chromatography.[10] Given the proposed biosynthetic model, we postulated that both the ManT and OMT recognize primarily the non-reducing end of the glycan. Hence, this substitution was not anticipated to be detrimental to catalysis by either enzyme.

Oligosaccharides **1**–**10** were assembled via a straightforward strategy involving sequential addition of monosaccharide residues to the growing chain (Scheme 2-3). The only difference between the unmethylated and methylated series was the non-reducing residues, which were attached at a late stage by reaction with unmethylated donor **11** or methylated donor **12**, respectively. Iterative coupling of thioglycoside donor **13** with an alcohol acceptor (e.g., **14**) provided the common core residues of both series of compounds.



Scheme 2-3. Retrosynthetic analysis for synthetic MMP fragments.

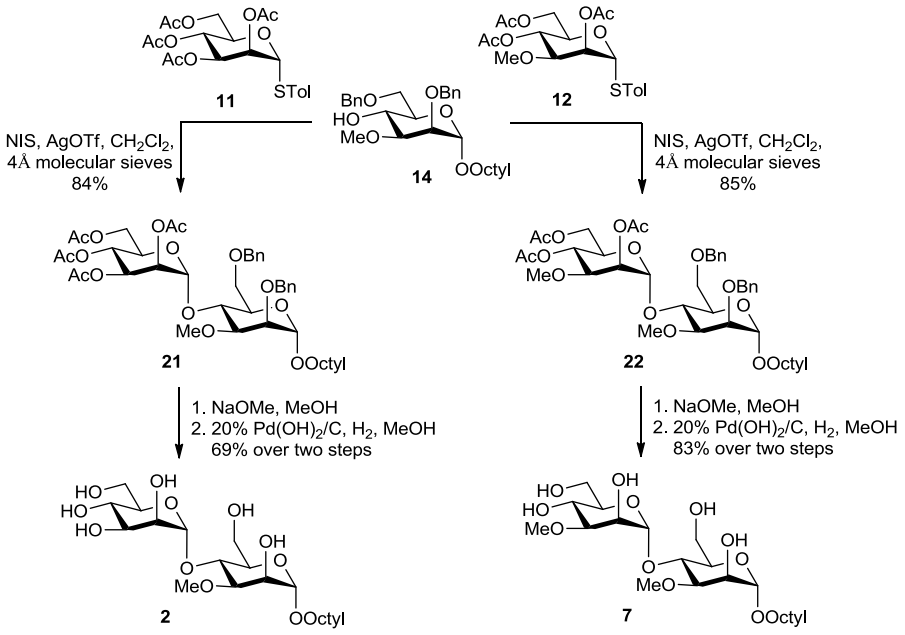
As depicted in Scheme 2-4A, thioglycoside **11** was easily synthesized from D-mannose via peracetylation with a catalytic amount of iodine in acetic anhydride[11] followed by coupling with *p*-thiocresol in the presence of BF_3•OEt_2.[12] The product was obtained in 81% yield over the two steps. Donor **12** was prepared from methyl α-D-mannopyranoside (Scheme 2-4B). First, selective methylation at O-3 hydroxyl group of methyl α-D-mannopyranoside was achieved in 66% yield via the formation of a di-*n*-butylstannane acetal intermediate.[13] The product, **15**, then underwent acetolysis to provide a 96% yield (α/β = 12:1) of **16**. Finally, reaction of **16** with *p*-thiocresol in the presence of BF_3•OEt_2 gave **12** in 76% yield (α/β = 6:1). Building block **13** was derived from **12** in five steps (Scheme 2-4C). First, thioglycoside **12** was deprotected and converted to benzylidene acetal **17** in 81% yield. The hydroxyl group of **17** was then protected as a benzoyl ester **18**. Subsequent regioselective opening of the benzylidene ring with BH_3•NMe_3 generated an alcohol, which was further acetylated to furnish the building block **13** in 81% overall yield.[14-15] Building block **14** was derived from octyl α-D-mannopyranoside (**1**) in five steps (Scheme 2-4D). Octyl glycoside **1** was synthesized directly from D-mannose and *n*-octanol, via Fisher glysosylation using sulfuric acid (immobilized on silica gel) as a catalyst.[16] Direct regioselective methylation of **1** mediated by an organotin reagent furnished **6** in 70% yield. Triol **6** was further converted to benzylidene acetal **19** and then benzyl ether **20** in 88% overall yield using standard methods. The same condition used for opening the benzylidene ring of **18** was applied to **20**, generating an 83% yield of the desired alcohol **14**.



Scheme 2-4. Synthesis of building blocks **11**–**14**.

With building blocks **11**–**14** in hand, oligosaccharide targets **1**–**10** were assembled using a linear strategy. All glycosylation reactions were performed using *N*-iodosuccinimide (NIS) and silver trifluoromethanesulfonate (AgOTf) activation conditions.[17] After each glycosylation step, the stereochemistry was confirmed by measuring the one-bond heteronuclear coupling constant at the anomeric centre ($^1J_{C\text{-}1,H\text{-}1}$)[18] of the newly formed linkage. For all the products, this value was between 167 and 177 Hz, clearly indicating the α-stereochemistry.

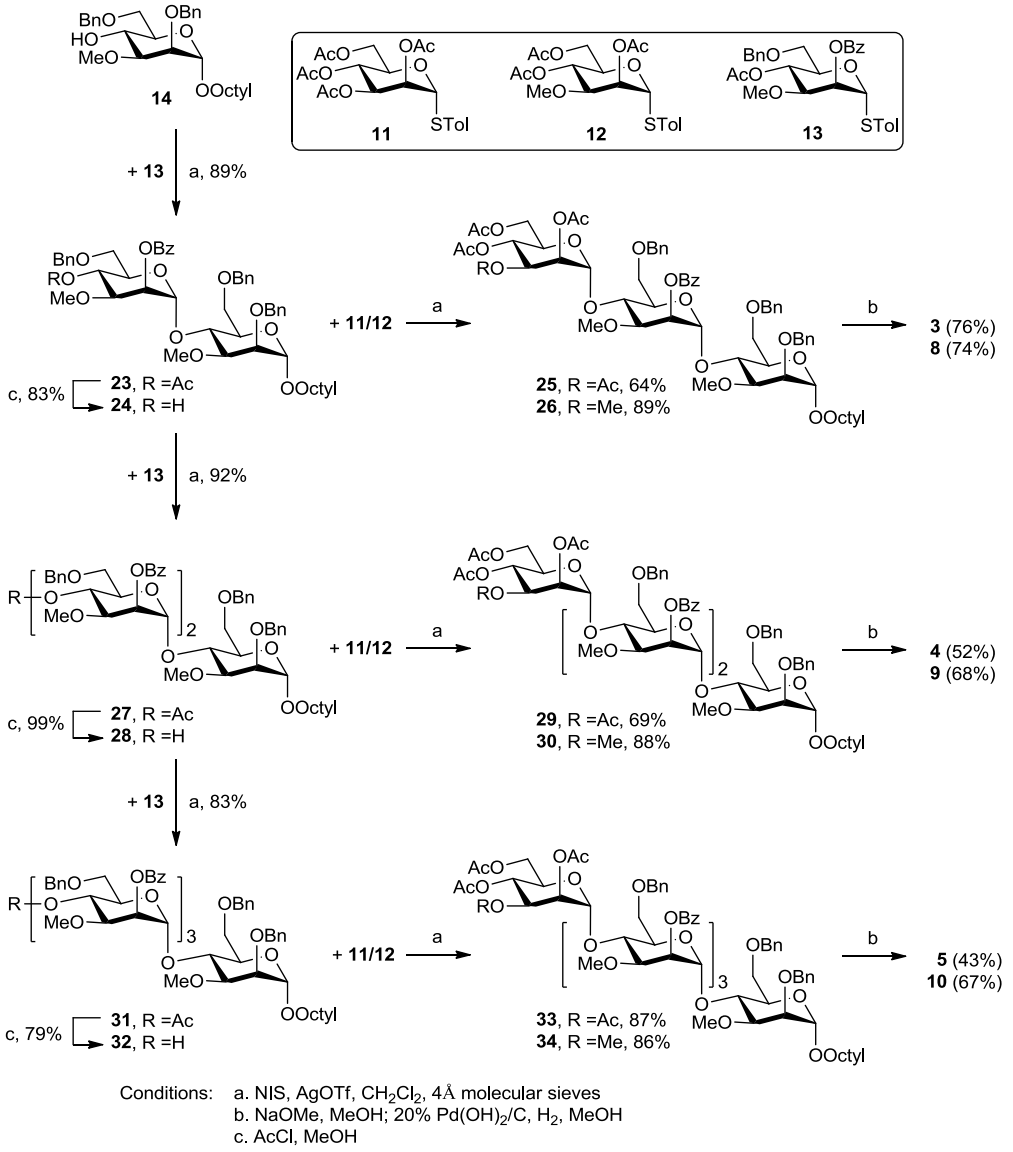
As illustrated in Scheme 2-5, disaccharides **2** and **7** were prepared by coupling alcohol **14** with either thioglycoside **11** or **12**. Both glycosylation reactions went smoothly to give the expected disaccharides **21** (84% yield) and **22** (85% yield). The acetyl groups of **21** and **22** were removed with sodium methoxide in methanol. Subsequent removal of benzyl groups under hydrogenolysis conditions provided the disaccharides **2** and **7** in 69% and 83% overall yields, respectively.



Scheme 2-5. Synthesis of disaccharides **2** and **7**.

As depicted in Scheme 2-6, the trisaccharides **3** and **8** were derived from disaccharide **23**, which was synthesized from coupling of alcohol **14** with thioglycoside **13** in 89% yield. Selective removal of the acetyl group of **23** in the presence of benzoyl group was achieved using acidic methanol in 83% yield. The resulting alcohol, **24**, was then glycosylated with **11** to generate a 64% yield of trisaccharide **25**. Deprotection of this compound, as described for the synthesis of

2 and **7**, provided a trisaccharide **3** in 76% overall yield. Coupling the alcohol **24** with donor **12** provided trisaccharide **26**, which was then deprotected to afford the trisaccharide **8**.



Scheme 2-6. Synthesis of trisaccharides **3** and **8**, tetrasaccharides **4** and **9**, and pentasaccharides **5** and **10**.

Using the same linear strategy, trisaccharide **27**, derived from disaccharide **24**, was further converted to tetrasaccharides **4** and **9** in four steps.

Similarly, pentasaccharides **5** and **10** were successfully synthesized from the tetrasaccharide **31**, which, in turn, obtained from a trisaccharide **28** (Scheme 2-6). In the synthesis of these larger oligosaccharides, all reactions proceeded without difficulty and in comparable yield to analogous reactions carried out to prepare the shorter glycans.

2.3 Recognition of synthetic MMPs by ManT and OMT

In their previous studies, Ballou and Weisman characterized the ManT (α-(1→4)-mannosyltransferase) and the OMT (3-*O*-methyltransferase) using MMP fragments degraded from the native polysaccharides as substrates.[7] The mixture of MMP isomers was subjected to partial methanolysis and the fragments were fractionated by gel filtration. All of the compounds, ranging in size from a disaccharide to an undecasaccharide, were shown to be recognized by the OMT.[9] However, only fragments larger than four mannose residues were acceptors for the ManT.[8] One question arising from this work is how the shorter oligomers (e.g., di- through tetrasaccharides) are assembled in the bacteria. In addition, the previous work showed that methylated OS were turned over by the ManT while the unmethylated OS were OMT substrates, but cross-reactivity between the two sets of substrates and enzymes (e.g., whether unmethylated OS are acceptors of ManT) had not been reported. We considered that the panel of homogenous and structurally well-defined substrates **1**–**10** synthesized above would allow us to address some of these questions.

In carrying out the assays, the first challenge was to identify a suitable mycobacterial strain. The *M. smegmatis* strain used in the earlier work (ATCC 356[8-9]) is no longer available, so we chose instead *M. smegmatis* ATCC 14468, which had been reported to produce MMPs and thus would also possess the biosynthetic enzymes.[8] We were also able to isolate mature MMPs from *M. smegmatis* ATCC 14468,[19] thus supporting the previous studies. Having chosen a strain, the enzyme extract fractions were obtained by centrifugation after cell lysis. The OMT was reported to exist in the cytoplasm of the bacteria while the ManT was proposed to be a peripheral membrane protein,[8] so we used the cytoplasmic fraction to serve as the OMT source and the membrane fraction as the ManT source.

The five unmethylated OS (**1**–**5**) were incubated with radiolabeled *S*-[methyl-^{3}H] adenosylmethionine in the presence of the OMT enzyme (cytoplasmic fraction). The remaining five methylated OS (**6**–**10**) were incubated with GDP-mannose-[2-^{3}H] in the presence of the ManT (membrane fraction). These reactions were monitored by measuring the radioactivity of the enzymatic products. A reported method employing C_{18} Sep-Pak cartridges was employed to remove the excess radioactive donor from the reaction mixture and the crude products were eluted with methanol.[10] Scintillation counting of the methanol eluent from the Sep-Pak provided a method for quantification of the enzyme activity.

Unfortunately, no radioactive enzymatic products were detected from the incubation of unmethylated OS (**1**–**5**) with OMT, indicating that either this

enzyme is absent from the cytoplasmic fraction, or this enzyme is unable to recognize the unmethylated OS. The first possibility was excluded by the evidence of isolated natural MMPs from *M. smegmatis* ATCC 14468,[19] indicating the OMT should exist in this bacterial strain. Failure to detect OMT from cytoplasmic fraction led us to believe this enzyme might be located in the membrane fraction. However, we were still unable to detect OMT activity when using the membrane fraction as the enzyme source. We are currently exploring alternative methods to measure OMT activity, and I focused my attention on characterizing the specificity of the ManT activity.

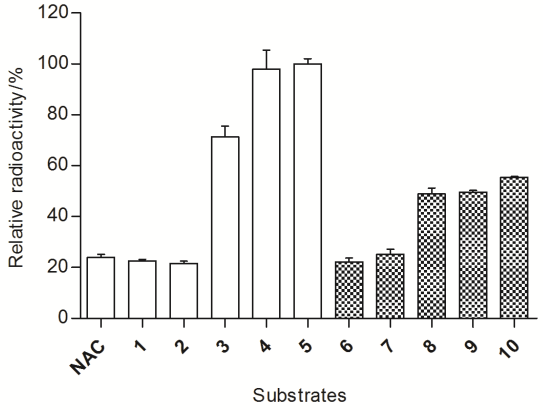


Figure 2-2. Relative activities of compounds **1**–**10** as substrates of ManT.

The radioactivity of the most active compound **5** was arbitrarily set to 100%. Unmethylated OS **1**–**5** are shown in open bars and methylated OS **6**–**10** are shown in shaded bars. NAC (no acceptor control) is the control assay without acceptors and the observed activities likely arise from endogenous acceptors present in the membrane fraction.

In contrast to the OMT, ManT activity was detected when using the bacterial membrane fraction as the enzyme source. The activity of the five methylated OS, **6**–**10** (Figure 2-2, shaded bars), were more or less equivalent to

those reported previously by Ballou using the mono- to pentasaccharides purified by gel filtration.[8] The only discrepancy occurred for the trisaccharide **8**, which Ballou and coworkers reported was not acceptor for the ManT. In our hands, the compound was a substrate. However, upon shortening the incubation time, the activity with **8** was scarcely detectable, indicating it is a relatively poor substrate. Our preliminary results supported Ballou and coworkers' hypothesis that the mono- and di- MMP fragments were not recognized by the enzyme whereas the tetrasaccharide and pentasaccharide MMP fragments acted as good substrates. In addition, our results further confirmed the trisaccaharide MMP fragment is a poor substrate of ManT.

2.4 Observation of an unexpected activity of ManT

To address the aforementioned possibility of the cross-reactivity between substrates (i.e., that the unmethylated oligosaccharides which possess free 4-OH at the non-reducing end could be potentially mannosylated by the ManT), unmethylated OS **1**–**5** were incubated with the ManT containing membrane fraction. To our suprise, radioactive enzymatic products were detected (Figure 2-2, open bars). Even more surprising was that these activities are high compared with methylated OS **6**–**10** (Figure 2-2, shaded bars). As shown in Figure 2-2, neither the monosaccharide **1** nor the disaccharide **2** exhibited significant activity. In contrast, the corresponding tri-, tetra- and pentasaccharides (**3**–**5**) possessed various levels of activities (71%, 98% and 100% activities relative to **5**, respectively). These values are, in fact, higher than those observed with their

methylated counterparts (49% for **8**, 50% for **9** and 56% for **10**, relative to **5**). According to the proposed biosynthetic pathway (Scheme 2-1), unmethylated MMP fragments (**1**–**5**) should not serve as substrates for the enzyme. However, contrary to this proposal, our results suggest the unmethylated MMP analogs are turned over by the ManT with even higher activities than the methylated analogs. Thus, these data suggest that MMPs elongation may not follow the alternating methylation and mannosylation pattern proposed earlier.[9]

To confirm our hypothesis, it was important to verify that the observed activity did indeed result from the action of the desired α-(1→4)-ManT, and not from the action of other glycosyltransferases that might also be present in the bacterial membrane fraction. For example, this fraction is also known to contain a membrane-associated α-(1→6)-ManT that is involved in the biosynthesis of lipoarabinomannan (LAM), a mycobacterial cell wall glycan.[20-22]

2.4.1 Does the activity come from incorporation of GDP-mannose?

It was first necessary to confirm that the activity seen with the unmethylated and methylated OS comes from the transfer of mannose from GDP-mannose to the substrates, and not to another endogenous acceptor that may also be retained on the C_{18} Sep-Pak cartridges. Tetrasaccharides **4** and **9**, which differ only in the substitution on the terminal mannose residues, were used as representative examples of unmethylated and methylated MMP analogs to analyze the formed products. As illustrated in Figure 2-3, each compound was incubated with GDP-mannose in the presence of the membrane fraction. The enzymatic

products were then purified by C_{18} Sep-Pak cartridge and the fractions collected were analyzed by MALDI mass spectrometry (MALDI-MS).

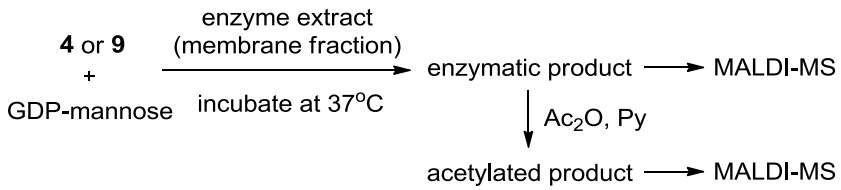


Figure 2-3. Incubation protocols for **4** and **9**, and separation of the enzymatic products.

The results were, again, surprising. Instead of introduction of single mannose residue to the starting materials, tetrasaccharides **4** and **9** both gave products with more than one mannose residue. When unmethylated **4** was incubated with the membrane fraction, a series of signals differing by the mass of a hexose residue (162 Da) were present in the MALDI spectrum (Figure 2-4A, $[M+Na]^+$: *m/z* = 1005, 1167, 1329, 1491, 1653 and 1815). Similar results were also observed in the products arising from incubation with methylated **9**. However, only three new peaks were detected (Figure 2-4B, $[M+Na]^+$: *m/z* = 1019, 1181 and 1343).

Postulating that the failure to detect longer products of **9** was due to low sensitivity of the MALDI-MS with unprotected neutral oligosaccharides,[23] the mixture of products was treated with acetic anhydride in pyridine to produce the corresponding acetylated derivatives (Figure 2-3). The crude products extracted from dichloromethane were characterized by MALDI-MS. This analysis revealed the formation of an additional four oligomers in the products formed from **9** (Figure 2-4C, $[M+Na]^+$: *m/z* = 1523, 1812, 2100, 2387, 2677, 2965, and 3253, mass difference = 288 Da, corresponding to an acetylated hexose residue).

Analogous results were obtained with the products arising from **4**. Taken together, these results suggest that ManT sequentially incorporates mannose residues onto **4** and **9** to form extended oligomers. This provides additional evidence that the enzyme does not require a methylated mannose residue at the terminus of the molecule, and that glycosylation and methylation may not necessarily alternate.

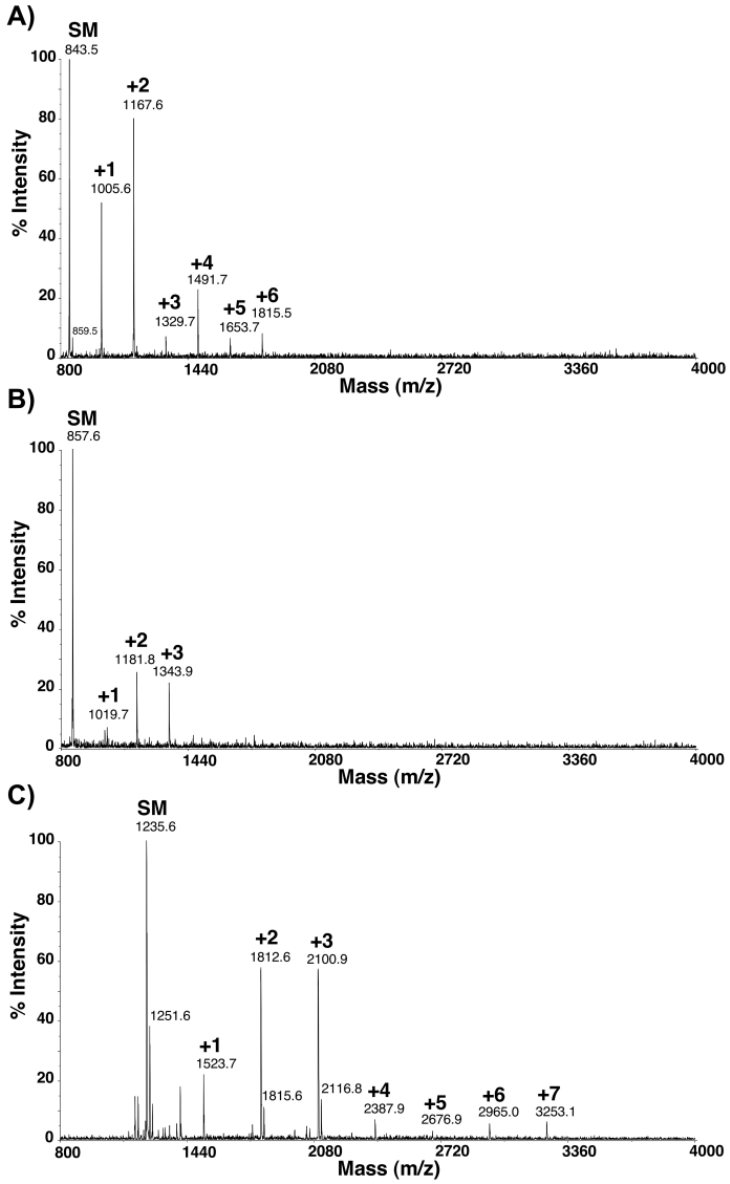


Figure 2-4. MALDI-MS spectra of the products resulting from incubation of **4** (A), **9** (B) and acetylated B (C). SM: starting material; numbers above each peak correspond to the number of additional mannose residues incorporated into the SM.

2.4.2 Are these products assembled by multiple mannosyltransferases?

After confirming that the observed enzymatic activity did indeed come from the transfer of mannose residues to the acceptor, the next issue that needed to be addressed was whether these observed polymer products are assembled by the desired α-(1→4)-ManT or other mannosyltransferases. This is necessary because, in addition to the α-(1→4)-ManT involved in MMPs biosynthesis, the membrane fraction harbours other ManT activities, in particular, an α-(1→6)-ManT involved in the assembly of LAM.[20-22] To address this question, *exo*-glycosidase digestion of the enzymatic products of both **4** and **9** were performed. Four *exo*-glycosidases were used: α-mannosidase from jack bean, β-mannosidase from *Helix pomatia*, α-(1→6)-mannosidase from *Chryseobacterium* (formerly named as *Flavobacterium*) *meningosepticum* and α-(1→2)-mannosidase from *Aspergillus saitoi*. The digestion reactions were monitored by MALDI-MS and the results are summarized in Table 2-1.

Table 2-1. Enzymatic digestion of the products formed from **4** and **9**.

Exo-glycosidase	products from **4**	products from **9**
α-mannosidase	√	√
β-mannosidase	×	×
α-(1→6)-mannosidase	×	×
α-(1→2)-mannosidase	×	×

Digestion was monitored by MALDI-MS. √: digestion cleavage was observed; ×: digestion cleavage was not observed.

When the enzymatic products from the reaction with **9** were treated with the α-mannosidase and the β-mannosidase, only the α-specific enzyme could digest the enzymatic products. After digestion, the peaks corresponding to the polymers ($[M+Na]^+$: *m/z* 1019, 1181, 1343) were converted back to starting

material ([M+Na]$^+$: *m/z* 857), which, due to the terminal methylated residue of **9**, was resistant to degradation. Moreover, when treating the products with the α-(1→6)-mannosidase and α-(1→2)-mannosidase, the products remained intact (Figure 2-5). Analogous results were also observed with the enzymatic products produced from **4**. Digestion was observed only with α-mannosidase. However, this time the digestion product was a trisaccharide ([M+Na]$^+$: *m/z* 681, from *m/z* 843–166 Da), which corresponded to further cleavage of the terminal mannose residue from **4** (Figure 2-6).

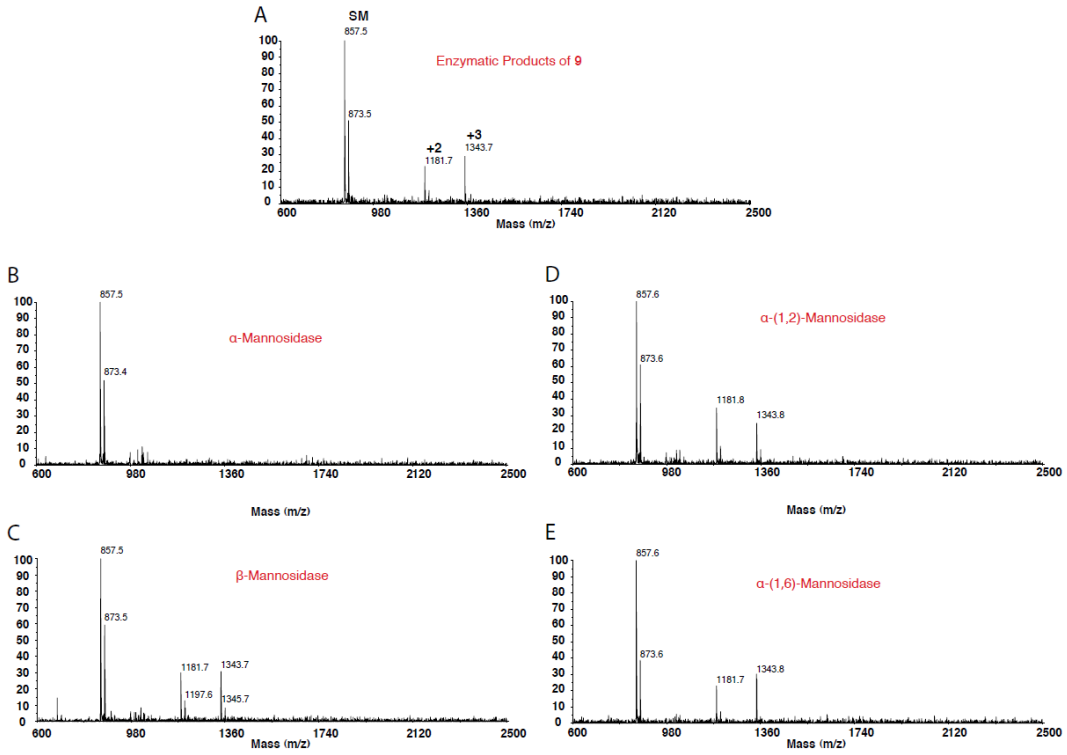


Figure 2-5. MS spectra of products from **9**, and those arising after enzymatic digestion.

Taken together, these digestion studies suggest that both sets of enzymatic products are α-linked (not β-linked) and, in addition, that they are neither α-(1→6) nor α-(1→2)-linked. Unfortunately, because there are no commercially available

enzymes capable of exclusively cleaving α-(1→3) and α-(1→4) mannopyranosyl linkages, it was not possible to distinguish between these possibilities using the digestion experiments. However, we surmised that an α-(1→4)-linkage more likely as, to date, no glycoconjugates containing an α-(1→3)-linked mannopyranosyl moiety have been reported in these mycobacterial strains. Nevertheless, additional investigations were carried out to prove the structure of the products.

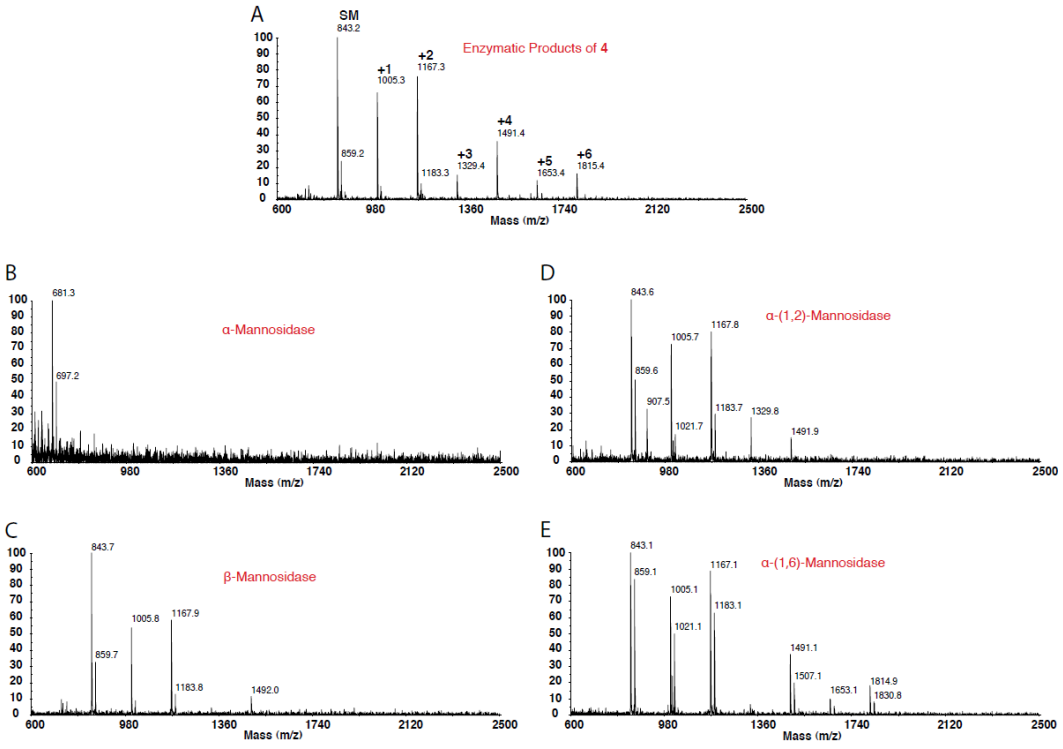
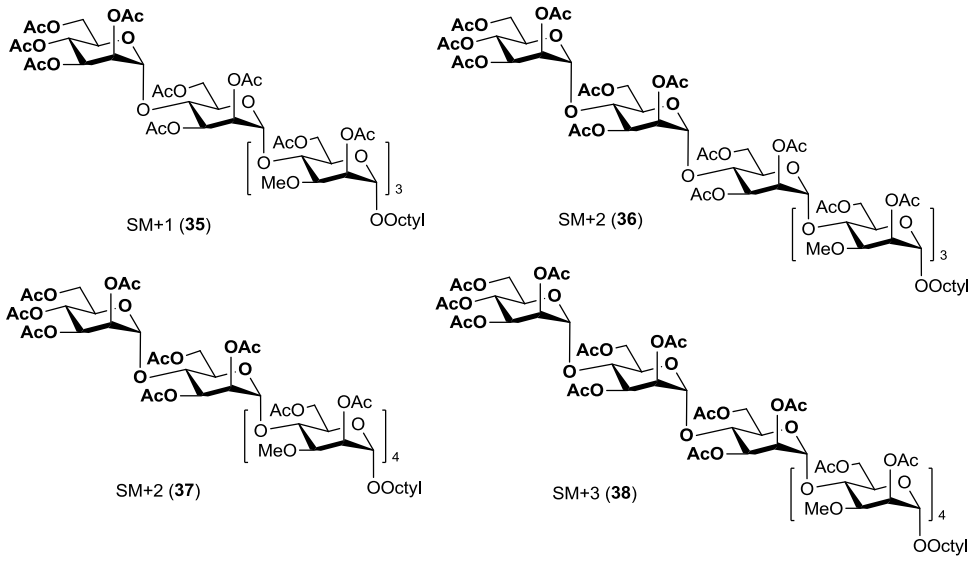


Figure 2-6. MS spectra of products from **4**, and those arising after enzymatic digestion.

2.4.3 Do the enzymatic products contain single or mixed linkages?

In addition to unequivocally determining the nature of the linkages between the monosaccharide residues introduced by the enzyme, it was also important to determine if a single linkage was formed, or if a mixture of α-(1→3)

and α-(1→4)-linkages were produced by the enzymatic activities present in the membrane preparation. To address the regiochemistry of the enzymatic reaction, scale-up incubations were performed using both **4** and **9**. A mixture of **4** (~10 mg) and non-radiolabeled GDP-mannose was incubated in the presence of membrane fraction, and the reaction was monitored by MALDI-MS. After the reaction was complete, the crude products were acetylated to facilitate separation. Two major products were separated by chromatography and their structures were confirmed by MALDI-MS and NMR to be a pentasaccharide (**35**) and a hexasaccharide (**36**) (Scheme 2-7). Incubation of **9** under the same conditions followed by per-acetylation led to similar results, with the major products being a hexasaccharide (**37**) and a heptasaccharide (**38**) (Scheme 2-7).



Scheme 2-7. Structures of four enzymatic products **35–37** after acetylation.

These four major products were obtained in 0.6–1 mg quantities, which allowed us to elucidate their structures by ^{1}H NMR spectroscopy. In addition to facilitating purification, acetylation also simplified the structural elucidation of the

products. Protons adjacent to acyl groups are deshielded significantly due to an anisotropic effect,[24] thus allowing them to be distinguished from those without neighbouring acyl groups. Figure 2-7 illustrates the ^{1}H NMR spectra of **4**, **35** and **36**. The resonances arising from H-1, H-2, H-3 and H-4 of the ring protons for each mannose residue were assigned based on two-dimensional experiments (^{1}H–^{1}H COSY and HSQC) together with coupling constant data characteristic of mannopyranose rings.[25]

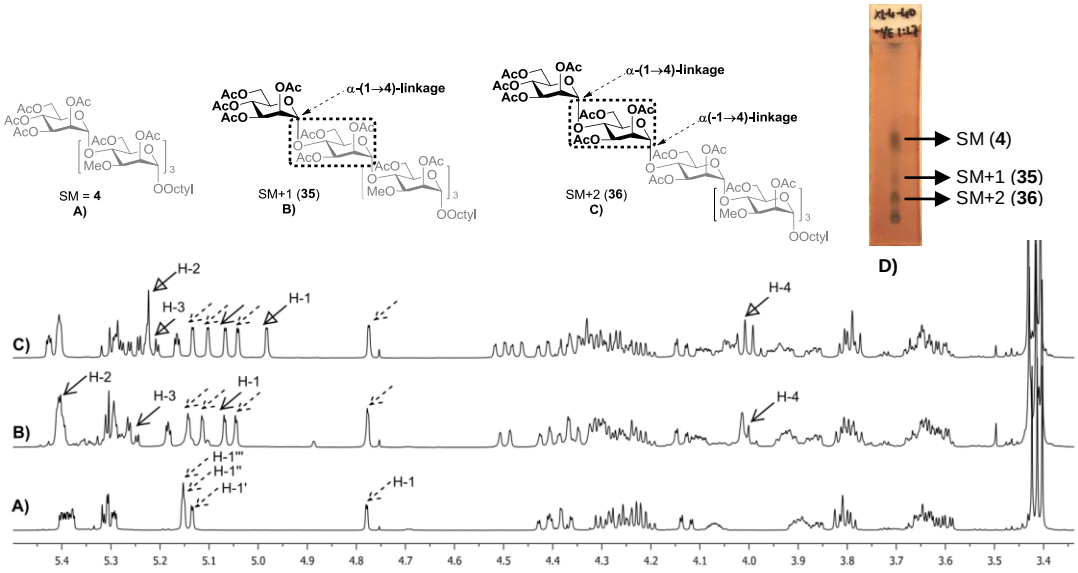


Figure 2-7. ^{1}H NMR spectra of the major products **35** and **36**, isolated from incubation of **4** with GDP-Man and ManT followed by acetylation. **A)** Tetrasaccharide **4** in its fully acetylated form. Dashed arrows indicate the four anomeric protons of **4**; **B)** Pentasaccharide **35**, solid arrows indicate the protons of the penultimate mannose (in dashed box); **C**) Hexasaccharide **36**, closed arrows indicate the protons of the penultimate mannose (in dashed box); **D)** TLC of the acetylated products formed from **4**.

Starting material **4** had four anomeric protons (Figure 2-7A), indicated by the dashed arrows, each as a doublet with $^{3}J_{\text{H-1,H-2}}$ = 1.0–1.5 Hz. Compared with **4**, the anomeric region of **35** showed an additional anomeric proton at 5.07 ppm

(Figure 2-7B, solid opened arrow), indicating the incorporation of one mannose residue. Moreover, inspection of the ^{1}H NMR spectrum of **35** revealed the presence of a peak for H-4 of the penultimate residue (Figure 2-7B, residue in dashed box) between 3.95–4.05 ppm. The chemical shift of this resonance indicates H-4 of this residue was glycosylated, as, after acetylation the chemical shift of this proton remained low. Had the newly introduced mannose been α-(1→3)-linked, H-4 of the penultimate mannose (in dashed box) would be deshielded after acetylation. Based on this information, and that obtained from the *exo*-glycosidase digestion, it was confirmed that the first residue added to **4** is α-(1→4)-linked.

Compared with **35**, the ^{1}H NMR spectrum arising from **36** had an additional resonance in the anomeric region at 4.98 ppm (Figure 2-7C, labeled with solid closed arrow). The protons of the penultimate mannose residue inside the dashed box had a similar pattern as the analogous residue in **35**, indicating that the second mannose was also incorporated via an α-(1→4)-linkage.

Using the same process, the two products formed from **9**, hexasaccharide **37** and pentasaccharide **38**, were also shown to result from the sequential incorporation of mannose residues to the O-4 position at the terminal end of the molecule (Figure 2-8). Based on the above information, it can be reasonably concluded that the other products resulting from the ManT mannosylation of **4** and **9**, as well as the other oligosaccharides tested above, also contain only α-(1→4)-linked mannopyranose residues.

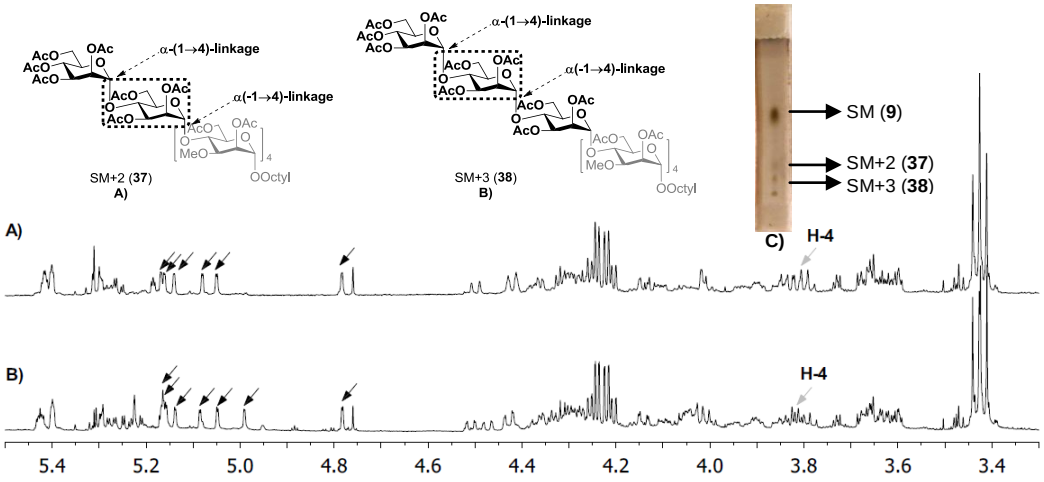


Figure 2-8. ^{1}H NMR spectra of the major products **37** and **38**, isolated from incubation of **9** with GDP-Man and ManT followed by acetylation. **A)** hexasaccharide **37**: six anomeric protons are indicated by black arrows; **B)** heptasaccharide **38**: seven anomeric protons are indicated by black arrows; Grey arrows indicate H-4 of the penultimate mannose reside circled with the dashed box. **C)** TLC of the acetylated products formed from **9**.

2.5 Kinetic characterization of tetrasaccharides 4 and 9

To compare the relative abilities of **4** and **9** to serve as substrates for the ManT, kinetic characterization was performed. For **4**, a K_m value of 20.7 ± 2.6 μM was obtained while for **9** the K_m is 7.9 ± 1.7 μM; the k_{cat}/K_m values were determined to be 0.28 ± 0.06 $min^{-1}g^{-1}$ and 0.10 ± 0.02 $min^{-1}g^{-1}$ for **4** and **9**, respectively (Figure 2-9). Thus, although the substrate **4** with an unmethylated terminal residue binds to the enzyme somewhat more weakly than its methylated counterpart **9**, it was turned over more rapidly. The observation that **4** serves as a better substrate compared to **9**, further calls into question the proposed biosynthetic pathway, where alternating mannosylation and methylation events occur.

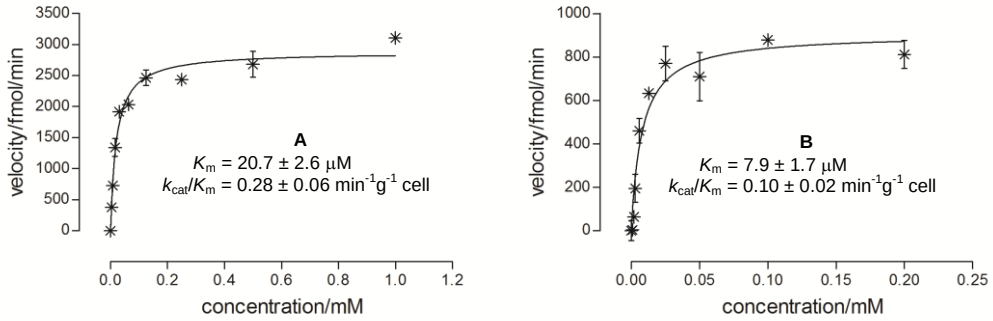


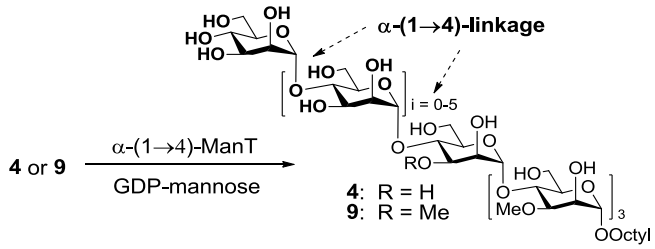
Figure 2-9. ManT kinetics with tetrasaccharide **4** and **9**.

A) Incorporation of radiolabeled GDP-mannose-[2-^{3}H] (1 mM) into **4** as a function of acceptor concentration. Assays were performed at 37 ℃ for 5 h, with substrate concentrations of 4, 8, 16, 31, 63, 125, 250, 500, 1000 μM. **B)** Incorporation of radiolabeled GDP-mannose-[2-^{3}H] (0.5 mM) into **9** as a function of acceptor concentration. Assays were performed at 37 ℃ for 10.5 h, with substrate concentrations 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, 100, 200 μM. Control experiments without the addition of acceptors were also performed in parallel. Each experiment was carried out in duplicate. The data obtained were subjected to nonlinear regression analysis using GraphPad Prism 4.0.

2.6 Conclusions

In our study, we used two series of synthetic mono- to pentasaccharides to probe the MMP biosynthetic pathway. When incubating unmethylated OS (**1**–**5**) and methylated OS (**6**–**10**) with *M. smegmatis* membrane fractions, the unmethylated OS served as better acceptors for the ManT. Moreover, the incubations of tetrasaccharides **4** and **9** with the crude ManT enzyme did not give a single product, but a homologous series of oligomers. Subsequent structural characterization confirmed that these products contain from one to six additional α-(1→4)-linked mannopyranose residues (Scheme 2-8). These results are inconsistent with the biosynthetic model previously proposed by Ballou and

Weisman, which involves alternating mannosylation and methylation reactions.[9] Instead, our results suggest that, MMP elongation does not require the cooperative activity between the ManT and OMT, but rather that the ManT can produce a linear polymer independent of OMT-catalyzed methylation.



Scheme 2-8. Observed activity of α-(1→4)-mannosyltransferase.

In contrast to the original pathway proposed for MMP biosynthesis involving alternating methylation and manosylation events,[9] our results suggest an alternative model whereby an unmethylated mannose chain (or MMP fragments) are first extended by ManT. These are then methylated at O-3 of each mannose residue by the OMT. Because ManT recognizes both methylated and unmethylated OS, these steps could be repeated to give full length MMPs, and could account for the observed length distribution of naturally occurring MMPs (Figure 2-10). When compared to the Ballou proposal, this proposal differs mainly in that ManT can introduce more than one mannose onto short oligomers in sequence and prior to methylation.

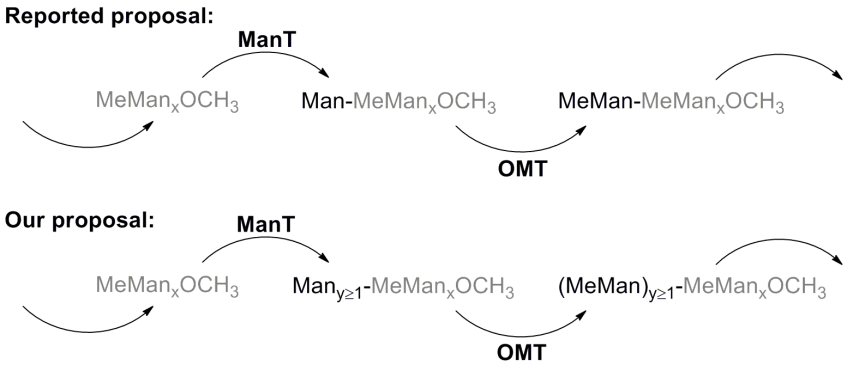


Figure 2-10. Revised proposal for MMP biosynthetic pathway.

This hypothesis is consistent with earlier data demonstrating that the OMT can recognize short chain length substrates,[9] and by analogy, short sequences within oligosaccharide chains. It is possible that in short substrates (trisaccharide or smaller) that methylation inhibits recognition by the ManT. This provides an explanation for the results obtained both by us and Ballou that these shorter α-(1→4)-linked mannose sequences containing methylation are not substrates for the ManT. To address these possibilities, studies with OMT and α-(1→4)-linked mannose oligomers lacking methyl groups are needed.

Although we could not detect OMT activity as previously reported, the OMT enzyme was present in the bacterial strains from which we derived the membrane fractions as we could successfully separate MMPs corresponding of 11–14 carbohydrate residues in length from this strain of *M. smegmatis*.[19] At this stage, we are unsure why we are unable to detect OMT activity. One possibility, which we consider unlikely given the data reported by Ballou is that the enzyme does not recognize synthetic MMP fragments with the octyl aglycone.

Alternatively, it is possible that the enzyme is unstable in the cell lysate. As such, efforts to identify the activity of the O-methyltransferase are ongoing.

2.7 Experimental details

General methods for chemical synthesis: All reagents were purchased from commercial sources and were used without further purification unless noted. Reaction solvents were purified by successive passage through columns of alumina and copper under an argon atmosphere. All reactions were carried out under positive pressure of argon at room temperature unless specified and were monitored by TLC on silica gel 60-F_{254} (0.25 mm, Silicycle, Quebec, Canada). Visualization of the reaction components was achieved using UV fluorescence (254 nm) and/or by charring with acidified anisaldehyde solution in ethanol. Organic solvents were evaporated under reduced pressure and the products were purified by column chromatography on silica gel (230–400 mesh, Silicycle, Quebec, Canada) or Iatrobeads (Iatron Laboratories Inc., Tokyo) if the eluent system contained greater than 10% methanol. The yields reported are after purification. Optical rotations were measured on Perkin–Elmer 241 polarimeter at ambient temperature and are in units of degree•mL/(g•dm). ^{1}H NMR spectra were recorded at 400, 500 or 600 MHz and chemical shifts were referenced to $CHCl_3$ (7.26 ppm), CHD_2OD (3.31 ppm), HOD (4.79 ppm). ^{13}C NMR spectra were recorded at 100 or 125 MHz and chemical shifts were referenced to $CDCl_3$ (77.1 ppm) or CD_3OD (49.0 ppm). Assignments of NMR spectra were based on two-dimensional experiments (^{1}H–^{1}H COSY, HMQC or HSQC, and HMBC) and

stereochemistry of the anomeric centers of the pyranose rings were confirmed by measuring $^{1}J_{C\text{-}1,H\text{-}1}$ via coupled HMQC or HSQC experiments. Electrospray mass spectra were recorded on Agilent Technologies 6220 TOF.

Octyl α-D-mannopyranoside (1): **1** was synthesized by Fisher glycosylation using sulfuric acid immobilized on silica (H_2SO_4–silica) as the catalyst.[26] Preparation of H_2SO_4–silica: To a stirring slurry of silica gel (10.4 g, 230-400 mesh) in dry diethyl ether (50 mL) was slowly added H_2SO_4 (3 mL). After stirring for 5 min, the solvent was removed and the resulting H_2SO_4–silica was dried in the oven for overnight. The Fisher glycosylation was performed with D-mannose (184.0 mg, 1.0 mmol) and H_2SO_4-silica (5 mg) in *n*-octanol (0.8 mL, 5.0 mmol). After stirring at 80 °C for two overnights, the reaction mixture was directly purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford **1** (172.0 mg, 58%) as pale yellow oil. R_f 0.35 (CH_2Cl_2–MeOH 6:1); ^{1}H NMR (500 MHz, CD_3OD): δ 4.72 (d, *J* = 1.5 Hz, 1H; H-1), 3.81 (dd, *J* = 11.7, 2.0 Hz, 1H; H-6a), 3.77 (dd, *J* = 1.5, 3.0 Hz, 1H; H-2), 3.75-3.70 (m, 2H; octyl OCH_2, H-6b), 3.68 (dd, *J* = 9.6, 3.9 Hz, 1H; H-3), 3.60 (t, *J* = 9.5 Hz, 1H; H-4), 3.51 (ddd, *J* = 9.0, 6.0, 2.5 Hz, 1H; H-5), 3.40 (dt, *J* = 9.6, 6.4 Hz, 1H; octyl OCH_2), 1.60 (m, 2H; octyl OCH_2CH_2), 1.40-1.20 (m, 10H; octyl CH_2), 0.90 (t, *J* = 6.8 Hz, 3H; octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD): δ =101.6 (C-1), 74.6, 72.7, 72.3, 68.7, 68.6 (C-6), 62.9 (octyl OCH_2), 33.0, 30.6, 30.5, 30.4, 27.4, 23.7 (6C; octyl CH_2), 14.4 (octyl CH_3); HRMS (ESI) *m/z* calcd $C_{14}H_{28}NaO_6$ $[M+Na]^+$ 315.1778, found 315.1775.

Octyl α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (2): Disaccharide **21** (121.1 mg, 0.15 mmol) was dissolved in MeOH (5 mL) and

NaOMe (1.0 M) was added until the pH of the solution was 9. After stirring at room temperature overnight, the mixture was neutralized with Amberlite IR120 H^+ ion exchange resin and then concentrated. The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford pale yellow syrup (95.5 mg, 99%). The partially deprotected compound (57.4 mg, 0.09 mmol) was then dissolved in MeOH (7 mL) before adding 20 wt. % $Pd(OH)_2/C$ (9.2 mg). The reaction mixture was stirred overnight under a H_2 atmosphere, and then the catalyst was removed by filtration through Celite. The filtrate was concentrated and the crude residue was purified by chromatography (CH_2Cl_2–MeOH 9:1) to afford **2** (29.2 mg, 70%) as a pale yellow syrup. R_f 0.08 (CH_2Cl_2–MeOH 9:1); $[\alpha]_D$ = +73.1 (*c* 2.6, MeOH); ^{1}H NMR (400 MHz, CD_3OD): δ 5.17 (d, *J* = 1.6 Hz, 1H, H-1'), 4.77 (d, *J* = 1.2 Hz, 1H, H-1), 4.00 (dd, *J* = 2.8, 1.6 Hz, 1H, H-2), 3.89 (app t, *J* = 9.5 Hz, 1H, H-4), 3.87–3.82 (m, 2 H, H-2', H-6a'), 3.78 (d, *J* = 3.2 Hz, 2H, H-6a, H-6b), 3.73-3.58 (m, 5H, H-6b', octyl OCH_2, H-3', H-4', H-5'), 3.54 (app dt, *J* = 9.5, 3.3 Hz, 1H, H-5), 3.46 (dd, *J* = 9.2, 3.1 Hz, 1H, H-3), 3.44–3.38 (m, 4H, octyl OCH_2, OMe-3), 1.55-1.65 (m, 2H, octyl OCH_2CH_2), 1.40–1.20 (m, 10H, octyl CH_2), 0.90 (t, *J* = 6.8 Hz, 3H, octyl CH_3); ^{13}C NMR (100 MHz, CD_3OD): δ 103.4 (C-1'), 101.4 (C-1), 83.5 (C-3), 75.6 (C-5'), 73.8 (C-4), 73.2 (C-5), 72.5 (C-3'), 72.4 (C-2'), 68.8 (octyl OCH_2), 68.5 (C-4'), 67.8 (C-2), 63.0 (C-6), 62.5 (C-6'), 56.8 (OMe), 33.0, 30.6, 30.5, 30.4, 27.4, 23.7 (6C; octyl CH_2), 14.5 (octyl CH_3); HRMS (ESI) *m/z* calcd $C_{21}H_{40}O_{11}$ $[M + Na]^+$ 491.2463, found 491.2461.

Octyl α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (3): The same procedure as that described for the synthesis of **2** was used. Deacylation was carried out on trisaccharide **25** (89.8 mg, 0.07 mmol) in CH_2Cl_2–MeOH (11 mL, 1:10 v/v). The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford colorless syrup (53.4 mg, 77%). The partially deprotected compound (49.4 mg, 0.05 mmol) was subsequently debenzylated using 20 wt. % $Pd(OH)_2$/C (10.0 mg) in MeOH (10 mL). Chromatographic purification (CH_2Cl_2–MeOH 5:1 → 3:1) gave **3** (34.5 mg, 99%) as a white foam. R_f 0.35 (CH_2Cl_2–MeOH 9:1); $[\alpha]_D$ = +124.0 (*c* 0.7, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 5.21 (d, *J* = 1.9 Hz, 1H, H-1'), 5.17 (d, *J* = 1.8 Hz, 1H, H-1''), 4.77 (d, *J* = 1.7 Hz, 1H, H-1), 4.08 (dd, *J* = 3.0, 2.0 Hz, 1H, H-2'), 4.01 (dd, *J* = 3.0, 1.9 Hz, 1H, H-2), 3.94–3.85 (m, 3H, H-4', H-4, H-2''), 3.85–3.58 (m, 11H, H-3'', H-4'', H-5', H-5'', H-6a, H-6b, H-6a', H-6b', H-6a'', H-6b'', octyl OCH_2), 3.56 (ddd, *J* = 9.8, 4.8, 2.2 Hz, 1H, H-5), 3.49 (dd, *J* = 9.3, 3.2 Hz, 1H, H-3), 3.46 (dd, *J* = 9.1, 3.1 Hz, 1H, H-3'), 3.42 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.41–3.38 (m, 1H, octyl OCH_2), 1.66–1.53 (m, 2H, octyl OCH_2CH_2), 1.45–1.25 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ 103.3(C-1''),103.1 (C-1'), 101.3 (C-1), 83.4 (C-3'), 83.2 (C-3), 75.6 (C-5''), 74.2 (H-5'), 73.9 (H-4), 73.6 (H-4'), 73.2 (H-5), 72.5 (H-2''), 72.4 (H-3''), 68.8 (octyl OCH_2), 68.5 (C-4''), 67.9 (C-2'), 67.7 (C-2''), 63.0, 62.7, 62.7 (3C, C-6'', C-6', C-6), 56.8 (OMe), 56.7 (OMe), 33.0 (octyl CH_2), 30.5 (octyl CH_2), 30.4, 30.4 (octyl CH_2), 27.4 (octyl CH_2), 23.7 (octyl CH_2), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{28}H_{52}O_{16}$ $[M + Na]^+$ 667.3148, found 667.3144.

Octyl α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (4): Tetrasaccharide **29** (104.3 mg, 0.07 mmol) was dissolved in MeOH (8 mL) and 1.0 M NaOMe was added until the pH of the solution was 9. After stirring at room temperature overnight, the mixture was neutralized with Amberlite IR120 H^+ ion exchange resin and then concentrated. The crude residue was purified by chromatography (CH_2Cl_2–MeOH 12:1) to afford pale yellow syrup (52.0 mg, 66%). The partially deprotected compound (52.0 mg, 0.04 mmol) was dissolved in MeOH (8 mL) before adding 20 wt. % $Pd(OH)_2/C$ (27.1 mg). The reaction was stirred overnight under a H_2 atmosphere and then the catalyst was removed by filtering through Celite. The filtrate was concentrated and the crude residue was purified by chromatography (CH_2Cl_2–MeOH 4:1) to afford **4** (28.4 mg, 79%) as a white foam. R_f 0.29 (CH_2Cl_2–MeOH 3:1); $[\alpha]_D$ = +140.9 (*c* 0.2, MeOH); ^{1}H NMR (600 MHz, CD_3OD) δ 5.21 (m, 2H, H-1', H-1''), 5.17 (d, *J* = 1.8 Hz, 1H, H-1'''), 4.77 (d, *J* = 1.7 Hz, 1H, H-1), 4.12–4.05 (m, 2H, H-2', H-2''), 4.01 (dd, *J* = 3.1, 1.9 Hz, 1H, H-2), 3.93–3.85 (m, 4 H, H-4', H-4'', H-2''', H-4), 3.85–3.69 (m, 9H, 8 × H-6, octyl OCH_2), 3.69–3.59 (m, 5H, H-3''', H-4''', H-5', H-5'', H-5'''), 3.56 (ddd, *J* = 9.9, 4.9, 2.2 Hz, 1H, H-5), 3.49 (dd, *J* = 9.6, 3.6 Hz, 1H, H-3), 3.48 (dd, *J* = 9.6, 3.0 Hz, 1H, H-3''), 3.46 (dd, *J* = 9.6, 3.6 Hz, 1H, H-3'), 3.43 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.42–3.39 (m, 1H, octyl OCH_2), 1.69–1.52 (m, 2H, octyl OCH_2CH_2), 1.45–1.23 (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 103.3 (C-1'''), 103.1, 103.0 (2C; C-1', C-1''), 101.3 (C-1), 83.4 (C-3), 83.2, 83.1 (2C; C-3', C-3''),

75.6 (C-5’’’), 74.2, 74.2 (2C; C-5’, C-5’’), 74.0 (C-4), 73.7 (C-4’’), 73.6 (C-4’), 73.2 (C-5), 72.5 (C-2’’’), 72.4 (C-3’’’), 68.7 (octyl OCH_2), 68.5 (C-4’’’), 67.9, 67.9 (2C; C-2’, C-2’’), 67.7 (3C; C-2), 63.0 , 62.7, 62.7, 62.7 (4C; 4 × C-6), 56.8, 56.7, 56.7 (3C; 3 × OMe), 33.0, 30.5, 30.4, 30.4, 27.4, 23.7 (6C; octyl CH_2), 14.5 ppm (octyl CH_3); HRMS (ESI) calcd $C_{35}H_{64}O_{21}$ $[M + Na]^+$ 843.3832, found 843.3834.

Octyl α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (5): Deprotection followed the same procedure as for **4**. Deacylation was carried out with pentasaccharide **33** (104.8 mg, 0.05 mmol) in MeOH (18 mL). The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford pale yellow syrup (43.2 mg, 55%). This partially deprotected compound (40.7 mg, 0.03 mmol) was subsequently debenzylated using 20 wt. % $Pd(OH)_2/C$ (30.8 mg) in MeOH (10 mL). Chromatography purification (CH_2Cl_2–MeOH 3:1) gave **5** (21.9 mg, 78%) as a white foam. R_f 0.24 (CH_2Cl_2–MeOH 2:1); $[\alpha]_D$ = +111.1 (*c* 1.0, MeOH); ^1H NMR (600 MHz, D_2O) δ 5.19 (brs, 1H, H-1’’’’), 5.17 (brs, 1H, H-1’’’), 5.11 (brs, 1H, H-1, H-1’’), 5.09 (brs, 1H, H-1; H-1’), 4.89 (brs, 1H, H-1), 4.23 (brs, 1H, H-2’), 4.22 (brs, 1H, H-2’’), 4.18 (brs, 1H, H-2’’’), 4.14 (brs, 1H, H-2), 3.97 (brs, 2H, H-2’’’’), 3.91–3.75 (m, 15H, H-4, H-4’, H-4’’, H-4’’’, 10×H-6, octyl OCH_2), 3.75–3.70 (m, 4H, H-3’’’’, H-5’, H-5’’, H-5’’’), 3.68 (app t, *J* = 9.6 Hz, 1H, H-4’’’’), 3.70–3.59 (m, 2H, H-5’’’’, H-5), 3.59–3.40 (m, 5H, H-3, H-3’, H-3’’, H-3’’’, octyl OCH_2), 3.48 (s, 3H, OMe), 3.48 (s, 6H, 2 × OMe), 3.46 (s, 3H, OMe),

1.72–1.58 (m, 2H, octyl OCH_2CH_2), 1.49–1.30 (m, 10H, octyl OCH_2), 0.96 ppm (t, *J* = 6.4 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, D_2O) δ 103.4 (C-1’’), 103.2 (C-1’), 102.6 (H-1’’’’), 102.4 (H-1’’’), 100.6 (C-1), 82.0, 82.0 (2C; C-3, C-3’’’), 81.9 (C-3’’), 81.7 (C-3’), 76.0 (C-4), 75.5 (C-4’), 74.6 (C-5’’’’), 74.3 (C-4’’), 73.3 (C-4’’’), 73.1 (3C; C-5’, C-5’’, C-5’’’), 72.3 (C-5), 71.4 (2C; C-2’’’’, C-3’’’’); 68.6 (octyl OCH_2), 67.4 (C-4’’’’), 67.0 (C-2’’’), 66.7 (3C; C-2, C-2’, C-2’’), 61.8, 61.7, 61.6, 61.5, 61.4 (5C; C-6, C-6’, C-6’’, C-6’’’, C-6’’’’), 57.3, 57.1, 57.0, 56.9 (4C; 4 × OMe); 32.5, 30.0, 29.9, 29.8, 26.8, 23.3 (6C; octyl CH_2), 14.8 ppm (octyl CH_3); HRMS (ESI) calcd $C_{42}H_{76}O_{26}$ $[M + Na]^+$ 1019.4517, found 1019.4515.

Octyl 3-*O*-methyl-α-D-mannopyranoside (6): Octyl α-D-mannopyranoside (4.70 g, 16.1 mmol) and *n*-Bu_2SnO (4.08 g, 16.1 mmol) in toluene (80 mL) were heated at reflux for 3 h. The reaction mixture was cooled to room temperature before *n*-Bu_4NI (6.06 g, 16.1 mmol) and MeI (11 mL, 174.6 mmol) were added. The reaction mixture was heated at 70 ℃ and stirring continued for 3 days. After cooling, the solvent was evaporated and the crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to give **6** (3.45 g, 70%) as yellow syrup. R_f 0.27 (CH_2Cl_2–MeOH 15:1); $[\alpha]_D$ = +39.8 (*c* 1.8, MeOH); ^1H NMR (500 MHz, D_2O): δ 4.86 (d, *J* = 1.5 Hz, 1H, H-1), 4.12 (dd, *J* = 3.0, 1.5 Hz, 1H, H-2), 3.82 (dd, *J* = 12.2, 2.3 Hz, 1H, H-6a), 3.77 (dd, *J* = 12.2, 5.2 Hz, 1H, H-6b), 3.74–3.69 (m, 2H, octyl OCH_2, H-4), 3.58 (ddd, *J* = 9.8, 5.0, 2.4 Hz, 1H, H-5), 3.48 (app dt, *J* = 9.9, 6.4 Hz, 1H, octyl OCH_2), 3.43 (dd, *J* = 3.0, 9.5 Hz, 1H, H-3), 3.43 (s, 3H, OMe), 1.65-1.55 (m, 2H, octyl OCH_2CH_2), 1.40–1.20 (m, 10H, octyl CH_2), 0.87

(t, J = 6.7 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, D_2O): δ 100.8 (C-1), 81.5 (C-3), 73.6 (C-5), 68.4 (octyl CH_2), 67.1 (C-4), 66.0 (C-2), 61.5 (C-6), 57.3 (OMe), 32.8 (octyl CH_2), 30.4 (octyl CH_2), 30.2 (octyl CH_2), 30.1 (octyl CH_2), 27.0 (octyl CH_2), 23.4 (octyl CH_2), 14.7 (octyl CH_3); HRMS (ESI) *m/z* calcd $C_{15}H_{30}O_6$ $[M + Na]^+$ 329.1935, found 329.1931.

Octyl 3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (7): The same procedure as that described for the synthesis of **2** was used. Deacylation was carried out on disaccharide **22** (293.3 mg, 0.37 mmol) in MeOH (10 mL). The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford pale yellow syrup (216.0 mg, 90%). The partially deprotected compound (216.0 mg, 0.33 mmol) was subjected to hydrogenolysis using 20 wt. % $Pd(OH)_2/C$ (27.8 mg) in MeOH (10 mL). Chromatographic purification (CH_2Cl_2–MeOH 9:1) gave **7** (148.4 mg, 92%) as a pale yellow syrup. R_f 0.17 (CH_2Cl_2–MeOH 9:1); $[\alpha]_D$ = +68.3 (*c* 0.6, MeOH); ^1H NMR (500 MHz, CD_3OD) δ 5.20 (d, J = 1.9 Hz, 1H, H-1'), 4.77 (d, J = 1.8 Hz, 1H, H-1), 4.07 (dd, J = 3.1, 2.0 Hz, 1H, H-2'), 4.00 (dd, J = 3.1, 1.9 Hz, 1H, H-2), 3.89 (app t, J = 9.6 Hz, 1H, H-4), 3.82 (dd, J = 11.7, 2.1 Hz, 1H, H-6a'), 3.80–3.77 (m, 2H, H-6a, H-6b), 3.75–3.65 (m, 3H, H-6b', H-4', octyl OCH_2), 3.61 (ddd, J = 9.7, 5.7, 2.1 Hz, 1H, H-5'), 3.55 (ddd, J = 9.9, 4.0, 3.0 Hz, 1H, H-5), 3.48 (dd, J = 9.6, 3.2 Hz, 1H, H-3), 3.45 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.44–3.38 (m, 1H, octyl OCH_2), 3.32 (dd, J = 9.1, 3.1 Hz, 1H, H-3'), 1.65–1.55 (m, 2H, octyl OCH_2CH_2), 1.40–1.25 (m, 10H, octyl CH_2), 0.90 (t, J = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ 103.3 (C-1'); 101.4 (C-1), 83.4 (C-3), 82.2 (C-3'), 75.6 (C-5'), 73.8

(C-4), 73.2 (C-5), 68.8 (octyl OCH_2), 68.2 (C-2'), 67.8 (C-2), 67.4 (C-4'), 63.0 (C-6'), 62.6 (C-6), 57.4 (OMe), 56.7 (OMe), 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), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{22}H_{42}O_{11}$ $[M + Na]^+$ 505.2619, found 505.2618.

Octyl 3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (8): The same procedure as that described for the synthesis of **2** was used. Deacylation was carried out on trisaccharide **26** (139.3 mg, 0.12 mmol) in MeOH (10 mL). The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford colorless syrup (87.5 mg, 78%). This partially deprotected compound (76.8 mg, 0.08 mmol) was subsequently debenzylated using 20 wt. % $Pd(OH)_2/C$ (24.5 mg) in MeOH (10 mL). Chromatographic purification (CH_2Cl_2–MeOH 5:1) gave **8** (52.1 mg, 96%) as a white foam. R_f 0.18 (CH_2Cl_2–MeOH 6:1); $[\alpha]_D$ = +99.2 (*c* 1.3, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 5.23–5.20 (m, 2H, H-1'', H-1'), 4.77 (d, *J* = 1.5 Hz, 1H, H-1), 4.09 (dd, *J* = 2.9, 2.2 Hz, 1H, H-2'), 4.07 (dd, *J* = 3.1, 2.0 Hz 1H, H-2''), 4.01 (dd, *J* = 3.0, 2.0 Hz, 1H, H-2), 3.91 (app t, *J* = 9.4 Hz, 1H, H-4'), 3.88 (app t, *J* = 9.8 Hz, 1H, H-4), 3.85–3.64 (m, 9H, H-4'', H-5', H-6a, H-6b, H-6a', H-6b', H-6a'', H-6b'', octyl OCH_2), 3.62 (ddd, *J* = 9.7, 5.7, 2.1 Hz, 1H, H-5''), 3.56 (ddd, *J* = 9.8, 4.8, 2.1 Hz, 1H, H-5), 3.50 (dd, *J* = 9.0, 3.0 Hz 1H, H-3), 3.47 (dd, *J* = 9.0, 3.0 Hz 1H, H-3'), 3.46 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.44-3.39 (m, 1H, octyl OCH_2), 3.33 (dd, *J* = 9.1, 3.1 Hz, 1H, H-3''), 1.69–1.51 (m, 2H, octyl OCH_2CH_2), 1.46–1.22 (m, 10H, octyl CH_2), 0.91 (t, *J* = 6.9 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ 103.2 (C-1''/C-1'),

103.1 (C-1’’/C-1’), 101.3 (C-1), 83.4,(C-3’), 83.2 (C-3), 82.2 (C-3’’), 75.6 (C-5’’), 74.2 (C-5’), 74.0 (C-4), 73.6 (C-4’), 73.2 (C-5), 68.8 (octyl OCH_2), 68.2 (C-2’’), 67.9 (C-2’), 67.7 (C-2), 67.4 (C-4’’), 63.0, 62.7, 62.6 (3C; C-6, C-6’, C-6’’), 57.4, 56.7, 56.7 (3C; 3 × OMe), 33.0 (octyl CH_2), 30.5 (octyl CH_2), 30.4 (octyl CH_2), 30.4 (octyl CH_2), 27.4 (octyl CH_2), 23.7 (octyl CH_2), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{29}H_{54}O_{16}$ $[M + Na]^+$ 681.3304, found 681.3302.

Octyl 3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (9): Deprotection followed the same procedure as for **4**. Deacylation was carried out with tetrasaccharide **30** (139.0 mg, 0.09 mmol) in MeOH (8 mL). The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford pale yellow syrup (102.0 mg, 94%). This partially deprotected compound (98.4 mg, 0.08 mmol) was subsequently debenzylated using 20 wt. % $Pd(OH)_2/C$ (49.5 mg) in MeOH (10 mL). Chromatography purification (CH_2Cl_2–MeOH 4:1) gave **9** (49.4 mg, 72%) as a white foam. R_f 0.19 (CH_2Cl_2–MeOH 4:1); $[\alpha]_D$ = +126.9 (*c* 0.3, MeOH); ^{1}H NMR (600 MHz, CD_3OD) δ 5.21 (d, *J* = 1.8 Hz, 1H, H-1’’), 5.20 (d, 2H, H-1’, H-1’’’), 4.77 (d, *J* = 1.8 Hz, 1H, H-1), 4.11–4.08 (m, 2H, H-2’, H-2’’), 4.07 (dd, *J* = 2.9, 2.1 Hz, 1H, H-2’’’), 4.01 (dd, *J* = 3.0, 1.9 Hz, 1H, H-2), 3.91 (app t, *J* = 9.6 Hz, 1H, H-4’’), 3.89, (app t, *J* = 9.6 Hz, 1H, H-4’), 3.87 (app t, *J* = 9.6 Hz, 1H, H-4), 3.84–3.64 (m, 12H, H-4’’’, H-5’, H-5’’, 8 × H-6, octyl OCH_2), 3.64–3.59 (ddd, *J* = 9.6, 6.0, 1.8 Hz, 1H, H-5’’’), 3.56 (ddd, *J* = 10.0, 5.0, 2.1 Hz, 1H, H-5), 3.49 (dd, *J* = 9.6, 3.6 Hz, 1H, H-3), 3.48 (dd, *J* = 9.0, 3.0 Hz, 2H, H-3’, H-3’’), 3.45 (s, 3H, OMe), 3.43 (s, 6H,

2 × OMe), 3.42 (s, 3H, OMe), 3.42–3.39 (m, 1H, octyl OCH_2), 3.32 (dd, *J* = 9.2, 3.1 Hz, 1H, H-3’’’), 1.66–1.52 (m, 2H, octyl OCH_2CH_2), 1.45–1.20 (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 103.2 (C-1’’’), 103.1, 103.1 (2C; C-1’, C-1’’), 101.3 (C-1), 83.4 (C-3), 83.1, 83.0 (2C; C-3’, C-3’’), 82.2 (C-3’’’), 75.6 (C-5’’’), 74.2 (2C; C-5’, C-5’’), 74.0 (C-4), 73.8 (C-4’’), 73.6 (C-4’), 73.2 (C-5), 68.7 (octyl OCH_2), 68.2 (C-2’’’), 67.9 (2C; C-2’, C-2’’), 67.7 (C-2), 67.3 (C-4’’’), 62.9, 62.8, 62.8, 62.7 (4C; 4 × C-6), 57.4, 56.7, 56.7, 56.6 (4C; 4 × OMe), 33.0, 30.5, 30.4, 30.4, 27.4, 23.7 (6C; octyl CH_2), 14.5 ppm (octyl CH_3); HRMS (ESI) calcd $C_{36}H_{66}O_{21}$ $[M + Na]^+$ 857.3989, found 857.3987.

Octyl 3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (10): Deprotection followed the same procedure as for **4**. Deacylation was carried out with pentasaccharide **34** (98.6 mg, 0.05 mmol) in MeOH (14 mL). The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford pale yellow syrup (56.2 mg, 74%). This partially deprotected compound (52.0 mg, 0.03 mmol) was subsequently debenzylated using 20 wt. % $Pd(OH)_2/C$ (25.6 mg) in MeOH (10 mL). Chromatography purification (CH_2Cl_2–MeOH 3:1) gave **10** (32.3 mg, 90%) as a white foam. R_f 0.19 (CH_2Cl_2–MeOH 3:1); $[\alpha]_D$ = +128.0 (*c* 0.3, MeOH); ^1H NMR (600 MHz, D_2O) δ 5.21 (brs, 1H, H-1’’’’), 5.14 (d, *J* = 1.2 Hz, 1H, H-1’), 5.09 (brs, 1H, H-1’’), 5.08 (d, *J* = 1.2 Hz, 1H, H-1’’’), 4.89 (brs, 1H, H-1), 4.24 (brs, 1H, H-2’’), 4.24 (brs, 1H, H-2’’’), 4.20 (brs, 1H, H-2’), 4.17

(brs, 1H, H-2’’’’), 4.14 (brs, 1H, H-2), 3.90–3.75 (m, 15H, H-4, H-4’, H-4’’, H-4’’’, 10×H-6, octyl OCH_2), 3.75–3.70 (m, 4H, H-4’’’’, H-5’, H-5’’, H-5’’’), 3.67 (ddd, *J* = 9.6, 5.4, 1.8 Hz, 1H, H-5’’’), 3.62 (brd, *J* = 10.2 Hz, 1H, H-5), 3.58–3.50 (m, 5H, H-3, H-3’, H-3’’, H-3’’’, octyl OCH_2), 3.49 (s, 3H, OMe), 3.48 (s, 3H, OMe), 3.48 (s, 3H, OMe), 3.47 (s, 3H, OMe), 3.47 (s, 3H, OMe), 3.44 (dd, *J* = 9.5, 3.1 Hz, 1H, H-3’’’’), 1.76–1.59 (m, 2H, octyl OCH_2CH_2), 1.48–1.27 (m, 10H, octyl CH_2), 0.97 ppm (t, *J* = 6.7 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, D_2O) δ 103.5, 103.3, 102.9 (3C; C-1’, C-1’’, C-1’’’), 102.4 (C-1’’’’), 100.7 (C-1), 82.0, 81.9, 81.8, 81.6 (4C; C-3, C-3’, C-3’’, C-3’’’), 80.9 (C-3’’’’), 76.2, 75.9, 74.9 (3C; C-4, C-4’’, C-4’’’), 74.6 (C-5’’’’), 73.5 (C-4’), 73.3, 73.2, 73.1 (3C; C-5’, C-5’’, C-5’’’), 72.3 (C-5), 68.7 (octyl OCH_2), 67.1 (C-2’’’’), 66.9 (C-1’), 66.7 (3C; C-2, C-2’’, C-2’’’), 66.3 (C-4’’’’), 61.8, 61.7, 61.7, 61.6, 61.4 (5C; C-6, C-6’, C-6’’, C-6’’’, C-6’’’’), 57.3, 57.2, 57.1, 57.0, 56.9 (5C; 5 × OMe), 32.7, 30.1, 30.0, 29.9, 26.8, 23.4 (6C; octyl CH_2), 14.9 ppm (6C; octyl CH_3); HRMS (ESI) calcd $C_{43}H_{78}O_{26}$ $[M + Na]^+$ 1033.4674, found 1033.4674.

***p*-Tolyl 2,3,4,6-tetra-*O*-acetyl-1-thio-α-D-mannopyranoside (11)**: To a stirring ice-cold solution of D-mannose (10.0 g, 55.8 mmol) in acetic anhydride (50 mL, 530.0 mmol) was added iodine (50 mg, 2.0 mmol). The resulting dark purple solution was continuously stirred for 6 h while warming up to room temperature. The reaction mixture was concentrated and then redissolved in CH_2Cl_2 (800 mL). After washing with water, saturated Na_2SO_3 and saturated $NaHCO_3$ followed by brine, the organic layer was dried over Na_2SO_4 and concentrated. The crude residue was purified by chromatography (hexane–EtOAc 2:1) to afford pale

yellow syrup (20.1 g, 92%, α/β = 2:1). This peracylated syrup (19.2 g, 49.2 mmol) was dissolved in CH_2Cl_2 (250 mL) and cooled to 0 ℃ before adding *p*-thiocresol (7.3 g, 59.1 mmol) followed by BF_3•OEt_2 (9.5 mL, 59.1 mmol). After stirring overnight, while warming to room temperature, the mixture was diluted with CH_2Cl_2 and washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the crude residue was purified by chromatography (hexane–EtOAc 3:1) to afford **11** (19.5 g, 88% for both isomers, α/β = 10:1) as pale yellow syrup. R_f 0.38 for α isomer (**11**) and 0.35 for β isomer (hexane–EtOAc 2:1); ^{1}H NMR (500 MHz, $CDCl_3$): δ 7.38 (d, *J* =8.1 Hz, 2 H; Tol), 7.12 (d, *J* =8.0 Hz, 2H; Tol), 5.49 (dd, *J* = 2.5, 1.5 Hz, 1H; H-2), 5.41 (d, *J* =1.5 Hz, 1 H; H-1), 5.33-5.32 (m, 2H; H-3, H-4), 4.55 (ddd, *J* =10.0, 6.0, 2.5 Hz, 1H; H-5), 4.30 (dd, *J* = 12.0, 6.0 Hz, 1H; H-6a), 4.10 (dd, *J* = 12.0, 2.5 Hz, 1H; H-6b), 2.33 (s, 3H; Tol(Me)), 2.14 (s, 3H; Ac), 2.07 (s, 3H; Ac), 2.06 (s, 3H; Ac), 2.01 (s, 3H; Ac); ^{13}C NMR (125 MHz, CDCl3): δ 170.5, 169.9, 169.8, 169.7 (4C; 4 × OAc), 138.4 (1C; Tol), 132.6 (2C; Tol), 129.9 (2C; Tol), 128.8 (1C; Tol), 86.0 (C-1), 70.9, 69.5 (C-5), 69.4 (C-2), 66.5 (C-4), 62.5 (C-6), 21.1, 20.8, 20.7, 20.7, 20.6 (5C; 4 × Ac, Tol(Me)); HRMS (ESI) *m/z* calcd $C_{21}H_{26}O_9SNa$ $[M+Na]^+$ 477.1190, found 477.1191.

***p*-Tolyl 2,4,6-tri-*O*-acetyl-3-*O*-methyl-1-thio-α-D-mannopyranoside (12)**: A solution of **16** (15.9 g, 44 mmol) and *p*-thiocresol (9.6 g, 75.7 mmol) in CH_2Cl_2 (150 mL) was cooled to 0 ℃ before BF_3•OEt_2 (13.2 mL, 106.8 mmol) was added. After stirring overnight, while warming to room temperature, the mixture was diluted with CH_2Cl_2 and washed with water, saturated aqueous $NaHCO_3$ and brine.

The organic layer was dried over Na_2SO_4, concentrated and the crude residue was purified by chromatography (hexane–EtOAc 4:1) to afford α isomer **12** (14.3 g, 76%) as a pale yellow solid, as well separable β isomer (pale yellow solid, 2.2 g, 12%). Data for α isomer (**12**): R_f 0.47 (hexane–EtOAc 1:2); $[\alpha]_D$ = +107.0 (*c* 0.85, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.37 (d, *J* = 8.0 Hz, 2H, Ar), 7.12 (d, *J* = 8.0 Hz, 2H, Ar), 5.57 (dd, *J* = 3.0, 1.5 Hz, 1H, H-2), 5.43 (d, *J* = 1.0 Hz, 1H, H-1), 5.22 (app t, *J* = 9.9 Hz, 1H, H-4), 4.56 (ddd, *J* = 9.5, 6.5, 2.0 Hz, 1H, H-5), 4.26 (dd, *J* = 12.1, 6.2 Hz, 1H, H-6a), 4.11 (dd, *J* = 12.2, 2.1 Hz, 1H, H-6b), 3.63 (dd, *J* = 9.6, 3.2 Hz, 1H, H-3), 3.38 (s, 3H, OMe), 2.33 (s, 3H, ArMe), 2.14 (s, 3H, OAc), 2.12 (s, 3H, OAc), 2.05 (s, 3H, OAc); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6 (C=O), 170.2 (C=O), 169.8 (C=O), 138.4 (Ar), 132.5 (Ar), 129.9 (Ar), 129.1 (Ar), 86.4 (C-1), 77.5 (C-3), 69.5 (C-5), 69.2 (C-2), 67.8 (C-4), 62.7 (C-6), 57.8 (OMe), 21.1, 21.0, 20.9, 20.7 (4C; 3 × OAc, ArMe); HRMS (ESI) *m/z* calcd $C_{20}H_{26}O_8S$ $[M + Na]^+$ 449.1241, found 449.1236.

***p*-Tolyl 4-*O*-acetyl-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-1-thio-α-D-mannopyranoside (13)**: Benzylidene acetal **18** (9.23 g, 18.7 mmol) and BH_3•NMe_3 (6.61 g, 87.9 mmol) were dissolved in THF (260 mL) and cooled to 0 ℃ before an ice-cold solution of $AlCl_3$ (10.1 g, 76.7 mmol) in THF (100 mL) was added. After stirring overnight while warming to room temperature, the solvent was evaporated and the crude residue was redissolved in EtOAc and washed with water, saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 4:1) to afford pale yellow syrup (8.69 g, 94%).

A portion of this material (310.9 mg, 0.63 mmol) was dissolved in pyridine (8 mL) and acetic anhydride (0.6 mL, 6.3 mmol) was added. After stirring overnight, the solution was diluted with CH_2Cl_2 and washed with 1M HCl, saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the residue was purified by chromatography (hexane–EtOAc 6:1) to afford **13** (289.4 mg, 86%) as a white foam. R_f 0.33 (hexane–EtOAc 5:1); $[\alpha]_D$ = +74.1 (*c* 1.56, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.03 (dd, *J* = 8.4, 1.3 Hz, 2H, Ar), 7.59–7.27 (m, 10H, Ar), 7.05 (d, *J* = 7.9 Hz, 2H, Ar), 5.82 (dd, *J* = 3.1, 1.8 Hz, 1H, H-2), 5.58 (d, *J* = 1.7 Hz, 1H, H-1), 5.46 (app t, *J* = 9.8 Hz, 1H, H-4), 4.68, 4.58 (AB q, *J* = 11.6 Hz, 2H, OCH_2Ph), 4.58–4.52 (m, 1H, H-5), 3.76 (dd, *J* = 9.6, 3.2 Hz, 1H, H-3), 3.72–3.66 (m, 2H, H-6a, H-6b), 3.43 (s, 3H, OMe), 2.30 (s, 3H, ArMe), 2.08 (s, 3H, OAc); ^{13}C NMR (100 MHz, $CDCl_3$) δ 169.9 (C=O), 138.2 (Ar), 138.1 (Ar), 133.3 (Ar), 132.6 (2C, Ar), 129.9 (2C, Ar), 129.8 (2C, Ar), 129.4 (2C, Ar), 128.4 (2C, Ar), 128.6 (2C, Ar), 127.7 (2C, Ar), 127.5 (Ar), 86.5 (C-1), 77.8 (C-3), 73.6 (OCH_2Ph), 71.0 (C-5), 69.8 (C-2), 69.4 (C-6), 68.6 (C-4), 57.7 (OMe), 21.0, 20.9 (2C, OAc, ArMe); HRMS (ESI) *m/z* calcd $C_{30}H_{32}O_7S$ $[M + Na]^+$ 559.1761, found 559.1763.

Octyl 2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (14): The same procedure used for the preparation of **13** was employed, using, benzylidene acetal **20** (841.3 mg, 1.74 mmol), BH_3•NMe_3 (525.1 mg, 7.0 mmol) and H_2O (64 μL, 3.5 mmol) in THF (25 mL). The presence of H_2O was reported to improve the reaction efficiency.[15] The crude product was purified by chromatography (hexane–EtOAc 4:1) to afford **14** (700.8 mg, 83%) as pale yellow syrup. R_f 0.19

(hexane–EtOAc 4:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44–7.17 (m, 10H, Ar), 4.90 (d, *J* = 1.7 Hz, 1H, H-1), 4.71, 4.68 (AB q, *J* = 12.5 Hz, 2H, OCH_2Ph), 4.65, 4.61 (AB q, *J* = 12.1 Hz, 2H, OCH_2Ph), 4.15 (brs, 1H, OH-4), 4.00 (app t, *J* = 9.4 Hz, 1H, H-4), 3.84–3.72 (m, 4H, H-2, H-5, H-6a, H-6b), 3.68 (app dt, *J* = 9.7, 6.9 Hz, 1H, octyl OCH_2), 3.50 (dd, *J* = 9.5, 3.1 Hz, 1H, H-3), 3.43–3.38 (m, 1H, octyl OCH_2), 3.38 (s, 3H, OMe), 1.62–1.54 (m, 2H, octyl OCH_2CH_2), 1.37–1.21 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.0 (2C, Ar), 128.4 (2C, Ar) 128.3 (2C, Ar), 127.9 (2C, Ar), 127.7 (Ar), 127.6 (2C, Ar), 127.6 (Ar), 97.8 (C-1), 81.2 (C-3), 73.6 (OCH_2Ph), 73.0 (C-2), 72.6 (OCH_2Ph), 71.1 (C-5), 70.4 (C-6), 67.9 (C-4), 67.8 (octyl OCH_2), 57.1 (OMe), 31.8 (octyl CH_2), 29.4 (octyl CH_2), 29.4 (octyl CH_2), 29.3 (octyl CH_2), 26.1 (octyl CH_2), 22.7 (octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) *m/z* calcd $C_{29}H_{42}O_6$ $[M + Na]^+$ 509.2874, found 509.2871.

Methyl 3-*O*-methyl-α-D-mannopyranoside (15): The same procedure used for the preparation of **6** was employed using methyl α-D-mannopyranoside (3.00 g, 15.3 mmol), *n*-Bu_2SnO (3.89 g, 15.3 mmol), *n*-Bu_4NI (5.77 g, 15.3 mmol) and MeI (3 mL, 47.6 mmol) in toluene (150 mL). To drive the reaction to completion, additional MeI (7 mL, 111.1 mmol) was added after 4 h at 80 ℃. The crude product was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford **15** (2.12 g, 66%) as yellowish syrup. R_f 0.27 (CH_2Cl_2–MeOH 9:1); ^{1}H NMR (500 MHz, CD_3OD): δ 4.67 (d, *J* = 2.0 Hz, 1H, H-1), 3.99 (dd, *J* = 3.0, 2.0 Hz, 1H, H-2), 3.82 (dd, *J* = 11.7, 2.2 Hz, 1H, H-6a), 3.70 (dd, *J* = 11.6, 5.6 Hz, 1H, H-6b), 3.67 (app t, *J* = 9.9 Hz, 1H, H-4), 3.49 (ddd, *J* = 9.8, 5.7, 2.3 Hz, 1H, H-5), 3.44 (s, 3H, C3-

OMe), 3.38 (s, 3H, C1-OMe), 3.32 (dd, *J* = 9.8, 3.0 Hz, 1H, H-3); ^{13}C NMR (125 MHz, CD_3OD): δ 102.6 (C-1), 82.4 (C-3), 74.5 (C-5), 67.9 (C-2), 67.4 (C-4), 62.8 (C-6), 57.5 (C3-OMe), 55.3 (C1-OMe); LRMS (ESI): *m/z* 231.1 $[M + Na]^+$.

1,2,4,6-*tetra*-*O*-acetyl-3-*O*-methyl-D-mannopyranose (16): Triol **15** (1.58 g, 7.6 mmol) was dissolved in a mixture of acetic anhydride-acetic acid and sulfuric acid (105:45:1 v/v/v, 30.2 mL). After stirring overnight the mixture was diluted with CH_2Cl_2 and washed with water, saturated aqueous $NaHCO_3$, and brine. The organic layer was dried over Na_2SO_4 and concentrated to afford **16** (2.64 g, 96%, α/β 12:1, inseparable) as yellow syrup. The crude product was used in the next step without further purification. Data for α isomer: ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.10 (d, *J* = 2.0 Hz, 1H, H-1), 5.34 (dd, *J* = 3.4, 2.1 Hz, 1H, H-2), 5.23 (app t, *J* = 10.0 Hz, 1H, H-4), 4.24 (dd, *J* = 12.4, 5.3 Hz, 1H, H-6a), 4.09 (dd, *J* = 12.4, 2.5 Hz, 1H, H-6b), 3.96 (ddd, *J* = 10.0, 5.2, 2.4 Hz, 1H, H-5), 3.66 (dd, *J* = 9.8, 3.4 Hz, 1H, H-3), 3.38 (s, 3H, OMe), 2.17 (s, 3H, OAc), 2.15 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.08 (s, 3H, OAc); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.7 (C=O), 169.9 (C=O), 169.6 (C=O), 168.0 (C=O), 100.0 (C-1), 76.8 (C-3), 70.7 (C-5), 67.0 (C-4), 66.6 (C-2), 62.4 (C-6), 57.9 (OMe), 20.9 (OAc), 20.8 (OAc), 20.8 (OAc), 20.7 (OAc); LRMS (ESI): *m/z* 385.1 $[M + Na]^+$.

***p*-Tolyl 4,6-*O*-benzylidene-3-*O*-methyl-1-thio-α-D-mannopyranoside (17)**: Thioglycoside **12** (11.6 g, 27.2 mmol) was deacylated following the procedure described for the synthesis of **2**. The crude residue was purified by chromatography (CH_2Cl_2–MeOH 12:1) to give a white solid (7.0 g, 86%). A portion of this solid (3.34 g, 11.1 mmol) was redissolved in DMF (30 mL) before

adding benzaldehyde dimethyl acetal (2.6 mL, 16.7 mmol) and *p*-TsOH (223.2 mg, 0.6 mmol). The reaction mixture was rotated under reduced pressure at 50 ℃ on a rotary evaporator. After 6 h, the solution was diluted with CH_2Cl_2 and washed with water, saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 6:1) to afford **17** (3.5 g, 81%) as a white foam. R_f 0.32 (hexane–EtOAc 3:1); $[\alpha]_D$ = +249.4 (*c* 6.71, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 (dd, *J* = 7.7, 1.8 Hz, 2H, Ar), 7.42–7.34 (m, 5H, Ar), 7.14 (d, *J* = 7.9 Hz, 2H, Ar), 5.60 (s, 1H, PhCH), 5.55 (d, *J* = 0.8 Hz 1H, H-1), 4.42–4.35 (m, 2H, H-2, H-5), 4.23 (dd, *J* = 10.3, 4.9 Hz, 1H, H-6a), 4.09 (app t, *J* = 9.6 Hz, 1H, H-4), 3.84 (app t, *J* = 10.3 Hz, 1H, H-6b), 3.73 (dd, *J* = 9.6, 3.4 Hz, 1H, H-3), 3.59 (s, 3H, OMe), 3.15 (brs, 1H, OH-2), 2.34 (s, 3H, ArMe); ^{13}C NMR (100 MHz, $CDCl_3$) δ 138.0 (Ar), 137.5 (Ar), 132.4 (2C, Ar), 130.0 (2C, Ar), 129.6 (Ar), 129.0 (Ar), 128.3 (2C, Ar), 126.2 (2C, Ar), 101.8 (PhCH), 88.4 (C-1), 79.0 (C-4), 77.7 (C-3), 70.6 (C-5), 68.6 (C-6), 64.5 (C-2), 58.8 (OMe), 21.1 (ArMe); HRMS (ESI) *m/z* calcd $C_{21}H_{24}O_5S$ $[M + Na]^+$ 411.1237, found 411.1233.

***p*-Tolyl 2-*O*-benzoyl-4,6-*O*-benzylidene-3-*O*-methyl-1-thio-α-D-mannopyranoside (18)**: To a solution of **17** (9.57 g, 24.6 mmol) and DMAP (309.9 mg, 2.5 mmol) in CH_2Cl_2 (200 mL) at 0 ℃ was added BzCl (4 mL, 31.9 mmol) and pyridine (5 mL). After stirring overnight, while warming to room temperature, the solution was diluted with CH_2Cl_2 and washed with HCl, saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated, and the crude residue was purified by chromatography (hexane–

EtOAc 12:1) to afford **18** (10.8 g, 89%) as a white foam. R_f 0.28 (hexane–EtOAc 12:1); $[\alpha]_D$ = +118.5 (*c* 1.52, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.09 (dd, *J* = 8.3, 1.2 Hz, 2H, Ar), 7.66–7.34 (m, 10H, Ar), 7.15 (d, *J* = 8.0 Hz, 2H, Ar), 5.85 (dd, *J* = 3.3, 1.4 Hz, 1H, H-2), 5.70 (s, 1H, PhCH), 5.56 (d, *J* = 1.3 Hz, 1H, H-1), 4.49 (app dt, *J* = 9.8, 4.8 Hz, 1H, H-5), 4.30 (dd, *J* = 10.3, 4.9 Hz, 1H, H-6a), 4.21 (app t, *J* = 9.7 Hz, 1H, H-4), 3.93 (dd, *J* = 9.6, 3.2 Hz, 1H, H-3), 3.92 (app t, *J* = 10.4 Hz, 1H, H-6b), 3.52 (s, 3H, OMe), 2.34 (s, 3H, ArMe); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.6 (C=O), 138.4 (Ar), 137.3 (Ar), 133.4 (Ar), 132.7 (2C, Ar), 130.0 (2C, Ar), 129.9 (2C, Ar), 129.6 (Ar), 129.3 (Ar), 129.1 (Ar), 128.4 (2C, Ar), 128.2 (2C, Ar), 126.2 (2C, Ar), 102.0 (PhCH), 87.6 (C-1), 79.1 (C-4), 76.4 (C-3), 71.3 (C-2), 68.6 (C-6), 65.0 (C-5), 58.4 (OMe), 21.1 (ArMe); HRMS (ESI) *m/z* calcd $C_{28}H_{28}O_6S$ $[M + Na]^+$ 515.1499, found 515.1502.

Octyl 4,6-*O*-benzylidene-3-*O*-methyl-α-D-mannopyranoside (19): Monosaccharide **6** (2.25 g, 7.4 mmol) was dissolved in DMF (25 mL) before adding benzaldehyde dimethyl acetal (1.7 mL, 11.2 mmol) and *p*-TsOH (71.1 mg, 0.4 mmol). The reaction mixture was rotated under reduced pressure at 50 ℃ on a rotary evaporator. After 3 h, the solvent was removed by evaporation and the crude residue was purified by chromatography (hexane–EtOAc from 4:1 to 2:1) to afford **19** (2.5 g, 88%) as colorless syrup. R_f 0.27 (hexane–EtOAc 3:1); $[\alpha]_D$ = +54.5 (*c* 0.85, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.51 (dd, *J* = 7.5, 1.9 Hz, 2H, Ar), 7.41–7.33 (m, 3H, Ar), 5.60 (s, 1H, PhCH), 4.88 (d, 1H, *J* = 1.6 Hz, H-1), 4.27 (AB q, *J* = 10.8 Hz, 1H, H-6a), 4.10 (dd, *J* = 3.3, 1.5 Hz, 1H, H-2), 3.98–4.04 (m, 1H, H-4), 3.89–3.79 (m, 2H, H-5, H-6b), 3.73-3.68 (m, 2H, H-3, octyl

OCH_2), 3.57 (s, 3H, OMe), 3.43 (app dt, *J* = 9.7, 6.6 Hz, 1H, octyl OCH_2), 2.58 (brs, 1H, OH-2), 1.68–1.52 (m, 2H, octyl OCH_2CH_2), 1.40–1.20 (m, 10H, octyl CH_2), 0.89 (t, *J* = 6.7 Hz, 3H, octyl CH_3); ^{13}C NMR (100 MHz, $CDCl_3$): δ 137.5 (Ar), 128.9 (Ar), 128.2 (2C, Ar), 126.1 (2C, Ar), 101.7 (PhCH), 99.9 (C-1), 78.8 (C-4), 77.3 (C-3), 69.3 (C-2), 68.9 (C-6), 68.0 (octyl OCH_2), 63.1 (C-5), 58.6 (OMe), 31.8 (octyl CH_2), 29.4 (octyl CH_2), 29.3 (octyl CH_2), 29.2 (octyl CH_2), 26.1 (octyl CH_2), 22.6 (octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) *m/z* calcd $C_{22}H_{34}O_6$ $[M + Na]^+$ 417.2248, found 417.2248.

Octyl 2-*O*-benzyl-4,6-*O*-benzylidene-3-*O*-methyl-α-D-mannopyranoside (20): Benzylidene acetal **6** (2.34 g, 5.9 mmol) was dissolved in DMF (30 mL) and cooled to 0 ℃. Then NaH (60% in mineral oil, 356.3 mg, 8.9 mmol) was added and stirred 0 ℃ for 0.5 h before adding BnBr (1.1 mL, 9.1 mmol). After 4 h, the reaction was warmed up to room temperature. MeOH (10 mL) was added and the mixture was then diluted with CH_2Cl_2 and washed with water, saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 12:1) to afford **20** (2.88 g, quantitative) as pale yellow syrup. R_f 0.53 (hexane–EtOAc 6:1); $[\alpha]_D$ = +33.8 (*c* 1.41, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.51 (dd, *J* = 7.7, 1.7 Hz, 2H, Ar), 7.44–7.28 (m, 8H, Ar), 5.63 (s, 1H, PhCH), 4.80 (d, *J* = 1.6 Hz, 1H, H-1), 4.83, 4.72 (AB q, *J* = 12.2 Hz, 2H, OCH_2Ph), 4.25 (dd, *J* = 9.9, 4.5 Hz, 1H, H-6a), 4.17 (app t, *J* = 9.9 Hz, 1H, H-4), 3.87 (app t, *J* = 10.2 Hz, 1H, H-6b), 3.86 (dd, *J* = 3.2, 1.7 Hz, 1H, H-2), 3.80 (app dt, *J* = 9.8, 4.5 Hz, 1H, H-5), 3.74 (dd, *J* = 10.0, 3.2 Hz, 1H, H-3), 3.65 (app dt, *J* = 9.6, 6.9 Hz, 1H, octyl OCH_2), 3.51 (s,

3H, OMe), 3.37 (app dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 1.60–1.52 (m, 2H, octyl OCH_2CH_2), 1.38–1.18 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.2 (Ar), 137.6 (Ar), 128.8 (Ar), 128.4 (2C, Ar), 128.2 (2C, Ar), 128.0 (2C, Ar), 127.7 (Ar), 126.1 (2C, Ar), 101.6 (PhCH), 99.2 (C-1), 79.1 (C-4), 78.1 (C-3), 75.8 (C-2), 73.5 (OCH_2Ph), 68.9 (C-6), 67.9 (octyl OCH_2), 64.0 (C-5), 58.8 (OMe), 31.8(octyl CH_2), 29.4 (octyl CH_2), 29.4 (octyl CH_2), 29.2 (octyl CH_2), 26.1 (octyl CH_2), 22.7 (octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) *m/z* calcd $C_{29}H_{40}O_6$ $[M + Na]^+$ 507.2727, found 507.2711.

Octyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (21): A mixture of thioglycoside **11** (240.9 mg, 0.53 mmol), alcohol **14** (235.9 mg, 0.48 mmol) and powdered 4Å molecular sieves were dissolved in CH_2Cl_2 (5 mL) and stirred at 0 ℃ for 0.5 h. Then *N*-iodosuccinimide (139.7 mg, 0.59 mmol) and silver trifluoromethanesulfonate (35.7 mg, 0.14 mmol) were added. After stirring overnight while warming to room temperature, triethylamine (1 mL) was added and the reaction mixture was filtered through Celite. The filtrate was concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 4:1) to afford **21** (332.5 mg, 84%) as pale yellow syrup. R_f 0.44 (hexane–EtOAc 3:1); $[\alpha]_D$ = +44.3 (*c* 2.09, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.40–7.20 (m, 10H, Ar), 5.37 (dd, *J* = 3.1, 1.9 Hz, 1H, H-2'), 5.32–5.21 (m, 2H, H-3', H-4'), 5.23 (d, *J* = 1.8 Hz, 1H, H-1'), 4.87 (d, *J* = 1.7 Hz, 1H, H-1), 4.75–4.49 (m, 4H, 2 × OCH_2Ph), 4.12 (dd, *J* = 12.2, 5.0 Hz, 1H, H-6a'), 3.98 (app t, *J* = 9.5 Hz, 1H, H-4), 3.94 (ddd, *J* = 9.6, 5.1, 2.3 Hz, 1H, H-5'), 3.84 (dd, *J* = 12.2, 2.3 Hz, 1H, H-6b'), 3.81–3.72 (m, 4H, H-2, H-5,

H-6a, H-6b), 3.70 (app dt, *J* = 9.7, 6.9 Hz, 1H, octyl OCH_2), 3.59 (dd, *J* = 9.3, 3.1 Hz, 1H, H-3), 3.40 (app dt, *J* = 9.7, 6.8 Hz, 1H, octyl OCH_2), 3.36 (s, 3H, OMe), 2.14 (s, 3H, OAc), 2.04 (s, 3H, OAc), 2.00 (s, 3H, OAc), 1.63–1.53 (m, 2H, octyl OCH_2CH_2), 1.38–1.22 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6 (C=O), 170.0 (C=O), 169.8 (C=O), 169.6 (C=O), 138.4 (Ar), 138.2 (Ar), 128.3 (2C, Ar), 128.3 (Ar), 127.7 (Ar), 127.6 (2C, Ar), 127.4 (2C, Ar), 127.4 (Ar), 99.4 (C-1'), 97.7 (C-1), 81.8 (C-3), 75.1 (C-4), 73.3 (OCH_2Ph), 73.2 (C-2), 72.6 (OCH_2Ph), 70.9 (C-5), 69.8 (C-6), 69.5 (C-2'), 69.2 (C-3'), 69.1 (C-5'), 68.0 (octyl OCH_2), 66.0 (C-4'), 62.4 (C-6'), 56.9 (OMe), 31.8 (octyl CH_2), 29.4 (octyl CH_2), 29.3 (octyl CH_2), 29.2 (octyl CH_2), 26.1 (octyl CH_2), 22.7 (octyl CH_2), 20.9 (OAc), 20.7 (OAc), 20.7 (2C; 2 × OAc), 14.1 (octyl CH_3); HRMS (ESI) *m/z* calcd $C_{43}H_{60}O_{15}$ $[M + Na]^+$ 839.3824, found 839.3824.

Octyl 2,4,6-tri-*O*-acetyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (22): Glycosylation was performed described for the synthesis of **21**, with thioglycoside **12** (49.8 mg, 0.12 mmol), alcohol **14** (52.7 mg, 0.11 mmol), *N*-iodosuccinimide (30.3 mg, 0.13 mmol) and silver trifluoromethanesulfonate (7.9 mg, 0.03 mmol) in CH_2Cl_2 (4 mL). The crude residue was purified by chromatography (hexane–EtOAc 3:1) to afford **22** (72.2 mg, 85%) as pale yellow syrup. R_f 0.19 (hexane–EtOAc 3:1); $[\alpha]_D$ = +26.3 (*c* 2.91, $CHCl_3$); ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.45–7.08 (m, 10H, Ar), 5.41 (dd, *J* = 3.2, 1.9 Hz, 1H, H-2'), 5.29 (d, *J* = 1.8 Hz, 1H, H-1'), 5.13 (app t, *J* = 10.0 Hz, 1H, H-4'), 4.87 (d, *J* = 1.8 Hz, 1H, H-1), 4.72, 4.67 (AB q, *J* = 12.3 Hz, 2H, OCH_2Ph), 4.68, 4.59 (AB q, *J* = 12.0 Hz, 2H, OCH_2Ph), 4.12 (dd, *J* = 12.3, 5.6 Hz,

1H, H-6a'), 3.97 (app t, *J* = 9.4 Hz, 1H, H-4), 3.91–3.82 (m, 2H, H-5', H-6b'), 3.81–3.67 (m, 5H, H-2, H-5, H-6a, H-6b, octyl OCH_2), 3.57 (dd, *J* = 9.1, 3.2 Hz, 1H, H-3), 3.56 (dd, *J* = 9.7, 3.4 Hz, 1H, H-3'), 3.41 (app dt, *J* = 9.7, 6.7 Hz, 1H, octyl OCH_2), 3.38 (s, 3H, OMe), 3.35 (s, 3H, OMe), 2.13 (s, 3H, OAc), 2.08 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.62–1.54 (m, 2H, octyl OCH_2CH_2), 1.38–1.20 (m, 10H, octyl CH_2), 0.89 (t, *J* = 6.9 Hz, 3H, octyl CH_3); ^{13}C NMR (400 MHz, $CDCl_3$) δ 170.7 (C=O), 170.0 (C=O), 169.7 (C=O), 138.4 (Ar), 138.1 (Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 127.7 (2C, Ar), 127.6 (Ar) 127.5 (Ar), 127.4 (2C, Ar), 99.3 (C-1', $^1J_{C\text{-}1,H\text{-}1}$ = 170.0 Hz), 97.7 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 169.8 Hz), 82.1 (C-3), 76.8 (C-3'), 74.1 (C-4), 73.3 (OCH_2Ph), 73.1 (C-2), 72.7 (OCH_2Ph), 71.1 (C-5), 70.0 (C-6), 69.3 (C-5'), 67.9 (octyl OCH_2), 67.7 (C-2'), 67.4 (C-4'), 62.7 (C-6'), 57.7 (OMe), 56.9 (OMe), 31.8 (octyl CH_2), 29.4 (octyl CH_2), 29.3 (octyl CH_2), 29.2 (octyl CH_2), 26.1 (octyl CH_2), 22.6 (octyl CH_2), 21.0, 20.9, 20.7 (3C; 3 × OAc), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{42}H_{60}O_{14}$ $[M + Na]^+$ 811.3875, found 811.3880.

Octyl 4-*O*-acetyl-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (23): Glycosylation was performed as described for the synthesis of **21**, with thioglycoside **13** (1.04 g, 1.94 mmol), alcohol **14** (857.4 mg, 1.76 mmol), *N*-iodosuccinimide (541.3 mg, 2.3 mmol) and silver trifluoromethanesulfonate (97.2 mg, 0.37 mmol) in CH_2Cl_2 (23 mL). The crude residue was purified by chromatography (hexane–EtOAc 6:1) to afford **23** (1.41 g, 89%) as pale yellow syrup. R_f 0.56 (hexane–EtOAc 3:1); $[\alpha]_D$ = +11.1 (*c* 1.55, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.02 (dd, *J* = 8.3, 1.2 Hz,

2H, Ar), 7.52 (t, *J* = 7.5 Hz, 1H, Ar), 7.39–7.20 (m, 17H, Ar), 5.66 (dd, *J* = 2.9, 2.1 Hz, 1H, H-2'), 5.40 (d, *J* = 2.0 Hz, 1H, H-1'), 5.39 (app t, *J* = 9.9 Hz, 1H, H-4'), 4.88 (d, *J* = 1.8 Hz, 1H, H-1), 4.72, 4.67 (AB q, *J* = 12.3 Hz, 2H, OCH_2Ph), 4.60, 4.53 (AB q, *J* = 11.8 Hz, 2H, OCH_2Ph), 4.55, 4.47 (AB q, *J* = 11.4 Hz, 2H, OCH_2Ph), 4.03 (app t, *J* = 9.3 Hz, 1H, H-4), 3.93 (app dt, *J* = 10.0, 3.8 Hz, 1H, H-5'), 3.86 (dd, *J* = 10.5, 1.5 Hz, 1H, H-6a), 3.83–3.78 (m, 2H, H-2, H-5), 3.75 (dd, *J* = 10.6, 6.2 Hz, 1H, H-6b), 3.73–3.68 (m, 2H, H-3', octyl OCH_2), 3.61 (dd, *J* = 9.2, 3.1 Hz, 1H, H-3), 3.52–3.46 (m, 2H, H-6a', H-6b'), 3.44 (s, 3H, OMe), 3.43–3.39 (m, 1H, octyl OCH_2), 3.39 (s, 3H, OMe), 2.01 (s, 3H, OAc), 1.66–1.55 (m, 2H, octyl OCH_2CH_2), 1.43–1.22 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.8 (OAc), 165.5 (OBz), 138.6 (Ar), 138.2 (Ar), 138.2 (Ar), 133.0 (Ar), 129.9 (2C, Ar), 129.8 (Ar), 128.4 (2C, Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 128.2 (2C, Ar), 127.8 (2C, Ar), 127.7 (2C, Ar), 127.6 (Ar), 127.4 (Ar), 127.4 (2C, Ar), 127.3 (Ar), 99.4 (C-1', $^1J_{C\text{-}1,H\text{-}1}$ = 174.3 Hz), 97.8 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 167.0 Hz), 82.1 (C-3), 77.1 (C-3'), 74.5 (C-4), 73.6 (OCH_2Ph), 73.3 (OCH_2Ph), 73.2 (C-2), 72.6 (OCH_2Ph), 71.3 (C-5'), 71.0 (C-5), 70.3 (C-6), 69.5 (C-6'), 68.4 (C-2'), 68.2 (C-4'), 67.9 (octyl OCH_2), 57.5 (OMe), 57.1 (OMe), 31.8 (octyl CH_2), 29.4 (octyl CH_2), 29.4 (octyl CH_2), 29.3 (octyl CH_2), 26.1 (octyl CH_2), 22.7 (octyl CH_2), 21.0 (OAc), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{52}H_{66}O_{13}$ $[M + Na]^+$ 921.4396, found 926.4407.

Octyl 2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (24): Disaccharide **23** (1.41 g, 1.57 mmol) was dissolved in CH_2Cl_2–MeOH (100 mL, 1:9 v/v) at 0 ℃ before

adding acetic chloride (2.6 mL, 2.6% v/v). After stirring overnight while the reaction warmed to room temperature, triethylamine (1 mL) was added and the solution was concentrated. The residue was dissolved in CH_2Cl_2 and washed with water, satd aq $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 5:1) to afford **23** (1.13 g, 83%) as yellow syrup. R_f 0.43 (hexane–EtOAc 3:1); $[\alpha]_D$ = +17.6 (*c* 0.44, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.02 (dd, *J* = 8.3, 1.2 Hz, 2H, Ar), 7.57–7.50 (m, 1H, Ar), 7.41–7.21 (m, 17H, Ar), 5.64 (dd, *J* = 2.7, 2.1 Hz, 1H, H-2'), 5.39 (d, *J* = 2.0 Hz, 1H, H-1'), 4.88 (d, *J* = 1.8 Hz, 1H, H-1), 4.72, 4.67 (AB q, *J* = 12.4 Hz, 2H, OCH_2Ph), 4.57 (m, 4H, 2 × OCH_2Ph), 4.11–4.00 (m, 2H, H-4, H-4'), 3.87–3.66 (m, 7H, H-2, H-5', H-5, H-6a', H-6b', H-6a, octyl OCH_2), 3.65–3.55 (m, 3H, H-3', H-3, H-6b), 3.46 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.41 (app dt, *J* = 9.7, 6.7 Hz, 1H, octyl OCH_2), 2.60 (d, *J* = 2.1 Hz, 1H, OH-4'), 1.61–1.52 (m, 2H, octyl OCH_2CH_2), 1.37–1.22 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 165.4 (C=O), 138.6 (Ar), 138.4 (Ar), 138.2 (Ar), 133.0 (Ar), 129.9 (3C, Ar), 128.3 (4C, Ar), 128.3 (2C, Ar), 128.2 (2C, Ar), 127.7 (2C, Ar), 127.6 (Ar), 127.5 (3C, Ar), 127.4 (2C, Ar), 127.3 (Ar), 99.7 (C-1'), 97.7 (C-1), 82.2 (C-3), 79.4 (C-3'), 74.1 (C-4), 73.7, 73.3, 72.6 (3C, 3 × OCH_2Ph), 73.2 (C-2), 72.2 (C-5'), 71.2 (C-5), 70.1 (C-6'/C-6), 70.0 (C-6'/C-6), 67.9 (octyl OCH_2), 67.7 (C-2'), 67.6 (C-4'), 57.3 (OMe), 57.1 (OMe), 31.8 (octyl CH_2), 29.4 (octyl CH_2), 29.3 (octyl CH_2), 29.2 (octyl CH_2), 26.1 (octyl CH_2), 22.7 (octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{50}H_{64}O_{12}$ $[M + Na]^+$ 879.4290, found 879.4302.

Octyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (25): Glycosylation was performed as described for the synthesis of **21**, using thioglycoside **11** (69.8 mg, 0.15 mmol), alcohol **24** (115.6 mg, 0.13 mmol) *N*-iodosuccinimide (41.6 mg, 0.18 mmol) and silver trifluoromethanesulfonate (11.6 mg, 0.04 mmol) in CH_2Cl_2 (6 mL). The crude residue was purified by chromatography (hexane–EtOAc 3:1) to afford **25** (103.1 mg, 64%) as pale yellow syrup. R_f 0.44 (hexane–EtOAc 2:1); $[\alpha]_D$ = +33.9 (*c* 0.97, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.96 (dd, *J* = 8.2, 1.1 Hz, 2H, Ar), 7.51 (app t, *J* = 7.5 Hz, 1H, Ar), 7.39–7.22 (m, 17H, Ar), 5.65 (dd, *J* = 2.9, 2.0 Hz, 1H, H-2'), 5.39–5.22 (m, 5H, H-2'', H-3'', H-4'', H-1'', H-1'), 4.89 (d, *J* = 1.7 Hz, 1H, H-1), 4.75–4.48 (m, 6H, 3 × OCH_2Ph), 4.17 (dd, *J* = 12.1, 4.3 Hz, 1H, H-6a''), 4.15 (app t, *J* = 9.6 Hz, 1H, H-4'), 4.04 (app t, *J* = 9.3 Hz, 1H, H-4), 4.00 (ddd, *J* = 9.7, 4.3, 2.4 Hz, 1H, H-5''), 3.90 (dd, *J* = 12.3, 2.3 Hz, 1H, H-6b''), 3.85–3.76 (m, 5H, H-2, H-5, H-5', H-6a', H-6b'), 3.75–3.69 (m, 2H, H-3', octyl OCH_2), 3.68 (dd, *J* = 11.3, 3.9 Hz, 1H, H-6a), 3.61 (dd, *J* = 9.3, 3.1 Hz, 1H, H-3), 3.54 (dd, *J* = 11.2, 1.4 Hz, 1H, H-6b), 3.44 (s, 3H, OMe), 3.41 (s, 3H, OMe), 3.42–3.38 (m, 1H, octyl OCH_2), 2.11 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.58–1.62 (m, 2H, octyl OCH_2CH_2), 1.38–1.23 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl OCH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6 (OAc), 170.1(OAc), 169.8 (OAc), 169.6 (OAc), 165.3 (OBz), 138.7 (Ar), 138.5 (Ar), 138.2 (Ar), 133.0 (Ar), 129.8 (2C, Ar), 129.8 (Ar), 128.4 (2C, Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 128.2 (2C, Ar), 127.7 (2C, Ar), 127.6 (Ar), 127.4

(3C, Ar), 127.3 (3C, Ar), 99.7 (C-1', $^{1}J_{C\text{-}1,H\text{-}1}$ = 176.3 Hz), 99.3 (C-1'', $^{1}J_{C\text{-}1,H\text{-}1}$ = 179.6 Hz), 97.7 (C-1, $^{1}J_{C\text{-}1,H\text{-}1}$ = 171.4 Hz), 82.0 (C-3), 80.0 (C-3'), 74.7 (C-4), 73.9 (C-4'), 73.6, 73.2, 72.6 (3C, 3 × OCH_2Ph), 73.2 (C-2), 71.8 (C-5'/C-5), 71.0 (C-5/C-5'), 69.9 (2C, C-6', C-6), 69.5, (2C, C-2'', C-5''), 69.2 (C-3''), 68.0 (octyl OCH_2), 67.8 (C-2'), 66.0 (C-4''), 62.3 (C-6''), 57.0 (OMe), 56.9 (OMe), 31.8 (octyl CH_2), 29.4 (octyl CH_2), 29.4 (octyl CH_2), 29.2 (octyl CH_2), 26.1 (octyl CH_2), 22.7 (octyl CH_2), 20.9 (OAc), 20.7 (2 × OAc), 20.7 (OAc), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{64}H_{82}O_{21}$ $[M + Na]^+$ 1209.5201, found 1209.5237.

Octyl 2,4,6-tri-*O*-acetyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (26): Glycosylation was performed as described for the synthesis of **21**, with thioglycoside **12** (69.2 mg, 0.16 mmol), alcohol **24** (124.4 mg, 0.15 mmol), *N*-iodosuccinimide (47.4 mg, 0.20 mmol) and silver trifluoromethanesulfonate (14.1 mg, 0.05 mmol) in CH_2Cl_2 (6 mL). The crude residue was purified by chromatography (hexane–EtOAc 3:1) to afford **26** (149.7 mg, 89%) as pale yellow syrup. R_f 0.38 (hexane–EtOAc 2:1); $[\alpha]_D$ = +33.1 (*c* 0.45, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.98 (dd, *J* = 8.3, 1.2 Hz, 2H, Ar), 7.55–7.50 (m, 1H, Ar), 7.38–7.24 (m, 17H, Ar), 5.65 (dd, *J* = 2.8, 2.1 Hz, 1H, H-2'), 5.41 (dd, *J* = 3.2, 2.0 Hz, 1H, H-2''), 5.36 (d, *J* = 2.0 Hz, 1H, H-1'), 5.30 (d, *J* = 2.0 Hz, 1H, H-1''), 5.18 (app t, *J* = 9.9 Hz, 1H, H-4''), 4.88 (d, *J* = 1.8 Hz, 1H, H-1), 4.77–4.51 (m, 6H, 3 × OCH_2Ph), 4.15 (dd, *J* = 12.8, 5.2 Hz, 1H, H-6a''), 4.12 (app t, *J* = 9.6 Hz, 1H, H-4'), 4.01 (app t, *J* = 9.4 Hz, 1H, H-4), 3.94–3.87 (m, 3H, H-5'', H-6a', H-6b''), 3.87–3.82 (m, 2H, H-5', H-5), 3.81 (dd, *J* = 3.1, 1.9 Hz,

1H, H-2), 3.76 (dd, J = 10.7, 6.3 Hz, 1H, H-6b'), 3.74–3.69 (m, 3H, H-3', H-6a, octyl OCH_2), 3.66–3.58 (m, 3H, H-3'', H-3, H-6b), 3.44 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.44–3.39 (m, 1H, octyl OCH_2), 3.37 (s, 3H, OMe), 2.10 (s, 3H, OAc), 2.09 (s, 3H, OAc), 2.02 (s, 3H, OAc), 1.66–1.54 (m, 2H, octyl OCH_2CH_2), 1.38–1.24 (m, 10H, octyl CH_2), 0.90 (t, J = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.7 (OAc), 167.0 (OAc), 169.7 (OAc), 165.3 (OBz), 138.6 (Ar), 138.4 (Ar), 138.2 (Ar), 133.1 (Ar), 129.8 (2C, Ar), 129.8 (Ar), 128.4 (2C, Ar), 128.4 (2C, Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 127.7 (2C, Ar), 127.6 (Ar), 127.5 (3C, Ar), 127.4 (Ar), 127.3 (2C, Ar), 99.6 (C-1', $^1J_{C\text{-}1,H\text{-}1}$ = 174.4 Hz), 99.3 (C-1'', $^1J_{C\text{-}1,H\text{-}1}$ = 175.2 Hz), 97.8 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 169.6 Hz), 82.1 (C-3), 80.1 (C-3'), 76.7 (C-3''), 74.7 (C-4), 73.6 (OCH_2Ph), 73.5 (OCH_2Ph), 73.3 (C-4'), 73.1 (C-2), 72.7 (OCH_2Ph), 71.9 (C-5'), 71.4 (C-5), 70.4 (C-6'), 69.4 (C-6), 69.4 (C-5''), 68.0 (octyl OCH_2), 67.8 (C-2'), 67.6 (C-2''), 67.4 (C-4''), 62.6 (C-6''), 57.6 (OMe), 57.1 (OMe), 56.9 (OMe), 31.8 (octyl CH_2), 29.4 (octyl CH_2), 29.4 (octyl CH_2), 29.2 (octyl CH_2), 26.1 (octyl CH_2), 22.7 (octyl CH_2), 21.0 (OAc), 20.9 (OAc), 20.7 (OAc), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{63}H_{82}O_{20}$ $[M + Na]^+$ 1181.5292, found 1181.5285.

Octyl 4-*O*-acetyl-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (27): A mixture of thioglycoside **13** (727.7 mg, 1.36 mmol), alcohol **24** (1.07 g, 1.12 mmol) and powdered 4 Å molecular sieves were dissolved in CH_2Cl_2 (20 mL) and stirred at 0 ℃ for 0.5 h. Then *N*-iodosuccinimide (226.4 mg, 0.96 mmol) and silver

trifluoromethanesulfonate (65.2 mg, 0.25 mmol) were added at 0 ℃. After stirring overnight while warming to room temperature, triethylamine (1 mL) was added and the reaction mixture was filtered through Celite. The filtrate was concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 6:1) to afford **27** (1.41 g, 92%) as pale yellow syrup. R_f 0.25 (hexane–EtOAc 5:1); $[\alpha]_D$ = +7.3 (*c* 3.5, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.99 (ddd, *J* = 9.8, 8.3, 1.3 Hz, 4H, Ar), 7.53–7.48 (m, 2H, Ar), 7.39–7.22 (m, 24H, Ar), 5.69–5.65 (m, 2H, H-2', H-2''), 5.45 (app t, *J* = 9.9 Hz, 1H, H-4''), 5.42 (d, *J* = 2.0 Hz, 1H, H-1'), 5.37 (d, *J* = 1.9 Hz, 1H, H-1''), 4.90 (d, *J* = 1.8 Hz, 1H, H-1), 4.76–4.41 (m, 8H, 4 × OCH_2Ph), 4.19 (app t, *J* = 9.6 Hz, 1H, H-4'), 4.02 (app t, *J* = 9.5 Hz, 1H, H-4), 3.98 (dd, *J* = 10.2, 3.1 Hz, 1H, H-5''), 3.94–3.91 (dd, *J* = 10.8, 1.2 Hz, 1H, H-6a), 3.89 (ddd, *J* = 9.9, 4.2, 1.6 Hz, 1H, H-5'), 3.87–3.83 (m, 1H, H-5), 3.82 (dd, *J* = 2.9, 2.1 Hz, 1H, H-2), 3.80–3.72 (m, 5H, H-3', H-3'', H-6b, H-6a', octyl OCH_2), 3.71 (dd, *J* = 10.9, 1.7 Hz, 1H, H-6b'), 3.63 (dd, *J* = 9.2, 3.1 Hz, 1H, H-3), 3.54–3.51 (m, 2H, H-6a'', H-6b''), 3.51 (s, 3H, OMe), 3.46 (s, 3H, OMe), 3.45–3.43 (m, 1H, octyl OCH_2), 3.42 (s, 3H, OMe), 2.04 (s, 3H, OAc), 1.64–1.57 (m, 2H, octyl OCH_2CH_2), 1.39–1.25 (m, 10H, octyl CH_2), 0.92 ppm (t, *J* = 7.1 Hz, 3H, octyl CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 169.8 (OAc), 165.4 (OBz), 165.4 (OBz), 138.7, 138.6, 138.2, 138.2, 133.0, 133.0, 129.9, 129.9, 129.8, 129.7, 128.3, 128.3, 128.2, 127.8, 127.7, 127.6, 127.5, 127.4, 127.3 (36C; Ar), 99.6 (C-1'), 99.5 (C-1'', $^1J_{C\text{-}1,H\text{-}1}$ = 173.9 Hz), 97.8 (C-1), 82.1 (C-3), 80.1 (C-3'), 77.0 (C-3''), 74.9 (C-4), 73.5 (C-4'), 73.5, 73.5, 73.4 (3C; 3 × OCH_2Ph), 73.1 (C-2), 72.6 (OCH_2Ph), 72.0 (C-5'), 71.4 (C-5), 70.9 (C-5''), 70.4 (C-6), 69.7 (C-6'), 69.5 (C-6''), 68.4

(C-4’’), 68.1 (C-2’/C-2’’), 67.9 (octyl OCH_2), 67.8 (C-2’’/C-2’), 57.5, 57.1, 57.1 (3C; 3 × OMe), 31.8, 29.4, 29.4, 29.2, 26.1, 22.7 (6C; octyl CH_2), 21.0 (OAc), 14.1 ppm (octyl CH_3); HRMS (ESI) calcd $C_{73}H_{88}O_{19}$ $[M + Na]^+$ 1291.5812, found 1291.5816.

Octyl 2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (28): Trisaccharide **27** (1.41 g, 1.1 mmol) was dissolved in CH_2Cl_2–MeOH (50 mL, 1:4 v/v) at 0 ℃ before acetyl chloride (1.3 mL, 2.6% v/v) was added. After stirring overnight while warming to room temperature, triethylamine (1 mL) was added and the solution was concentrated. The residue was dissolved in CH_2Cl_2 and washed with water, saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 4:1) to afford **28** (1.50 g, 99%) as a white foam. R_f 0.33 (hexane–EtOAc 3:1); $[\alpha]_D$ = +26.3 (*c* 1.7, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.98 (m, 4H, Ar), 7.50 (m, 2H, Ar), 7.38–7.24 (m, 24H, Ar), 5.67 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2’), 5.63 (dd, *J* = 3.0, 2.0 Hz, 1H, H-2’’), 5.42 (d, *J* = 1.9 Hz, 1H, H-1’’), 5.36 (d, *J* = 1.9 Hz, 1H, H-1’), 4.89 (d, *J* = 1.7 Hz, 1H, H-1), 4.76–4.44 (m, 8H, 4 × OCH_2Ph), 4.21 (app t, *J* = 9.6 Hz, 1H, H-4’), 4.11 (app td, *J* = 9.6, 1.9 Hz, 1H, H-4’’), 4.02 (app t, *J* = 9.5 Hz, 1H, H-4), 3.94–3.82 (m, 4H, H-5, H-5’, H-5’’, H-6a’), 3.82 (dd, *J* = 3.1, 2.0 Hz, 1H, H-2), 3.79–3.75 (m, 4H, H-3’, H-6b’, H-6a’’, H-6a), 3.73 (app dt, 1H, *J* = 9.7, 6.9 Hz, octyl OCH_2), 3.67–3.63 (m, 3H, H-3’’, H-6b’’, H-6b), 3.62 (dd, *J* = 9.2, 3.1 Hz, 1H, H-3), 3.50 (s, 3H, OMe), 3.49 (s, 3H, OMe), 3.45 (s, 3H, OMe),

3.43 (app dt, J = 9.8, 6.8 Hz, 1H, octyl OCH_2), 2.67 (d, J = 2.0 Hz, 1H, 4''-OH), 1.65–1.56 (m, 2H, octyl OCH_2CH_2), 1.39–1.21 (m, 10H, octyl CH_2), 0.91 ppm (t, J = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (100 MHz, $CDCl_3$) δ 165.4 (OBz), 165.3 (OBz), 138.7, 138.6, 138.3, 138.2, 133.1, 133.0, 129.9, 129.8, 128.3, 128.2, 127.7, 127.6, 127.5, 127.3 (36C; Ar), 99.7 (2C; C-1', C-1''), 97.8 (C-1), 82.1 (C-3), 80.3 (C-3'), 79.3 (C-3''), 74.8 (C-4), 73.7, 73.5, 73.4 (3C; 3 × OCH_2Ph), 73.2, 73.1 (2C; C-2, C-4'), 72.6 (OCH_2Ph), 72.1 (C-5'/C-5''), 72.0 (C-5''/C-5'), 71.4 (C-5), 70.3, 70.1, 69.4 (3C; C-6, C-6', C-6''), 68.0 (octyl OCH_2), 67.9, 67.7, 67.6 (3C; C-2', C-2'', C-4''), 57.3, 57.1, 57.1 (3C; 3 × OMe), 31.8, 29.4, 29.4, 29.2, 26.1, 22.7 (6C; octyl CH_2), 14.1 ppm (octyl CH_3); HRMS (ESI) calcd $C_{71}H_{86}O_{18}$ [M + Na]$^+$ 1249.5706, found 1249.5704.

Octyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (29): Glycosylation was performed as for **27**, with thioglycoside **11** (1.04 g, 1.94 mmol), alcohol **28** (857.4 mg, 1.76 mmol), *N*-iodosuccinimide (52.3 mg, 0.22 mmol) and silver trifluoromethanesulfonate (15.5 mg, 0.06 mmol) in CH_2Cl_2 (6 mL). The crude residue was purified by chromatography (hexane–EtOAc 5:1) to afford **29** (127.3 mg, 69%) as pale yellow syrup. R_f 0.24 (hexane–EtOAc 5:2); $[\alpha]_D$ = +26.6 (*c* 0.8, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.96 (dd, J = 20.2, 7.4 Hz, 4H, Ar), 7.49 (t, J = 7.4 Hz, 2H, Ar), 7.43–7.22 (m, 24H, Ar), 5.69 (dd, J = 2.9, 2.1 Hz, 1H, H-2'), 5.65 (dd, J = 2.7, 2.2 Hz, 1H, H-2''), 5.40–5.35 (m, 4H, H-1', H-1'', H-2''', H-3'''), 5.34–5.25

(m, 2H, H-1’’’, H-4’’’), 4.90 (d, J = 1.1 Hz, 1H, H-1), 4.77–4.47 (m, 8H, 4 × OCH_2Ph), 4.24 (app t, J = 9.6 Hz, 1H, H-4’), 4.21 (app t, J = 9.7 Hz, 1H, H-4’’), 4.16 (dd, J = 12.3, 4.3 Hz, 1H, H-6a’’’), 4.06 (app t, J = 9.3 Hz, 1H, H-4), 4.01 (ddd, J = 9.9, 4.3, 2.5 Hz, 1H, H-5’’’), 3.95–3.69 (m, 12H, H-2, H-3’, H-3’’, H-5, H-5’, H-5’’, 5 × H-6, octyl OCH_2), 3.69–3.57 (m, 3H, H-3, 2 × H-6), 3.51 (s, 3H, OMe), 3.46 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.43–3.40 (m, 1H, octyl OCH_2), 2.12 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.01 (s, 6H, 2 × OAc), 1.65–1.54 (m, 2H, octyl OCH_2CH_2), 1.38–1.24 (m, 10H, octyl CH_2), 0.91 ppm (t, J = 6.8 Hz, 3H, octyl OCH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.6, 170.0, 169.8, 169.6 (4C; 4 × OAc), 165.3 (2C; 2 × OBz), 138.8, 138.7, 138.5, 138.3, 133.0, 133.0, 129.9, 129.8, 129.7, 128.3, 128.3, 128.3, 127.7, 127.6, 127.5, 127.4, 127.4, 127.3 (36C; Ar), 99.8 (C-1’), 99.5 (C-1’’), 99.3 (C-1’’’, $^1J_{C\text{-}1,H\text{-}1}$ = 175.1 Hz), 97.8 (C-1), 82.1 (C-3), 80.2 , 80.0 (2C; C-3’’, C-3’), 74.9 (C-4), 73.7 (C-4’’), 73.6, 73.5, 73.4 (3C; 3 × OCH2Ph), 73.3 (C-4’), 73.2 (C-2), 72.6 (OCH_2Ph), 72.0, 71.9 (2C, C-5’, C-5’’), 71.3 (C-5), 70.2 69.3, 69.3 (3C; C-6, C-6’, C-6’’’), 69.5 (2C; C-2’’’, C-3’’’), 69.2 (C-5’’’), 68.0 (octyl OCH_2), 67.9 (C-2’), 67.8 (C-2’’), 66.0 (C-4’’’), 62.3 (C-6’’), 57.1, 57.0, 56.9 (3C; 3 × OMe), 31.8, 29.4, 29.4, 29.3, 26.1, 22.7 (6C; octyl CH_2), 20.9, 20.7 (4C; 4 × OAc), 14.1 ppm (octyl CH_3); HRMS (ESI) calcd $C_{85}H_{104}O_{27}$ $[M + Na]^+$ 1579.6657, found 1579.6651.

Octyl 2,4,6-tri-*O*-acetyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (30): Glycosylation was performed as for **27**,

with thioglycoside **12** (56.5 mg, 0.13 mmol), alcohol **28** (140.7 mg, 0.11 mmol), *N*-iodosuccinimide (36.4 mg, 0.15 mmol) and silver trifluoromethanesulfonate (9.7 mg, 0.04 mmol) in CH_2Cl_2 (8 mL). The crude residue was purified by chromatography (hexane–EtOAc 5:2) to afford **30** (148.3 mg, 88%) as pale yellow syrup. R_f 0.25 (hexane–EtOAc 2:1); $[\alpha]_D$ = +28.0 (*c* 0.9, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.97 (dd, *J* = 8.3, 1.2 Hz, 2H, Ar), 7.95 (dd, *J* = 8.3, 1.2 Hz, 2H, Ar), 7.54–7.46 (m, 2H, Ar), 7.40–7.23 (m, 24H, Ar), 5.68 (dd, *J* = 3.1, 2.1 Hz, 1H, H-2'), 5.64 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2''), 5.45 (dd, *J* = 3.1, 2.0 Hz, 1H, H-2'''), 5.37 (d, *J* = 1.9 Hz, 1H, H-1''), 5.36 (d, *J* = 1.9 Hz, 1H, H-1'), 5.33 (d, *J* = 1.8 Hz, 1H, H-1'''), 5.19 (app t, *J* = 9.9 Hz, 1H, H-4'''), 4.89 (d, *J* = 1.7 Hz, 1H, H-1), 4.76–4.50 (m, 8H, 4 × OCH_2Ph), 4.20–4.12 (m, 3H, H-4', H-4'', H-6'''), 4.01 (app t, *J* = 9.5 Hz, 1H, H-4), 3.95–3.87 (m, 5H, H-5', H-5'', H-5''', 2 × H-6), 3.85 (ddd, *J* = 9.6, 6.6, 1.6 Hz, 1H, H-5), 3.82 (dd, *J* = 2.8, 2.1 Hz, 1H, H-2), 3.81–3.70 (m, 7H, H-3', H-3'', 4 × H-6; octyl OCH_2), 3.66 (dd, *J* = 11.1, 1.9 Hz, 1H, H-6), 3.64 (dd, *J* = 10.0, 3.2 Hz, 1H, H-3'''), 3.62 (dd, *J* = 9.5, 3.2 Hz, 1H, H-3), 3.51 (s, 3H, OMe), 3.47 (s, 3H, OMe), 3.46 (s, 3H, OMe), 3.43 (app dt, *J* = 9.8, 6.8 Hz, 1H, octyl OCH_2), 3.34 (s, 3H, OMe), 2.10 (s, 6H, 2 × OAc), 2.01 (s, 3H, OAc), 1.65–1.54 (m, 2H, octyl OCH_2CH_2), 1.39–1.24 (m, 10H, octyl CH_2), 0.91 ppm (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.7, 167.0, 169.7 (3C; 3 × OBz), 165.3 (2C; 2 × OAc), 138.7, 138.7, 138.4, 138.2, 133.1, 133.0, 129.9, 129.8, 129.7, 128.3, 128.3, 127.7, 127.6, 127.6, 127.5, 127.4, 127.3, 127.3 (36C; Ar), 99.7 (C-1'), 99.6 (C-1''), 99.4 (C-1''', $^1J_{C\text{-}1,H\text{-}1}$ = 173.4 Hz), 97.8 (C-1), 82.1 (C-3), 80.1 (C-3'), 80.0 (C-3''), 76.8 (C-3'''), 74.9 (C-4), 73.9 (C-4'),

73.6, 73.6, 73.5 (3C; 3 × OCH_3Ph), 73.3 (C-4’’), 73.1(C-2), 72.7 (OCH_3Ph), 72.0 (C-5’), 71.9 (C-5’’), 71.5 (C-5), 70.4, 69.7, 69.5 (3C; 3 × C-6), 69.4(C-5’’’), 68.0 (octyl OCH_2), 67.8 (C-2’), 67.7 (C-2’’), 67.6 (C-2’’’), 67.4 (C-4’’’), 62.6 (C-6’’’), 57.6, 57.1, 57.0 (3C; 3 × OAc), 31.8, 29.4, 29.4, 29.2, 26.1, 22.7 (6C; octyl CH_2), 21.0, 20.9, 20.7 (3C; 3 × OAc), 14.1 ppm (octyl CH_3); HRMS (ESI) calcd $C_{84}H_{104}O_{26}$ $[M + Na]^+$ 1551.6708, found 1551.6711.

Octyl 4-*O*-acetyl-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (31): Glycosylation was performed as for **27**, with thioglycoside **13** (299.3 mg, 0.55 mmol), alcohol **28** (611.1 mg, 0.50 mmol), *N*-iodosuccinimide (157.4 mg, 0.66 mmol) and silver trifluoromethanesulfonate (38.9 mg, 0.15 mmol) in CH_2Cl_2 (10 mL). The crude residue was purified by chromatography (hexane–EtOAc 6:1) to afford **31** (676.0 mg, 83%) as pale yellow syrup. R_f 0.38 (hexane–EtOAc 4:1); $[\alpha]_D$ = +19.4 (*c* 0.2, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.09–7.89 (m, 6H, Ar), 7.48 (m, 3H, Ar), 7.41–7.18 (m, 31H, Ar), 5.70 (dd, 1H, *J* = 2.9, 2.2 Hz, H-2’’’), 5.67 (app t, *J* = 2.5 Hz, 1H, H-2’), 5.65 (app t, *J* = 2.5 Hz, 1H, H-2’’), 5.45 (app t, *J* = 9.9 Hz, 1H, H-4’’’), 5.44 (d, 1H, *J* = 1.9 Hz, H-1’’’), 5.37–5.35 (m, 2H, H-1’, H-1’’), 4.90 (d, *J* = 1.6 Hz, 1H, H-1), 4.76–4.39 (m, 10H, 5 × OCH_2Ph), 4.22 (app t, *J* = 9.6 Hz, 1H, H-4’’), 4.16 (app t, *J* = 9.6 Hz, 1H, H-4’), 4.03–3.97 (m, 2H, H-4, H-5’’’), 3.96–3.83 (m, 4H, H-5, H-5’, H-5’’, H-6), 3.83–3.70 (m, 10H, H-2, H-3’, H-3’’, H-3’’’, 5 × H-6, , octyl OCH_2), 3.63 (dd, *J* = 9.2, 3.0, 1H, H-3), 3.52 (s, 3H, OMe), 3.51 (s, 3H, OMe),

3.50–3.49 (m, 2H, H-6’’’a, H-6’’’b), 3.46 (s, 3H, OMe), 3.45–3.41 (m, 1H, , octyl OCH_2), 3.38 (s, 3H, OMe), 2.03 (s, 3H, OAc), 1.69–1.49 (m, 2H, octyl OCH_2CH_2), 1.36–1.19 (m, 10H, octyl CH_2), 0.91 ppm (t, J = 7.0 Hz, 3H, octyl CH_3) ; ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.8 (OAc), 165.5 (OBz), 165.3 (2C; 2 × OBz), 138.7, 138.7, 138.6, 138.2, 138.1, 133.0, 130.0, 129.9, 129.8, 129.7, 128.3, 128.3, 128.3, 128.2, 127.8, 127.8, 127.7, 127.6, 127.6, 127.5, 127.4, 127.3 (48C; Ar), 99.8, 99.7 (2C; C-1’, C-1’’), 99.6 (C-1’’’, $^1J_{C\text{-}1,H\text{-}1}$ = 175.2 Hz), 97.8 (C-1), 82.1 (C-3), 80.1 (2C; C-3’, C-3’’), 77.0 (C-3’’’), 75.0 (C-4), 74.1 (C-4’), 73.7 (C-4’’), 73.6, 73.6, 73.5, 73.4 (4C; 4 × OCH_2Ph), 73.1 (C-2), 72.7 (OCH_2Ph), 72.0 (2C; C-5’, C-5’’), 71.5 (C-5), 70.9 (C-5’’’), 70.4, 69.7, 69.7, 69.5 (4C; C-6, C-6’, C-6’’, C-6’’’), 68.5 (C-4’’’), 68.1 (2’’’), 68.0 (octyl OCH_2), 67.8 (C-2’), 67.7 (C-2’’), 57.5 (OMe), 57.1(3C; 3 × OMe), 31.8, 29.5, 29.4, 29.3, 26.1, 22.7 (6C; octyl CH_2), 21.0 (OAc), 14.1 ppm (octyl CH_3); HRMS (ESI) calcd $C_{94}H_{110}O_{25}$ $[M + Na]^+$ 1661.7228, found 1661.7221.

Octyl 2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (32): Deacetylation was performed as for **27**, with tetrasaccharide **31** (676.0 mg, 0.41 mmol), acetyl chloride (0.8 mL, 2.6% v/v) in CH_2Cl_2–MeOH (30 mL, 1:5 v/v). The crude residue was purified by chromatography (hexane–EtOAc 3:1) to afford **32** (520.6 mg, 79%) as a white foam. R_f 0.32 (hexane–EtOAc 3:1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05–7.89 (m, 6H, Ar), 7.49 (m, 3H, Ar), 7.44–7.13 (m, 31H, Ar), 5.71–5.68 (m, 2H, H-2’, H-

2’’’), 5.66 (dd, *J* = 3.0, 2.1 Hz,1H, H-2’’), 5.45 (d, *J* = 1.6, 1H, H-1’’’), 5.37 (d, *J* = 1.7, 1H, H-1’’), 5.36 (d, *J* = 1.7, 1H, H-1’), 4.91 (d, *J* = 1.5 Hz, 1H, H-1), 4.78–4.46 (m, 10H, 5 × OCH_2Ph), 4.26 (app t, *J* = 9.6 Hz, 1H, H-4’’), 4.17 (app t, *J* = 9.6 Hz, 1H, H-4’), 4.13 (app t, *J* = 9.6 Hz, 1H, H-4’’’), 4.01 (app t, *J* = 9.5 Hz, 1H, H-4), 3.96–3.82 (m, 7H, H-2, H-3’’, H-5, H-5’, H-5’’, H-5’’’, H-6), 3.82–3.72 (m, 6H, H-3’, 4 × H-6, otcyl OCH_2), 3.72–3.61 (m, 5H, H-3, H-3’’’, 3 × H-6), 3.53 (s, 3H, OMe), 3.51 (s, 3H, OMe), 3.47 (s, 3H, OMe), 3.46 (s, 3H, OMe), 3.45–3.40 (m, 1H, octyl OCH_2), 2.69 (s, 1H, 4’’’-OH), 1.67–1.58 (m, 2H, octyl OCH_2CH_2), 1.39–1.22 (m, 10H, octyl CH_2), 0.92 ppm (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (101 MHz, $CDCl_3$) δ 165.4 (OBz), 165.3 (2C; 2 × OBz), 149.5, 138.7, 138.6, 138.6, 138.3, 138.2, 133.0, 129.9, 129.8, 128.3, 128.3, 128.2, 127.7, 127.6, 127.5, 127.4, 127.3 (48C; Ar), 99.8, 99.7 (2C; C-1’’, C-1’’’), 99.7 (C-1’), 97.8 (C-1), 82.1(C-3), 80.2 (C-3’’), 80.1 (C-3’), 79.3 (C-3’’’), 75.1 (C-4), 74.0 (C-4’), 73.7, 73.6, 73.5, 73.4 (4C; 4 × OCH_2Ph), 73.2 (C-4’’), 73.1 (C-2), 72.6 (OCH_2Ph), 72.2 (C-5’’’), 72.0 (2C; C-5’, C-5’’), 71.5 (C-5), 70.4, 70.1, 69.6, 69.4 (4C; C-6, C-6’, C-6’’, C-6’’’), 68.0 (octyl OCH_2), 67.8, 67.7, 67.6, 67.5 (4C; C-2’, C-2’’, C-2’’’, C-4’’’), 57.3 (OMe), 57.1 (3C; 3 × OMe), 31.8, 29.4, 29.4, 29.2, 26.1, 22.7 (6C; octyl CH_2), 14.1 ppm (octyl CH_3); HRMS (ESI) calcd $C_{92}H_{108}O_{24}$ $[M + Na]^+$ 1619.7123, found 1619.7113.

Octyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-

mannopyranoside (33): Glycosylation was performed as for **27**, with thioglycoside **11** (37.4 mg, 0.07 mmol), alcohol **32** (102.3 g, 0.06 mmol), *N*-iodosuccinimide (30.5 mg, 0.13 mmol) and silver trifluoromethanesulfonate (9.6 mg, 0.03 mmol) in CH_2Cl_2 (3 mL). The crude residue was purified by chromatography (hexane–EtOAc 2:1) to afford **33** (107.2 mg, 87%) as a pale yellow syrup. R_f 0.19 (hexane–EtOAc 2:1); ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.05–7.90 (m, 6H), 7.52–7.21 (m, 34H), 5.68 (m, 3H, H-2', H-2'', H-2'''), 5.44 (d, *J* = 1.7 Hz, 1H, H-1''), 5.41–5.34 (m, 4H, H-1', H-1''', H-2'''', H-3'''), 5.33–5.27 (m, 2H, H-1'''', H-4''''), 4.90 (d, *J* = 1.7 Hz, 1H, H-1), 4.77–4.44 (m, 10H, 5 × OCH_2Ph), 4.29 (app t, *J* = 9.6 Hz, 1H, H-4'''), 4.233 (app t, *J* = 9.6 Hz, 1H, H-4''), 4.228 (m app t, *J* = 9.6 Hz, 1H, H-4'), 4.16 (dd, *J* = 12.4, 4.2 Hz, 1H, H-6''''),4.03 (app t, *J* = 9.5 Hz, 1H, H-4), 4.01 (ddd, *J* = 10.1, 4.1, 2.6 Hz, 1H, H-5''''), 3.96–3.90 (m, 3H, H-5'', H-5'''', H-6), 3.90–3.76 (m, 10H, H-2, H-3', H-3'', H-3''', H-5, H-5', 4 × H-6), 3.76–3.68 (m, 4H, 3 × H-6, otcyl OCH_2), 3.63 (dd, *J* = 9.2, 3.0 Hz, 1H, H-3), 3.57 (dd, *J* = 11.1, 1.4 Hz, 1H, H-6), 3.53 (s, 3H, OMe), 3.52 (s, 3H, OMe), 3.47 (s, 3H, OMe), 3.46–3.42 (m, 1H, otcyl OCH_2), 3.42 (s, 3H, OMe), 2.12 (s, 3H, OAc), 2.06 (s, 3H, OAc), 2.02 (s, 3H, OAc), 2.01 (s, 3H, OAc), 1.65–1.57 (m, 2H, octyl OCH_2CH_2), 1.38–1.25 (m, 10H, octyl CH_2), 0.92 ppm (t, *J* = 7.1 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.6, 170.0, 169.8, 169.6 (4C; 4 × OAc), 165.4 (OBz), 165.3 (2C; 2 × OBz), 138.7, 138.7, 138.6, 138.4, 138.2, 133.0, 133.0, 130.1, 129.9, 129.8, 129.8, 129.7, 128.3, 128.3, 127.7, 127.6, 127.6, 127.5, 127.4, 127.3, 127.3, 127.2 (48 C; Ar), 99.8 (H-1'), 99.7 (H-1'''), 99.5 (H-1''), 99.3 (C-1'''', $^1J_{C\text{-}1,\ H\text{-}1}$ = 176.2 Hz), 97.8 (C-1),

82.1 (C-3), 80.2 (2C; C-3', C-3'''), 80.0 (C-3''), 75.0 (C-4), , 73.9 (C-4'), 73.6 (3C; 3 × OCH_2Ph), 73.5 (OCH_2Ph), 73.5 (C-4''), 73.2, 73.1 (2C; C-2 or C-4'''), 73.4 (OCH_2Ph), 72.0, 72.0, 71.9 (3C; C-5', C-5'', C-5'''), 71.5 (C-5), 70.3, 69.6, 69.3, 69.1 (4C; C-6, C-6', C-6'', C-6'''), 69.5 (C-2''''), 69.2 (2C; C-3'''', C-5''''), 68.0 (octyl OCH_2), 67.8 (3C; C-2', C-2'', C-2'''), 66.0 (C-4''''), 62.3 (C-6''''), 57.1 (3C; 3 × OMe), 57.0 (OMe), 31.8, 29.4, 29.4, 29.3, 26.1, 22.7 (6C; octyl CH_2), 20.9, 20.8, 20.7 (4C; 4 × OAc), 14.1 ppm (octyl CH_3); HRMS (ESI) calcd $C_{106}H_{126}O_{33}$ $[M + Na]^+$ 1949.8074, found 1949.8064.

Octyl 2,4,6-tri-*O*-acetyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2,6-di-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (34): Glycosylation was performed as for **27**, with thioglycoside **12** (36.9 mg, 0.08 mmol), alcohol **32** (102.1 mg, 0.06 mmol), *N*-iodosuccinimide (23.6 mg, 0.10 mmol) and silver trifluoromethanesulfonate (5.3 mg, 0.02 mmol) in CH_2Cl_2 (3 mL). The crude residue was purified by chromatography (hexane–EtOAc 2:1) to afford **34** (104.9 mg, 86%) as pale yellow syrup. R_f 0.21 (hexane–EtOAc 2:1); ^1H NMR (600 MHz, $CDCl_3$) δ 8.01–7.90 (m, 6H), 7.53–7.22 (m, 34H), 5.71–5.69 (m, 2H, H-2', H-2''), 5.69 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2'''), 5.45 (dd, *J* = 3.0, 2.0 Hz, 1H, H-2''''), 5.43 (d, *J* = 1.7 Hz, 1H, H-1''), 5.40 (d, *J* = 2.1 Hz, 1H, H-1'''), 5.39 (d, *J* = 2.1 Hz, 1H, H-1'), 5.35 (d, *J* = 1.6 Hz, 1H, H-1''''), 5.21 (app t, *J* = 9.9 Hz, 1H, H-4''''), 4.91 (d, *J* = 1.5 Hz, 1H, H-1), 4.77–4.58 (m, 10H, 5 × OCH_2Ph), 4.24 (app t, *J* = 9.6 Hz, 1H,

H-4’’’), 4.20 (app t, J = 9.7 Hz, 1H, H-4’’), 4.19 (app t, J = 9.6 Hz, 1H, H-4’), 4.17 (dd, J = 12.0, 4.3 Hz, 1H, H-6’’’’), 4.04 (app t, J = 9.5 Hz, 1H, H-4), 3.98–3.86 (m, 7H, H-5, H-5’, H-5’’, H-5’’’, H-5’’’’, 2 × H-6), 3.86–3.73 (m, 11H, H-2, H-3’, H-3’’, H-3’’’, 6 × H-6, otcyl OCH_2), 3.69–3.62 (m, 3H, H-3, H-3’’’’, H-6), 3.55 (s, 3H, OMe), 3.53 (s, 3H, OMe), 3.48 (s, 3H, OMe), 3.46 (s, 3H, OMe), 3.43–3.46 (m, 1H, octyl OCH_2), 3.34 (s, 3H, OMe), 2.11 (s, 6H, 2 × OAc), 2.11 (OAc), 2.02 (s, 3H, OAc), 1.66–1.59 (m, 2H, octyl OCH_2CH_2), 1.38–1.24 (m, 10H, octyl CH_2), 0.93 ppm (t, J = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.7, 170.0, 169.7 (3C; 3 × OAc), 165.3 (2 × OBz), 165.3 (OBz), 138.8, 138.7, 138.4, 138.2, 133.1, 133.0, 130.0, 129.9, 129.7, 128.3, 128.3, 127.8, 127.7, 127.7, 127.6, 127.6, 127.5, 127.4, 127.3, 127.3 (48C; Ar), 99.7, 99.7 (3C; C-1’, C-1’’, C-1’’’), 99.4 (C-1’’’’, $^1J_{C\text{-}1,H\text{-}1}$ = 175.6 Hz), 97.8 (C-1), 82.1 (C-3), 80.1 (3C; C-3’, C-3’’, C-3’’’), 76.8 (C-3’’’’), 75.0 (C-4), 74.1 (C-4’), 73.9 (C-4’’’), 73.6 (2C; 2 × OCH_2Ph), 73.6 (2C; 2 × OCH_2Ph), 73.3 (C-4’’), 73.1 (C-2), 72.7 (OCH_2Ph), 72.1, 72.1, 72.0 (3C; C-5’, C-5’’, C-5’’’), 71.5 (C-5), 70.4, 69.7, 69.6, 69.4 (4C; C-6, C-6’, C-6’’, C-6’’’), 69.4 (C-5’’’’), 68.0 (octyl OCH_2), 67.8, 67.7, 67.7 (3C; C-2’, C-2’’, C-2’’’), 67.5 (C-2’’’’), 67.4 (C-4’’’’), 62.6 (C-6’’’’), 57.5 (OMe), 57.3 (3C; 3 × OMe), 57.0 (OMe), 31.8, 29.4, 29.3, 26.2, 24.7, 22.7 (6C; octyl CH_2), 20.7 (3C; 3 × OAc), 14.1 ppm (octyl CH_3); HRMS (ESI) calcd $C_{105}H_{126}O_{32}$ $[M + Na]^+$ 1921.8124, found 1921.8134.

Growth of bacteria strains: *M. smegmatis* strain ATCC 14468 was purchased from American Type Culture Collection (ATCC). The bacteria were grown at 37 ℃ to mid-log phase (48 h) in a medium containing yeast extract (0.3 g),

peptone (0.5 g), glycerol (2 g) and Tween 80 (0.2 mL) in Milli-Q water (100 mL). The culture (50 mL) was then transferred to 2 × 1 L of fresh media and cultured for another 48 h. Cells were harvested by centrifugation and stored at –20 ℃ until use.

Preparation of ManT and OMT fractions: All of the following steps were carried out at 0 ℃ on ice. Wet cell pellets (10 g from 1 L culture) of *M. smegmatis* was resuspended in 50 mM *N*-[Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) buffer (30 mL, containing 10 mM $MgCl_2$, pH 8.2) and supplemented with one complete protease inhibitor cocktail tablet (Roche). Cell lysis was performed by passing through a cell disruptor three times at 24 Kpsi and then the cell debris was removed by centrifugation at 20,000 × g for 30 min. The obtained cell lysate was further centrifuged at 200,000 × g for 1.5 h. The bottom brown pellet was resuspended in 50 mM TAPS buffer (2 mL, containing 10 mM $MgCl_2$, 5 mM EGTA, pH 8.2) and was used as the ManT source, while the top supernatant fraction was used directly as the OMT source. All enzyme preparations were made fresh made and used immediately after preparation.

Radioactive enzymatic assay: The incubation protocols were established based on earlier work by Ballou and Weisman.[8] A preliminary enzymatic study for the ManT was performed using both unmethylated OS (**1**–**5**) and methylated OS (**6**–**10**) as acceptors. Each assay contained GDP-mannose (20 mM, 1μL) with guanosine diphosphate mannose-[2-^{3}H] (0.04 μCi, American Radiolabeled

Chemicals, Inc.), acceptor (20 mM, 1 μL) and the ManT source (18 μL), in a total volume of 20 μL. All assays were performed in duplicate and control assays without acceptors were performed in parallel to correct for activity arising from endogenous acceptors present in the membrane fraction. Enzymatic products were purified with a C_{18} Sep-Pak cartridge (Waters).[10] After incubation at 37 ℃ for 21 h, the assays were stopped by adding Milli-Q water (0.6 mL) before loading onto the C_{18} cartridge (prewashed with MeOH followed by H_2O). The unreacted radioactive donor guanosine diphosphate mannose-[2-^{3}H] was removed by washing the cartridge with Milli-Q water (50 mL). The enzymatic products containing a hydrophobic octyl chain were then eluted with MeOH (4 mL), mixed with Ecolite cocktail (10 mL) and counted on a scintillation counter. The raw dpm (decay per minute) values obtained from scintillation counter were used to calculate the enzymatic activity (result shown in Figure 2-2).

Kinetic analysis of 4 and 9: For kinetic study of **4**, 1.2 mL fresh ManT fraction obtained from 9.9 g wet cell pellets (from 1 L culture) was used. For kinetic study of **9**, 1.5 mL fresh ManT fraction obtained from 7.1 g wet cell pellets (from 1 L culture) was used. ManT activities were determined by using a range of acceptor concentrations (4–1000 μM for **4**, 0.8–200 μM for **9**). Assays were incubated 5 h 10 min for **4** and 10.5 h for **9**. All other conditions were the same as described above. The kinetic parameters K_m and V_{max} were obtained from the Michaelis–Menten equation, by nonlinear regression analysis with GraphPad Prism 4.0 software (result shown in Figure 2-9).

Calculation of V_{max} and k_{cat}/K_m for 4:

K_m = 20.7±2.6 μM

V_{max} = (2881.0 ±80.2 fmol•min^{-1})/ 10μL total volume = 288.1 ± 8.0 nM•min^{-1}

[enzyme amount] = 9.9 g cell/1.2mL buffer × 6μL = 49.5 mg cell in each assay

$k_{cat} = V_{max}/[E]$ = 5.82 ± 0.16 μM• min^{-1}g^{-1} cell

k_{cat}/K_m = 0.28 ± 0.06 min^{-1}g^{-1} cell

Calculation of V_{max} and k_{cat}/K_m for 9:

K_m = 7.9 ± 1.7 μM

V_{max} = (906.3 ± 50.8 fmol•min^{-1})/ 20 μL total volume = 45.3 ± 2.5 nM•min^{-1}

[enzyme amount] = 7.1 g cell/1.5 mL buffer × 12 μL = 56.8 mg cell in each assay

$k_{cat} = V_{max}/[E]$ = 0.79 ± 0.04 μM•min^{-1}g^{-1} cell

k_{cat}/K_m = 0.10 ± 0.02 min^{-1}g^{-1} cell

Incubation with tetrasaccharides 4 and 9 for MALDI-MS analysis: Tetrasaccharides **4** or **9** (3 mg), non-radiolabeled GDP-mannose (3 mg, ~1.2 mol equivalent) and alkaline phosphatase (0.5 μL) was incubated in the presence of the membrane fraction (5 mL, obtained from 10 g wet pellet resulting from a single 1 L cell culture). After incubation at 37 ℃ for 21 h, the assay reaction was stopped by centrifugation at 200,000 × g for 1 h to remove the enzyme. The supernatant was applied to C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA), washed with Milli-Q water (10 mL), then eluted with 10%, 30%, 50%, 70%, 90%, 100% MeOH in Milli-Q water (10 mL each). The fractions containing products, as detected by MALDI-MS (2,5-dihydroxybenzoic acid as matrix), were

combined and the relative product distributions were analyzed using MALDI-MS (results shown in Figure 2-4).

Peracetylation of enzymatic products: The enzymatic product mixtures were acylated with acetic anhydride (200 μL) in pyridine (0.5 mL). After stirring at room temperature for two days, the reaction mixtures were diluted with CH_2Cl_2. This solution was then washed with H_2O, saturated aqueous $NaHCO_3$ and brine. The organic layer containing per-acetylated products were dried over Na_2SO_4, concentrated and examined by MALDI-MS directly. Result for **9** was shown in Figure 2-4.

***Exo*-glycosidase digestions**: Both enzymatic product mixtures obtained from incubation with **4** or **9** were redissoved in Milli-Q water (200 μL) individually. These two solutions were used as digestion samples. *Exo*-glycosidases used were α-(1→2, 3, 6)-mannosidase from jack bean (Glyko-5010), β-mannosidase from *Helix pomatia* (Sigma M9400), α-(1→2)-mannosidase from *Aspergillus saitoi* (Glyko-5009) and α-(1→6)-mannosidase from *Chryseobacterium* (formerly *Flavobacterium*) *meningosepticum* (New England Biolabs, P0727S). In each digestion assay, 1 μL of the sample was treated with 10–50 U of glycosidase at 37 ℃ in the digestion buffer (supplied together with Glyko-5010), in a total volume of 10 μL. After incubating at 37 °C overnight, fresh enzyme (10–50U) was added and the incubation was extended for another overnight. Each digestion mixture was examined directly by MALDI-MS after desalting with a ZipTip (results shown in Figure 2-5 and Figure 2-6 and summarized in Table 2-1).

Scale-up incubation and enzymatic products separation: Tetrasaccharide **4** or **9** (10 mg), GDP-mannose (10 mg, ~1.2 mol equivalent) and alkaline phosphatase (2 μL) were incubated with membrane fraction for overnight as described above. The unreacted GDP-mannose was removed by passing through C_{18} Sep-Pak cartridge. The crude enzymatic product was peracetylated with acetic anhydride as described above. The acetylated products were examined by thin layer chromatography (TLC) with an eluent system of hexane and ethyl acetate (hexane–EtOAc 1:1.9 for **4**, hexane–EtOAc 1:1.7 for **9**). Visualization of products was achieved by charring with acidified anisaldehyde solution in ethanol. Two major products **35** (R_f = 0.16) and **36** (R_f = 0.08) were observed for **4**.Two major products **37** (R_f = 0.16) and **38** (R_f = 0.11) were observed for **9**. The crude acetylated products were loaded onto a silica-containing column (0.6 mm diameter × 20 mm length) and eluted with gradient of ethyl acetate in hexane (50% to 100%). The fractions were detected by MALDI-MS and fractions containing products were combined and concentrated. All of the major products were successfully separated and their ^{1}H NMR and ^{1}H–^{1}H COSY spectra were recorded on a Varian i600 MHz instrument in $CDCl_3$. The results were shown in Figure 2-7 and Figure 2-8.

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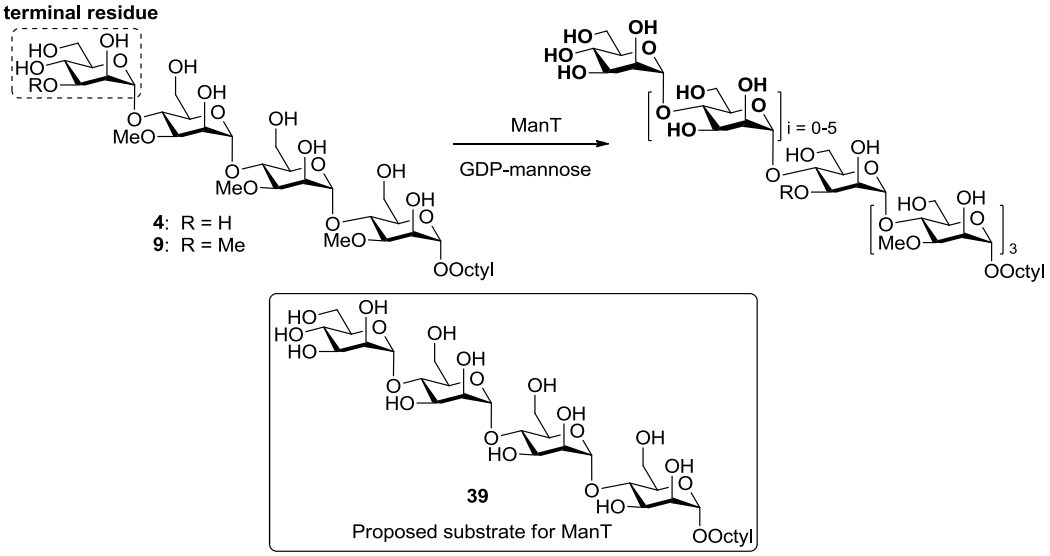
Chapter 3: Evaluation of an unmethylated tetrasaccharide as a substrate of α-(1→4)-mannosyltransferase

3.1 Introduction

3-*O*-methyl-mannose polysaccharides (MMPs) are methylated polysaccharides found in the cytoplasm of some mycobacterial species.[1-3] They are a mixture of four homologs containing 11–14 α-(1→4)-linked mannopyranose residues. Their unique α-(1→4)-linkage indicates the existence of an α-(1→4)-mannosyltransferase (ManT). The activity of ManT, along with a 3-*O*-methyltransferase (OMT), was detected by Ballou and coworkers in 1984.[4-5] At this time, they also proposed a MMP biosynthetic pathway, which, to date, has not been fully verified.

In our efforts to verify the biosynthetic pathway of MMPs, we discovered an unexpected activity for ManT. Previous reports proposed that ManT recognizes only substrates that have terminal 3-*O*-methyl-mannopyranosyl residues (*e.g.*, tetraasaccharide **9**, Scheme 3-1).[4] On the other hand, the work described in Chapter 2, demonstrated that ManT is also able to recognize substrates without a terminal methylated residue (*e.g.*, tetrasaccharide **4**, Scheme 3-1), with higher efficiency when compared with the fully methylated tetrasaccharide **9**.[6] Moreover, when either **4** or **9** were incubated with ManT, the products identified contained not only the product arising from addition of a single mannose. Instead a series of polymers incorporating multiple α-(1→4)-linked mannose residues were observed (Scheme 3-1).



Scheme 3-1. Observed activity of ManT and proposed new substrate **39** for ManT.

These results were inconsistent with the proposed pathway for MMP biosynthesis. In the proposed pathway ManT activity is dependent on methylation of the 3-OH of the terminal mannose residue catalyzed by 3-*O*-methyltransferase (OMT), which results in alternating methylation and mannosylation reactions.[5] Instead, our results suggest an alternative model where ManT may independently carry out repetitive mannosylation to give full length MMPs before installation of methyl groups by OMT (Figure 3-1). Therefore, α-(1→4)-linked mannopyranose oligosaccharides lacking any methyl groups (*e.g.*, tetrasaccharide **39**, Scheme 3-1) could be a substrate of ManT, and thus may serve as a better substrate when compared with methylated tetrasaccharides **4** and **9**. To test this hypothesis, we carried out the chemical synthesis of tetrasaccharide **39**, and evaluated its activity as a ManT substrate.

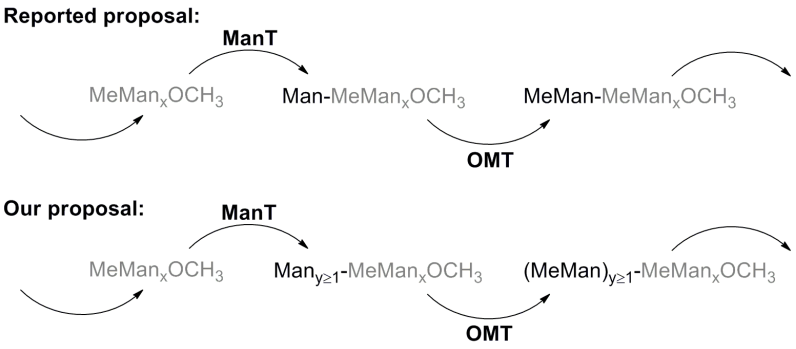
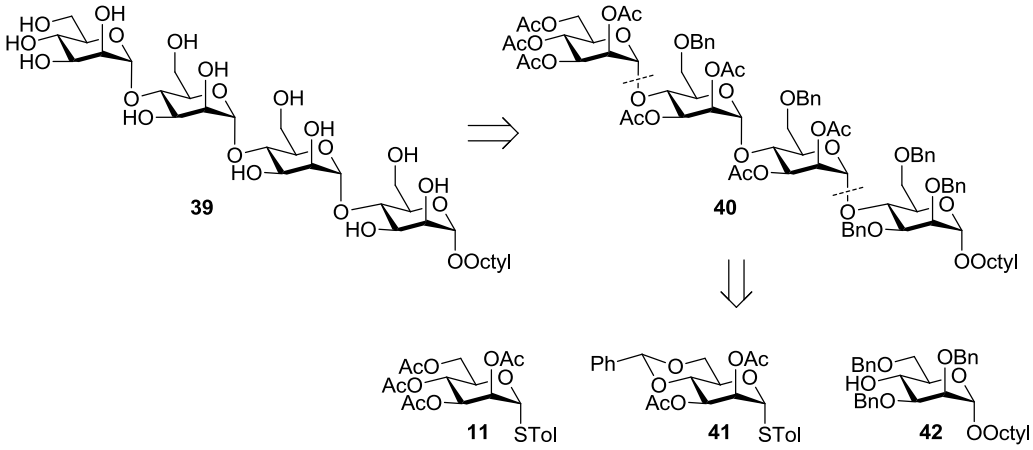


Figure 3-1. The reported and our revised proposal for MMP biosynthetic pathway.

3.2 Synthesis of a tetrasaccharide 39

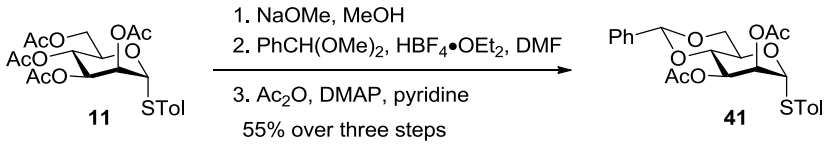
We envisioned that tetrasaccharide **39** could be derived from **40**, which, in turn, could be assembled from three different building blocks **11**, **41** and **42** (Scheme 3-2).



Scheme 3-2. Retrosynthetic analysis for the tetrasaccharide **39** without methyl groups.

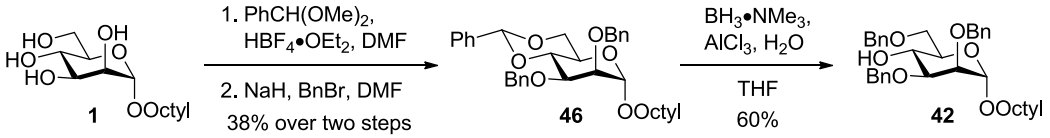
The synthesis of thioglycoside **11** was described in Chapter 2. The thioglycoside building block **41** was derived from **11** in three steps (Scheme 3-3). After removal of acetyl groups with sodium methoxide, **11** was regioselectively protected as 4,6-benzylidene acetal using benzaldehyde dimethyl acetal in the

presence of $HBF_4 \cdot OEt_2$.[7] The remaining two hydroxyl groups were then acetylated to furnish donor **41** in 55% yield over the three steps.



Scheme 3-3. Synthesis of thioglycoside **41**.

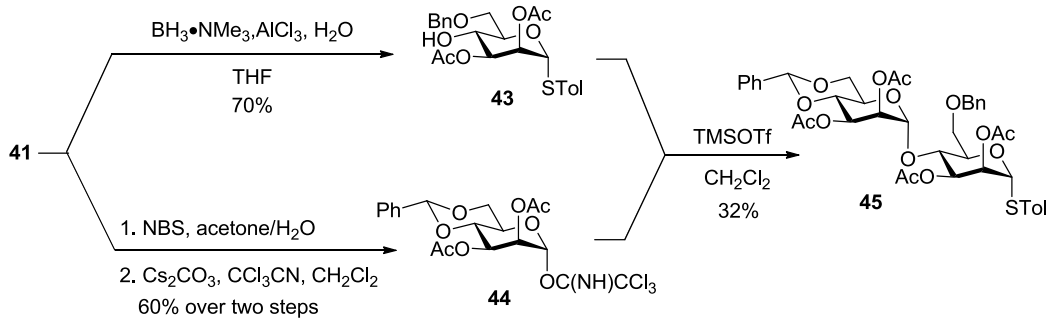
The octyl glycoside **42** was derived from monosaccharide **1** in three steps. (Scheme 3-4). First, selective protection of the 4-OH and 6-OH groups of **1** as the benzylidene acetal, followed by protection of the remaining hydroxyl groups as benzyl ethers gave the protected **46** in 38% yield over two steps. Regioselective opening of the benzylidene acetal using $BH_3 \cdot NMe_3$ and $AlCl_3$ then gave the desired alcohol **42** in 60% yield.



Scheme 3-4. Synthesis of acceptor **42**.

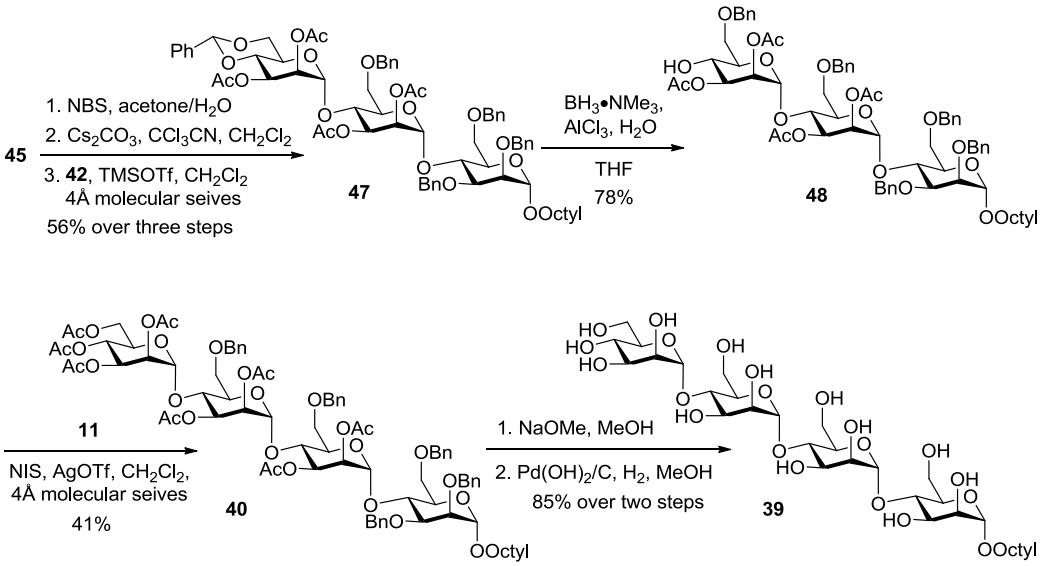
With the three building blocks in hand, the tetrasaccharide was assembled. First, regioselective opening of the benzylidene ring of **41** mediated by $BH_3 \cdot NMe_3$ gave thioglycoside acceptor **43** with a free alcohol in 70% yield.[8-9] Alternatively, thioglycoside **41** was converted, in 60% overall yield, into the trichloroacetimidate donor **44** in two steps.[10-11] Glycosylation of acceptor **43** with **44** under TMSOTf activating conditions gave the desired disaccharide **45**

(Scheme 3-5), albeit in modest (32%) yield.[12] The low yield was due to formation of two byproducts: an orthoester from the donor and a TMS-protected acceptor.



Scheme 3-5. Synthesis of disaccharide donor **45**.

To form trisaccharide **47**, thioglycoside **45** was first converted to the more active imidate donor and then coupled with alcohol **42**, giving the trisaccharide in a 56% yield over three steps. Regioselective opening of the benzylidene acetal of product trisaccharide **47**, again using $BH_3 \cdot NMe_3$ and $AlCl_3$, gave acceptor alcohol **48** in 78% yield. Coupling **48** with thioglycoside **11** under *N*-iodosuccinimide and silver trifluoromethanesulfonate activating conditions then generated tetrasaccharide **40** in 41% yield.[13] Final removal of the acetyl groups with sodium methoxide and benzyl groups under hydrogenolysis conditions produced the desired tetrasaccharide **39** in 85% yield over two steps (Scheme 3-6).[14] With **39** in hand, we set out to evaluate its activity with ManT and compare it with tetrasaccharides **4** and **9**, which were reported in Chapter 2.



Scheme 3-6. Synthesis of tetrasaccharide **39**.

3.3 Biochemical Evaluation of tetrasaccharide 39

3.3.1 Evaluation of tetrasaccharide 39 as a substrate of ManT

Based on our previous studies, we proposed a biosynthetic pathway of MMP assembly in which methylation at O-3 of the mannose residues is not required for the activity of the ManT enzyme. Thus, we speculated that tetrasaccharide **39**, which contains no methyl groups, would serve as a preferential substrate of ManT when compared with methylated tetrasaccharides **4** and **9**, which were examined in Chapter 2. To test this hypothesis, all of the three tetrasaaccharide analogs were incubated with radiolabeled GDP-mannose in the presence of the ManT-containing membrane fraction of *M. smegmatis* (strain ATCC 14468). The relative activity of ManT in these incubations was monitored by measuring the radioactivity incorporated into the enzymatic products. As illustrated in Figure 3-2, compound **39** did serve as substrate for ManT. However,

the observed activity was lower (42%, relative to **4**) than that observed for both tetrasaccharide **4** and **9** (100% and 55% relative to **4**, respectively). These observations were opposite to what we expected based on our proposed biosynthetic model. Instead, they suggest methyl substitution does play a role in the substrate recognition and turnover of the ManT catalyzed reaction.

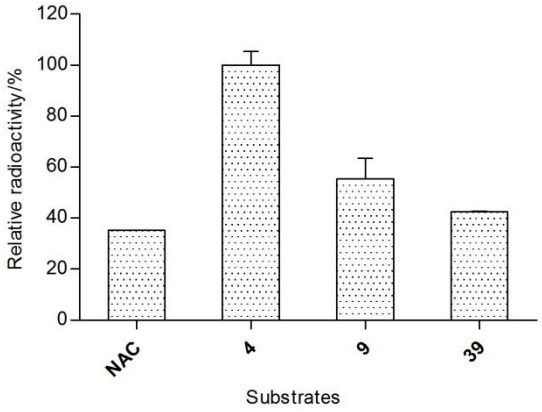


Figure 3-2. Comparison of relative activities of three tetrasaccharides **4**, **9** and **39**. The radioactivity of the most active compound, **4**, was arbitrarily set to 100%. NAC (no acceptor control) is the control assay without added acceptor; We propose that the observed activities arise from endogenous acceptors present in the bacteria.

The enzymatic products of **39** were also monitored by MALDI-MS and formation of three additional oligomers was observed (Figure 3-3, $[M+Na]^+$: *m/z* = 964, 1126 and 1450, mass difference = 162 Da, corresponding to a hexose unit), which corresponds to incorporation of one, two and four additional mannose residues. This result was analogous to the enzymatic products of both **4** and **9**, therefore, implying compound **39** indeed serves as a substrate for ManT, although with poor activity.

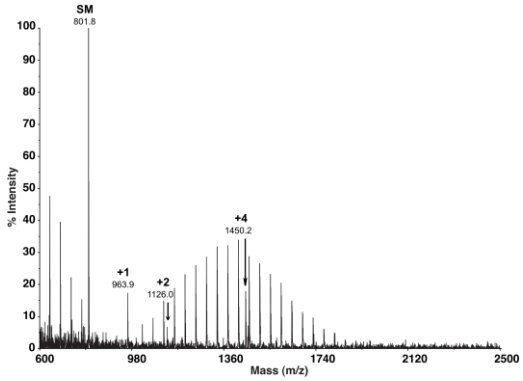


Figure 3-3. MALDI-MS spectrum of the products resulting from incubation of **39**.

SM: starting material; numbers above each peak correspond to the number of additional mannose residues incorporated into the SM. The cluster of peaks between *m/z* 1000–2000 are from Tween 80 used in the cell culture media.

3.3.2 Kinetic characterization

To better understand why **39** served as a poor substrate for ManT, compared with **4** and **9**, we analyzed the kinetics of the reaction. Due to the low activity with this substrate, however, it was difficult to obtain meaningful results from the kinetic analysis, and the results showed relatively large errors for the observed kinetic parameters. Nevertheless, an approximate K_m value of 444.9 ± 180.8 μM and k_{cat} value of 0.44 ± 0.07 μM·$min^{-1}g^{-1}$ could be calculated from the resulting data (Figure 3-4). The k_{cat} value for tetrasaccharide **39** was in the same order of magnitude as that for tetrasaccharide **9** (k_{cat} = 0.79 ± 0.04 μM·$min^{-1}g^{-1}$ cell) and only one order of magnitude lower then **4** (k_{cat} = 5.82 ± 0.16 μM·$min^{-1}g^{-1}$ cell), which implies the lack of methyl groups does not substantially influence the maximum turnover rate. However, the large observed K_m value implies only weak

binding interactions between **39** with ManT, and helps to explain the low relative activity of ManT with this substrate.

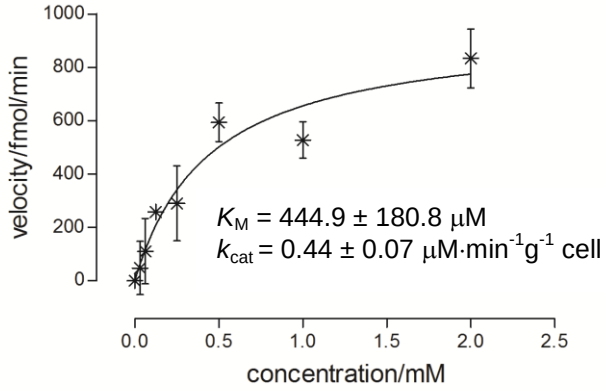


Figure 3-4. ManT kinetics of tetrasaccharide **39**.

Incorporation of radiolabeled GDP-mannose-[2-^{3}H] (4 mM) into **39** as a function of acceptor concentration. Assays were performed at 37 ℃ for 4 h, with substrate concentrations of 8, 16, 31, 63, 125, 250, 500, 1000, 2000 μM. A control experiment without the addition of acceptor was also performed in parallel. Each experiment was carried out in duplicate. The data obtained were subjected to nonlinear regression analysis using GraphPad Prism 5.0.

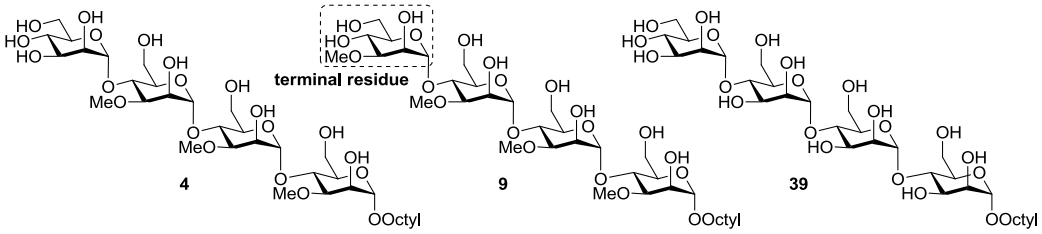
3.3.3 Comparison of three substrates with different methylation patterns

The tetrasaccharides **4**, **9** and **39**, differing only in their degree of methylation, exhibited different activities against ManT. To explain the origin of this difference, a more detailed analysis was done by comparing the, K_m, k_{cat} and k_{cat}/K_m, of the enzymatic reactions. As illustrated in Table 3-1, with an increased number of methyl groups present on the substrates (**9** has four methyl groups, **4** has three and **39** has none), the binding affinity with ManT increases. The K_m values for **9**, **4**, and **39**, are 7.9 ± 1.7 μM, 20.7 ± 2.6 μM, and 444.9 ± 180.8 μM, respectively. Of these three substrates, **4** and **9** have comparable K_m values (within

the same order of magnitude), while the K_m for **39** is significantly higher (**9**<**4**<<**39**). This suggests that the methyl group at the terminal non-reducing end does not improve binding affinity with ManT. However, the presence of methyl groups on the remaining mannose residues contributes substantially to the binding of the enzyme.

In contrast, although the methyl group on the non-reducing terminal residue is not essential for binding, its presence appears to hinder turnover. When comparing the k_{cat} values, **4** shows higher maximum turnover when compared with the fully methylated or unmethylated substrates **9** and **39**, respectively. Finally, a more than 100-fold decreased k_{cat}/K_m value of **39** relative to **4** and **9** demonstrates the poor ability of **39** to serve as substrate of ManT.

Table 3-1 Comparison of three tetrasaccharides **4**, **9** and **39**.



Substrate	K_m (μM)	V_{max} (nM•min^{-1})	k_{cat} (μM•min^{-1}g^{-1} cell)	k_{cat}/K_m (min^{-1}g^{-1} cell)
4	20.7 ± 2.6	288.1 ± 8.0	5.82 ± 0.16	0.28 ± 0.06
9	7.9 ± 1.7	45.3 ± 2.5	0.79 ± 0.04	0.10 ± 0.02
39	444.9 ± 180.8	47.4 ± 7.2	0.44 ± 0.07	0.001 ± 0.0004

3.4 Conclusions

Based on our previous studies, we proposed a model for MMP biosynthesis in which methylation of the mannose residues is not required for ManT activity. Instead we suggested that the full length mannose polymer could

be constructed before addition of the methyl groups. To probe this hypothesis, we sought to evaluate whether mannose oligomers with no methyl groups could serve as substrates for ManT. To that end, we synthesized and evaluated tetrasaccharide **39** having no methyl group substitutions. Although this compound did not serve as good substrate of ManT, it did still show turnover.

Comparison of this substrate analog with tetrasaccharides **4** and **9** provided us with important information regarding the specificity of ManT. In particular, comparing the kinetic parameters of the enzymatic reaction with **4**, **9** and **39** revealed the importance of the methyl group located on the terminal non-reducing end residue of the substrate. This methyl group does not contribute substantially to the binding between the substrate and ManT, but, if present, results in a decrease in the maximum catalytic activity of the reaction. On the other hand, methyl groups on the other non-reducing mannose residues appear to be required for activity as removing these groups, as in substrate **39**, resulted in a 100-fold decrease in activity (k_{cat}/K_m).

Based on these results, we propose that the substrate binding pocket of the ManT recognizes not only the terminal residue at the non-reducing end of the substrate but has an extended binding pocket that interacts with some of the other methylated mannose residues of the molecule. To address these possibilities, studies with OMT and α-(1→4)-linked mannose oligomers lacking methyl groups at different positions are required. Thus, access to a panel of structurally more diverse MMP analogs will be necessary to further address the activity of these enzymes.

3.5 Experimental details

General methods for chemical synthesis: All reagents were purchased from commercial sources and were used without further purification unless noted. Reaction solvents were purified by successive passage through columns of alumina and copper under an argon atmosphere. All reactions were carried out under a positive pressure of argon at room temperature unless otherwise specified and were monitored by TLC on silica gel 60-F_{254} (0.25 mm, Silicycle, Quebec, Canada). Visualization of the reaction components was achieved using UV fluorescence (254 nm) and/or by charring with acidified anisaldehyde solution in ethanol. Organic solvents were evaporated under reduced pressure and the products were purified by column chromatography on silica gel (230–400 mesh, Silicycle, Quebec, Canada) or Iatrobeads (Iatron Laboratories Inc., Tokyo) if the eluent system contained greater than 10% methanol. The yields reported are after purification. Optical rotations were measured on Perkin–Elmer 241 polarimeter at ambient temperature and are in units of degree·mL/(g·dm). ^{1}H NMR spectra were recorded at 500 or 600 MHz and chemical shifts are referenced to $CHCl_3$ (7.26 ppm), HOD (4.79 ppm). ^{13}C NMR spectra were recorded at 125 MHz and chemical shifts were referenced to $CDCl_3$ (77.1 ppm). Assignments of NMR spectra were based on two-dimensional experiments (^{1}H–^{1}H COSY, HMQC or HSQC, and HMBC) and stereochemistry of the anomeric centers of the pyranose rings were confirmed by measuring $^{1}J_{C\text{-}1,H\text{-}1}$ *via* coupled HMQC or HSQC experiments. Electrospray mass spectra were recorded on Agilent Technologies 6220 TOF.

Octyl α-D-mannopyranosyl-(1→4)-α-D-mannopyranosyl-(1→4)-α-D-mannopyranosyl-(1→4)-D-mannopyranoside (39): To a solution **40** (9.0 mg, 0.006 mmol) in MeOH (5 mL) was added NaOMe (1.0 M) until the pH of the solution was 9. After stirring at room temperature overnight, the mixture was neutralized with Amberlite IR120 H^+ ion exchange resin and then concentrated. The crude residue was purified by chromatography (CH_2Cl_2–MeOH 6:1) to afford a white solid (6.4 mg, 90%). This partially deprotected compound was then dissolved in MeOH (1 mL) before adding 20 wt. % $Pd(OH)_2$–C (10 mg). The reaction mixture was stirred overnight under a H_2 atmosphere, and then the catalyst was removed by filtration through Celite. The filtrate was concentrated and the crude residue was purified by reverse phase C_{18} chromatography (MeOH in H_2O 0→100%) to afford target tetrasaccharide **39** (3.8 mg, 94%) as a white foam. $[\alpha]_D$ = +99.4 (*c* 0.1, H_2O); ^{1}H NMR (500 MHz, D_2O) δ 5.30 (s, 1H, H-1), 5.27 (s, 1H, H-1), 5.25 (s, 1H, H-1), 4.92 (s, 1H, H-1), 4.18–4.05 (m, 3H, 3 × H-2), 4.05–3.69 (m, 22H, H-2, 4 × H-3, 4 × H-4, 4 × H-5, 8 × H-6, octyl OCH_2), 3.67–3.56 (m, 1H, octyl OCH_2), 1.81–1.57 (m, 2H, octyl $OCH_2C\underline{H}_2$), 1.55–1.22 (m, 10H, octyl CH_2), 0.95 (t, *J* = 5.6 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, D_2O) δ 102.7, 102.6, 102.5, 100.4 (4C, 4 × C-1), 76.2, 75.6, 75.2, 74.7, 73.2, 73.2, 72.2, 72.1, 71.9, 71.8, 71.7 (2C), 71.5, 71.4, 71.3, 68.5, 67.5, 62.0, 61.9 (2C), 61.9, 32.0, 29.4, 29.3, 29.1, 26.3, 23.0 (6C, octyl CH_2), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{32}H_{58}O_{21}$ $[M+Na]^+$ 801.3363, found 801.3363.

Octyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzyl-α-D-

mannopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-mannopyranoside (40): A mixture of thioglycoside **11** (12.3 mg, 0.03 mmol), alcohol **48** (17.5 mg, 0.01 mmol) and powdered 4Å molecular sieves were dissolved in CH_2Cl_2 (2.5 mL) and stirred at 0 ℃ for 1 h. Then *N*-iodosuccinimide (15.0 mg, 0.06 mmol) and silver trifluoromethanesulfonate (2.0 mg, 0.01 mmol) were added. After stirring overnight while warming to room temperature, triethylamine (1 mL) was added and the reaction mixture was filtered through Celite. The filtrate was concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 2:1) to afford **40** (9.0 mg, 41%) as a pale yellow syrup. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39–7.18 (m, 25H, Ar), 5.36 (dd, *J* = 3.2, 1.9 Hz, 1H, H-2‴), 5.30 (d, *J* = 1.7 Hz, 1H, H-1‴), 5.28–5.24 (m, 3H, H-3‴, H-4‴, H-3′), 5.22 (dd, *J* = 9.7, 3.2 Hz, 1H, H-3″), 5.18 (dd, *J* = 3.2, 2.1 Hz, 1H, H-2″), 5.15 (dd, *J* = 2.9, 2.1 Hz, 1H, H-2′), 5.07 (d, *J* = 2.0 Hz, 1H, H-1′), 5.03 (d, *J* = 2.0 Hz, 1H, H-1″), 4.83 (d, *J* = 1.8 Hz, 1H, H-1), 4.65–4.38 (m, 10H, 5 × OCH_2Ph), 4.21–4.11 (m, 4H, H-4″, H-4, H-4′, H-6a‴), 3.93 (ddd, *J* = 8.7, 5.3, 2.7 Hz, 1H, H-5‴), 3.91 (dd, *J* = 9.4, 3.0 Hz, 1H, H-3), 3.88 (dd, *J* = 12.3, 2.4 Hz, 1H, H-6b‴), 3.85–3.74 (m, 5H, H-5, H-5′, H-5″, H-6a, H-6b), 3.72–3.65 (m, 4H, H-2, H-6a″, H-a′, octyl OCH_2), 3.63 (dd, *J* = 11.4, 3.5 Hz, 1H, H-6a′), 3.51 (dd, *J* = 11.3, 1.7 Hz, 1H, H-6b″), 3.45 (dd, *J* = 11.3, 1.5 Hz, 1H, H-6b′), 3.40–3.32 (m, 1H, octyl OCH_2), 2.12 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.03 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.97 (s, 3H, Ac), 1.67–1.57 (m, 2H, octyl OCH_2CH_2), 1.30–1.25 (m, 10H, octyl CH_2), 0.87 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 177.9, 177.7, 177.6, 170.6, 170.2, 169.9, 169.7, 169.6 (8C, 8 × OAc),

138.7, 138.6, 138.4, 138.2, 138.1, 128.3, 128.2, 128.0, 127.6, 127.5, 127.4, 127.2 (30C, Ar), 99.4 (C-1‴, $^1J_{C\text{-}1,H\text{-}1}$ = 173.8 Hz), 99.2 (2C, C-1′, $^1J_{C\text{-}1,H\text{-}1}$ = 173.2 Hz; C-1″, $^1J_{C\text{-}1,H\text{-}1}$ = 174.6 Hz), 97.5 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 168.4 Hz), 80.3 (C-3), 74.7 (C-2), 74.3 (C-4), 73.4 (2C, 2 × OCH_2Ph), 73.2 (C-4″), 73.1 (OCH_2Ph), 72.9 (C-4′), 72.5 (OCH_2Ph), 71.9 (3C, C-3′, C-5′, C-5″), 71.9 (OCH_2Ph), 71.2 (C-3″), 71.0 (C-5), 69.9 (2C, C-2″, C-2‴), 69.7 (C-6), 69.6 (C-2′), 69.5 (C-5‴), 68.7 (C-6′), 68.7 (C-3‴), 68.6 (C-6″), 67.9 (octyl OCH_2), 65.9 (C-4‴), 62.3 (C-6‴), 31.9, 29.6, 29.4, 29.2, 26.1, 22.7 (6C, octyl CH_2), 21.1, 20.8, 20.8, 20.7, 20.6 (8C, 8 × Ac), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{83}H_{104}O_{29}$ $[M+Na]^+$ 1587.6555, found 1587.6533.

***p*-Tolyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-1-thio-α-D-mannopyranoside (41)**: Thioglycoside **11** (1.93 g, 4.26 mmol) was dissolved in CH_2Cl_2 (3 mL) and MeOH (15 mL). NaOMe (1.0 M) was added until the pH of the solution was 9. After stirring at room temperature for 6 h, the mixture was neutralized with Amberlite IR120 H^+ ion exchange resin and then concentrated. This crude product was then dissolved in DMF (6 mL) cooled to 0 °C and to this solution was added $PhCH(OMe)_2$ (0.7 mL, 4.26 mmol) followed by HBF_4 (54% in Et_2O, 0.5 mL, 3.41 mmol). After stirring overnight while warming to room temperature, Et_3N (2 mL) was added and the mixture was concentrated. The crude residue was purified by chromatography (hexane–EtOAc 2:1) to afford a white solid (3.5 g). Acetylation of this product was carried out in pyridine (6 mL) with acetic anhydride (2 mL, 21.3 mmol) and 4-dimethylaminopyridine (97.0 mg, 0.85 mmol). After stirring at room temperature overnight, the reaction mixture was diluted with CH_2Cl_2 and

washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the crude residue was purified by chromatography (hexane–EtOAc 4:1) to afford **41** (1.07 g, 55% over three steps) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.54 (dd, *J* = 7.6, 1.9 Hz, 2H, Ar), 7.50–7.23 (m, 5H, Ar), 7.17 (d, *J* = 8.0 Hz, 2H, Ar), 5.66 (dd, *J* = 3.4, 1.4 Hz, 1H, H-2), 5.64 (s, 1H, PhCH), 5.49 (dd, *J* = 10.4, 3.4 Hz, 1H, H-3), 5.42 (d, *J* = 1.2 Hz, 1H, H-1), 4.53 (app td, *J* = 9.9, 4.9 Hz, 1H, H-5), 4.30 (dd, *J* = 10.4, 4.9 Hz, 1H, H-6a), 4.18 (app t, *J* = 10.0 Hz, 1H, H-4), 3.91 (app t, *J* = 10.3 Hz, 1H, H-6b), 2.37 (s, 3H, ArC$\underline{H}_3$), 2.20 (s, 3H, Ac), 2.08 (s, 3H, Ac); ^{13}C NMR (126 MHz, $CDCl_3$) δ 169.8 (OAc), 169.8 (OAc), 138.5 (1C, Ar), 137.1 (1C, Ar), 132.9 (2C, Ar), 130.1 (2C, Ar), 129.2 (1C, Ar), 129.0 (1C, Ar), 128.33 (2C, Ar), 126.30 (2C, Ar), 102.0 (PhCH), 87.2 (C-1), 76.3 (C-4), 71.5 (C-2), 68.6 (C-3), 68.5 (C-6), 65.2 (C-5), 21.2 (Ar$\underline{C}H_3$), 20.9 (Ac), 20.8 (Ac); HRMS (ESI) calcd $C_{24}H_{26}O_7S$ $[M+Na]^+$ 481.1291, found 481.1289.

Octyl 2,3,6-tri-*O*-benzyl-α-D-mannopyranoside (42): Benzylidene acetal **46** (32.9 mg, 0.06 mmol) and $BH_3{\cdot}NMe_3$ (30.0 mg, 0.38 mmol) were dissolved in THF (1 mL) and cooled to 0 ℃ before an ice-cold solution of $AlCl_3$ (45.0 mg, 0.25 mmol) in THF (1 mL) was added followed by H_2O (2 μL, 0.11 mmol). After stirring overnight while warming to room temperature, the solvent was evaporated and the crude residue was purified by chromatography (hexane–EtOAc 6:1) to afford **42** (19.7 mg, 60%) as a colorless syrup. ^{1}H NMR (498 MHz, $CDCl_3$) δ 7.45–7.19 (m, 15H, Ar), 4.87 (d, *J* = 1.5 Hz, 1H, H-1), 4.74–4.49 (m, 6H, 3 × OCH_2Ph), 4.05 (app td, *J* = 9.2, 1.5 Hz, 1H, H-4), 3.84–3.71 (m, 5H, H-2, H-3, H-

5, H-6a, H-6b), 3.67 (dt, J = 9.6, 6.9 Hz, 1H, octyl OCH_2), 3.38 (dt, J = 9.6, 6.5 Hz, 1H, octyl OCH_2), 2.51 (d, J = 1.6 Hz, 1H, OH-4), 1.69–1.45 (m, 2H, octyl OCH_2CH_2), 1.41–1.19 (m, 10H, octyl CH_2), 0.89 (t, J = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.3 (1C, Ar), 138.3 (1C, Ar), 138.2 (1C, Ar), 128.4 (2C, Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 127.8 (2C, Ar), 127.7 (3C, Ar), 127.6 (1C, Ar), 127.6 (2C, Ar), 127.5 (1C, Ar), 98.0 (C-1), 79.7 (C-3), 74.1 (C-2), 73.5 (OCH_2Ph), 72.6 (OCH_2Ph), 71.8 (OCH_2Ph), 71.4 (C-5), 70.5 (C-6), 68.0 (C-4), 67.7 (octyl OCH_2), 31.8, 29.4, 29.4, 29.2, 26.1, 22.7 (octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{35}H_{46}O_6$ $[M+Na]^+$ 585.3187, found 585.3181.

***p*-Tolyl 2,3-di-*O*-acetyl-6-*O*-benzyl-1-thio-α-D-mannopyranoside (43)**: Benzylidene acetal **39** (490.0 mg, 1.18 mmol) and $BH_3 \cdot NMe_3$ (365 mg, 4.72 mmol) were dissolved in THF (10 mL) and cooled to 0 ℃ before an ice-cold solution of $AlCl_3$ (1.0 g, 7.08 mmol) in THF (5 mL) was added followed by H_2O (20 μL, 1.11 mmol). After stirring overnight while warming to room temperature, the solvent was evaporated and the crude residue was redissolved in CH_2Cl_2 and washed with brine. The organic layer was dried over Na_2SO_4, concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 5:2) to afford **43** (346.5 mg, 70%) as a colorless syrup. ^1H NMR (500 MHz, $CDCl_3$) δ 7.43–7.33 (m, 7H, Ar), 7.09 (d, J = 7.9 Hz, 2H, Ar), 5.50 (dd, J = 3.3, 1.6 Hz, 1H, H-2), 5.41 (d, J = 1.4 Hz, 1H, H-1), 5.21 (dd, J = 9.9, 3.3 Hz, 1H, H-3), 4.66 (d, J = 11.9 Hz, 1H, OCH_2Ph), 4.57 (d, J = 11.9 Hz, 1H, OCH_2Ph), 4.41 (app dt, J = 9.3, 4.5 Hz, 1H, H-5), 4.11 (app td, J = 9.8, 3.8 Hz, 1H, H-4), 3.87 (dd, J = 10.4, 4.9 Hz, 1H, H-6a), 3.82 (dd, J = 10.4, 4.2 Hz, 1H, H-6b), 2.72 (d, J = 4.1 Hz, 1H,

OH-4), 2.33 (s, 3H, ArC$\underline{H}_3$), 2.13 (s, 3H, Ac), 2.11 (s, 3H, Ac); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.7 (OAc), 167.0 (Ac), 138.1 (1C, Ar), 137.8 (1C, Ar), 132.6 (2C, Ar), 129.9 (2C, Ar), 129.4 (1C, Ar), 128.4 (2C, Ar), 127.8 (1C, Ar), 127.7 (2C, Ar), 86.3 (C-1), 73.7 (OCH_2Ph), 72.1 (C-3), 72.0 (C-5), 71.2 (C-2), 70.0 (C-6), 67.5 (C-4), 21.1 (Ar$\underline{C}H_3$), 20.9 (Ac), 20.8 (Ac); HRMS (ESI) calcd $C_{24}H_{28}O_7S$ $[M+Na]^+$ 483.1448, found 483.1446.

***p*-Tolyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzyl-1-thio-α-D-mannopyranoside (45)**: To a solution of **43** (278.0 mg, 0.60 mmol) and **44** (300.0 mg, 0.60 mmol) in CH_2Cl_2 with 4Å molecular sieves at −60 °C was added TMSOTf (10% v/v, 0.5 mL, 0.28 mmol). The reaction mixture was stirred for 3 h while warming to −20 °C. After Et_3N was added, the mixture was filtered through Celite and the filtrate was concentrated. The crude product was purified by chromatography (hexane–EtOAc 2:1) to afford **45** (148.2 mg, 32%) as a white foam. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53–7.27 (m, 12H, Ar), 7.11 (d, *J* = 8.0 Hz, 2H, Ar), 5.60 (s, 1H, PhCH), 5.58 (dd, *J* = 3.1, 1.6 Hz, 1H, H-2), 5.45 (d, *J* = 1.4 Hz, 1H, H-1), 5.41 (dd, *J* = 9.8, 3.4 Hz, 1H, H-3′), 5.37–5.33 (m, 2H, H-3, H-2′), 5.10 (d, *J* = 1.6 Hz, 1H, H-1′), 4.66 (d, *J* = 11.8 Hz, 1H, OCH_2Ph), 4.59 (d, *J* = 11.8 Hz, 1H, OCH_2Ph), 4.49 (dd, *J* = 9.7, 2.5 Hz, 1H, H-5), 4.31 (app t, *J* = 9.7 Hz, 1H, H-4), 4.17 (dd, *J* = 10.4, 4.4 Hz, 1H, H-6a′), 4.13–4.03 (m, 2H, H-4′, H-5′), 3.96 (dd, *J* = 11.2, 4.1 Hz, 1H, H-6a), 3.85–3.76 (m, 2H, H-6b, H-6b′), 2.34 (s, 3H, ArC$\underline{H}_3$), 2.20 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.05 (s, 3H, Ac); ^{13}C NMR (125 MHz, $CDCl_3$) δ 167.0, 169.9, 169.8, 169.7 (4C, 4 × OAc), 138.2 (1C, Ar), 138.1 (1C, Ar), 137.1 (1C, Ar), 132.6

(2C, Ar), 129.9 (2C, Ar), 129.3 (1C, Ar), 129.2 (1C, Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 127.5 (1C, Ar), 127.5 (2C, Ar), 126.3 (2C, Ar), 102.0 (PhCH), 100.4 (C-1′, $^{1}J_{C\text{-}1,H\text{-}1}$ = 172.3 Hz), 85.9 (C-1, $^{1}J_{C\text{-}1,H\text{-}1}$ = 169.1 Hz), 76.0 (C-4′), 73.8 (C-4), 73.5 (OCH_2Ph), 72.0 (C-5), 71.9 (C-2), 71.3 (C-3), 70.3 (C-2′), 69.0 (C-6), 68.5 (C-6′), 68.0 (C-3′), 65.0 (C-5′), 21.2 ($ArCH_3$), 20.9 (Ac), 20.9 (Ac), 20.8 (Ac), 20.7 (Ac); HRMS (ESI) calcd $C_{41}H_{46}O_{14}S$ $[M+Na]^+$ 817.2500, found 817.2499.

Octyl 2,3-di-*O*-benzyl-4,6-*O*-benzylidene-1-thio-α-D-mannopyranoside (46): To a solution of **1** (49.7 mg, 0.17 mmol) in DMF (1 mL) at 0 °C was added $PhCH(OMe)_2$ (30 μL, 0.20 mmol) followed by HBF_4 (54% in Et_2O, 20 μL, 0.15 mmol). After stirring overnight while warming to room temperature, the mixture was cooled down 0 °C and NaH (60% in mineral oil, 187.0 mg, 4.7 mmol) was added followed by benzyl bromide (0.22 mL, 1.8 mmol). After stirring overnight while warming to room temperature, MeOH (2 mL) was added and the solution was concentrated. The crude residue was purified by chromatography (hexane–EtOAc 15:1) to afford **46** (36.6 mg, 38% over two steps) as a colorless syrup. NMR data are consistent as reported.[15] ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.64–7.19 (m, 15H, Ar), 5.68 (s, 1H, PhCH), 4.80 (d, *J* = 1.5 Hz, 1H, H-1), 4.91–4.66 (m, 4H, 2 × OCH_2Ph), 4.30–4.24 (m, 2H, H-4, H-6a), 4.00 (dd, *J* = 10.0, 3.2 Hz, 1H, H-3), 3.91 (app t, *J* = 10.3 Hz, 1H, H-6b), 3.85 (dd, *J* = 3.2, 1.6 Hz, 1H, H-2), 3.85–3.79 (m, 1H, H-5), 3.65 (dt, *J* = 9.6, 6.8 Hz, 1H, octyl OCH_2), 3.37 (dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 1.65–1.46 (m, 2H, OCH_2CH_2), 1.43–1.22 (m, 10H, CH_2), 0.91 (t, *J* = 7.0 Hz, 3H, CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.8 (1C, Ar), 138.2 (1C, Ar), 137.7 (1C, Ar), 128.8 (1C, Ar), 128.4 (2C, Ar), 128.3 (2C,

Ar), 128.2 (2C, Ar), 128.1 (2C, Ar), 127.7 (1C, Ar), 127.5 (2C, Ar), 127.4 (1C, Ar), 126.1 (2C, Ar), 101.4 (PhCH), 99.4 (C-1), 79.3 (C-4), 76.6 (2C, C-2, C-3), 73.6 (OCH_2Ph), 73.2 (OCH_2Ph), 68.9 (C-6), 67.9 (octyl OCH_2), 64.2 (C-5), 31.8, 29.4, 29.4, 29.4, 29.2, 26.1, 22.7 (6C, octyl CH_2), 14.1 (octyl CH_3).

Octyl 2,3-di-*O*-acetyl-4,6-*O*-benzylidene-α-D-mannopyranosyl-(1→4)-2,3-*di*-*O*-acetyl-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-2,3,6-*tri*-*O*-benzyl-α-D-mannopyranoside (47): Thioglycoside **45** was converted into imidate in two steps. First, to an ice-cold solution of **45** (57.6 mg, 0.09 mmol) in acetone and water (2 mL, v/v 4:1) was added *N*-bromosuccinimide (215 mg, 1.20 mmol). After stirring for 1 h, the reaction mixture was concentrated and purified by chromatography (hexane–EtOAc 3:2) to give the corresponding reducing sugar. Then, to this compound in ice-cold CH_2Cl_2 (2 mL) was added Cs_2CO_3 (140.0 mg, 0.43 mmol) and CCl_3CN (0.1 mL, 0.98 mmol). After stirring for 1 h at 0 °C, the mixture was filtered through Celite and concentrated to afford the crude imidate, which was used without further purification. The imidate donor was stirred with acceptor **42** (19.7 mg, 0.03 mmol) in ice-cold CH_2Cl_2 with 4Å molecular sieves for 1 h, and then cooled down to –60 °C before adding TMSOTf (10% v/v, 20 μL, 0.01 mmol). After stirring for 2 h while warming to room temperature, Et_3N (0.5 mL) was added and the mixture was filtered through Celite. The filtrate was concentrated and purified by chromatography (hexane–EtOAc 5:2) to afford trisaccharide **47** (24.2 mg, 56%) as a pale yellow syrup. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.65–7.13 (m, 25H, Ar), 5.58 (s, 1H, PhCH), 5.42–5.36 (m, 2H, H-3″, H-2′), 5.35 (d, *J* = 1.6 Hz, 1H, H-1′), 5.29–5.23 (m, 2H, H-2″, H-3′), 5.02 (d, *J* =

1.5 Hz, 1H, H-1″), 4.87 (d, *J* = 1.5 Hz, 1H, H-1), 4.72–4.40 (m, 6H, 3 × OCH_2Ph), 4.18 (app t, *J* = 9.5 Hz, 1H, H-4), 4.17 (app t, *J* = 9.8 Hz, 1H, H-4′), 4.09 (dd, *J* = 10.2, 4.7 Hz, 1H, H-6a″), 4.04 (app t, *J* = 9.9 Hz, 1H, H-4″), 3.99–3.91 (m, 2H, H-3, H-5″), 3.85 (ddd, *J* = 9.5, 5.3, 1.5 Hz, 1H, H-5), 3.82–3.69 (m, 6H, H-2, H-5′, H-6a, H-6b, H-6b″, octyl OCH_2), 3.55 (dd, *J* = 11.3, 3.0 Hz, 1H, H-6a′), 3.43–3.33 (m, 2H, H-6b′, octyl OCH_2), 2.17 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.94 (s, 3H, Ac), 1.70–1.56 (m, 2H, octyl OCH_2CH_2), 1.40–1.26 (m, 10H, octyl CH_2), 0.93 (t, *J* = 7.1 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.1, 169.7, 169.6, 169.6 (4C, 4 × OAc), 138.6, 138.5, 138.4, 138.1, 137.2, 129.1, 128.3, 128.3, 128.3, 128.2, 127.9, 127.6, 127.5, 127.5, 127.4, 127.4, 126.2 (30C, Ar), 102.0 (PhCH), 100.2 (C-1″, $^1J_{C\text{-}1,H\text{-}1}$ = 172.3 Hz), 99.3 (C-1′, $^1J_{C\text{-}1,H\text{-}1}$ = 172.7 Hz), 97.6 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 168.7 Hz), 80.4 (C-3), 76.1 (C-4″), 74.6 (C-2), 74.0 (C-4), 73.6 (OCH_2Ph), 73.1 (OCH_2Ph), 73.0 (C-4′), 72.5 (OCH_2Ph), 72.0 (C-3′), 71.7 (OCH_2Ph), 71.7 (C-5′), 70.9 (C-5), 70.3 (C-2″), 69.9 (C-2′), 69.6 (C-6), 68.6, 68.5 (2C, C-6′, C-6″), 68.0 (C-3″), 67.9 (octyl OCH_2), 64.8 (C-5″), 31.9, 29.5, 29.4, 29.3, 26.2, 22.7 (6C, octyl CH_2), 20.9, 20.8, 20.8, 20.7 (4C, 4 × Ac), 14.2 (octyl CH_3); HRMS (ESI) calcd $C_{69}H_{84}O_{20}$ $[M+Na]^+$ 1255.5448, found 1255.5442.

Octyl 2,3-di-*O*-acetyl-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-2,3-di-*O*-acetyl-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-2,3,6-tri-*O*-benzyl-α-D-mannopyranoside (48): Benzylidene acetal **47** (24.2 mg, 0.04 mmol) and $BH_3 \cdot NMe_3$ (25.0 mg, 0.21 mmol) were dissolved in THF (1.5 mL) and cooled to 0 ℃ before an ice-cold solution of $AlCl_3$ (20.0 mg, 0.14 mmol) in THF (1 mL) was added followed by H_2O (2 μL, 0.11 mmol). After stirring overnight while

warming to room temperature, the solvent was evaporated and the crude residue was purified by chromatography (hexane–EtOAc 3:2) to afford **48** (18.9 mg, 78%) as a colorless syrup. ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43–7.15 (m, 25H, Ar), 5.37 (dd, *J* = 3.1, 1.9 Hz, 1H, H-2′), 5.33 (d, *J* = 1.7 Hz, 1H, H-1′), 5.27 (dd, *J* = 9.8, 3.2 Hz, 1H, H-3′), 5.17–5.11 (m, 2H, H-2″, H-3″), 5.08 (d, *J* = 1.4 Hz, 1H, H-1″), 4.86 (d, *J* = 1.7 Hz, 1H, H-1), 4.70–4.37 (m, 10H, 5 × OCH_2Ph), 4.18 (app t, *J* = 9.9 Hz, 1H, H-4), 4.16 (app t, *J* = 9.4 Hz, 1H, H-4′), 4.04 (app td, *J* = 9.8, 3.9 Hz, 1H, H-4″), 3.94 (dd, *J* = 9.3, 3.0 Hz, 1H, H-3), 3.86–3.83 (m, 1H, H-5), 3.83–3.75 (m, 4H, H-5′, H-5″, H-6a, H-6b), 3.75–3.68 (m, 2H, H-2, octyl OCH_2), 3.65 (dd, *J* = 10.2, 4.2 Hz, 1H, H-6a″), 3.62 (dd, *J* = 11.4, 3.6 Hz, 1H, H-6a′), 3.52 (dd, *J* = 10.2, 4.5 Hz, 1H, H-6b″), 3.44 (dd, *J* = 11.2, 1.4 Hz, 1H, H-6b′), 3.38 (dt, *J* = 9.6, 6.8 Hz, 1H, octyl OCH_2), 2.57 (d, *J* = 3.9 Hz, 1H, OH-4″), 2.10 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.03 (s, 3H, Ac), 1.94 (s, 3H, Ac), 1.67–1.57 (m, 2H, octyl OCH_2CH_2), 1.40–1.25 (m, 10H, octyl CH_2), 0.92 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, $CDCl_3$) δ 170.5, 170.1, 169.8, 169.5 (4C, 4 × OAc), 138.6, 138.6, 138.3, 138.1, 137.7, 128.4, 128.4, 128.2, 128.2, 127.9, 127.8, 127.6, 127.6, 127.5, 127.4, 127.4, 127.3 (30C, Ar), 99.3 (C-1′) , 99.1 (C-1″), 97.5 (C-1), 80.3 (C-3), 74.6 (C-2), 74.1 (C-4), 73.6, 73.4, 73.1, 72.5 (4C, 4 × OCH_2Ph), 72.3 (C-4′), 72.1 (C-3′), 71.8 (2C, C-5′, C-5″), 71.7 (OCH_2Ph), 71.4 (C-3″), 71.0 (C-5), 69.9 (C-6″), 69.9 (2C, C-2′, C-2″), 69.7 (C-6), 68.7 (C-6′), 67.9 (octyl OCH_2), 67.2 (C-4″), 31.9, 29.4, 29.4, 29.2, 26.1, 22.7 (6C, octyl CH_2), 20.9, 20.8, 20.8, 20.7 (4C, 4 × Ac), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{69}H_{86}O_{20}$ $[M+Na]^+$ 1257.5605, found 1257.5602.

Growth of bacteria strains: *M. smegmatis* strain ATCC 14468 was grown at 37 °C for 48 h in a medium containing yeast extract (3 g), peptone (5 g), glycerol (20 g) and Tween 80 (2 mL) in Milli-Q water (1 L). Cells were harvested by centrifugation and stored at –20 ℃ until use.

Preparation of ManT fraction for radioactive assay: All of the following steps were carried out at 0 ℃ on ice. Wet cell pellets (6.1 g from 1 L culture) of *M. smegmatis* was resuspended in 50 mM TAPS buffer (24 mL, containing 10 mM $MgCl_2$, pH 8.2) and supplemented with one complete protease inhibitor cocktail tablet (Roche). Cell lysis was performed by passing through a cell disruptor three times at 24 Kpsi and then the cell debris was removed by centrifugation at 20,000 × g for 30 min. The obtained cell lysate was further centrifuged at 200,000 × g for 1 h. The bottom brown pellet was resuspended in 50 mM TAPS buffer (1 mL, containing 10 mM $MgCl_2$, 5 mM EGTA, pH 8.2) and was used as the ManT source.

Radioactive enzymatic assay: Tetrasaccharide **39** was incubated with ManT using the same protocols described for tetrasaccharides **4** and **9** in Chapter 2. Tetrasaccharides **4** and **9** were incubated simultaneously with **39** to compare their relative activities. Each assay contained GDP-mannose (20 mM, 1μL) with guanosine diphosphate mannose-[2-^{3}H] (0.04 μCi, American Radiolabeled Chemicals, Inc.), acceptor (20 mM, 1 μL) and the ManT source (18 μL), in a total volume of 20 μL. All assays were performed in duplicate and control assays without acceptors were performed in parallel to correct for activity arising from

endogenous acceptors present in the membrane fraction. Enzymatic products were purified with a C_{18} Sep-Pak cartridge (Waters). The enzymatic products eluted with MeOH (4 mL) were mixed with Ecolite cocktail (10 mL) and counted on a scintillation counter. The raw dpm (decay per minute) values obtained from scintillation counter were used to calculate the enzymatic activity (result shown in Figure 3-2.).

MALDI-MS analysis of the enzymatic products from 39: Tetrasaccharide was incubated as above, expect non-radiolabeled GDP-mannose was used. The enzymatic products were purified by C_{18} Sep-Pak cartridge (Waters) and analyzed by MALDI-MS using 2,5-dihydroxybenzoic acid as the matrix (results are shown in Figure 3-3).

Kinetic characterization of 39: 1.0 mL fresh ManT fraction obtained from 6.8 g wet cell pellets (from 0.7 L culture) was used. ManT activities were determined by using fixed donor concentration (4 mM GDP-mannose) and various acceptor concentrations (8, 16, 31, 63, 125, 250, 500, 1000, 2000 μM). Each assay has donor (40 mM, 2 μL, contain 0.15 μL radioactive GDP-mannose-[2-^{3}H]), acceptor (2μL) and enzyme (16 μL) in a total volume of 20 μL. Assays were incubated 4 h at 37 °C. A control experiment without the addition of the acceptor was also performed in parallel. Each experiment was done in duplicate. The kinetic parameters K_m and V_{max} were obtained from the Michaelis–Menten equation, by nonlinear regression analysis with GraphPad Prism 5.0 software (result shown in Figure 3-4).

Calculation of V_{max} and k_{cat}/K_m for 39:

K_m = 444.9 ± 180.8 μM

V_{max} = (947.1 ± 144.0 fmol·min^{-1})/ 20 μL total volume = 47.4 ± 7.2 nM·min^{-1}

[enzyme amount] = 6.8 g cell/1 mL buffer × 16 μL = 108.8 mg cell in each assay

k_{cat} = V_{max}/[E] = 0.44 ± 0.07 μM·min^{-1}g^{-1} cell

k_{cat}/K_m = 0.001 ± 0.0004 min^{-1}g^{-1} cell

3.6 Bibliography

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Chapter 4: Regioselective Polymethylation of α-(1→4)-linked Mannopyranose Oligosaccharides

4.1 Introduction

Total synthesis plays an important role in providing compounds that can be used for understanding the biological role of complex natural products. However, this approach is generally laborious and time-consuming. To overcome this problem, semisynthetic approaches can provide a more rapid solution by using readily available natural product skeletons as templates.[1-2] A recent example of semi-synthesis was MacMillan and coworker's installation of a trifluoromethyl moiety onto the drug Lipitor, via direct C–H functionalization.[3]

Although appealing, direct and selective functionalization of carbohydrates, particularly glycans more complicated than monosaccharides, is difficult. Although it is often possible to exploit steric differences to selectively functionalize primary over secondary carbohydrate alcohols, differentiating secondary hydroxyl groups on oligosaccharides that are larger than monosaccharides is challenging.[4-5]

Regioselective acylations have been successfully carried out directly on unprotected disaccharides, *e.g.*, sucrose **49**[6] and lactose **50**[7] (Figure 4-1), using organotin reagents. However, further functionalization of these molecules is often difficult, as the introduced acyl groups are base-labile and thus prone to migrate under the basic conditions typically used in alkylation reactions.[8] In another example, sulfate groups have been introduced onto unprotected oligosaccharides, however, in low yield, *e.g.*, xylose trisaccharide **51** (Figure 4-1).[9] On the other hand, regioselective alkylation reactions, which require harsher conditions than acylations, appear to be even more challenging to carry out on large

oligosaccharides. Indeed, only limited examples report the regioselective alkylation of unprotected oligosaccharides, *e.g.*, **52**,[10] **53**,[11] **54**[12] (Figure 4-1). The regioselectivities of the reactions leading to **52** and **53** were achieved by deprotonating the most acidic hydroxyl groups with strong bases, and lactoside **54** was obtained *via* the organotin-mediated selective activation of vicinal diols. These methods are generally substrate-specific; thus, their applications are limited.

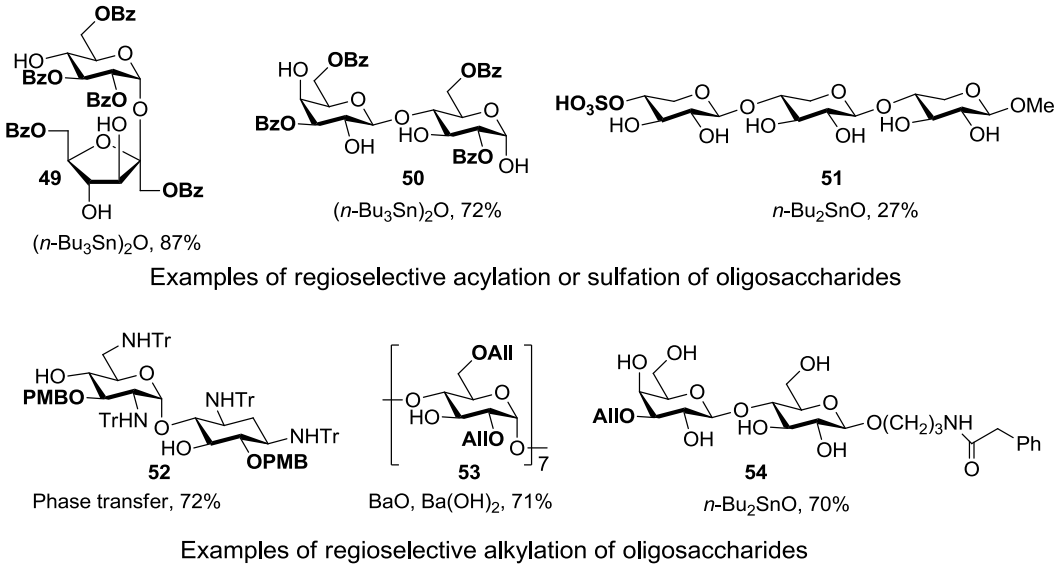


Figure 4-1. Examples of regioselective functionalization of oligosaccharides.

During studies of the α-(1→4)-mannosyltransferase involved in the biosynthesis of 3-*O*-methyl-mannose polysaccharides (MMPs),[13] we had the need to access a panel of α-(1→4)-linked mannopyranose oligosaccharides with or without methyl groups (**A** and **B**, Figure 4-2). Although these analogs are structurally similar, traditional routes for their synthesis require different building blocks, or multiple functional group interconversions on oligosaccharides. For example, to obtain tetrasaccharides of both analogs (n = 2 for both **A** and **B**), 15

steps are required for **A** and 24 steps for **B** in Figure 4-2. We were interested to see whether it was possible to access **B** *via* functionalization of **A**. We report here our studies towards achieving this goal.

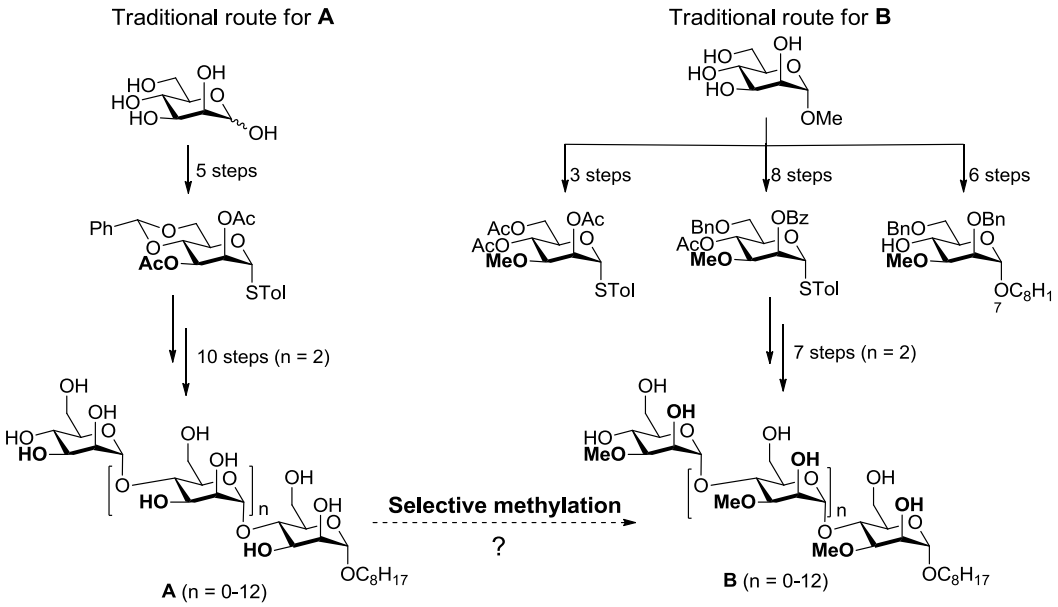
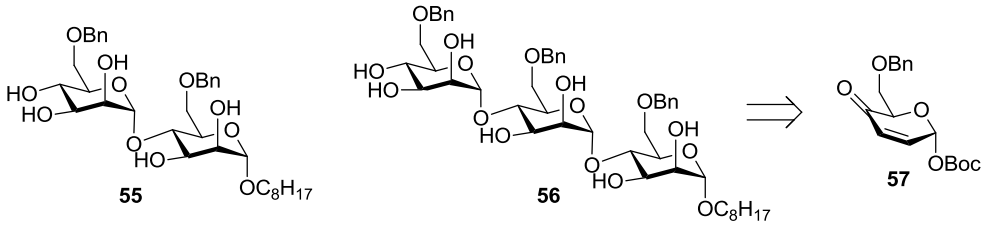


Figure 4-2. Conventional methods to synthesize analogs **A** and **B**.

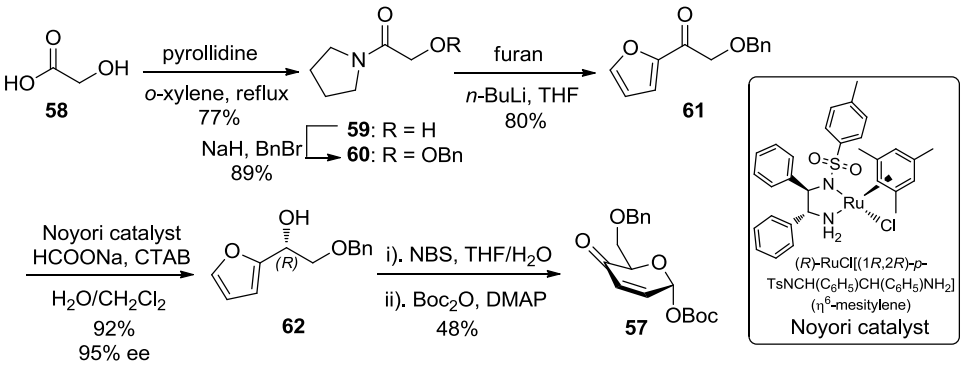
4.2 Attempt of regioselective alkylation of oligosaccharides

We first investigated the selective methylation of α-(1→4)-linked mannopyranose disaccharide **55** and trisaccharide **56** (Scheme 4-1). These substrates were synthesized from pyranone 57[14] using the Pd-catalyzed iterative glycosylation methodology developed by O'Doherty and coworkers.[15]



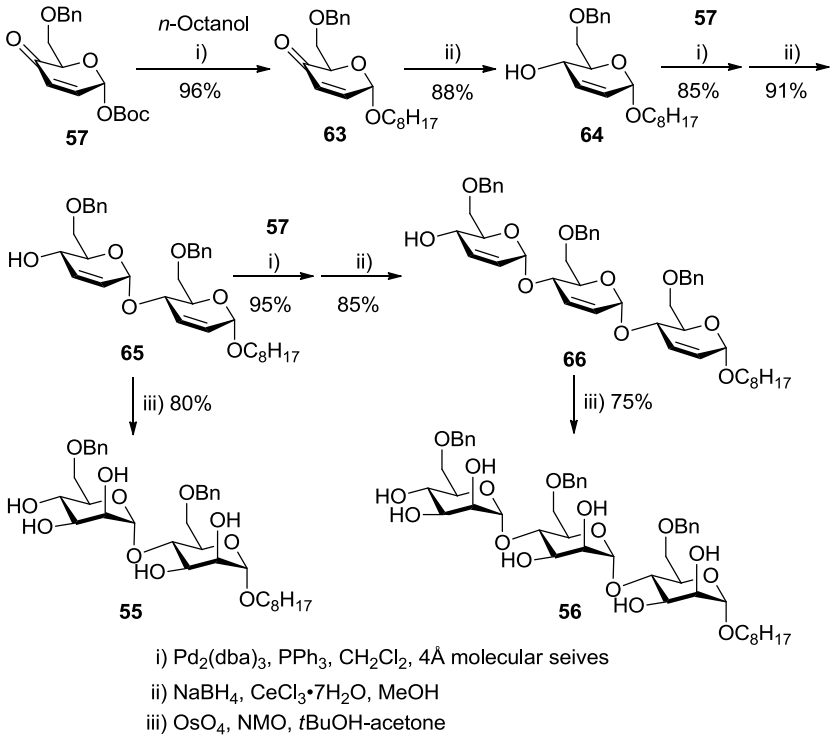
Scheme 4-1. Retrosynthetic analysis for disaccharide **55** and trisaccharide **56**.

Pyranone **57** was prepared according to the reported method with revision of the protecting groups (Scheme 4-2).[14] The synthesis started with glycolic acid **58**, which was converted to amide **59** via heating at reflux with pyrrolidine. The hydroxyl group was protected as benzyl ether, giving **60** in 69% yield over the two steps. Nucleophilic addition of 2-lithiofuran onto **60** produced acylfuran **61** in 80% yield. The ketone **61** then underwent asymmetric hydrogenation in the presence of Noyori's catalyst[16] to furnish alcohol **62** in 92% yield and 95% enantioselectivity. To determine the enantiomeric purity of the reaction, the alcohol **62** and its enantiomer were reacted with (*S*)-+-O-acetyl mandelic acid to produce a pair of diasteromers.[17] Their ratio was analyzed using ^{1}H NMR spectroscopy by integrating the methylene protons of the benzyl ethers in the corresponding diastereomers. Treatment of furan alcohol **62** with *N*-bromosuccinimide generated an Achmatowicz-rearrangement adduct, which was further protected with di-*t*-butyl dicarbonate furnishing pyranone **57** in 48% yield over the two steps.



Scheme 4-2. Synthesis of pyranone **57**.

Pd-catalyzed glycosylation[15] was then employed to couple pyranone **57** with octanol (Scheme 4-3). The generated ketone **63** was stereoselectively reduced by treating with sodium borohydride in presence of cerium trichloride (Luche reduction),[18] producing an 84% yield of alcohol **64** over the two steps. The two reactions, Pd-catalyzed glycosylation and Luche reduction, were used iteratively to produce disaccharide and trisaccharide precursors **65** and **66**, respectively. Stereoselective dihydroxylation of **65** and **66** with osmium tetroxide[19] provided the desired disaccharide **55** and trisaccharide **56** in excellent overall yields (Scheme 4-3).

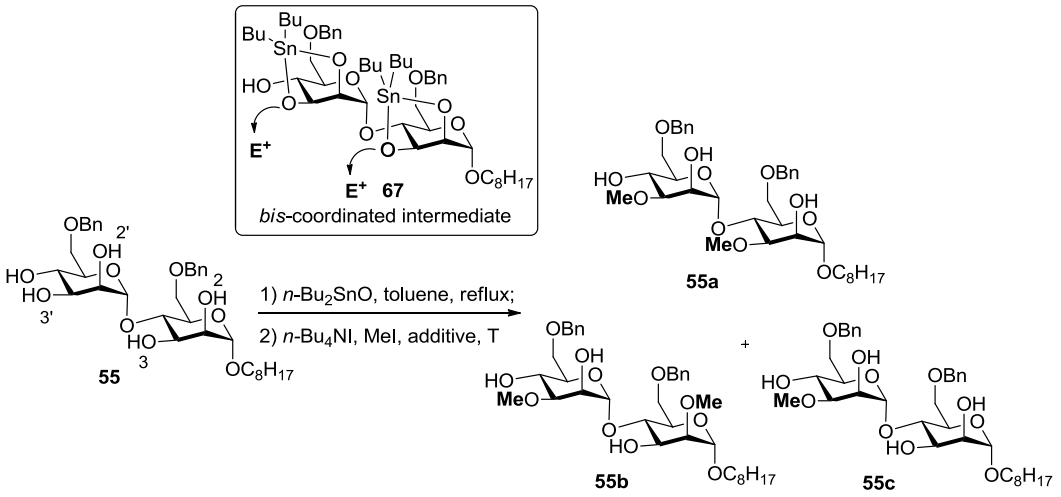


Scheme 4-3. Synthesis of disaccharide **55** and trisaccharide **56**.

After obtaining both model compounds **55** and **56**, we started to investigate the possibility of direct introduction of alkyl groups onto these

substrates. We first tested this idea on disaccharide **55**. Because *n*-Bu_2SnO has been reported to selectively activate the equatorial hydroxyl groups of *cis*-diols via a cyclic stannylene acetal,[20] we anticipated that using two equivalents of this reagent would result in formation of a bis-coordinated intermediate **67** from disaccharide **55**. Subsequent addition of the alkylating reagent would lead to the formation of the di-substituted product **55a** (Table 4-1), with alkylation at both equatorial positions (O-3 and O-3′).

Table 4-1. Direct regioselective alkylation of disaccharide **55**.[a]



Entry	T (℃)	Additive	Products distribution (NMR yield[b])		Recovered **55**
			disubstituted	monosubstituted	
1	40	-	< 5%	15% mixture	80%
2	70	-	**55a** (19%), **55b** (3%)	**55c** (35%), 15% other isomers	28%
3	40	CsF	**55a** (29%), **55b** (45%), 26% other isomers	-	-

a. Reaction conditions: **55** (1 equiv), *n*-Bu_2SnO (2 equiv), *n*-Bu_4NI (2 equiv), MeI (20 equiv), additive (4 equiv), toluene (0.01 M);

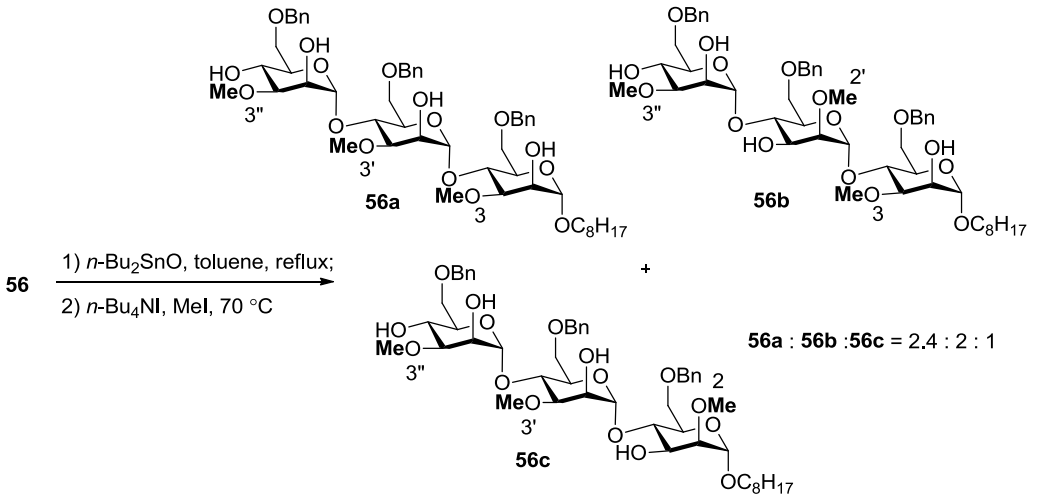
b. Yields were obtained by integration of anomeric protons of the products observed in crude ^1H NMR spectrum.

To explore this possibility, disaccharide **55** was heated at reflux in toluene with two equivalents of *n*-Bu_2SnO and then treated with methyl iodide at various temperatures (Table 4-1). Formation of products was monitored by ^1H NMR

spectroscopy of the crude reaction mixtures, and the yield was determined by integrating the anomeric protons in the newly formed products. Three major products were observed: two disubstituted regioisomers (**55a** and **55b**) as well as a monosubstituted derivative, **55c**. When the alkylation was performed at 40 ℃ (entry 1, Table 4-1), 80% of starting material **55** remained. Raising the reaction temperature to 70 ℃ increased the amount of dimethylated products, **55a** (19%) and **55b** (3%). However, the reaction was incomplete and a number of monomethylated products were produced (entry 2, Table 4-1). Addition of CsF drove the reaction to completion with the formation of exclusively dimethylated products (entry 3, Table 4-1).[21] However, the desired regioisomer **55a** was formed in only 29% yield, while the major product was the undesired isomer **55b** in 45% yield. The remaining mass balance was the other dimethylated isomers. The structures of **55a**, **55b** and **55c** were assigned unambiguously after isolation of the corresponding products and characterization by ^{1}H NMR spectroscopy. Regioisomers **55a** and **55b** were distinguished by comparison of the ring protons adjacent to the alkylated hydroxyl groups. After alkylation, both H-3 and H-3′ of **55a** shifted upfield significantly. In the case of **55b**, the shielded protons were H-2 and H-3′, indicating that alkylation took place at the 2-OH and 3′-OH of **55**. Finally, for **55c**, only the signal for H-3′ was deshielded.

Despite the rather modest results obtained with disaccharide **55** as the substrate, we applied the *n*-Bu_2SnO-mediated alkylation conditions to trisaccharide **56** (Scheme 4-4). With this substrate, tri-substitution took place predominantly. No starting material was detected after the reaction. However, a

minimum of five products were observed. The desired 3, 3′, 3′′-trisubstituted **56a** was isolated as the major product; however, it accounted only for 41% of all the products. Two other isomers were identified: the O-3, O-2′, O-3′′ tri-*O*-methylated **56b** and O-2, O-3′, O-3′′ tri-*O*-methylated **56c**. The structures of the products were established as described above for the reactions with disaccharide **55**.



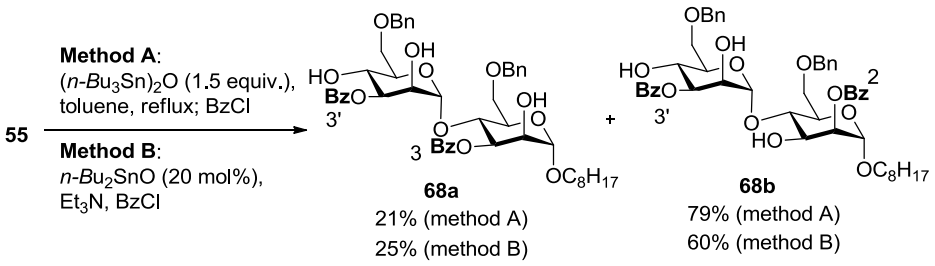
Scheme 4-4. Direct regioselective alkylation of **56**.

These studies suggest that the degree of alkylation of carbohydrate polyol systems can be controlled with *n*-Bu_2SnO-coordination. However, consistent regioselectivity was difficult to achieve with substrates such as disaccharide **55** and trisaccharide **56**. We decided to explore if regioselectivity could be improved for acylation reactions. We first tested acylation of disaccharide **55**. Because *n*-Bu_2SnO-mediated acylation of *cis*-diols usually activates the equatorial hydroxyl group,[20] we expected to see predominantly the O-3, O-3′-disubstituted product **55**.

4.3 Regioselective acylation of oligosaccharides

4.3.1 Regioselective acylation of disaccharide 55

Regioselective acylation of **55** was explored using BzCl at room temperature after heating the substrate at reflux with a tin reagent (2 equiv *n*-Bu_2SnO or 1.5 equiv $(n\text{-}Bu_3Sn)_2O$) in toluene. Of the two reagents used, *n*-Bu_2SnO gave only trace amounts of disubstituted products. Isolation was not attempted due to the low yield. In contrast, to our delight, when $(n\text{-}Bu_3Sn)_2O$ was used, di-*O*-benzoylated products were obtained exclusively (method A, Scheme 4-5). In addition, only two of the four possible regioisomers were produced. Unexpectedly, the expected 3,3′-disubstituted **68a** was only formed as a minor product (21%). Instead, **68b**, in which O-2 and O-3′ were benzoylated, was the major product, and was produced in a 79% yield as observed from the ^{1}H NMR spectrum of the crude reaction mixture. This regiochemistry was supported by the significant downfield shift of H-2 and H-3′ in the ^{1}H NMR spectrum for **68b** compared to that for **55**. In addition to the method described above, which involed an excess of tin reagent, the reaction was also performed with a catalytic amount of *n*-Bu_2SnO (method B, Scheme 4-5).[22] However, this catalytic method also produced the 3,3′-disubstituted product **68a** in a low yield (25%). The major product of this reaction was 2,3′-disubstituted product **68b**, which was obtained in 60% yield; The remaining 15% were other unidentified disubstituted isomers.



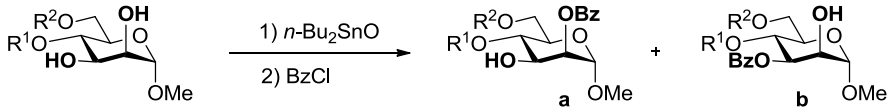
Scheme 4-5. Regioselective acylation of **55**.

4.3.2 Rationale for the regioselectivity and substrate modification

Before proceeding to try more acylation conditions, we considered the origin of the regioselectivity. Noticeably, the two diols of **55** (2′,3′-*cis*-diol and 2,3-*cis*-diol) gave different regioselectivities to produce **68b** as the major product in both methods A and B (Scheme 4-5). The diol at the non-reducing moiety (2′,3′-*cis*-diol) underwent substitution preferentially at the equatorial 3′-OH group. In contrast, the diol at the reducing end residue (2,3-*cis*-diol) gave the opposite selectivity, resulting in preferential functionalization of the axial 2-OH group.

The latter result is inconsistent with many reported tin-mediated acylation reactions in which functionalization of the equatorial hydroxyl group of *cis*-diols on six-membered rings is preferential.[20] For example, benzoylation of **69**, when carried out in the presence of *n*-Bu_2SnO, produced only **69b** with substitution at the equatorial hydroxyl group (Figure 4-3).[23] More recently developed methods, using either borinic acid derivatives[24] or Me_2SnCl_2,[25] also give the same selectivity with *cis*-diols. Nevertheless, it should be noted that examples giving different regioselectivity have been previously reported in the case of mannose. As examples, **69**–**72**, when reacted under the same conditions, resulted in different regioselectivities (Figure 4-3).[23,26-28] These compounds differ only in the

protecting groups, suggesting that these substituents play an important role in controlling the regioselectivity.



69: R^1 = H, R^2 = Trityl resin	none	98%
70: R^1, R^2 = benzylidene	major	minor
71: R^1, R^2 = Bn	9-41%	43-67%
72: R^1 = Bn, R^2 = per-benzoyl Man*p* α-(1$\rightarrow$6)	32%	63%

Figure 4-3. Regioselective acylation of 2,3-*cis*-diol of mannose residues.

To rationalize the selectivity of the acylation reaction of **55**, we compared the steric environment of the two diols. We postulated that the selectivity of the diol at the reducing end (2,3-*cis*-diol) was influenced by the bulky mannose substituent at O-4, which rendered O-3 less accessible for reaction than O-2 (Figure 4-4A). On the other hand, the diol at the non-reducing end of **55** (2′,3′-*cis*-diol) is not hindered and the acylation occurs on the equatorial O-3′ position preferentially. This rationalization also explains the inconsistent regioselectivity observed in **69**–**72** (Figure 4-3). With no substituent at O-4, benzoylation occurred exclusively at O-3 to give **69b**.[23] However, the presence of any substituent at O-4 (**70**,[26] **71**,[27] or **72**[28]) decreased the regioselectivity and significant amounts of O-2 substitution was also observed. Therefore, we hypothesized that introducing a bulky substituent at O-4′ of disaccharide **55** would reverse the selectivity at the non-reducing residue diol, and give an enhanced yield of the O-2 and O-2′ di-*O*-acylated products (Figure 4-4B).

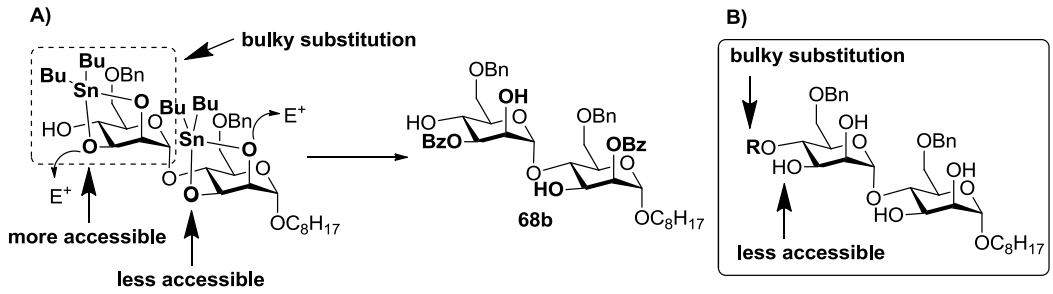
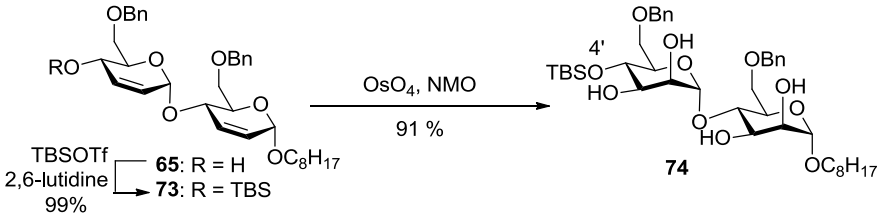


Figure 4-4. Rationale for the observed regioselectivity of the benzoylation of **55** with *n*-Bu_2SnO.

To test this hypothesis, a disaccharide modified at O-4′ with a *t*-butyldimethylsilyl (TBS) group (**74**, Scheme 4-6) was synthesized. The TBS group was introduced by treating alcohol **65** with TBSOTf and the resulting product, **73**, was then dihydroxylated with OsO_4 to provide **74** in 91% yield overall yield. With **74** in hand, the regioselective acylation reaction was examined. We investigated three reported catalytic methods for regioselective acylation of diols, including two tin reagents, Me_2SnCl_2[25] and *n*-Bu_2SnO,[22] as well as a borinic acid derivative $Ph_2BOCH_2CH_2NH_2$.[24] All these methods were reported to efficiently catalyze the regioselective acylation of monosaccharides, but their application to oligosaccharide systems had not been investigated.



Scheme 4-6. Preparation of TBS-modified disaccharide **74**.

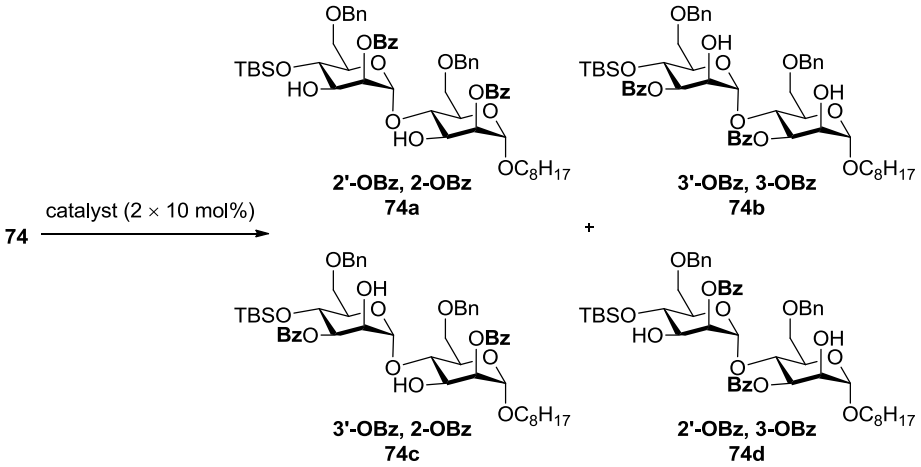
4.3.3 Acylation of the modified disaccharide 74 and optimizations

When **74** was treated with 2.4 equivalents BzCl (1.2 equiv per diol) in the presence of 20 mol% Me_2SnCl_2, *n*-Bu_2SnO or $Ph_2BOCH_2CH_2NH_2$ (10 mol% per diol), both tin reagents gave satisfactory conversions (entries 1 and 2, Table 4-2). However, the use of $Ph_2BOCH_2CH_2NH_2$ led only to ~10% conversion of **74** (entry 3, Table 4-2). Using Me_2SnCl_2 gave 60% of a mixture of mono-*O*-benzoylated products and 40% of di-*O*-benzoylated products (entry 1, Table 4-2). Of the disubstituted products, 22% was the expected 2,2′-di-*O*-benzoylated **74a** and 18% was the 3,2′-di-*O*-benzoylated isomer **74d** as determined by ^{1}H NMR spectroscopy. The other two isomers, **74b** and **74c**, were not detected. With *n*-Bu_2SnO, three di-*O*-benzoylated products were formed: 63% of the desired **74a**, 9% of **74c** and 16% of **74d** (entry 2, Table 4-2). These results support our hypothesis that a bulky substituent at O-4′ of **74** alters the regioselectivity of the non-reducing end diol to favor substitution at O-2′.

The regioselectivity employing *n*-Bu_2SnO (**74a**:**74d** = 3.9:1) was better than with Me_2SnCl_2 (**74a**:**74d** = 1.2:1), suggesting that the size of the alkyl group in the catalyst also influence acylation regioselectivity. Indeed, replacing Me_2SnCl_2 with the more hindered *n*-Bu_2SnCl_2 gave **74a** as the only observed disubstituted isomer (entry 4, Table 4-2). However, 57% of the monobenzoylated products remained when this catalyst was used. When the *n*-Bu_2SnCl_2-catalyzed reaction of **74** was repeated with 10 equiv of BzCl, (5 equiv per diol), an 87% yield of **74a** was produced (entry 5, Table 4-2). However, when the same amount

of BzCl was employed in reactions using *n*-Bu_2SnO, mainly tribenzoylated products were produced (entry 6, Table 4-2).

Table 4-2. Regioselective benzoylation of **74** with different catalysts.[a]



Entry	BzCl amount	Conditions	Products distribution (NMR yield)					Unreacted **74**
			disubstituted				monosubstituted	
			74a	**74b**	**74c**	**74d**		
1	2.4 eq	Me_2SnCl_2, DIPEA, THF	22	-	-	18	60	-
2	2.4 eq	*n*-Bu_2SnO, Et_3N, CH_2Cl_2	63	-	9	16	-	-
3	2.4 eq	$Ph_2BOCH_2CH_2NH_2$, CH_3CN	-	-	-	-	<10	90
4	2.4 eq	*n*-Bu_2SnCl_2, DIPEA, THF	43	-	-	-	57	-
5	10 eq	*n*-Bu_2SnCl_2, DIPEA, THF	87	-	6	7	-	-
6	10 eq	*n*-Bu_2SnO, Et_3N, CH_2Cl_2	tribenzoylated products observed					

[a] Reaction conditions: **74** (1 equiv, 0.1 M), catalyst (20 mol%), base (4 equiv for entry 1–4, 10 equiv for entry 5–6,), BzCl (2.4–10 equiv), room temperature, 3 h.

Encouraged by the success of these preliminary studies, the regioselective benzoylation of **74** catalyzed by *n*-Bu_2SnCl_2 was optimized using various solvents (Table 4-3). In all solvents, a similar trend in the regioselectivity (**74a** >> **74c**, **74d** > **74b**) was observed, with the exception of pyridine, where the substrate formed a mixture of products containing more than two benzoyl groups. These

results demonstrate that, aside from pyridine, the reaction solvent is not critical to the regioselectivity of the reaction. Among all the solvents tested, THF gave the least amount of undesired isomers (entry 1). Lowering the temperature to 0 ℃ did not improve the selectivity but, as expected, slowed the reaction (entry 6). Thus, future reactions employed THF as the solvent and the reactions were carried out at room temperature.

Table 4-3. Solvent optimization for regioselective benzoylation of **74**[a]

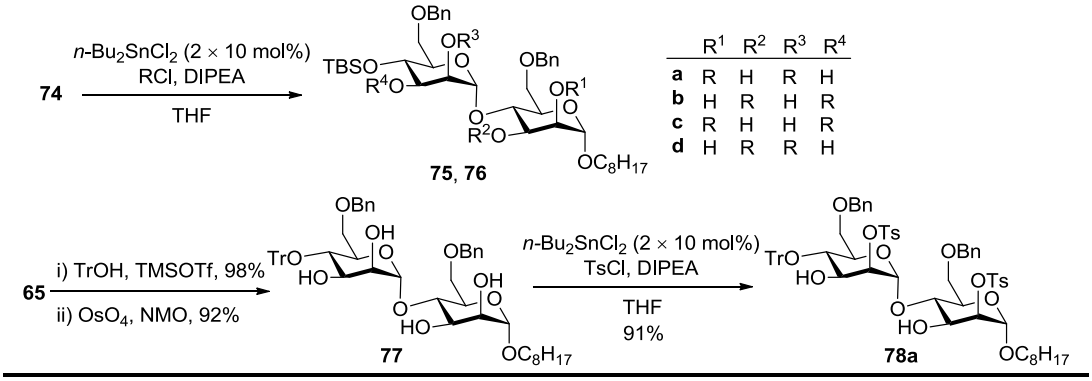
Entry	Solvent	T (℃)	Product distribution (%)				
			Disubstituted				Monosubstituted
			74a	**74b**	**74c**	**74d**	
1	THF	RT	87	-	6	7	-
2	CH_2Cl_2	RT	68	-	10	20	-
3	MeCN	RT	61	-	< 4	28	
4	Et_2O	RT	76	-	12	12	-
5	Pyridine	RT	overbenzoylation				
6	THF	0 ℃	66	-	8	7	19

[a] Reaction conditions: **74** (1 equiv 0.1 M), *n*-Bu_2SnCl_2 (20 mol%), DIPEA (10 equiv), BzCl (10 equiv), 3 h.

Next, we tested the optimized method with other reagents (Table 4-4). The relatively unhindered acylation reagent AcCl gave no regioselectivity (entry 1). The bulky PivCl produced a single disubstituted isomer **75a** with the expected 2,2′-disubstitution; however, 51% of the mono-2-*O*-pivaloyated product remained, as well as 36% of starting material **74** (entry 2). Replacing BzCl with *p*-TsCl gave the expected di-*O*-tosylated product **76a** in excellent yield and regioselectivity (entry 3), although the equivalents of TsCl and reaction time had to be increased to drive the reaction to completion. When tosylation was performed with 15 equivalents of TsCl for 24 hours, 89% of **76a** was observed (entry 4).

Having established that the introduction of a sterically-demanding protecting group on O-4′ of **74** successfully switched the regioselectivity of the non-reducing end residue, we next replaced the TBS group of **74** with a trityl substituent (disaccharide **77**, Table 4-4). The trityl group was introduced by reacting alcohol **65** with TrOTf, which was generated *in situ* from TrOH and TMSOTf.[29] The adduct then underwent dihydroxylation to give trityl-modified disaccharide **77**.[29] This compound was found to be a better substrate than **74**. For example, regioselective tosylation of **77** efficiently furnished a 91% yield of the desired **78a** (entry 5, Table 4-4). Only a trace amount of other isomers was produced from this reaction. Based on these results and for reasons discussed in greater detail below, we shifted our focus to tosylation reactions.

Table 4-4. Protecting group scope of regioselective acylation.[a]

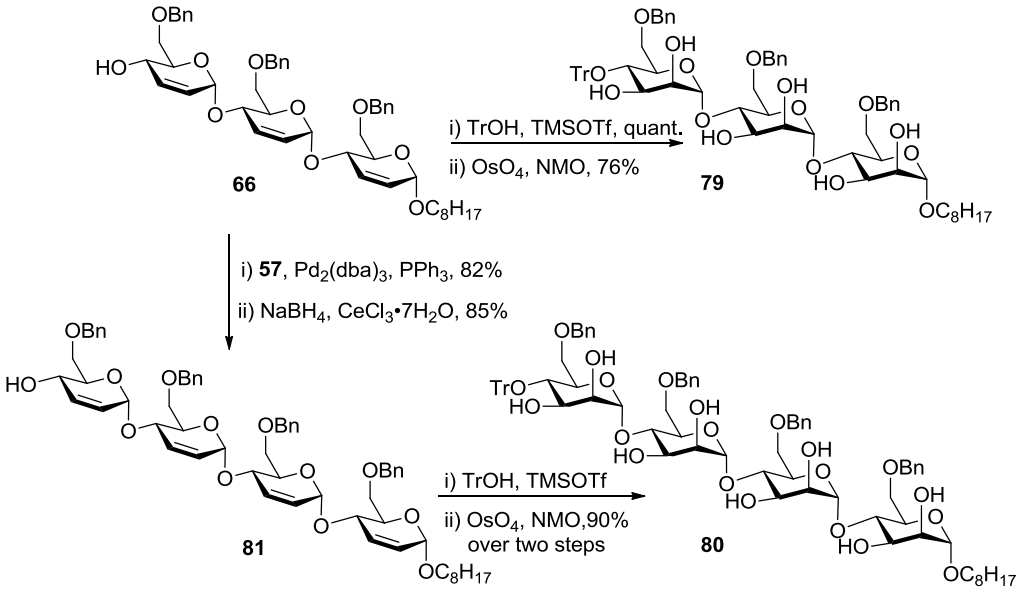


Entry	Substrate	RCl (amount)	Products (NMR yield)		Unreacted starting material
			disubstituted	monosubstituted	
1	**74** (R^1 = TBS)	AcCl (2.4 eq)	trace	> 80%	10%
2		PivCl (10 eq)	**75a** (13%)	51%	36%
3		TsCl (10 eq)	**76a** (62%)	31%	trace
4		TsCl (15 eq)	**76a** (89%), **76d** (11%)	-	-
5	**77** (R^1 = Tr)	TsCl (10 eq)	**78a** (91%), **78d** (4%)	-	-

[a] Reaction conditions: substrate (1 equiv 0.1 M), *n*-Bu_2SnCl_2 (20 mol%), DIPEA (10 equiv), RCl (2.4–15 equiv), room temperature, 24h.

4.3.4 Application to trisaccharide and tetrasaccharide substrates

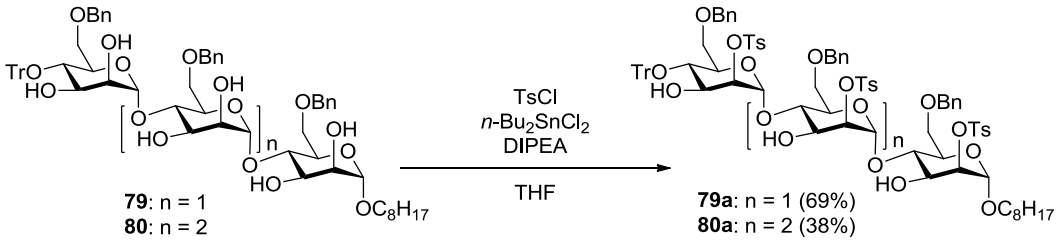
With a set of optimized conditions developed, this methodology was applied to more challenging substrates: trisaccharide **79** and tetrasaccharide **80** (Scheme 4-7). These compounds were synthesized from triene **66**. First, a trityl group was introduced onto **66** before dihydroxylation leading to **79** in 76% yield. Alternatively, glycosylation of **66** with **57**, and then Luche reduction, yielded tetraene **81** in 70% yield over the two steps. Subsequent tritylation of **81** and then dihydroxylation provided tetrasaccharide **80** in 90% overall yield.



Scheme 4-7. Synthesis of trisaccharide **79** and tetrasaccharide **80**.

Considering that regioselective acylation of each diol generates two possible regioisomers, trisaccharide **79**, with three diol pairs, would have eight possible tri-*O*-sulfonylated isomers and tetrasaccharide **80** would have 16 possible products. When **79** and **80** were subjected to the optimized regioselective sulfonation conditions, the desired trisubstituted product **79a** and tetrasubstituted

product **80a** were obtained, in 69% and 38% yield, respectively (Scheme 4-8). In both cases, these products were the major regioisomers. Although the isolated yield of the desired regioisomers dropped when the substrates went from the disaccharide to the tetrasaccharide, the efficiency of the regioselectivity is still remarkable. In the case of the reaction with **79**, a 69% yield of **79a** corresponds to an 88% sulfonylation selectivity for each diol pair. For the reaction with **80**, a 38% yield is equivalent to sulfonylating each diol with 79% selectivity.

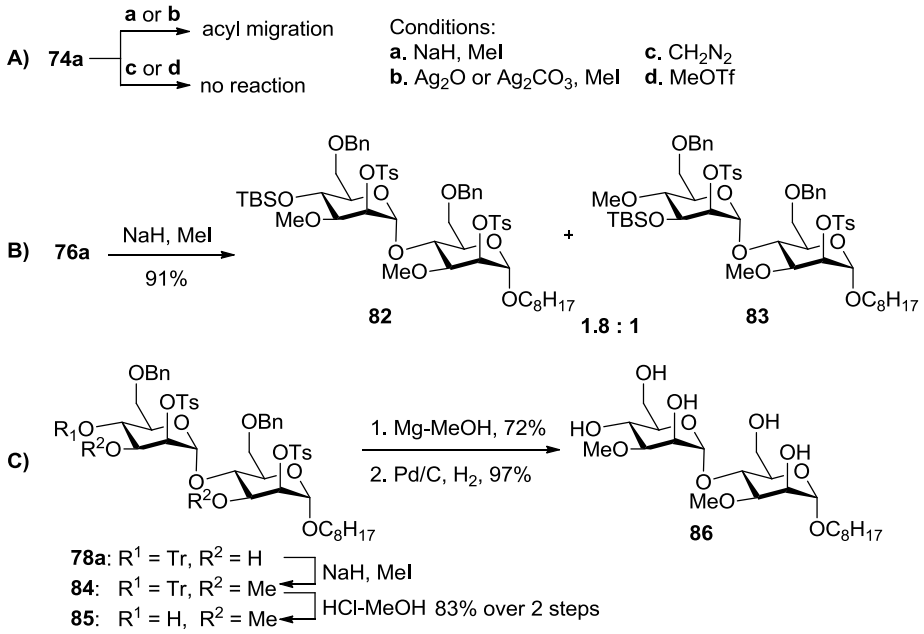


Scheme 4-8. Regioselective modification of trisaccharide **79** and tetrasaccharide **80**.

4.4 Subsequent functionalization of oligosaccharides by alkylation

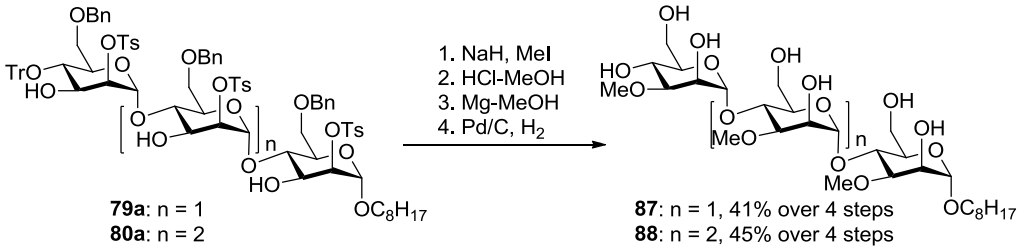
After successfully introducing multiple protecting groups onto the di-, tri- and tetrasaccharides with good regioselectivity, the ability to further functionalize these compounds was investigated. Attempts to methylate **74a** using NaH resulted in benzoyl group migration (condition a, Scheme 4-9A), a common problem during alkylation under basic conditions.[8] The use of milder bases such as Ag_2O and Ag_2CO_3 failed to prevent acyl migration (condition b, Scheme 4-9A).[30-31] In addition, methylation of **74a** under acidic conditions using CH_2N_2 and neutral conditions with MeOTf also proved unsuccessful (conditions c and d, Scheme 4-9A).[32-33]

To overcome this acyl migration problem, the substrates with the sulfonyl groups were used.[34] As expected, the tosyl groups of **76a** were stable to alkylation with NaH and MeI. However, in addition to the desired product **82**, an unexpected byproduct, **83** (Scheme 4-9B), presumably resulting from intramolecular silyl group migration under the basic reaction conditions, was formed. The use of an alternative substrate with a base-stable trityl group on O-4′ (**78a**) avoided this problem giving the desired di-*O*-methyl disaccharide **84**. The trityl group in **84** was easily removed using HCl in methanol to give **85** in 83% yield over the two steps. Finally, the tosyl groups of **85** were cleaved with magnesium in methanol at reflux.[35] Subsequent hydrogenolysis removed the remaining benzyl groups, furnishing disaccharide **86** in four steps from **78a** (Scheme 4-9C).



Scheme 4-9. Attempted Methylation with disaccharides **74a**, **76a** and **78a**.

Using the same four-step sequence, the methylated trisaccharide **87** and tetrasaccharide **88** were obtained within four steps from **79a** and **80a**, in 41% and 45% overall yields, respectively (Scheme 4-10). Preparation of these compounds, if using traditional routes from monosaccharides as outlined in Figure 4-2, would take much longer and proceed in lower overall yield.



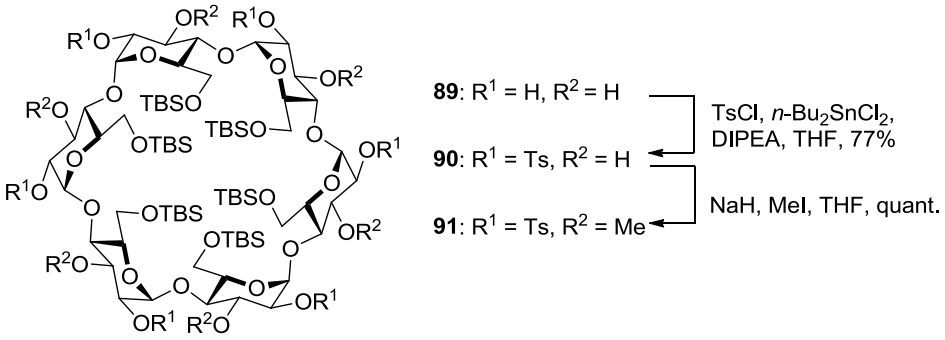
Scheme 4-10. Methylation of trisaccharide **79a** and tetrasaccharide **80a**.

4.5 Application to α-cyclodextrin

Glycans with single sugar repeating units are common in nature. For example, amylose[36] and α-, β-, γ-cyclodextrins[37] consist of α-(1→4)-linked D-glucopyranose units, and cellulose is a polymer of repeating β-(1→4)-D-glucopyranose units.[38] In addition to the 3-*O*-methylmannose polysaccharides described above,[39] mycobacteria produce other polysaccharides, containing domains of the same monosaccharide attached in sequence. For example, lipoarabinomannan contains α-(1→6)-linked D-mannopyranose units,[40] and 6-*O*-methyl-glucose lipopolysaccharides, have an amylose-like structure of α-(1→4)-linked D-glucopyranose units.[41]

To explore the generality of the methodology, it was applied to α-cyclodextrin. Chemical modifications of cyclodextrins are of significant interest

and have been widely used to tune their physical properties.[42] However, methods to selectively functionalize the secondary face of these cyclic oligosaccharides are still limited. In particular, derivatives that are functionalized at O-3 positions are difficult to prepare.[43] Noticing of the similarity between the α-(1→4)-linked mannose oligosaccharides described above and the α-(1→4)-linked glucopyranose residues present in α-cyclodextrin, we envisioned our methodology might be able to provide the 3-*O*-alkylated analogs. As expected, the TBS-protected α-cyclodextrin **89**[44] underwent tosylation with high regioselectivity (Scheme 4-11). The expected fully 2-*O*-tosylated product **90** was obtained in 77% isolated yield. Of the other isomers produced from this reaction, the major one, isolated in 5% yield, had five of the residues exclusively tosylated at O-2. After protection of all the O-2 positions of cyclodextrin, methyl groups were installed quantitatively onto O-3 of **90** to give **91**. This substrate has *trans*- instead of *cis*-1,2-diols, which suggested the approach does not require *cis*-diol. This methodology thus provides a new method for the selective functionalization of cyclodextrins.



Scheme 4-11. Regioselective functionalization of α-cyclodextrin.

4.6 Conclusions

In conclusion, we demonstrate here a method that can be used to regioselectively functionalize multiple hydroxyl groups in oligosaccharides. Key to the development of this approach was the realization that the regioselectivity of organotin-mediated acylation and sulfonation of diols can be altered by tuning the size of protecting groups on the substrate. Although such effects can, in retrospect, be identified in previous examples of such functionalizations,[23,26-28] rational optimization of these steric effects appears not to have been carried out previously. The focus of this paper has been on the selective methylation of *cis*-diols in α-(1→4)-linked mannopyranose oligomers, which are an interesting class of lipid-binding oligosaccharides.[45-47] When coupled with O'Doherty's palladium-catalyzed pyranone glycosylation methodology,[15] we consider this approach to be a viable alternative to more traditional approaches to these targets, which rely on the preparation of a number of selectively protected monosaccharides derivatives.[13,48-54] We also provide examples showing that the method is effective for selectively functionalizing not only *cis*-diols but also *trans*-diols (in α-cyclodextrin) and further work in applying this method to other oligosaccharide systems is ongoing. We note that during the course of our studies a similar method, also employing *n*-Bu_2SnCl_2 as the catalyst, was reported for the regioselective monosulfonylation of an unprotected disaccharide by Muramatsu.[55] The major difference is that whereas Muramatsu aimed to protect only one of the hydroxyl groups of an oligosaccharide, our approach targets the simultaneous functionalization of multiple hydroxyl groups.

4.7 Experimental details

General experimental methods: All reagents were purchased from commercial sources and were used without further purification unless noted. All reactions were carried out under a positive pressure of argon or nitrogen at room temperature unless specified and were monitored by TLC on silica gel 60-F_{254} (0.25 mm, Silicycle, Quebec, Canada). Visualization of the reaction components was achieved using UV fluorescence (254 nm) and/or by charring with acidified anisaldehyde solution in ethanol. Organic solvents were evaporated under reduced pressure and the products were purified by column chromatography on silica gel (230–400 mesh, Silicycle, Quebec, Canada). Optical rotations were measured on a Perkin–Elmer 241 polarimeter with a microcell (10 cm, 1 mL) at ambient temperature and are in units of degree·mL/(g·dm). Nuclear magnetic resonance (NMR) spectra were recorded on either Varian Inova 500 or Varian Inova 600 spectometers. ^{1}H NMR spectra were recorded at 500 MHz or 600 MHz and chemical shifts are referenced to residual $CHCl_3$ (7.26 ppm, $CDCl_3$), $CHDCl_2$ (5.32 ppm, CH_2Cl_2), or CHD_2OD (3.30 ppm, CD_3OD). ^{13}C NMR spectra were recorded at 125 MHz and chemical shifts are referenced to $CDCl_3$ (77.0 ppm) or $CHDCl_2$ (53.8 ppm). Reported splitting patterns are abbreviated as s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, app = apparent. Assignments of NMR spectra were based on two-dimensional experiments (^{1}H–^{1}H COSY, HSQC, and HMBC) and stereochemistry of the anomeric centers of the pyranose rings were confirmed by measuring $^{1}J_{C\text{-}1,H\text{-}1}$ via coupled HSQC experiments. Electrospray mass spectra were recorded on Agilent Technologies 6220 TOF.

Octyl 6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranoside (**55**): To a solution of **65** (561.8 mg, 0.97 mmol) in *t*-butanol and acetone (4 mL, v/v 1:1) was added OsO_4 (2.5 wt.% in *t*-butanol, 250 uL, 0.02 mmol) and *N*-methyl-morpholine *N*-oxide (NMO, 50% w/v in water, 0.3 mL). After stirring at room temperature overnight, saturated aqueous Na_2SO_3 solution was added. The mixture was concentrated to remove *t*-butanol and then the residue was extracted with CH_2Cl_2 three times. The combined organic layer was concentrated and the resultig residue was purifed by chromatography (CH_2Cl_2–methanol 15:1) to afford **55** (492.3 mg, 80%) as a pale yellow syrup. R_f 0.14 (CH_2Cl_2–methanol 15:1); $[\alpha]_D$ = +59.5 (*c* 1.2, methanol); ^{1}H NMR (498 MHz, CD_3OD) δ 7.34–7.20 (m, 10H, Ar), 5.22 (d, *J* = 1.8 Hz, 1H, H-1′), 4.69 (d, *J* = 1.6 Hz, 1H, H-1), 4.53–4.41 (m, 4H, 4 × OC*H*$_2$Ph), 3.94 (dd, *J* = 3.1, 1.9 Hz, 1H, H-2′), 3.83–3.59 (m, 12H, H-2, H-3, H-3′, H-4, H-4′, H-5, H-5′, 2 × H-6, 2 × H-6′, octyl OCH_2), 3.39 (dt, *J* = 9.6, 6.4 Hz, 1H, octyl OCH_2), 1.61–1.52 (m, 2H, octyl OCH_2C*H*$_2$), 1.38–1.21 (m, 10H, octyl CH_2), 0.88 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ 139.8, 139.7 (2C, Ar), 129.3 (2C, Ar), 129.3 (2C, Ar), 129.0 (2C, Ar), 128.9 (2C, Ar), 128.6 (Ar), 128.6 (Ar), 103.4 (C-1′, $^1J_{C\text{-}1,H\text{-}1}$ = 171.4 Hz), 101.4 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 167.0 Hz), 76.6 (C-4), 74.6, 74.4 (2C, 2 × O*C*H$_2$Ph), 74.4, 73.2, 72.7, 72.5, 72.2, 72.1 (6C, C-2, C-2′, C-3, C-3′, C-5, C-5′), 71.4, 71.4 (2C, C-6, C-6′), 68.8 (octyl OCH_2), 68.7 (C-4′), 33.0, 30.6, 30.4, 30.4, 27.3, 23.7 (6C, octyl CH_2), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{34}H_{50}O_{11}$ $[M+Na]^+$ 657.3245, found 657.3239.

Synthesis of octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside **(55a)**, octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-methyl-α-D-mannopyranoside (**55b**) and octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranoside (**55c**): Compound **55** (0.01–0.04 mmol, 1 equiv) and *n*-Bu_2SnO (2 equiv) were heated at reflux in toluene (0.01 M) overnight. The resulting yellowlish solution was cooled to room teperature before MeI (20 equiv), *t*-Bu_4NI (2 equiv) and CsF (4 equiv) were added. The mixture was stirred at designated temperature (see Table 4-1) overnight before concentrated. The crude products were then purifed by chromatography (CH_2Cl_2–methanol 30:1 to 20:1) to afford an inseparable mixture of **55a** and **55b** as a yellow syrup, as well as separable **55c** as a yellow syrup. R_f 0.51 for **55a** and **55b**, 0.43 for **55c** (CH_2Cl_2–methanol 12:1); Isomers **55a** and **55b** were distinguished by an ^1H–^1H COSY experiment. H-2, H-2′, H-3 and H-3′ of **55** are between 3.94–3.59 ppm. After alkylation, H-3 and H-3′ of **55a** shifted significantly upfield to 3.46 and 3.33 ppm, respectively, while H-2 and H-2′ were slightly downfield-shifted to 4.00 and 4.08 ppm, respectively. This result indicates **55a** is alkylated at O-3 and O-3′. In **55b**, H-2 and H-3′ are shifted upfield shifted to 3.37 and 3.33 ppm, indicating substitution at these two positions.

Octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (**55a**): ^1H NMR (600 MHz, CD_3OD) δ 7.39–7.18 (m, 10H, Ar), 5.16 (d, *J* = 1.9 Hz, 1H, H-1′), 4.76 (d, *J* = 1.8 Hz, 1H, H-1), 4.53–4.40 (m, 4H, 4 × OC*H*$_2$Ph), 4.08 (dd, *J* = 3.1, 2.1 Hz, 1H, H-2′), 4.00 (dd, *J* = 3.2, 1.9 Hz, 1H, H-2), 3.87–3.55 (m, 9H, H-4, H-4′, H-5, H-5′, 4 × H-6, octyl OCH_2),

3.46 (dd, *J* = 9.2, 3.3 Hz, 1H, H-3), 3.45 (s, 3H, OMe), 3.43–3.40 (m, 1H, octyl OCH_2), 3.41 (s, 3H, OMe), 3.33 (dd, *J* = 9.3, 3.1 Hz, 1H, H-3′), 1.62–1.55 (m, 2H, octyl OCH_2CH_2), 1.40–1.21 (m, 10H, octyl CH_2), 0.84 (t, *J* = 7.5 Hz, 3H, octyl CH_3); HRMS (ESI) calcd $C_{36}H_{54}O_{11}$ $[M+Na]^+$ 685.3558, found 685.3555.

Octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-methyl-α-D-mannopyranoside (55b): ^1H NMR (600 MHz, CD_3OD) δ 7.39–7.18 (m, 10H, Ar), 5.24 (d, *J* = 1.9 Hz, 1H, H-1′), 4.87 (d, *J* = 1.6 Hz, 1H, H-1), 4.53–4.40 (m, 4H, 4 × OCH_2Ph), 4.16 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2′), 3.87–3.55 (m, 10H, H-3, H-4, H-4′, H-5, H-5′, 4 × H-6, octyl OCH_2), 3.44 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.43–3.40 (m, 1H, octyl OCH_2), 3.37 (dd, *J* = 3.5, 1.7 Hz, 1H, H-2), 3.33 (dd, *J* = 9.3, 3.1 Hz, 1H, H-3′), 1.62–1.55 (m, 2H, octyl OCH_2CH_2), 1.40–1.21 (m, 10H, octyl CH_2), 0.84 (t, *J* = 7.5 Hz, 3H, octyl CH_3); HRMS (ESI) calcd $C_{36}H_{54}O_{11}$ $[M+Na]^+$ 685.3558, found 685.3555;

Octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranoside (55c): ^1H NMR (600 MHz, CD_3OD) δ 7.36–7.19 (m, 10H, Ar), 5.25 (d, *J* = 2.0 Hz, 1H, H-1′), 4.70 (d, *J* = 1.6 Hz, 1H, H-1), 4.54–4.40 (m, 4H, 2 × OCH_2Ph), 4.17 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2′), 3.85–3.60 (m, 11H, H-2, H-3, H-4, H-4′, H-5, H-5′, 4 × H-6, octyl OCH_2), 3.45 (s, 3H, OMe), 3.42–3.38 (m, 1H, octyl OCH_2), 3.35 (dd, *J* = 9.3, 3.2 Hz, 1H, H-3′), 1.62–1.53 (m, 2H, octyl OCH_2CH_2), 1.38–1.23 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.1 Hz, 3H, octyl CH_3); HRMS (ESI) calcd $C_{35}H_{52}O_{11}$ $[M+Na]^+$ 671.3402, found 671.3402.

Octyl 6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranoside (**56**): The

dihydroxylation reaction was performed as described for the synthesis of **55**, with **66** (243.3 mg, 0.31 mmol), OsO_4 (2.5 wt.% in *t*-butanol, 119 uL, 0.01 mmol), NMO (50% w/v in water, 0.9 mL) in *t*-butanol and acetone (1 mL, v/v 1:1). The crude residue was purified by chromatography (CH_2Cl_2–methanol 12:1) to afford trisaccharide **56** (205.1 mg, 75%) as a colorless foam. R_f 0.61 (CH_2Cl_2–methanol 6:1); $[\alpha]_D$ = +64.8 (*c* 1.0, methanol); ^{1}H NMR (498 MHz, CD_3OD) δ 7.33–7.17 (m, 15H, Ar), 5.25 (d, *J* = 1.6 Hz, 1H, H-1′/H-1″), 5.23 (d, *J* = 1.7 Hz, 1H, H-1′/H-1″), 4.71 (d, *J* = 1.6 Hz, 1H, H-1), 4.52–4.33 (m, 6H, 6 × OC*H*$_2$Ph), 3.98 (dd, *J* = 3.0, 1.9 Hz, 1H, H-2′/H-2″), 3.90 (dd, *J* = 2.9, 2.2 Hz, 1H, H-2′/H-2″), 3.86–3.60 (m, 17H, H-2, 3 × H-3, 3 × H-4, 3 × H-5, 6 × H-6, octyl OCH_2), 3.38 (dt, *J* = 9.6, 6.3 Hz, 1H, octyl OCH_2), 1.61–1.52 (m, 2H, octyl OCH_2CH_2), 1.39–1.21 (m, 10H, OCH_2), 0.88 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ 139.6 (Ar), 139.5 (2C, Ar), 129.3 (2C, Ar), 129.3 (2C, Ar), 129.2 (2C, Ar), 129.0 (2C, Ar), 129.0 (2C, Ar), 128.8 (2C, Ar), 128.6 (Ar), 128.6 (Ar), 128.5 (Ar), 103.3 (C-1′/ C-1″, $^1J_{C\text{-}1,H\text{-}1}$ = 170 Hz), 103.0 (C-1′/ C-1″, $^1J_{C\text{-}1,H\text{-}1}$ = 171 Hz), 101.3 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 168.8 Hz), 76.4, 76.2 (2C, C-4, C-4′), 74.5, 74.4 (2C, 2 × O*C*H$_2$Ph), 74.3 (C-5″), 74.2 (OCH_2Ph), 73.2, 72.9, 72.8, 72.6, 72.6, 72.5, 72.1, 71.9 (9C, 3 × C-2, 3 × C-3, C-4″, C-5, C-5′), 71.3, 71.3, 71.1 (3C, 3 × C-6), 68.7 (octyl OCH_2), 32.9, 30.5, 30.3, 30.3, 27.2, 23.6 (6C, octyl CH_2), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{47}H_{66}O_{16}$ $[M+Na]^+$ 909.4243, found 909.4244.

Synthesis of octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (56a), octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-

(1→4)-6-*O*-benzyl-2-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (56b) and octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-methyl-α-D-mannopyranoside (56c): Compound **56** (33.3 mg, 0.04 mmol) and *n*-Bu_2SnO (31.8 mg, 0.12 mmol, 3 equiv) were heated at reflux in toluene (4 mL) for 14 h. The resulting yellowish solution was cooled to room teperature before MeI (70 μL, 1.2 mmol), *t*-Bu_4NI (44.0 mg, 0.12 mmol, 3 equiv) were added. After stirring at 70 °C for 7 h, additional MeI (150 μL, 2.4 mmol) was added and the reaction was stirred overnight. The crude products were purifed by chromatography (CH_2Cl_2–methanol 30:1 to 25:1) to afford three major products: an inseparable mixture (11.7 mg, 34%) of **56a** and **56c**, as well as **56b** (6.0 mg, 17%) both as colorless syrups. R_f 0.64 for **56b**, 0.61 for **56a** and **56c** (CH_2Cl_2–methanol 15:1); **56a** and **56c** were successfully separated after per-acetylation with acetic anhydride (0.1 mL) in pyridine (0.1 mL) and CH_2Cl_2 (1 mL). Chromatography purification gave acetylated **56a** (6.9 mg) and acetylated **56c** (3.5 mg) as colorless syrup.

Octyl 2,4-di-*O*-acetyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-acetyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-acetyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (acetylated **56a**): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39–7.16 (m, 15H, Ar), 5.39 (dd, *J* = 3.1, 2.0 Hz, 1H, H-2′), 5.38 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2″), 5.28 (dd, *J* = 3.2, 1.8 Hz, 1H, H-2), 5.25 (d, *J* = 1.8 Hz, 1H, H-1′), 5.19 (app t, *J* = 10.0 Hz, 1H, H-4″), 5.19 (d, *J* = 1.8 Hz, 1H, H-1″), 4.80 (d, *J* = 1.7 Hz, 1H, H-1), 4.64–4.38 (m, 6H, 6 × OC*H*$_2$Ph), 3.90 (app t, *J*

= 10.0 Hz, 1H, H-4′), 3.89–3.79 (m, 5H, H-4, H-5, H-5′, H-5″, H-6), 3.76–3.68 (m, 3H, H-6, H-6, octyl OCH_2), 3.67–3.62 (m, 2H, H-3, H-6, H-6), 3.61–3.56 (m, 2H, H-3′, H-3″), 3.46–3.41 (m, 3H, H-6, H-6, octyl OCH_2), 3.41 (s, 3H, OMe), 3.40 (s, 3H, OMe), 3.36 (s, 3H, OMe), 2.11 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.06 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.66–1.58 (m, 2H, octyl OCH_2CH_2), 1.40–1.23 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.1 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.3, 170.1, 170.0, 169.9 (4C, 4 × OAc), 138.5 (Ar), 138.2 (Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 128.2 (2C, Ar), 127.8 (2C, Ar), 127.6 (Ar), 127.4 (3C, Ar), 127.4 (2C, Ar), 127.4 (Ar), 99.7 (C-1″), 99.4 (C-1′), 97.5 (C-1), 80.0 (C-3), 79.9 (C-3′), 76.5 (C-3″), 74.5 (C-4), 73.6 (C-4′), 73.6, 73.4, 73.3 (3 × OCH_2Ph), 71.9, 71.0, 70.8 (3C, C-5, C-5′, C-5″), 70.0, 69.9, 69.5 (3C, C-6, C-6′, C-6″), 68.4 (C-4″), 68.3 (octyl OCH_2), 67.7 (2C, C-2, C-2′), 67.4 (C-2″), 57.6, 57.2, 57.1 (3C, 3 × OMe), 31.8, 29.4, 29.4, 29.2, 26.1, 22.7 (6C, octyl CH_2), 21.0, 21.0, 21.0, 21.0 (4C, 4 × Ac), 14.1 (octyl CH_3).

Octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (**56b**): ^{1}H NMR (600 MHz, CD_3OD) δ 7.41–7.13 (m, 15H, Ar), 5.24 (d, *J* = 2.0 Hz, 1H, H-1″), 5.24 (d, *J* = 1.7 Hz, 1H, H-1′), 4.76 (d, *J* = 1.8 Hz, 1H, H-1), 4.53–4.32 (m, 6H, 6 × OCH_2Ph), 4.17 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2″), 4.01 (dd, *J* = 3.2, 1.9 Hz, 1H, H-2), 3.86–3.56 (m, 14H, H-3′, H-4, H-4′, H-4″, H-5, H-5′, H-5″,6 × H-6, octyl OCH_2), 3.48–3.40 (m, 3H, H-2′, H-3, octyl OCH_2), 3.45 (s, 3H, OMe), 3.43 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.36 (dd, *J* = 9.2, 3.2 Hz, 1H, H-3″), 1.65–1.52 (m, 2H, octyl OCH_2CH_2), 1.42–1.20 (m, 10H, octyl

CH_2), 0.89 (t, *J* = 7.1 Hz, 3H, octyl CH_3); HRMS (ESI) calcd $C_{50}H_{72}O_{16}$ $[M+Na]^+$ 951.4713, found 951.4708.

Octyl 2,4-di-*O*-acetyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-2-*O*-acetyl-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-acetyl-6-*O*-benzyl-2-*O*-methyl-α-D-mannopyranoside (acetylated **56c**): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.39–7.13 (m, 15H, Ar), 5.37 (dd, *J* = 3.1, 2.1 Hz, 1H, H-2″), 5.22 (dd, *J* = 9.6, 3.3 Hz, 1H, H-3), 5.21 (d, *J* = 1.9 Hz, 1H, H-1″), 5.18 (app t, *J* = 9.8 Hz, 1H, H-4″), 5.16 (dd, *J* = 2.9, 2.1 Hz, 1H, H-2′), 4.99 (d, *J* = 1.9 Hz, 1H, H-1′), 4.86 (d, *J* = 1.7 Hz, 1H, H-1), 4.61–4.39 (m, 6H, 6 × OC*H*$_2$Ph), 4.01 (app t, *J* = 9.6 Hz, 1H, H-4), 3.91–3.60 (m, 10H, H-2, H-4′, H-5, H-5′, H-5″, 4 × H-6, 1 × octyl OCH_2), 3.59 (dd, *J* = 9.8, 3.2 Hz, 1H, H-3″), 3.56 (dd, *J* = 9.0, 3.1 Hz, 1H, H-3′), 3.43 (s, 3H, OMe), 3.47–3.39 (m, 3H, 2 × H-6, 1 × octyl OCH_2), 3.37 (s, 3H, OMe), 3.36 (s, 3H, OMe), 2.20 (s, 3H, OAc), 2.10 (s, 3H, OAc), 2.06 (s, 3H, OAc), 1.99 (s, 3H, OAc), 1.66–1.58 (m, 2H, octyl OCH_2C*H*$_2$), 1.40–1.21 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.7 (OAc), 170.1 (OAc), 170.0 (OAc), 169.9 (OAc), 138.7, 138.4, 138.0, 128.3, 128.2, 128.2, 127.8, 127.6, 127.5, 127.4, 127.4, 127.3 (18C, Ar), 100.2 (C-1′), 99.5 (C-1″), 97.0 (C-1), 79.5 (C-3′), 78.4 (C-2), 76.5 (C-3″), 75.7 (C-4), 73.8 (2C, C-3, C-4′), 73.6, 73.3, 73.3 (3C, 3 × O*C*H$_2$Ph), 71.9 (C-5″), 71.2 (C-5′), 70.8 (C-5), 69.9, 69.8, 69.5 (3C, C-6, C-6′, C-6″), 68.4 (C-4″), 68.2 (octyl OCH_2), 67.7 (C-2′), 67.6 (C-2″), 59.3, 57.6, 57.2 (3C, 3 × OMe), 31.8, 29.5, 29.4, 29.2, 26.1, 22.7 (6C, octyl CH_2), 21.2, 21.0, 20.9, 20.9 (4C, 4 × Ac), 14.1 (octyl CH_3);

(2*R*,6*R*)-6-((benzyloxy)methyl)-5-oxo-5,6-dihydro-2*H*-pyran-2-yl *tert*-butyl carbonate (**57**): To a stirring ice-cold solution of **62** (2.9 g, 13.2 mmol) in THF and H_2O (21 mL, THF–H_2O 4:1) was add $NaHCO_3 \cdot 3H_2O$ (2.3 g, 26.5 mmol) and NaOAc (1.1 g, 13.2 mmol). Then, *N*-bromosuccinimide (2.4 g, 13.2 mmol) was added and the resulting yellow solution was stirred at 0 °C for 1.5 h. The reaction mixture was concentrated to remove THF and the residue was extracted with CH_2Cl_2. After washing with saturated aqueous $NaHCO_3$ and brine, the organic layer was dried over Na_2SO_4 and concentrated to afford yellow liquid (3.4 g). This yellow liquid (1.5 g, 5.7 mmol) was then dissolved in CH_2Cl_2 (8 mL) and cooled to −78 °C. Di-*t*-butyl dicarbonate (1.5 g, 6.8 mmol) was added into this solution followed by 4-dimethylamino-pyridine (70.8 mg, 0.6 mmol). The solution was stirred for 1 h while warming to room temperature. The resulting black solution was concentrated and purified by chromatography (hexane–EtOAc 9:1) to afford α isomer **57** (919.4 mg, 48% over two steps) as a pale yellow syrup. This reaction also produced β isomer (212.1 mg, 11%) as yellow syrup. The following data are for α isomer **57** only. R_f 0.57 (hexane–EtOAc 3:1); $[\alpha]_D = -72.2$ (*c* 3.0, $CHCl_3$); IR: 1751.6 (C=O), 1702.3 (Boc C=O); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.27 (m, 5H, Ar), 6.94 (dd, *J* = 10.3, 3.7 Hz, 1H, H-2), 6.48 (d, *J* = 3.7 Hz, 1H, H-1), 6.28 (d, *J* = 10.3 Hz, 1H, H-3), 4.72 (dd, *J* = 4.4, 2.7 Hz, 1H, H-5), 4.60 (s, 2H, 2 × OC*H*$_2$Ph), 3.97 (dd, *J* = 10.9, 4.4 Hz, 1H, H-6a), 3.92 (dd, *J* = 10.9, 2.6 Hz, 1H, H-6b), 1.54 (s, 9H, Boc); ^{13}C NMR (126 MHz, $CDCl_3$) δ 193.0 (C-4), 151.7 (Boc C=O), 141.4 (C-2), 137.8 (Ar), 129.1 (C-3), 128.4 (2C, Ar), 127.7 (2C, Ar), 127.7

(Ar), 89.2 (C-1), 83.7 (Boc *t*-Bu), 76.4 (C-5), 73.7 (O*C*H_2Ph), 68.5 (C-6), 27.7 (Boc *t*-Bu); HRMS (ESI) calcd $C_{18}H_{22}O_6$ $[M+Na]^+$ 357.1309, found 357.1311.

2-hydroxy-1-(1-pyrrolidinyl)ethanone (**59**): Glycolic acid **58** (29.6 g, 389.2 mmol) and pyrrolidine (32.5 mL, 389.2 mmol) in *o*-xylene (40 mL) were heated at reflux overnight with azeotropic removal of water (6.9 mL collected). Amide **59** (38.9 g, 77%) was then isolated by reduced-pressure distillation (165–175 °C, 30 mm Hg) as a pale yellow solid. ^{1}H NMR (498 MHz, $CDCl_3$) δ 4.07 (s, 2H, OCH_2), 3.53 (app t, J = 6.9 Hz, 2H, NCH_2), 3.27 (app t, J = 6.8 Hz, 2H, NCH_2), 1.98 (app p, J = 6.8 Hz, 2H, $NCH_2C\mathit{H}_2$), 1.88 (app p, J = 6.8 Hz, 2H, $NCH_2C\mathit{H}_2$); ^{13}C NMR (125 MHz, $CDCl_3$) δ 169.9 (C=O), 60.5 (OCH_2), 46.0 (NCH_2), 44.3 (NCH_2), 25.8 ($NCH_2\mathit{C}H_2$), 24.0 ($NCH_2\mathit{C}H_2$); HRMS (ESI) calcd $C_6H_{11}O_2N$ $[M+Na]^+$ 152.0682, found 152.0682.

2-benzyloxy-1-(1-pyrrolidinyl)ethanone (**60**): To a stirring ice-cold solution of **59** (9.25 g, 71.6 mmol) in DMF (17 mL) was added NaH (60% in mineral oil, 3.85 g, 96.3 mmol). The solution was stirred at 0 ℃ for 0.5 h before (10.4 mL, 85.9 mmol) was added. After stirring overnight while warming to room temperature, methanol (3 mL) was added to the mixture, which was then diluted with CH_2Cl_2, before being washed with water followed by saturated aqueous $NaHCO_3$ and brine. The separated organic layer was dried over Na_2SO_4, concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 1:1 to 1:3) to afford **60** (13.9 g, 89%) as bright yellow liquid. R_f 0.36 (EtOAc); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.42–7.27 (m, 5H, Bn), 4.66 (s, 2H, O$C\mathit{H}_2$Ph), 4.12 (s, 2H, OCH_2), 3.52 (app t, J = 6.9 Hz, 2H, NCH_2), 3.40 (app t, J

= 6.8 Hz, 2H, NCH_2), 1.94 (app p, *J* = 6.8 Hz, 2H, NCH_2CH_2), 1.85 (app p, *J* = 6.7 Hz, 2H, NCH_2CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 167.8 (C=O), 137.5 (Ar), 128.4 (2C, Ar), 128.1 (2C, Ar), 127.9 (Ar), 73.2 (OCH_2Ph), 69.6 (OCH_2), 45.9 (NCH_2), 45.7 (NCH_2), 26.2 (NCH_2CH_2), 23.9 (NCH_2CH_2); HRMS (ESI) calcd $C_{13}H_{17}O_2N$ $[M+Na]^+$ 242.1151, found 242.1151.

2-benzyloxy-1-(2-furanyl)ethanone (**61**): To a stirring ice-cold solution of furan (10 mL, 136 mmol) in THF (40 mL) was very slowly and carefully added *n*-BuLi (2.5M in hexane, 27.5 mL, 68.8 mmol). The resulting yellow solution was stirred for 20 min at 0 °C before being cooled to −78 °C. A solution of **60** (13.0 g, 59.3 mmol) in THF (35 mL) was then added into the solution and the reaction mixture was stirred vigorously at −78 °C for another 1 h before saturated aqueous NH_4Cl was added. After warming to room temperature, the mixture was diluted with EtOAc and the organic layer was washed with saturated aqueous $NaHCO_3$ and brine. The organic layer was dried over Na_2SO_4, concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 8:1) to afford **61** (10.3 g, 80%) as a yellow liquid. R_f 0.38 (hexane–EtOAc 3:1); ^1H NMR (498 MHz, $CDCl_3$) δ 7.54 (dd, *J* = 1.7, 0.7 Hz, 1H, furan OCH), 7.39–7.23 (m, 6H, 5 × Ar, furan CH), 6.49 (dd, *J* = 3.6, 1.7 Hz, 1H, furan CH), 4.65 (s, 2H, OCH_2Ph), 4.55 (s, 2H, OCH_2); ^{13}C NMR (125 MHz, $CDCl_3$) δ 185.5 (C=O), 150.9 (furan OCH), 146.6 (furan OCH), 137.2 (Ar), 128.5 (2C, Ar), 128.0 (2C, Ar), 128.0 (Ar), 118.2 (furan CH), 112.3 (furan CH), 73.5 (OCH_2Ph), 72.2 (OCH_2); HRMS (ESI) calcd $C_{13}H_{12}O_3$ $[M+Na]^+$ 239.0679, found 239.0681.

(*R*)-2-(benzyloxy)-1-(furan-2-yl)ethanol (**62**): To a solution of **61** (7.3 g, 33.8 mmol) in mixed water and CH_2Cl_2 (74 mL, H2O–CH_2Cl_2 40:1) was added HCOONa (11.6 g, 169 mmol), cetyltrimethylammonium bromide (CTAB) (1.3 g, 3.4 mmol) and Noyori's catalyst ((*R*)-RuCl[(1*R*,2*R*)-*p*-TsNCH(C_6H_5)CH(C_6H_5)NH_2](η^6-mesitylene)) (210.7 mg, 0.34 mmol). The reaction flask was de-gassed and recharged with Argon. After stirring at room temperature overnight, the reaction mixture was extracted three times with CH_2Cl_2. The combined organic layer was then washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and then concentrated. The resulting residue was purified by chromatography (hexane–EtOAc 8:1 to 7:1) to afford **62** (4.5 g, 92%, 95% ee) as a pale yellow liquid. The enantiomeric purity was determined by NMR analysis of the ester obtained from the reaction of **62** and (*S*)-+-O-acetyl mandelic acid (based on integration of methylene protons from the benzyl ether). R_f 0.30 (toluene–EtOAc 5:1); $[\alpha]_D$ = +14.0 (*c* 0.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.43–7.29 (m, 6H, 5 × Ar, furan OCH), 6.40–6.30 (m, 2H, furan CH), 4.96 (ddd, *J* = 5.5, 5.5, 3.4 Hz, 1H, OCH), 4.65 (d, *J* = 12.0 Hz, 1H, OC*H*$_2$Ph), 4.62 (d, *J* = 12.0 Hz, 1H, OC*H*$_2$Ph), 3.79 (d, *J* = 5.8 Hz, 2H, OCH_2), 2.81 (d, *J* = 3.2 Hz, 1H, OH); ^{13}C NMR (126 MHz, $CDCl_3$) δ 153.5 (furan OCH), 142.2 (furan OCH), 137.7 (Ar), 128.5 (2C, Ar), 127.9 (Ar), 127.8 (2C, Ar), 110.3, 107.1 (2C, furan CH), 73.5 (O*C*H_2Ph), 72.5 (OCH_2), 66.9 (OCH); HRMS (ESI) calcd $C_{13}H_{14}O_3$ $[M+Na]^+$ 241.0835, found 241.0837.

(2*R*,6*S*)-2-((benzyloxy)methyl)-6-(octyloxy)-2*H*-pyran-3(6*H*)-one (**63**): A solution of CH_2Cl_2 (3 mL) containing **57** (352.9 mg, 1.1 mmol) and 1-octanol (0.5

mL, 3.2 mmol) with 4 Å molecular sieves was stirred at room temperature for 0.5 h before 2.5 mol% tris(dibenzylideneacetone)dipalladium(0) $Pd_2(dba)_3$ (26.8 mg, 0.03 mmol) and 10 mol% triphenylphosphine PPh_3 (28.0 mg, 0.12 mmol) were added. After stirring for 1.5 h, the resulting purple solution was concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 15:1) to afford **63** (351.4 mg, 96%) as a colorless liquid. R_f 0.59 (hexane–EtOAc 3:1); $[\alpha]_D$ = –32.7 (*c* 0.9, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.39–7.26 (m, 5H, Ar), 6.91 (dd, *J* = 10.2, 3.5 Hz, 1H, H-2), 6.15 (d, *J* = 10.3 Hz, 1H, H-3), 5.33 (d, *J* = 3.5 Hz, 1H, H-1), 4.65–4.60 (m, 3H, H-5, 2 × OC*H*$_2$Ph), 3.95 (dd, *J* = 10.8, 4.8 Hz, 1H, H-6a), 3.92 (dd, *J* = 10.9, 3.0 Hz, 1H, H-6b), 3.88 (dt, *J* = 9.6, 6.8 Hz, 1H, octyl OCH_2), 3.62 (dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 1.70–1.59 (m, 2H, octyl OCH_2CH_2), 1.44–1.21 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 194.4 (C-4), 144.1 (C-2), 138.0 (Ar), 128.3 (2C, Ar), 127.8 (Ar), 127.6 (3C, Ar (2C), C-3), 93.2 (C-1), 74.5 (C-5), 73.7 (O*C*H$_2$Ph), 69.7 (octyl OCH_2), 68.7 (C-6), 31.8, 29.7, 29.4, 29.3, 26.2, 22.7 (6C, octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{21}H_{30}O_4$ $[M+Na]^+$ 369.2036, found 369.2040.

(2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(octyloxy)-3,6-dihydro-2*H*-pyran-3-ol (**64**): To a solution of ketone **63** (343.0 mg, 1.1 mmol) in methanol (2.5 mL) at −78 °C was added $NaBH_4$ (42.3 mg, 1.1 mmol) and $CeCl_3$•$7H_2O$ (392.7 mg, 1.1 mmol). The solution was stirred overnight while warming to room temperature. The mixture was concentrated to remove the methanol and then the residue was redissolved in CH_2Cl_2. After washing with water and brine, the organic layer was

dried over Na_2SO_4, and then concentrated. The resulting residue was purified by chromatography (hexane–EtOAc 6:1) to afford alcohol **64** (307.3 mg, 88%) as a colorless syrup. R_f 0.35 (hexane–EtOAc 3:1); $[\alpha]_D$ = +20.0 (*c* 0.8, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.40–7.27 (m, 5H, Ar), 5.94 (d, *J* = 10.2 Hz, 1H, H-3), 5.76 (ddd, *J* = 10.2, 2.7, 2.2 Hz, 1H, H-2), 4.99 (d, *J* = 2.6 Hz, 1H, H-1), 4.65 (d, *J* = 12.0 Hz, 1H, OC*H*$_2$Ph), 4.61 (d, *J* = 12.0 Hz, 1H, OC*H*$_2$Ph), 4.23 (dddd, *J* = 9.2, 5.6, 3.5, 1.8 Hz, 1H, H-4), 3.89–3.83 (m, 1H, H-5), 3.82–3.75 (m, 2H, H-6a, octyl OCH_2), 3.72 (dd, *J* = 10.0, 4.9 Hz, 1H, H-6b), 3.50 (dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 2.67 (d, *J* = 5.9 Hz, 1H, OH-4), 1.67–1.55 (m, 2H, octyl OCH_2C*H*$_2$), 1.43–1.22 (m, 10H, octyl CH_2), 0.91 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 137.9 (Ar), 133.0 (C-3), 128.5 (2C, Ar), 127.8 (Ar), 127.7 (2C, Ar), 126.2 (C-2), 94.3 (C-1), 73.7 (O*C*H$_2$Ph), 70.7 (C-6), 70.0 (C-5), 68.8 (octyl OCH_2), 65.6 (C-4), 31.9, 29.8, 29.4, 29.3, 26.2, 22.7 (6C, octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{21}H_{32}O_4$ $[M+Na]^+$ 371.2193, found 371.2189.

(2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(((2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(octyloxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-3,6-dihydro-2*H*-pyran-3-ol (**65**): The reaction was performed as described for the synthesis of **63**, with alcohol **64** (611.8 mg, 1.8 mmol) and donor **57** (818.5 mg, 2.6 mmol) in the presence of $Pd_2(dba)_3$ (41.8 mg, 0.05 mmol) and PPh_3 (60.0 mg, 0.23 mmol) in CH_2Cl_2 (6 mL). The crude residue was purified by chromatography (hexane–EtOAc 7:1) to afford a ketone (842.1 mg, 85%) as a colorless syrup. This ketone was then reduced as described for **9**, with $NaBH_4$ (68.6 mg, 1.8 mmol) and $CeCl_3$•$7H_2O$ (656.2 mg, 1.8 mmol) in methanol (6 mL). Chromatographic purification of the

crude reaction mixture (hexane–EtOAc 6:1) furnished alcohol **65** (770.3 mg, 91%) as a colorless syrup. R_f 0.38 (hexane–EtOAc 2:1); $[\alpha]_D$ = +35.3 (*c* 0.9, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.40–7.24 (m, 10H, Ar), 6.05 (d, *J* = 10.3 Hz, 1H, H-3), 5.97 (d, *J* = 10.2 Hz, 1H, H-3′), 5.84 (ddd, *J* = 10.3, 2.7, 1.8 Hz, 1H, H-2), 5.68 (ddd, *J* = 10.2, 2.4, 2.4 Hz, 1H, H-2′), 5.20 (d, *J* = 2.4 Hz, 1H, H-1′), 5.03 (d, *J* = 2.1 Hz, 1H, H-1), 4.62–4.41 (m, 5H, H-4, 4 × OC*H*$_2$Ph), 4.26 (dddd, *J* = 10.3, 3.4, 3.4, 1.7 Hz, 1H, H-4′), 4.01 (ddd, *J* = 9.4, 3.6, 3.6 Hz, 1H, H-5), 3.81 (dt, *J* = 9.5, 6.8 Hz, 1H, octyl OCH_2), 3.77–3.73 (m, 2H, H-6a, H-6b), 3.73–3.68 (m, 1H, H-5′), 3.66 (dd, *J* = 9.6, 4.3 Hz, 1H, H-6a′), 3.55 (dd, *J* = 9.6, 5.7 Hz, 1H, H-6b′), 3.50 (dt, *J* = 9.5, 6.6 Hz, 1H, octyl OCH_2), 2.33 (d, *J* = 4.9 Hz, 1H, OH-4′), 1.67–1.57 (m, 2H, octyl $OCH_2C\mathit{H}_2$), 1.40–1.21 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.5, 137.6 (2C, Ar), 133.2 (C-3′), 129.3 (C-3), 128.5 (2C, Ar), 128.3 (2C, Ar), 127.9 (Ar), 127.8 (2C, Ar), 127.4 (3C, Ar), 127.3 (C-2), 125.8 (C-2′), 94.3 (C-1), 91.1 (C-1′), 73.7, 73.21 (2C, 2 × O*C*H_2Ph), 70.6 (C-6′), 69.9 (C-5′), 69.7 (C-6), 69.1 (C-5), 68.7 (octyl OCH_2), 67.3 (C-4), 66.0 (C-4′), 31.9, 29.8, 29.4, 29.3, 26.3, 22.7 (6C, octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{34}H_{46}O_7$ $[M+Na]^+$ 589.3136, found 589.3136.

(2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(((2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(((2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(octyloxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-3,6-dihydro-2*H*-pyran-3-ol (**66**): The reaction was performed as described for the synthesis of **63**, with alcohol **65** (978.0 mg, 1.73 mmol) and donor **57** (1.02 g, 3.05 mmol) in the presence of $Pd_2(dba)_3$ (69.0 mg, 0.08 mmol) and PPh_3 (80.0 mg, 0.31 mmol) in CH_2Cl_2 (22

mL). The crude residue was purified by chromatography (hexane–EtOAc 9:1) to afford a ketone (1.28 g, 95%) as a pale yellow syrup. $[\alpha]_D$ = +5.1 (*c* 0.6, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.38–7.21 (m, 15H, Ar), 6.83 (dd, *J* = 10.2, 3.5 Hz, 1H, H-2″), 6.18 (d, *J* = 10.3 Hz, 1H, H-3″), 6.11 (d, *J* = 10.3 Hz, 1H, H-3′), 6.05 (d, *J* = 10.3 Hz, 1H, H-3), 5.85 (ddd, *J* = 10.3, 2.7, 1.8 Hz, 1H, H-2), 5.78 (ddd, *J* = 10.3, 2.7, 1.9 Hz, 1H, H-2′), 5.57 (d, *J* = 3.5 Hz, 1H, H-1″), 5.27 (d, *J* = 2.1 Hz, 1H, H-1′), 5.03 (d, *J* = 2.2 Hz, 1H, H-1), 4.63 (dd, *J* = 9.3, 1.3 Hz, 1H, H-4′), 4.60–4.37 (m, 8H, H-4, H-5″, 6 × OC*H*$_2$Ph), 4.02 (ddd, *J* = 9.3, 5.6, 1.8 Hz, 1H, H-5), 3.85–3.75 (m, 4H, H-5′, H-6a, H-6a″, octyl OCH_2), 3.71 (dd, *J* = 10.9, 5.7 Hz, 1H, H-6b), 3.63–3.58 (m, 2H, H-6a′, H-6b″), 3.54 (dd, *J* = 10.8, 1.7 Hz, 1H, H-6b′), 3.50 (dt, *J* = 9.5, 6.6 Hz, 1H, octyl OCH_2), 1.66–1.57 (m, 2H, octyl $OCH_2C\mathit{H}_2$), 1.41–1.22 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 194.0 (C-4″), 143.6 (C-2″), 138.5, 138.0, 137.8 (3C, Ar), 129.2 (2C, C-3, C-3′), 128.3 (2C, Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 128.2 (C-3″), 127.7 (2C, Ar), 127.6 (2C, Ar), 127.6 (2C, Ar), 127.4 (4C, 3 × Ar, C-2), 127.3 (C-2′), 94.2 (C-1), 91.4 (C-1′), 90.0 (C-1″), 75.0 (C-5″), 73.7, 73.5, 73.2 (3C, 3 × O*C*H_2Ph), 69.9 (C-6), 69.7 (C-5′), 69.1 (C-5), 69.0 (C-6′), 68.7 (octyl OCH_2), 68.5 (C-6″), 67.5 (C-4), 67.4 (C-4′), 31.9, 29.8, 29.4, 29.3, 26.3, 22.7 (6C, octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{47}H_{58}O_{10}$ $[M+Na]^+$ 805.3922, found 805.3915. This ketone (208.9 mg, 0.21 mmol) was then reduced as described for **9**, with $NaBH_4$ (8.0 mg, 0.21 mmol) and $CeCl_3{\cdot}7H_2O$ (80.2 mg, 0.21 mmol) in methanol (2.5 mL). Chromatographic purification of the crude reaction mixture (hexane–EtOAc 3:1) furnished alcohol **66** (177.2 mg, 85%) as a

colorless syrup. R_f 0.30 (hexane–EtOAc 3:1); $[\alpha]_D$ = +35.6 (*c* 0.8, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.38–7.23 (m, 15H, Ar), 6.07 (d, *J* = 10.1 Hz, 1H, H-3′), 6.05 (d, *J* = 9.3 Hz, 1H, H-3), 5.99 (d, *J* = 10.2 Hz, 1H, H-3″), 5.84 (ddd, *J* = 10.3, 2.7, 1.8 Hz, 1H, H-2), 5.74 (ddd, *J* = 10.3, 2.7, 1.9 Hz, 1H, H-2′), 5.70 (ddd, *J* = 10.2, 2.7, 2.3 Hz, 1H, H-2″), 5.25 (d, *J* = 2.0 Hz, 1H, H-1′), 5.20 (d, *J* = 2.4 Hz, 1H, H-1″), 5.03 (d, *J* = 2.1 Hz, 1H, H-1), 4.60–4.39 (m, 8H, H-4, H-4′, 3 × OCH_2Ph), 4.27 (ddd, *J* = 8.6, 4.4, 1.7 Hz, 1H, H-4″), 4.02 (ddd, *J* = 9.2, 6.0, 1.7 Hz, 1H, H-5), 3.88–3.76 (m, 3H, H-5′, H-6a, octyl OCH_2), 3.74–3.62 (m, 4H, H-5″, H-6a, H-6a′, H-6a″), 3.60 (dd, *J* = 10.8, 2.0 Hz, 1H, H-6b′), 3.55–3.46 (m, 2H, H-6b″, octyl OCH_2), 2.41 (d, *J* = 4.6 Hz, 1H, OH-4″), 1.67–1.55 (m, 2H, octyl OCH_2CH_2), 1.40–1.20 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.5, 138.4, 137.6 (3C, 3 × Ar), 133.3 (C-3″), 129.9, 129.3 (2C, C-3, C-3′), 128.5 (2C, Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 127.9 (Ar), 127.8 (2C, Ar), 127.4 (3C, Ar), 127.4 (2C, Ar), 127.4 (Ar), 127.3 (C-2), 126.7 (C-2′), 125.7 (C-2″), 94.2 (C-1), 91.3 (C-1′), 91.0 (C-1″), 73.7, 73.4, 73.2 (3C, 3 × OCH_2Ph), 70.7 (C-6″), 69.9 (C-6), 69.8 (2C, C-5′, C-5″), 69.2 (C-6′), 69.1 (C-5), 68.7 (octyl OCH_2), 67.5 (C-4), 66.7 (C-4′), 66.1 (C-4″), 31.9, 29.8, 29.4, 29.3, 26.3, 22.7 (6C, octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{47}H_{60}O_{10}$ $[M+Na]^+$ 807.4079, found 807.4070.

Synthesis of octyl 3-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-3-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranoside (68a) and octyl 3-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranoside (68b): Method A: Disaccharide **55** (13.3 mg, 0.02 mmol) and

(n-$Bu_3Sn)_2O$ (175 μL, 0.03 mmol) were heated at reflux in toluene (1.2 mL) for 15 h. The resulting yellowish solution was cooled to room temperature before BzCl (48 μL, 0.4 mmol) was added. After stirring at room temperature for 6 h, the reaction mixture was concentrated and the crude products were confirmed by ^{1}H NMR spectroscopy to be a mixture of **68a** (21% NMR yield) and **68b** (79% NMR yield). **Method B**: To a solution of **55** (14.1 mg, 0.02 mmol) in CH_2Cl_2 (0.5 mL) was added n-Bu_2SnO (1.3 mg, 0.004 mmol), Et_3N (9 μL, 0.06 mmol) followed by BzCl (6.5 μL, 0.05 mmol). The reaction mixture was stirred at room temperature overnight. The crude NMR spectrum indicated a mixture of **68a** (25%) and **68b** (60%).

Octyl 3-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-3-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranoside (**68a**): ^{1}H NMR (600 MHz, $CDCl_3$) δ 8.23–6.99 (m, 20H, Ar), 5.49 (dd, J = 9.6, 3.0 Hz, 1H, H-3), 5.23 (dd, J = 9.8, 3.2 Hz, 1H, H-3′), 5.22 (d, J = 1.4 Hz, 1H, H-1′), 4.87 (d, J = 1.9 Hz, 1H, H-1), 4.61–4.49 (m, 4H, 4 × OCH_2Ph), 4.38 (app t, J = 9.5 Hz, 1H, H-4), 4.20–4.18 (m, 1H, H-2), 4.10 (app td, J = 9.6, 4.3 Hz, 1H, H-4′), 4.01–3.71 (m, 6H, H-2′, H-5, H-5′, H-6a, H-6b, octyl OCH_2), 3.69 (dd, J = 10.2, 3.6 Hz, 1H, H-6a′), 3.61 (dd, J = 10.2, 4.2 Hz, 1H, H-6b′), 3.50–3.43 (m, 1H, octyl OCH_2), 2.71 (d, J = 4.3 Hz, 1H, OH-4′), 2.09 (d, J = 6.6 Hz, 1H, OH-2), 1.89 (d, J = 5.2 Hz, 1H, OH-2′), 1.78–1.58 (m, 2H, octyl OCH_2CH_2), 1.55–1.18 (m, 10H, octyl CH_2), 0.90 (t, J = 7.1 Hz, 3H, octyl CH_3).

Octyl 3-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranoside (**68b**): ^{1}H NMR (600 MHz, CD_3OD) δ 8.23–6.99 (m, 20H, Ar), 5.38 (d, J = 2.0 Hz, 1H, H-1′), 5.28 (dd, J = 9.7, 3.2 Hz, 1H, H-3′),

5.22 (dd, *J* = 2.8, 1.8 Hz, 1H, H-2), 4.87 (d, *J* = 1.8 Hz, 1H, H-1), 4.61–4.49 (m, 4H, 4 × OC*H*$_2$Ph), 4.25 (dd, *J* = 3.1, 2.0 Hz, 1H, H-2′), 4.23–4.19 (m, 1H, H-3), 4.07 (app t, *J* = 9.9 Hz, 1H, H-4′), 4.01–3.92 (m, 3H, H-5, H-5′, H-6), 3.90–3.83 (m, 2H, H-4, H-6), 3.79–3.67 (m, 3H, 2 × H-6, octyl OCH$_2$), 3.49 (app dt, *J* = 9.7, 6.4 Hz, 1H, octyl OCH$_2$), 1.78–1.58 (m, 2H, octyl OCH$_2$C*H*$_2$), 1.55–1.18 (m, 10H, octyl CH$_2$), 0.90 (t, *J* = 7.1 Hz, 3H, octyl CH$_3$).

(((2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(((2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(octyloxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)(tert-butyl)dimethylsilane (**73**): To a solution of alcohol **65** (302.3 mg, 0.53 mmol) in CH_2Cl_2 (6 mL) at −10 °C was added 2,6-lutidine (280 μL, 2.40 mmol) followed by TBSOTf (380 μL, 1.60 mmol). The resulting solution was stirred for 0.5 h before being concentrated. The crude product was purified by chromatography (hexane–EtOAc 12:1) to afford trisaccharide **73** (360.4 mg, 99%) as a colorless syrup. R_f 0.65 (hexane–EtOAc 3:1); $[\alpha]_D$ = +58.8 (*c* 1.1, $CHCl_3$); ^{1}H NMR (498 MHz, $CDCl_3$) δ 7.36–7.20 (m, 10H, Ar), 6.02 (d, *J* = 10.3 Hz, 1H, H-3), 5.87 (d, *J* = 10.2 Hz, 1H, H-3′), 5.81 (ddd, *J* = 10.3, 2.6, 1.9 Hz, 1H, H-2), 5.60 (ddd, *J* = 10.2, 2.7, 2.1 Hz, 1H, H-2′), 5.21 (d, *J* = 2.8 Hz, 1H, H-1′), 5.01 (d, *J* = 2.0 Hz, 1H, H-1), 4.58 (d, *J* = 12.2 Hz, 1H, OC*H*$_2$Ph), 4.53 (d, *J* = 12.2 Hz, 1H, OC*H*$_2$Ph), 4.47 (s, 2H, OC*H*$_2$Ph), 4.38 (dd, *J* = 9.5, 1.2 Hz, 1H, H-4), 4.34 (dd, *J* = 9.1, 1.4 Hz, 1H, H-4′), 4.02 (ddd, *J* = 9.0, 6.0, 1.7 Hz, 1H, H-5), 3.84–3.73 (m, 2H, H-6a, octyl OCH$_2$), 3.71–3.62 (m, 2H, H-5′, H-6b), 3.55 (dd, *J* = 10.5, 3.7 Hz, 1H, H-6a′), 3.51–3.44 (m, 2H, H-6b′, octyl OCH$_2$), 1.68–1.52 (m, 2H, octyl OCH$_2$C*H*$_2$), 1.38–1.18 (m, 10H, octyl CH$_2$), 0.97–0.81 (m, 12H, octyl CH$_3$,

TBS(*t*-Bu)), 0.08 (s, 3H, TBS(Me)), 0.02 (s, 3H, TBS(Me)); ^{13}C NMR (125 MHz, $CDCl_3$) δ 138.5, 138.1 (2C, Ar), 134.8 (C-3′), 129.4 (C-3), 128.3 (2C, Ar), 128.2 (2C, Ar), 127.7 (2C, Ar), 127.5 (Ar), 127.4 (2C, Ar), 127.3 (Ar), 127.2 (C-2), 125.1 (C-2′), 94.2 (C-1), 91.6 (C-1′), 73.5, 73.2 (2C, 2 × O*C*H_2Ph), 71.6 (C-5′), 69.9 (C-6), 69.1 (C-5), 68.6, 68.6 (2C, C-6′, octyl OCH_2), 67.5 (C-4), 63.8 (C-4′), 31.8, 29.8, 29.4, 29.3, 26.3 (5C, octyl CH_2), 25.7 (TBS(*t*-Bu), 22.7 (octyl CH_2), 17.9 (TBS(*t*-Bu)), 14.1 (octyl CH_3), –4.2, –4.9 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{40}H_{60}O_7Si$ $[M+Na]^+$ 703.4001, found 703.3994.

Octyl 6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranoside (**74**): The dihydroxylation reaction was performed as described for the synthesis of **55**, with **73** (588.6 mg, 0.86 mmol), OsO_4 (2.5 wt.% in *t*-butanol, 213 μL, 0.017 mmol), NMO (50% w/v in water, 1.3 mL) in *t*-butanol and acetone (9 mL, v/v 1:1). The crude residue was purified by chromatography (CH_2Cl_2–methanol 30:1) to afford **74** (68.4 mg, 91%) as a colorless syrup. R_f 0.49 (CH_2Cl_2–methanol 15:1); $[\alpha]_D$ = +58.7 (*c* 0.7, $CHCl_3$); ^{1}H NMR (500 MHz, CD_3OD) δ 7.33–7.22 (m, 10H, Ar), 5.24 (d, *J* = 1.9 Hz, 1H, H-1′), 4.71 (d, *J* = 1.5 Hz, 1H, H-1), 4.57–4.41 (m, 4H, 4 × O*C*H_2Ph), 3.89 (dd, *J* = 3.1, 2.2 Hz, 1H, H-2), 3.84–3.77 (m, 3H, H-4, H-6a, H-6b), 3.77–3.67 (m, 6H, H-2′, H-3, H-4′, H-5, H-5′, octyl OCH_2), 3.60 (dd, *J* = 8.5, 3.3 Hz, 1H, H-3′), 3.59–3.55 (m, 2H, H-6a′, H-6b′), 3.40 (dt, *J* = 9.6, 6.3 Hz, 1H, octyl OCH_2), 1.65–1.52 (m, 2H, octyl OCH_2C*H*$_2$), 1.41–1.23 (m, 10H, octyl CH_2), 0.89 (t, *J* = 6.9 Hz, 3H, octyl CH_3), 0.86 (s, 9H, TBS(*t*-Bu)), 0.14 (s, 3H, TBS(Me)), 0.05 (s, 3H, TBS(Me)); ^{13}C NMR (126 MHz, CD_3OD) δ 139.7 (Ar), 139.5 (Ar), 129.4 (2C,

Ar), 129.4 (2C, Ar), 129.2 (2C, Ar), 128.8 (2C, Ar), 128.7 (Ar), 128.6 (Ar), 103.1 (C-1′, $^1J_{C\text{-}1,H\text{-}1}$ = 171.7 Hz), 101.5 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 167.3 Hz), 76.1 (C-4), 74.6 (C-5′), 74.5, 74.4 (2C, 2 × O*C*H$_2$Ph), 73.3 (C-4′), 72.9, 72.7, 72.7, 72.1 (4C, C-2, C-2′, C-3, C-5), 71.4 (C-6), 71.0 (C-6′), 70.5 (C-3′), 68.9 (octyl OCH$_2$), 33.0, 30.6, 30.4, 30.4, 27.4 (5C, octyl CH$_2$), 26.7 (TBS(*t*-Bu)), 23.7 (octyl CH$_2$), 19.2 (TBS(*t*-Bu)), 14.5 (octyl CH$_3$), –3.4, –4.7 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{40}H_{64}O_{11}Si$ $[M+Na]^+$ 771.4110, found 771.4106.

General procedures for reactions in Table 4-2: To a solution of **74** (1 equiv, 0.1 M in the indicated solvent (0.1 M) was added catalyst (20 mol%) and base (4 equiv for entry 1–4, 10 equiv for entry 5–6) followed by BzCl (2.4–10 equiv). The resulting mixture was stirred at room temperature for 3 h before concentrated. The crude products **74a**, **74c** and **74d** were purified by chromatography as indicated below.

Octyl 2-*O*-benzoyl-6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranoside (**74a**): The reaction was performed as the general procedure described above. Chromatographic purification (hexane–EtOAc 7:1) gave **74a** as a colorless film. R_f 0.66 (toluene–EtOAc 5:1); $[\alpha]_D$ = +13.9 (*c* 0.4, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.05 (dd, *J* = 8.4, 1.3 Hz, 2H, Ar), 8.01 (dd, *J* = 8.4, 1.3 Hz, 2H, Ar), 7.60–7.52 (m, 2H, Ar), 7.44–7.26 (m, 14H, Ar), 5.49 (d, *J* = 1.8 Hz, 1H, H-1′), 5.36 (dd, *J* = 3.4, 1.8 Hz, 2H, H-2, H-2′), 4.97 (d, *J* = 1.8 Hz, 1H, H-1), 4.73–4.54 (m, 4H, 4 × OC*H*$_2$Ph), 4.42 (dd, *J* = 9.6, 3.5 Hz, 1H, H-3), 4.27 (app t, *J* = 9.6 Hz, 1H, H-4), 4.12–4.03 (m, 1H, H-3′), 4.01–3.90 (m, 4H, H-4′, H-5, H-5′, H-6a), 3.88 (dd, *J* = 10.4, 1.5

Hz, 1H, H-6b), 3.76 (dt, *J* = 9.6, 6.8 Hz, 1H, octyl OCH_2), 3.67 (dd, *J* = 10.3, 1.0 Hz, 1H, H-6a′), 3.63 (dd, *J* = 10.3, 5.1 Hz, 1H, H-6b′), 3.49 (dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 1.68–1.57 (m, 2H, octyl OCH_2CH_2), 1.44–1.22 (m, 10H, octyl CH_2), 0.91 (t, *J* = 7.0 Hz, 3H, octyl CH_3), 0.87 (s, 9H, TBS(*t*-Bu)), 0.13 (s, 3H, TBS(Me)), 0.05 (s, 3H, TBS(Me)); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.3 (C=O), 166.2 (C=O), 138.6 (Ar), 138.1 (Ar), 133.3 (Ar), 133.2 (Ar), 129.9 (2C, Ar), 129.8 (2C, Ar), 129.7 (Ar), 129.6 (Ar), 128.5 (2C, Ar), 128.4 (2C, Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 127.5 (3C, Ar), 127.4 (3C, Ar), 97.3 (C-1), 96.9 (C-1′), 75.2 (C-4), 73.5, 73.5 (2C, C-2, C-2′), 73.4, 73.3 (2C, 2 × O*C*H_2Ph), 73.2 (C-5′), 70.8 (C-3′), 69.9, 69.6 (2C, C-4′, C-5), 69.5 (C-6), 69.3 (C-6′), 69.2 (C-3), 68.3 (octyl OCH_2), 31.8, 29.4, 29.4, 29.2, 26.2 (5C, octyl CH_2), 25.9 (TBS(*t*-Bu)), 22.7 (octyl CH_2), 18.3 (TBS(Me)), 14.1 (octyl CH_3), –4.0, –4.9 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{54}H_{72}O_{13}Si$ $[M+Na]^+$ 979.4634, found 979.4628.

Octyl 3-*O*-benzoyl-6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-α-D-mannopyranosyl-(1→4)-2-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranoside (**74c**): The reaction was performed as the general procedure described above. Chromatographic purification (hexane–EtOAc 2:1) gave **74c** as a colorless film. R_f 0.14 (toluene–EtOAc 5:1); $[\alpha]_D$ = +17.2 (*c* 0.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.15–7.97 (m, 4H, Ar), 7.62–7.17 (m, 16H, Ar), 5.37 (dd, *J* = 8.0, 3.0 Hz, 1H, H-3′), 5.35 (dd, *J* = 3.4, 1.8 Hz, 1H, H-2), 5.32 (d, *J* = 2.7 Hz, 1H, H-1′), 4.97 (d, *J* = 1.7 Hz, 1H, H-1), 4.70–4.49 (m, 4H, 4 × O*C*H_2Ph), 4.40 (dd, *J* = 9.5, 3.4 Hz, 1H, H-3), 4.24–4.14 (m, 3H, H-2′, H-4, H-4′), 4.02–3.92 (m, 3H, H-5, H-5′, H-6a), 3.91–3.85 (m, 1H, H-6b), 3.76 (dt, *J* = 9.6, 6.7 Hz, 1H, octyl OCH_2), 3.59 (d, *J* = 3.7 Hz,

2H, H-6a′, H-6b′), 3.48 (dt, *J* = 9.6, 6.7 Hz, 1H, octyl OCH_2), 1.74–1.57 (m, 2H, octyl OCH_2CH_2), 1.47–1.23 (m, 10H, octyl CH_2), 0.92 (t, *J* = 6.9 Hz, 3H, octyl CH_3), 0.76 (s, 9H, TBS(*t*-Bu)), –0.01 (s, 3H, TBS(Me)), –0.05 (s, 3H, TBS(Me); ^{13}C NMR (126 MHz, $CDCl_3$) δ 166.3 (C=O), 166.0 (C=O), 138.6 (Ar), 137.9 (Ar), 133.2 (2C, Ar), 130.0 (Ar), 129.9 (2C, Ar), 129.9 (2C, Ar), 129.7 (Ar), 128.4 (2C, Ar), 128.4 (2C, Ar), 128.4 (2C, Ar), 128.3 (2C, Ar), 127.7 (2C, Ar), 127.6 (Ar), 127.3 (3C, Ar), 100.5 (C-1′), 97.4 (C-1), 75.9 (C-4), 75.4 (C-3′), 73.7 (C-5′), 73.4 (O*C*H_2Ph), 73.4 (C-2), 73.3 (O*C*H_2Ph), 70.3 (C-5), 69.8, 69.7 (2C, C-3, C-4′), 69.6 (C-6), 69.1 (C-6′), 68.3 (octyl OCH_2), 66.6 (C-2′), 31.9, 29.5, 29.4, 29.3, 26.2 (5C, octyl CH_2), 25.7 (TBS(*t*-Bu)), 22.7 (octyl CH_2), 18.0 (TBS(*t*-Bu)), 14.1 (octyl CH_3), –4.2, –4.9 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{54}H_{72}O_{13}Si$ $[M+Na]^+$ 979.4634, found 979.4629.

Octyl 2-*O*-benzoyl-6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-α-D-mannopyranosyl-(1→4)-3-*O*-benzoyl-6-*O*-benzyl-α-D-mannopyranoside (**74d**): The reaction was performed as the general procedure described above. Chromatographic purification (hexane–EtOAc 4:1) gave **74d** as a colorless film. R_f 0.43 (toluene–EtOAc 5:1); $[\alpha]_D$ = +42.9 (*c* 0.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.11 (dd, *J* = 8.4, 1.3 Hz, 2H, Ar), 7.81 (dd, *J* = 8.4, 1.3 Hz, 2H, Ar), 7.60–7.22 (m, 16H, Ar), 5.53 (dd, *J* = 9.6, 3.2 Hz, 1H, H-3), 5.25 (d, *J* = 1.8 Hz, 1H, H-1′), 5.14 (dd, *J* = 3.1, 2.0 Hz, 1H, H-2′), 4.89 (d, *J* = 1.8 Hz, 1H, H-1), 4.70–4.53 (m, 4H, 4 × OC*H*$_2$Ph), 4.36 (app t, *J* = 9.6 Hz, 1H, H-4), 4.21 (dd, *J* = 3.1, 2.0 Hz, 1H, H-2), 4.02–3.95 (m, 2H, H-4′, H-5), 3.94–3.89 (m, 2H, H-3′, H-6a), 3.85 (dd, *J* = 10.8, 1.8 Hz, 1H, H-6b), 3.83–3.74 (m, 2H, H-5′, octyl OCH_2), 3.73 (dd, *J* = 10.7, 4.5

Hz, 1H, H-6a′), 3.62 (dd, *J* = 10.6, 1.7 Hz, 1H, H-6b′), 3.49 (dt, *J* = 9.6, 6.7 Hz, 1H, octyl OCH_2), 1.72–1.60 (m, 2H, octyl OCH_2CH_2), 1.49–1.24 (m, 10H, octyl CH_2), 0.92 (t, *J* = 6.8 Hz, 3H, octyl CH_3), 0.87 (s, 9H, TBS(*t*-Bu)), 0.07 (s, 3H, TBS(Me)), 0.03 (s, 3H, TBS(Me)); ^{13}C NMR (126 MHz, $CDCl_3$) δ 165.6 (C=O), 165.6 (C=O), 138.6 (Ar), 138.3 (Ar), 133.3 (Ar), 133.1 (Ar), 129.8 (2C, Ar), 129.7 (2C, Ar), 129.5 (Ar), 129.4 (Ar), 128.5 (Ar), 128.4 (2C, Ar), 128.30 (2C, Ar), 128.28 (2C, Ar), 128.2 (2C, Ar), 127.6 (2C, Ar), 127.4 (Ar), 127.3 (2C, Ar), 99.5 (C-1), 99.4 (C-1′), 74.9 (C-3), 73.9 (C-5′), 73.5 (OCH_2Ph), 73.4 (C-4), 73.3 (OCH_2Ph), 73.0 (C-2′), 70.9, 70.6, 69.6 (3C, C-3′, C-4′, C-5), 69.3 (C-6), 69.2 (C-6′), 69.0 (C-2), 68.3 (octyl OCH_2), 31.9, 29.4, 29.4, 29.3, 26.2 (5C, octyl CH_2), 26.0 (TBS(*t*-Bu)), 22.7 (octyl CH_2), 18.3 (TBS(*t*-Bu)), 14.1 (octyl CH_3), –4.1, –5.0 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{54}H_{72}O_{13}Si$ $[M+Na]^+$ 979.4634, found 979.4628.

Octyl 6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-2-*O*-pivaloyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-pivaloyl-α-D-mannopyranoside

(75a): The reaction was performed as the general procedure for Table 4-2 described above. The amounts for each reagent was listed in Table 4-4. The reaction mixture were concentrated and examinated by crude ^{1}H NMR. ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.34–7.23 (m, 10H, Ar), 5.27 (d, *J* = 1.8 Hz, 1H, H-1′), 5.04 (dd, *J* = 3.2, 1.7 Hz, 1H, H-2′), 4.98 (dd, *J* = 3.7, 1.8 Hz, 1H, H-2), 4.78 (d, *J* = 2.0 Hz, 1H, H-1), 4.62–4.44 (m, 4H, 4 × OCH_2Ph), 4.22 (dd, *J* = 9.5, 3.5 Hz, 1H, H-3), 4.06 (dd, *J* = 9.5, 3.2 Hz, 1H, H-3′), 4.00–3.35 (m, 10H, H-4, H-4′, H-5, H-5′, 4 × H-6, 2 × octyl OCH_2), 1.72–1.60 (m, 2H, octyl OCH_2CH_2), 1.35–1.24 (m,

10H, octyl CH_2), 0.87 (t, *J* = 6.8 Hz, 3H, octyl CH_3), 1.26 (s, 9H, Piv), 0.83 (s, 9H, TBS(*t*-Bu)), 0.09 (s, 3H, TBS(Me)), 0.00 (s, 3H, TBS(Me));

Synthesis of 76a and 76d: To a solution of **76** (50.5 mg, 0.067 mmol) in THF (0.7 mL) was added *n*-Bu_2SnCl_2 (20 mol%, 4.2 mg, 0.0013 mmol) and DIPEA (240 μL, 1.34 mmol) followed by TsCl (190.7 mg, 0.013 mmol). The reaction mixture was stirred at room temperature for 24 h before concentrated. The crude products **76a** and **76d** were purified by chromatography as described below.

Octyl 6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**76a**): Chromatographic purification (hexane–EtOAc 5:1) to give **76a** (47.7 mg, 67%) as a colorless syrup. R_f 0.66 (CH_2Cl_2–methanol 30:1); $[\alpha]_D$ = +27.2 (*c* 0.9, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.3 Hz, 2H, Ar), 7.77 (d, *J* = 8.3 Hz, 2H, Ar), 7.35–7.22 (m, 14H, Ar), 5.09 (d, *J* = 1.8 Hz, 1H, H-1′), 4.80 (d, *J* = 1.7 Hz, 1H, H-1), 4.68 (dd, *J* = 3.1, 2.0 Hz, 1H, C-2′), 4.65 (dd, *J* = 3.4, 1.8 Hz, 1H, H-2), 4.53–4.40 (m, 4H, 4 × OC*H*$_2$Ph), 4.02 (dd, *J* = 8.8, 3.2 Hz, 1H, H-3), 3.78–3.73 (m, 2H, H-3′, H-5′), 3.72–3.65 (m, 3H, H-4, H-5, H-6a), 3.65–3.58 (m, 3H, H-4′, H-6b, octyl OCH_2), 3.56 (dd, *J* = 10.3, 1.7 Hz, 1H, H-6a′), 3.44 (dd, *J* = 10.3, 6.8 Hz, 1H, H-6b′), 3.36 (dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 2.43 (s, 3H, ArC*H*$_3$), 2.40 (s, 3H, ArC*H*$_3$), 1.58–1.48 (m, 2H, octyl OCH_2CH_2), 1.33–1.22 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.1 Hz, 3H, octyl CH_3), 0.79 (s, 9H, TBS(*t*-Bu)), 0.06 (s, 3H, TBS(Me)), –0.04 (s, 3H, TBS(Me)); ^{13}C NMR (125 MHz, $CDCl_3$) δ 145.3, 145.1, 138.4, 137.8, 133.2, 133.0, 129.9, 129.8, 128.3, 128.2, 128.1, 127.6, 127.5, 127.4 (24C, Ar), 97.4 (C-1′), 97.1 (C-1), 79.4

(C-2′), 79.2 (C-2), 75.8 (C-4), 73.3 (2C, C-5′, O*C*H_2Ph), 73.2 (O*C*H_2Ph), 70.0, 69.9 (2C, C-3′, C-5), 69.4 (C-4′), 69.3 (C-6), 69.1 (C-6′), 68.5 (C-3), 68.2 (octyl OCH_2), 31.8, 29.3, 29.2, 29.2, 26.0 (5C, octyl CH_2), 25.8 (TBS(*t*-Bu)), 22.6 (octyl CH_2), 21.7 (Ar*C*H_3), 21.6 (Ar*C*H_3), 18.1 (TBS(*t*-Bu)), 14.1 (octyl CH_3), –4.0, –5.0 (2C, TBS(Me)); HRMS (ESI) calcd $C_{54}H_{76}O_{15}S_2Si$ $[M+Na]^+$ 1079.4287, found 1079.4794.

Octyl 6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-toluenesulfonyl-α-D-mannopyranoside (**76d**): Chromatographic purification to give **76d** as a colorless syrup. R_f 0.37 (CH_2Cl_2–methanol 30:1); $[\alpha]_D$ = +27.5 (*c* 1.5, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.92 (d, *J* = 8.2 Hz, 2H, Ar), 7.76 (d, *J* = 8.2 Hz, 2H, Ar), 7.42 (d, *J* = 8.2 Hz, 2H, Ar), 7.39–7.23 (m, 12H, Ar), 7.20 (d, *J* = 8.1 Hz, 2H, Ar), 4.76 (d, *J* = 1.7 Hz, 1H, H-1), 4.72 (dd, *J* = 9.5, 3.1 Hz, 1H, H-3), 4.64 (dd, *J* = 3.3, 1.7 Hz, 1H, H-2′), 4.61 (d, *J* = 1.4 Hz, 1H, H-1′), 4.56–4.36 (m, 4H, 4 × OC*H*$_2$Ph), 4.04 (br s, 1H, H-2), 3.90 (dd, *J* = 11.0, 3.6 Hz, 1H, H-6a), 3.76 (t, *J* = 9.6 Hz, 1H, H-4), 3.73–3.53 (m, 8H, H-3′, H-4′, H-5, H-5′, H-6b, H-6a′, H-6b′, octyl OCH_2), 3.38 (dt, *J* = 9.6, 6.8 Hz, 1H, octyl OCH_2), 2.48 (s, 3H, ArC*H*$_3$), 2.39 (s, 3H, ArC*H*$_3$), 2.17 (br d, *J* = 5.0 Hz, 1H, OH-2), 1.91 (d, *J* = 6.8 Hz, 1H, OH-3′), 1.64–1.53 (m, 2H, octyl OCH_2C*H*$_2$), 1.40–1.24 (m, 10H, octyl CH_2), 0.92 (t, *J* = 6.7 Hz, 3H, octyl CH_3), 0.86 (s, 9H, TBS(*t*-Bu)), 0.12 (s, 3H, TBS(Me)), 0.03 (s, 3H, TBS(Me)); ^{13}C NMR (126 MHz, $CDCl_3$) δ 145.5, 145.1, 138.3, 138.2, 133.3, 132.8, 130.3, 129.9 (8C, Ar), 128.3 (3C, Ar), 128.3 (3C, Ar), 128.2 (2C, Ar), 128.0 (2C, Ar), 127.9 (2C, Ar), 127.6 (2C, Ar), 127.6 (Ar), 127.5 (Ar), 100.6

(C-1′), 99.2 (C-1), 81.0 (C-3), 79.1 (C-2′), 75.2 (C-4), 73.9 (C-5′), 73.3, 73.2 (2C, 2 × O*C*H_2Ph), 71.2 (C-5), 69.6 (C-6′), 69.6, 69.5, 69.2 (3C, C-2, C-3′, C-4′), 68.6 (C-6), 68.2 (octyl OCH_2), 31.8, 29.4, 29.3, 29.3, 26.1 (5C, octyl CH_2), 26.0 (TBS(*t*-Bu)), 22.7 (octyl CH_2), 21.8, 21.6 (2C, 2 × Ar*C*H_3), 18.3 (TBS(*t*-Bu)), 14.1 (octyl CH_3), –3.9, –5.0 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{54}H_{76}O_{15}S_2Si$ $[M+Na]^+$ 1079.4287, found 1079.4791.

Octyl 6-*O*-benzyl-4-*O*-triphenylmethyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranoside (**77**): TrOTf was prepared *in situ* by adding a CH_2Cl_2 solution of TMSOTf (10% v/v, 1 mL, 0.53 mmol) into stirring ice-cold TfOH (137 mg, 0.53 mmol) in CH_2Cl_2 (4 mL). The bright yellow solution of TrOTf was formed after stirring at 0 °C for 5 min. This fresh-made TrOTf solution was slowly added into a stirring ice-cold solution of alcohol **65** (107 mg, 0.19 mmol) and 2,4,6-collidine (0.11 mL, 0.79 mmol) in CH_2Cl_2 (2 mL) over 0.5 h. Methanol (0.5 mL) was then added and then the reaction mixture was concentrated, and the crude residue was purified by chromatography (hexane–EtOAc 8:1) to afford the expected trityl ether (150.4 mg, 98%) as a colorless syrup. R_f 0.69 (hexane–EtOAc 3:1); $[\alpha]_D$ = +96.8 (*c* 0.8, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.55–7.10 (m, 25H, Ar), 6.01 (d, *J* = 10.3 Hz, 1H, H-3), 5.83 (ddd, *J* = 10.3, 2.7, 1.8 Hz, 1H, H-2), 5.75 (d, *J* = 10.5 Hz, 1H, H-3′), 5.44 (ddd, *J* = 10.5, 2.7, 1.9 Hz, 1H, H-2′), 5.10 (d, *J* = 2.3 Hz, 1H, H-1′), 5.04 (d, *J* = 2.3 Hz, 1H, H-1), 4.69 (d, *J* = 12.2 Hz, 1H, OC*H*$_2$Ph), 4.61 (d, *J* = 12.2 Hz, 1H, OC*H*$_2$Ph), 4.38 (ddd, *J* = 9.5, 2.9, 1.6 Hz, 1H, H-4), 4.34 (s, 2H, OC*H*$_2$Ph), 4.16–4.08 (m, 2H, H-5, H-5′), 3.93–3.88 (m, 2H, H-4′, H-6a), 3.86 (dt, *J* = 9.5, 6.9 Hz, 1H, octyl

OCH_2), 3.77 (dd, *J* = 10.9, 6.4 Hz, 1H, H-6b), 3.58–3.47 (m, 3H, H-6a, H-6b, octyl OCH_2), 1.73–1.56 (m, 2H, octyl OCH_2CH_2), 1.45–1.19 (m, 10H, octyl CH_2), 0.92 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.8 (3C, Ar), 138.6 (Ar), 138.2 (Ar), 133.0 (C-3′), 129.5 (C-3), 129.0 (6C, Ar), 128.3 (2C, Ar), 128.2 (2C, Ar), 127.7 (6C, Ar), 127.6 (2C, Ar), 127.5 (2C, Ar), 127.4 (Ar), 127.4 (Ar), 127.2 (C-2), 127.2 (3C, Ar), 124.7 (C-2′), 94.2 (C-1), 91.8 (C-1′), 86.7 (Ph_3C), 73.4, 73.2 (2C, 2 × OCH_2Ph), 70.5 (C-5′), 70.0, 69.3 (2C, C-6, C-6′), 69.2 (C-5), 68.7 (octyl OCH_2), 67.9 (C-4), 66.1 (C-4′), 31.9, 29.9, 29.5, 29.3, 26.3, 22.7 (6C, octyl CH_2), 14.2 (octyl CH_3); HRMS (ESI) calcd $C_{53}H_{60}O_7$ $[M+Na]^+$ 831.4231, found 831.4223. The dihydroxylation of the trityl ether (165.3 mg, 0.20 mmol) was carried out with OsO_4 (2.5 wt.% in *t*-butanol, 40 μL, 0.004 mmol) and NMO (50% w/v in water, 0.3 mL) in *t*-butanol and acetone (2 mL, v/v 1:1). After stirring at room temperature overnight, the resulting saturated aqueous Na_2SO_3 solution was added. The mixture was concentrated to remove *t*-butanol and then the residue was extracted with CH_2Cl_2 three times. The combined organic layer was concentrated and the residue was purifed by chromatography (CH_2Cl_2–methanol 30:1) to afford **77** (187.3 mg, 92%) as a white foam. R_f 0.26 (CH_2Cl_2–methanol 20:1); $[\alpha]_D$ = +33.5 (*c* 0.6, methanol); ^1H NMR (600 MHz, $CDCl_3$) δ 7.43–7.14 (m, 25H, Ar), 4.96 (d, *J* = 4.8 Hz, 1H, H-1′), 4.83 (d, *J* = 1.4 Hz, 1H, H-1), 4.63–4.58 (m, 2H, OCH_2Ph), 4.37 (d, *J* = 4.8 Hz, 1H, OH-3), 4.33 (d, *J* = 12.0 Hz, 1H, OCH_2Ph), 4.27 (d, *J* = 12.0 Hz, 1H, OCH_2Ph), 4.11–4.03 (m, 1H, H-3), 3.95 (app t, *J* = 9.8 Hz, 1H, H-4), 3.92–3.88 (m, 2H, H-5′, H-2), 3.88–3.83 (m, 3H, H-5, H-2′, H-3′), 3.81–3.77 (m, 2H, H-6a, H-6b), 3.69 (dt, *J* = 9.6, 6.8 Hz, 1H,

octyl OCH_2), 3.50 (app t, J = 5.1 Hz, 1H, H-4′), 3.41 (dt, J = 9.6, 6.6 Hz, 1H, octyl OCH_2), 3.36 (dd, J = 10.3, 2.8 Hz, 1H, H-6a′), 3.16 (s, 1H, OH-3′), 3.06 (dd, J = 10.3, 7.5 Hz, 1H, H-6b′), 2.66 (d, J = 2.6 Hz, 1H, OH-2), 2.51 (d, J = 3.7 Hz, 1H, OH-2′), 1.59–1.53 (m, 2H, octyl OCH_2CH_2), 1.37–1.22 (m, 10H, octyl CH_2), 0.89 (t, J = 7.1 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, CD_3OD) δ 146.0 (3C, Ar), 139.9 (Ar), 139.5 (Ar), 130.3 (3C, Ar), 129.4 (Ar), 129.3 (Ar), 128.87 (3C, Ar), 128.86 (3C, Ar), 128.6 (Ar), 128.5 (Ar), 128.4 (2C, Ar), 101.48, 101.47 (2C, C-1′, $^1J_{C\text{-}1,H\text{-}1}$ = 169.0 Hz; C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 167.6 Hz), 89.0 (Ph_3C), 77.5 (C-4), 76.2 (C-5′), 74.4 (OCH_2Ph), 73.9 (OCH_2Ph), 73.6, 72.9, 72.4, 72.3, 72.2 (5C, C-2, C-3, C-3′, C-4′, C-5), 72.0 (C-2′), 71.3 (C-6), 70.5 (C-6′), 68.7 (octyl OCH_2), 33.0, 30.6, 30.5, 30.4, 27.4, 23.8 (6C, octyl CH_2), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{53}H_{64}O_{11}$ $[M+Na]^+$ 899.4341, found 899.4334.

Octyl 6-*O*-benzyl-2-*O*-toluenesulfonyl-4-*O*-triphenylmethyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**78a**): To a solution of **77** (36.5 mg, 0.04 mmol) in THF (0.4 mL) was added *n*-Bu_2SnCl_2 (20 mol%, 2.5 mg, 0.008 mmol) and DIPEA (85 μL, 0.48 mmol) followed by TsCl (80.9 mg, 0.42 mmol). The reaction mixture was stirred at room temperature for 24 h before concentrated. The crude product was purified by chromatography (hexane–EtOAc 7:2) to give **78a** (45.0 mg, 91%) as a colorless film. R_f 0.43 (toluene–EtOAc 5:1); ^{1}H NMR (500 MHz, acetone) δ 7.91 (d, J = 8.3 Hz, 2H, Ar), 7.87 (d, J = 8.3 Hz, 2H, Ar), 7.58–7.24 (m, 29H), 5.32 (d, J = 5.4 Hz, 1H, H-1′), 4.92 (d, J = 1.7 Hz, 1H, H-1), 4.79 (dd, J = 5.4, 2.9 Hz, 1H, H-2′), 4.65 (dd, J = 3.5, 1.7 Hz, 1H, H-2), 4.63–4.55 (m, 2H, OCH_2Ph), 4.40–4.33

(m, 2H, OC*H*$_2$Ph), 4.10 (dd, *J* = 5.0, 2.9 Hz, 1H, H-3′), 4.02 (dd, *J* = 9.5, 3.4 Hz, 1H, H-3), 3.96 (dd, *J* = 9.8, 6.6 Hz, 1H, H-5′), 3.90 (dd, *J* = 11.0, 1.6 Hz, 1H, H-6a), 3.87–3.79 (m, 2H, H-4, octyl OCH$_2$), 3.73 (dd, *J* = 10.9, 6.1 Hz, 1H, H-6b), 3.70–3.62 (m, 2H, H-5, H-4′), 3.55 (dt, *J* = 9.8, 6.7 Hz, 1H, octyl OCH$_2$), 3.41 (dd, *J* = 10.5, 3.0 Hz, 1H, H-6a′), 3.37 (dd, *J* = 10.8, 6.9 Hz, 1H, H-6b′), 2.53 (s, 3H, ArC*H*$_3$), 2.51 (s, 3H, ArC*H*$_3$), 1.78–1.67 (m, 2H, octyl OCH$_2$C*H*$_2$), 1.56–1.36 (m, 10H, octyl CH$_2$), 0.99 (t, *J* = 7.0 Hz, 3H, octyl CH$_3$); ^{13}C NMR (101 MHz, CDCl$_3$) δ 144.9, 144.9, 144.1, 138.6, 137.6, 133.5, 133.4, 130.0, 129.9, 129.7, 129.6, 129.0, 128.5, 128.4, 128.4, 128.3, 128.2, 128.0, 128.0, 128.0, 127.8, 127.7, 127.7, 127.5, 127.5, 127.4, 127.3.0 (42C, Ar), 97.3 (C-1), 97.0 (C-1′), 88.0 (Ph$_3$P), 79.4 (C-2), 79.3 (C-2′), 75.3 (C-4), 73.3 (O*C*H$_2$Ph), 73.2 (O*C*H$_2$Ph), 73.1 (C-5′), 71.6 (C-4′), 70.1 (C-5), 69.6 (C-6′), 69.5 (C-6), 69.4 (C-3′), 68.4 (C-3), 68.3 (octyl OCH$_2$), 31.9, 29.4, 29.3, 29.3, 26.1, 22.7 (6C, octyl CH$_2$), 21.7 (Ar*C*H$_3$), 21.7 (Ar*C*H$_3$), 14.2 (octyl CH$_3$). This compound is not stable in CDCl$_3$. A ninor component could be seen in ^{13}C NMR spectrum, which arose from decomposition of the unstable trityl group in NMR tube. HRMS (ESI) calcd $C_{67}H_{76}O_{15}S_2$ $[M+Na]^+$ 1207.4518, found 1207.4530.

Octyl 6-*O*-benzyl-4-*O*-triphenylmethyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranoside (**79**):

The synthesis was performed as described for **77**. Installation of the trityl group was carried out with **66** (72.5 mg, 0.09 mmol), 2,4,6-collidine (75 μL, 0.56 mmol), TrOH (73.2 mg, 0.28 mmol) and TMSOTf (10% v/v in CH$_2$Cl$_2$, 0.5 mL, 0.28 mmol) in CH$_2$Cl$_2$ (2 mL). The crude residue was purified by chromatography

(hexane–EtOAc 7:1) to afford the trityl ether (100 mg, quantitative) as a pale yellow syrup. $[\alpha]_D$ = +78.6 (*c* 0.9, CH_2Cl_2); ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.47–7.10 (m, 30H, Ar), 6.02 (dd, *J* = 10.3, 3.8 Hz, 2H, H-3, H-3′), 5.80 (ddd, *J* = 10.2, 2.8, 1.8 Hz, 1H, H-2), 5.72 (ddd, *J* =10.4, 2.7, 1.9 Hz, 1H, H-2′), 5.71 (d, *J* = 10.3 Hz, 1H, H-3), 5.45 (ddd, *J* = 10.4, 2.6, 1.9 Hz, 1H, H-2″), 5.21 (d, *J* = 2.3 Hz, 1H, H-1′), 5.05 (d, *J* = 2.4 Hz, 1H, H-1″), 4.97 (d, *J* = 2.5 Hz, 1H, H-1), 4.54–4.45 (m, 4H, 4 × OC*H*$_2$Ph), 4.36–4.30 (m, 2H, H-4, H-4′), 4.27–4.22 (d, *J* = 11.9 Hz, 1H, OC*H*$_2$Ph), 4.24 (d, *J* = 11.9 Hz, 1H, OC*H*$_2$Ph), 4.11–4.07 (m, 1H, H-5″), 3.95 (ddd, *J* = 8.5, 6.4, 1.6 Hz, 1H, H-5), 3.88 (ddd, *J* = 7.6, 5.5, 1.6 Hz, 1H, H-5′), 3.84–3.72 (m, 4H, H-4″, H-6a, H-6a′, octyl OCH_2), 3.70–3.63 (m, 2H, H-6b, H-6b′), 3.54–3.48 (m, 2H, H-6a″, H-6b″), 3.46 (dt, *J* = 9.5, 6.6 Hz, 1H, octyl OCH_2), 1.64–1.48 (m, 2H, octyl OCH_2CH_2), 1.38–1.21 (m, 10H, octyl CH_2), 0.88 (t, *J* = 7.0 Hz, 1H, octyl CH_3); ^{13}C NMR (126 MHz, CD_2Cl_2) δ 145.3 (3C, Ar), 139.3, 139.1, 138.8 (3C, Bn), 133.0 (C-3″), 130.5 (C-3′), 129.5 (C-3), 129.3, 128.6, 128.6, 128.5, 128.1, 127.9, 127.9, 127.8, 127.7, 127.7, 127.6 (31C, 30 × Ar, C-2), 127.0 (C-2′), 125.3 (C-2″), 94.5 (C-1), 92.1 (C-1″), 91.7 (C-1′), 87.0 (Ph_3C), 73.7, 73.5, 73.4 (3C, 3 × OC*H*$_2$Ph), 71.0 (C-5″), 70.7 (C-6), 70.3 (C-5′), 70.3 (C-6), 69.8 (C-6), 69.6 (C-5), 68.9 (octyl OCH_2), 68.1 (C-4′), 67.8 (C-4), 66.5 (C-4″), 32.2, 30.2, 29.8, 29.7, 26.65, 23.1 (6C, octyl CH_2), 14.3 (octyl CH_3); HRMS (ESI) calcd $C_{66}H_{74}O_{10}$ $[M+Na]^+$ 1049.5174, found 1049.5168. The dihydroxylation of the trityl ether was carried out with OsO_4 (2.5 wt.% in *t*-butanol, 75 μL, 0.006 mmol) and NMO (50% w/v in water, 0.3 mL) in *t*-butanol and acetone (3 mL, v/v 1:1). The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to

afford trisaccharide **79** (79.5 mg, 76%) as a white foam. R_f 0.26 (CH_2Cl_2–methanol 15:1); $[\alpha]_D$ = +63.2 (*c* 0.1, CH_2Cl_2); ^{1}H NMR (500 MHz, CD_3OD) δ 7.46–7.11 (m, 30H, Ar), 5.27 (d, *J* = 1.6 Hz, 1H, H-1′), 5.03 (d, *J* = 5.7 Hz, 1H, H-1″), 4.71 (d, *J* = 1.3 Hz, 1H, H-1), 4.58–4.17 (m, 6H, 6 × OC*H*$_2$Ph), 3.98–3.91 (m, 2H, H-2′, H-2″), 3.91–3.77 (m, 9H, H-3, H-3′, H-3″, H-4, H-4′, H-5, H-5′, H-5″, H-6), 3.76–3.63 (m, 5H, H-2, 3 × H-6, octyl OCH_2), 3.55 (app t, *J* = 4.9 Hz, 1H, H-4″), 3.44–3.37 (m, 2H, H-6a″, octyl OCH_2), 3.30–3.24 (m, 1H, H-6b″), 1.63–1.48 (m, 2H, octyl OCH_2CH_2), 1.43–1.18 (m, 10H, octyl CH_2), 0.90 (t, *J* = 6.9 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, CD_3OD) δ 146.0 (3C, Ar), 139.9, 139.6, 139.5, 130.4, 129.5, 129.4, 129.3, 129.1, 129.0, 128.9, 128.87, 128.7, 128.6, 128.5, 128.4 (33C, Ar), 102.9 (C-1′), 101.7 (C-1″), 101.5 (C-1), 89.1 (Ph_3C), 77.5 (C-4), 76.2, 76.0 (2 × C-5), 74.5, 74.4, 73.9 (3C, 3 × O*C*H$_2$Ph), 73.6, 73.4, 73.0, 72.8, 72.7, 72.4, 72.3, 72.1, 72.0 (9C, 3 × C-2, 3 × C-3, 2 × C-4, C-5), 71.3 (C-6″), 70.6 (C-6/C-6′), 68.7 (octyl OCH_2), 67.4 (C-6′/C-6), 33.0, 30.6, 30.5, 30.5, 27.4, 23.8 (6C, octyl CH_2), 14.6 (octyl CH_3); HRMS (ESI) calcd $C_{66}H_{80}O_{16}$ $[M+Na]^+$ 1151.5339, found 1151.5333.

Octyl 6-*O*-benzyl-2-*O*-toluenesulfonyl-4-*O*-triphenylmethyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside 79a: To a solution of **79** (29.3 mg, 0.026 mmol) in THF (0.3 mL) was added *n*-Bu_2SnCl_2 (30 mol%, 2.0 mg, 0.008 mmol) and DIPEA (83 μL, 0.47 mmol) followed by TsCl (75.2 mg, 0.39 mmol). The reaction mixture was stirred at room temperature for 24 h before concentrated. Chromatographic

purification (hexane–EtOAc 5:2) gave **79a** (28.3 mg, 69%) as a colorless film. $[\alpha]_D$ = +36.2 (*c* 0.1, CH_2Cl_2); ^{1}H NMR (500 MHz, CD_2Cl_2) δ 7.83–7.62 (m, 6H, Ar), 7.37–7.08 (m, 36H, Ar), 5.13 (d, *J* = 1.7 Hz, 1H, H-1′), 5.09 (d, *J* = 3.9 Hz, 1H, H-1″), 4.75 (d, *J* = 1.7 Hz, 1H, H-1), 4.69 (dd, *J* = 3.8, 3.2 Hz, 1H, H-2″), 4.64–4.60 (m, 2H, H-2, H-2′), 4.55–4.39 (m, 4H, 2 × OCH_2Ph), 4.23 (s, 2H, OCH_2Ph), 4.06 (app dt, *J* = 8.4, 3.4 Hz, 1H, H-3), 3.99–3.91 (m, 2H, H-3′, H-5″), 3.86 (ddd, *J* = 9.9, 6.1, 1.6 Hz, 1H, H-5′), 3.78–3.59 (m, 8H, H-3″, H-4, H-4′, H-5, H-6a, H-6b, H-6a′, octyl OCH_2), 3.56 (dd, *J* = 10.7, 6.2 Hz, 1H, H-6b′), 3.43–3.34 (m, 2H, H-4″, octyl OCH_2), 3.33 (dd, *J* = 10.7, 2.2 Hz, 1H, H-6a″), 3.00 (dd, *J* = 10.4, 6.2 Hz, 1H, H-6b″), 2.96 (d, *J* = 7.2 Hz, 1H, OH-3′), 2.71 (d, *J* = 8.0 Hz, 1H, OH-3), 2.42 (s, 3H, ArCH_3), 2.40 (s, 3H, ArCH_3), 2.40 (s, 3H, ArCH_3), 1.59–1.49 (m, 2H, octyl OCH_2CH_2), 1.35–1.22 (m, 10H, octyl CH_2), 0.89 (t, *J* = 6.9 Hz, 1H, octyl CH_3); ^{13}C NMR (126 MHz, CD_2Cl_2) δ 145.8, 145.8, 145.7, 144.6, 138.9, 138.6, 138.1, 133.7, 133.5, 133.4, 130.3, 130.2, 130.1, 129.3, 128.7, 128.6, 128.4, 128.4, 128.3, 128.2, 128.1, 128.0, 128.0, 128.0, 127.9, 127.8 (54C, Ar), 97.8, 97.6, 97.5 (3C, C-1, C-1′, C-1″), 88.3 (Tr), 80.0, 79.9, 79.8 (3C, C-2, C-2′, C-2″), 75.9 (C-4), 75.2 (C-4′), 73.7 (OCH_2Ph), 73.6 (OCH_2Ph), 73.6 (C-5″), 73.5 (OCH_2Ph), 72.1 (C-4″), 71.3 (C-5′), 70.2 (C-5), 69.9 (C-6″), 69.8 (C-3″), 69.7 (C-6′), 69.4 (C-6), 68.9 (C-3′), 68.8 (C-3), 68.6 (octyl OCH_2), 32.2, 29.7, 29.6, 29.6, 26.4, 23.0 (6C, octyl CH_2), 21.79 (2C, 2 × ArCH_3), 21.78 (ArCH_3), 14.3 (octyl CH_3); HRMS (ESI) calcd $C_{87}H_{98}O_{22}S_3$ $[M+Na]^+$ 1613.5604, found 1613.5602.

Octyl 6-*O*-benzyl-4-*O*-triphenylmethyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-

6-*O*-benzyl-α-D-mannopyranoside (**80**): The synthesis was performed as described for **77**. Installation of the trityl group was carried out with **81** (24 mg, 0.02 mmol), 2,4,6-collidine (25 μL, 0.19 mmol), TrOH (23.4 mg, 0.09 mmol) and TMSOTf (10% v/v in CH_2Cl_2, 160 μL, 0.09 mmol) in CH_2Cl_2 (0.6 mL). The crude residue from CH_2Cl_2 extraction was used for next step without further purification. The dihydroxylation of the trityl ether was carried out with OsO_4 (2.5 wt.% in *t*-butanol, 30 μL, 0.002 mmol) and NMO (50% w/v in water, 0.12 mL) in *t*-butanol and acetone (1 mL, v/v 1:1). The crude residue was purified by chromatography (CH_2Cl_2–MeOH 12:1) to afford tetrasaccharide **80** (29.7 mg, 90% over two steps) as a white foam. R_f 0.40 (CH_2Cl_2–methanol 9:1); $[\alpha]_D$ = +63.2 (*c* 0.2, methanol); ^{1}H NMR (600 MHz, CD_3OD) δ 7.45–7.13 (m, 35H, Ar), 5.26 (d, *J* = 1.8 Hz, 1H, H-1′/H-1″), 5.25 (d, *J* = 1.8 Hz, 1H, H-1′/H-1″), 5.04 (d, *J* = 5.5 Hz, 1H, H-1‴), 4.72 (d, *J* = 1.7 Hz, 1H, H-1), 4.58–4.18 (m, 8H, 8 × OCH_2Ph), 3.99 (dd, *J* = 5.5, 3.1 Hz, 1H, H-2‴), 3.96 (dd, *J* = 3.2, 2.0 Hz, 1H, H-2′/H-2″), 3.95–3.90 (m, 2H, H-5‴, H-5′/H-5″), 3.89–3.75 (m, 11H, H-2′/H-2″, 4 × H-3, 3 × H-4, H-5, 2 × H-6), 3.75–3.67 (m, 5H, H-2, H-5′/H-5″, 2 × H-6, octyl OCH_2), 3.58 (d, *J* = 3.4 Hz, 2H, 2 × H-6), 3.54 (app t, *J* = 5.3 Hz, 1H, H-4‴), 3.41 (dt, *J* = 9.7, 6.3 Hz, 1H, octyl OCH_2), 3.38 (dd, *J* = 11.3, 8.2 Hz, 1H, H-6‴), 3.32–3.30 (m, 1H, H-6‴), 1.64–1.54 (m, 2H, octyl OCH_2CH_2), 1.41–1.23 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.1 Hz, 1H, octyl CH_3); ^{13}C NMR (126 MHz, CD_3OD) δ 146.0, 139.8, 139.7, 139.6, 139.5, 130.4, 129.5, 129.4, 129.4, 129.3, 129.3, 129.1, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4 (42C, Ar), 103.0, 102.9 (2C, C-1′, C-1″, average $^1J_{C-1,H-1}$ = 174.6 Hz), 102.1 (C-1‴, $^1J_{C-1,H-1}$ = 169.4 Hz), 101.5 (C-1, $^1J_{C-1,H-1}$ = 169.3

Hz), 89.1 (Ph_3P), 77.9 (C-4), 76.0, 76.0, 75.9 (3C), 74.5 (2C, 2 × O*C*H_2Ph), 74.3 (O*C*H_2Ph), 74.0 (O*C*H_2Ph), 73.6, 73.4, 73.1, 73.0, 72.8, 72.7, 72.7, 72.4, 72.3, 72.2, 72.0 (15C), 71.4, 71.4, 71.2, 70.8 (4C, 4 × C-6), 68.7 (octyl OCH_2), 33.0, 30.6, 30.4, 30.4, 27.4, 23.8 (6C, octyl CH_2), 14.6 (octyl CH_3); HRMS (ESI) calcd $C_{79}H_{96}O_{21}$ $[M+Na]^+$ 1403.6336, found 1403.6340.

Octyl 6-*O*-benzyl-2-*O*-toluenesulfonyl-4-*O*-triphenylmethyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**80a**): To a solution of **80** (21.1 mg, 0.015 mmol) in THF (0.3 mL) was added *n*-Bu_2SnCl_2 (40 mol%, 2.0 mg, 0.006 mmol) and DIPEA (65 μL, 0.36 mmol) followed by TsCl (56.2 mg, 0.30 mmol). The reaction mixture was stirred at room temperature for 22 h before concentrated. Chromatographic purification (hexane–EtOAc 3:2) gave **80a** (11.7 mg, 38%) was a colorless film. $[\alpha]_D$ = +35.8 (*c* 0.6, methanol); ^{1}H NMR (600 MHz, CD_3OD) δ 7.88–7.72 (m, 8H, Ar), 7.45–7.07 (m, 43H, Ar), 5.20 (d, *J* = 5.5 Hz, 1H, H-1‴), 5.01 (d, *J* = 1.9 Hz, 1H, H-1′), 4.95 (d, *J* = 1.7 Hz, 1H, H-1″), 4.78 (dd, *J* = 3.2, 2.1 Hz, 1H, H-2′), 4.73 (d, *J* = 1.8 Hz, 1H, H-1), 4.71–4.67 (m, 2H, H-2″, H-2‴), 4.53 (dd, *J* = 3.4, 1.8 Hz, 1H, H-2), 4.46–4.30 (m, 4H, 2 × OCH_2Ph), 4.21 (d, *J* = 12.0 Hz, 1H, OCH_2Ph), 4.16 (d, *J* = 12.0 Hz, 1H, OCH_2Ph), 3.91 (dd, *J* = 9.8, 2.9 Hz, 1H, H-3′), 3.89 (dd, *J* = 9.5, 3.0 Hz, 1H, H-3), 3.79 (brs, 1H, H-3‴), 3.76 (dd, *J* = 9.3, 3.2 Hz, 1H, H-3″), 3.75–3.72 (m, 1H, H-5‴), 3.72–3.68 (m, 1H, H-5′), 3.68 (app t, *J* = 9.5 Hz, 1H, H-4″), 3.65–3.56 (m, 7H, H-4, H-4′, H-5, H-6a, H-6b, H-6a″, octyl

OCH_2), 3.57–3.45 (m, 5H, H-4‴, H-5″, H-6b″, H-6a′, H-6b′), 3.35 (dt, *J* = 9.9, 6.3 Hz, 1H, octyl OCH_2), 3.27–3.21 (m, 1H, H-6a‴), 3.20–3.14 (m, 1H, H-6b‴), 2.43 (s, 3H, ArCH_3), 2.39 (s, 3H, ArCH_3), 2.36 (s, 3H, ArCH_3), 2.34 (s, 3H, ArCH_3)), 1.55–1.46 (m, 2H, octyl OCH_2CH_2), 1.35–1.17 (m, 10H, octyl CH_2), 0.86 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (125 MHz, CD_3OD) δ 146.6, 146.5, 146.3, 145.6, 139.8, 139.7, 139.7, 139.4, 135.2, 135.1, 135.0, 131.1, 131.0, 131.0, 130.9, 130.3, 129.5, 129.4, 129.4, 129.4, 129.3, 129.3, 129.0, 128.9, 128.9, 128.8, 128.8, 128.7, 128.6, 128.5, 128.5 (66C, Ar), 100.2 (C-1′), 100.0 (C-1″), 98.5 (C-1), 97.4 (C-1‴), (octyl OCH_2), 33.0, 30.4, 30.4, 30. 89.2 (Ph_3C), 81.4, 81.23, 81.20, 81.17 (4C, C-2, C-2′, C-2″, C-2‴), 76.72 (C-4), 76.68 (C-4′), 75.7 (C-5‴), 74.4 (3C, 3 × OCH_2Ph), 74.0 (OCH_2Ph), 73.1 (C-5′), 73.0 (C-4″), 72.3 (C-5″), 71.0 (C-3‴), 70.9 (C-6″), 70.5 (C-6), 70.4 (2C, C-6′, C-6‴), 70.2 (C-3), 69.9 (C-3″), 69.7 (C-3′), 69.3 4, 27.2, 23.7 (6C, octyl CH_2), 22.1 (ArCH_3), 21.7 (ArCH_3), 21.6 (2C, 2 × ArCH_3), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{107}H_{120}O_{29}S_4$ $[M+Na]^+$ 2019.6690, found 2019.6684.

(2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(((2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(((2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(((2*R*,3*S*,6*S*)-2-((benzyloxy)methyl)-6-(octyloxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-3,6-dihydro-2*H*-pyran-3-ol (**81**): The coupling step was performed as described for the synthesis of **63**, with alcohol **66** (219.2 mg, 0.28 mmol) and donor **57** (184.3 mg,0.55 mmol) in the presence of $Pd_2(dba)_3$ (12.9 mg, 0.01 mmol) and PPh_3 (16.0 mg, 0.06 mmol) in CH_2Cl_2 (5 mL). The crude residue was purified by chromatography (hexane–EtOAc 3.5:1) to

afford a ketone (208.9 mg, 82%) as pale yellow syrup. $[\alpha]_D$ = +7.7 (*c* 2.0, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.36–7.18 (m, 20H, Ar), 6.82 (dd, *J* = 10.2, 3.5 Hz, 1H, H-2‴), 6.17 (d, *J* = 10.3 Hz, 1H, H-3‴), 6.12 (d, *J* = 10.3 Hz, 1H, H-3″), 6.06 (d, *J* = 10.4 Hz, 1H, H-3′), 6.03 (d, *J* = 10.4 Hz, 1H, H-3), 5.82 (ddd, *J* = 10.3, 2.6, 1.9 Hz, 1H, H-2), 5.78 (ddd, *J* = 10.3, 2.6, 2.0 Hz, 1H, H-2″), 5.73 (ddd, *J* = 10.3, 2.6, 2.0 Hz, 1H, H-2′), 5.56 (d, *J* = 3.5 Hz, 1H, H-1‴), 5.25 (d, *J* = 2.3 Hz, 1H, H-1″), 5.23 (d, *J* = 2.3 Hz, 1H, H-1′), 5.00 (d, *J* = 2.3 Hz, 1H, H-1), 4.64 (dd, *J* = 9.3, 1.3 Hz, 1H, H-4″), 4.46 (dd, *J* = 9.3, 1.3 Hz, 1H, H-4′), 4.56–4.32 (m, 8H, 8 × OC*H*$_2$Ph), 4.38 (dd, *J* = 9.4, 1.2 Hz, 1H, H-4), 4.01 (ddd, *J* = 9.1, 6.2, 1.7 Hz, 1H, H-5), 3.85 (ddd, *J* = 9.3, 3.4, 3.4 Hz, 1H, H-5′), 3.82–3.76 (m, 4H, H-5″, H-6a, H-6a‴, octyl OCH_2), 3.68 (dd, *J* = 11.0, 6.2 Hz, 1H, H-6b), 3.63–3.55 (m, 4H, H-6a′, H-6b′, H-6a″, H-6b‴), 3.50 (dd, *J* = 10.7, 1.8 Hz, 1H, H-6b″), 3.48 (dt, *J* = 9.5, 6.6 Hz, 1H, octyl OCH_2), 1.64–1.54 (m, 2H, octyl $OCH_2C\mathit{H}_2$), 1.38–1.20 (m, 10H, octyl CH_2), 0.88 (t, *J* = 7.1 Hz, 3H, octyl CH_3); ^{13}C NMR (151 MHz, $CDCl_3$) δ 194.0 (C-4‴), 143.6 (C-2‴), 138.6, 138.3, 137.9, 137.8 (4C, Ar), 129.8, 129.3, 129.3 (3C, C-3, C-3′, C-3″), 128.3, 128.3, 128.3, 128.2, 127.6, 127.6, 127.6, 127.4, 127.4, 127.4, 127.3, 127.2, 126.8 (24C, 20 × Ar, C-2, C-2′, C-2″, C-3‴), 94.1 (C-1), 91.3, 91.3 (2C, C-1′, C-1″), 90.0 (C-1‴), 75.0 (C-5‴), 73.7, 73.5, 73.4, 73.1 (4C, 4 × O*C*H$_2$Ph), 70.0 (C-6), 69.8, 69.7 (2C, C-5′, C-5″), 69.4 (C-6′), 69.1 (C-5), 68.9 (C-6″), 68.7 (octyl OCH_2), 68.4 (C-6‴), 67.6 (C-4), 67.2 (C-4″), 67.0 (C-4′), 31.8, 29.8, 29.4, 29.3, 26.2, 22.7 (octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{60}H_{72}O_{13}$ $[M+Na]^+$ 1023.4865, found 1023.4856. This ketone (208.9 mg, 0.21 mmol) was then reduced as described for **9**, with $NaBH_4$ (8.0 mg, 0.21 mmol)

and $CeCl_3 \cdot 7H_2O$ (80.2 mg, 0.21 mmol) in MeOH (2.5 mL). Chromatographic purification (hexane–EtOAc 3:1) furnished alcohol **81** (177.2 mg, 85%) as a colorless syrup. R_f 0.37 (hexane–EtOAc 2:1); $[\alpha]_D$ = +32.2 (*c* 1.0, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44–7.17 (m, 20H, Ar), 6.12–6.03 (m, 3H, H-3, H-3′, H-3″), 6.00 (d, *J* = 10.2 Hz, 1H, H-3‴), 5.84 (ddd, *J* = 10.3, 2.7, 1.8 Hz, 1H, H-2), 5.79–5.69 (m, 3H, H-2′, H-2″, H-2‴), 5.29–5.24 (m, 2H, H-1′, H-1″), 5.22 (d, *J* = 2.6 Hz, 1H, H-1‴), 5.02 (d, *J* = 2.2 Hz, 1H, H-1), 4.58–4.36 (m, 11H, 8 × OC*H*$_2$Ph, H-4, H-4′, H-4″), 4.28 (dddd, *J* = 6.2, 3.6, 1.8, 1.8 Hz, 1H, H-4‴), 4.03 (ddd, *J* = 9.3, 6.3, 1.7 Hz, 1H, H-5), 3.91–3.78 (m, 4H, H-5′, H-5″, H-6a, octyl OCH_2), 3.76–3.56 (m, 7H, H-5‴, H-6b, H-6a′, H-6b′, H-6a″, H-6b″, H-6a‴), 3.55–3.46 (m, 2H, H-6b‴, octyl OCH_2), 2.46 (d, *J* = 4.5 Hz, 1H, OH-4‴), 1.66–1.56 (m, 2H, octyl OCH_2CH_2), 1.40–1.21 (m, 10H, octyl CH_2), 0.90 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.6, 138.4, 138.4, 137.6 (4C, Ar), 133.3, 130.0, 129.9, 129.3, 128.5, 128.3, 128.2, 128.0, 127.8, 127.5, 127.4, 127.4, 127.3, 127.3 (20C, Ar), 126.7 (C-2), 126.6 (2C,C-2′, C-2″), 125.7 (C-2‴), 94.1 (C-1), 91.3, 91.2 (C-1′, C-1″), 91.0 (C-1‴), 73.7, 73.4, 73.4, 73.1 (4C, 4 × O*C*H$_2$Ph), 70.7 (C-6), 70.1 (C-6), 69.8 (3C, C-5′, C-5″, C-5‴), 69.5 (C-6), 69.2 (C-6), 69.1 (C-5), 68.7 (octyl OCH_2), 67.6 (C-4), 67.0, 66.6 (2C, C-4′, C-4″), 66.0 (C-4‴), 31.9, 29.8, 29.4, 29.3, 26.3, 22.7 (6C, octyl CH_2), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{60}H_{74}O_{13}$ $[M+Na]^+$ 1025.5022, found 1025.5027.

Synthesis of octyl 6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**82**) and octyl 6-*O*-benzyl-3-*O*-*t*-

butyldimethylsilyl-4-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**83**): To a stirring ice-cold solution of alcohol **76a** (9.3 mg, 0.01 mmol) and MeI (10 μL, 0.16 mmol) in DMF (0.7 mL) was added NaH (60% in mineral oil, 6.3 mg, 0.15 mmol). The resulting solution was stirred at 0 ℃ for 15 min before methanol (0.1 mL) was added. The resulting reaction mixture was diluted with CH_2Cl_2 and washed with brine. The separated organic layer was dried over Na_2SO_4, concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 8:1) to afford an inseparable mixture of **82** and **83** (8.7 mg, 91%, **82**/**83** = 1.8:1) as a colorless syrups. R_f 0.63 (hexane–EtOAc 4:1); Distinguishing between **82** and **83** was assisted by both ^{1}H-^{1}H COSY and 1D-TOCSY NMR spectroscopy.

Octyl 6-*O*-benzyl-4-*O*-*t*-butyldimethylsilyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (82): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.87–7.78 (m, 4H, Ar), 7.36–7.21 (m, 14H, Ar), 5.00 (d, *J* = 2.1 Hz, 1H, H-1′), 4.95 (dd, *J* = 2.9, 2.2 Hz, 1H, H-2′), 4.91 (d, *J* = 1.9 Hz, 1H, H-1), 4.79 (dd, *J* = 3.2, 1.9 Hz, 1H, H-2), 4.55–4.38 (m, 4H, 4 × OC*H*$_2$Ph), 3.75–3.53 (m, 9H); 3.51 (dd, *J* = 9.4, 3.3 Hz, 1H, H-3), 3.41–3.38 (m, 1H, octyl OCH_2); 3.25 (dd, *J* = 8.7, 3.2 Hz, 1H, H-3′), 3.19 (s, 3H, OMe), 3.14 (s, 3H, OMe), 2.44 (s, 3H, ArC*H*$_3$), 2.40 (s, 3H, ArC*H*$_3$), 1.60–1.53 (m, 2H, octyl OCH_2CH_2), 1.34–1.24 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.0 Hz, 3H, octyl CH_3), 0.80 (s, 9H, TBS(*t*-Bu)), –0.02 (s, 3H, TBS(Me)), –0.06 (s, 3H, TBS(Me)); HRMS (ESI) calcd $C_{56}H_{80}O_{15}S_2Si$ $[M+Na]^+$ 1107.4600, found 1107.4594.

Octyl 6-*O*-benzyl-3-*O*-*t*-butyldimethylsilyl-4-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (83): ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.87–7.78 (m, 4H, Ar), 7.36–7.21 (m, 14H, Ar), 4.85 (br d, *J* = 1.9 Hz, 2H, H-1, H-1′), 4.78–4.76 (m, 2H, H-2, H-2′), 4.55–4.38 (m, 4H, 4 × OC*H*$_2$Ph), 3.94 (dd, *J* = 8.9, 2.7 Hz, 1H, H-3′), 3.75–3.53 (m, 5H); 3.47–3.42 (m, 2H, H-3, H-6a′), 3.41–3.38 (m, 1H, octyl OCH_2), 3.39 (s, 3H, OMe-4′), 3.35 (app t, *J* = 8.7 Hz, 1H, H-4′), 3.20 (s, 3H, OMe-3), 2.43 (s, 3H, ArC*H*$_3$), 2.40 (s, 3H, ArC*H*$_3$), 1.60–1.53 (m, 2H, octyl OCH_2CH_2), 1.34–1.24 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.0 Hz, 3H, octyl CH_3), 0.94 (s, 9H, TBS(*t*-Bu)), 0.13 (s, 3H, TBS(Me)), 0.12 (s, 3H, TBS(Me)); HRMS (ESI) calcd $C_{56}H_{80}O_{15}S_2Si$ $[M+Na]^+$ 1107.4600, found 1107.4594.

Octyl 6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**85**): To a stirring ice-cold solution of alcohol **78a** (56.3 mg, 0.05 mmol) and MeI (24 μL, 0.38 mmol) in THF (0.6 mL) was added NaH (60% in mineral oil, 24 mg, 0.6 mmol). The resulting solution was stirred at 0 ℃ for 20 min before methanol (1 mL) was added. Then a methanolic solution of HCl (0.1 mL, 10% v/v) was added and the reaction mixture was stirred for 0.5 h. The yellowish solution was concentrated and redissolved in CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, followed by saturated Na_2SO_3 and brine. The separated organic layer was dried over Na_2SO_4, concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 2.5:1) to afford **85** (38.2 mg, 83% over two steps) as a colorless syrup. R_f 0.31 (hexane–EtOAc 2:1); $[\alpha]_D$ = +6.2 (*c* 0.4,

$CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.88 (d, *J* = 8.3 Hz, 2H, Ar), 7.82 (d, *J* = 8.3 Hz, 2H, Ar), 7.38–7.23 (m, 14H, Ar), 5.15 (d, *J* = 1.7 Hz, 1H, H-1′), 4.96–4.92 (m, 2H, H-1, H-2′), 4.81 (dd, *J* = 3.0, 2.0 Hz, 1H, H-2), 4.54–4.44 (m, 4H, 4 × OC*H*$_2$Ph), 3.82–3.64 (m, 7H, H-4′, H-4, H-5, H-5′, H-6, H-6, octyl OCH_2), 3.62–3.52 (m, 3H, H-3, H-6, H-6), 3.42 (dt, *J* = 9.7, 6.7 Hz, 1H, octyl OCH_2), 3.39 (dd, *J* = 9.5, 3.0 Hz, 1H, H-3′), 3.25 (s, 3H, OMe), 3.21 (s, 3H, OMe), 2.63 (brs, 1H, OH-4′), 2.46 (s, 3H, ArC*H*$_3$), 2.44 (s, 3H, ArC*H*$_3$), 1.65–1.54 (m, 2H, octyl OCH_2C*H*$_2$), 1.37–1.25 (m, 10H, octyl CH_2), 0.91 (t, *J* = 6.9 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 144.9, 144.8, 138.4, 138.0, 134.0, 133.7, 129.8, 129.7, 128.4, 128.3, 128.1, 127.9, 127.7, 127.5, 127.4 (24C, Ar), 99.6 (C-1′), 97.3 (C-1), 79.4 (C-3), 78.4 (C-3′), 74.2 (C-2), 73.9 (C-2′), 73.6 (O*C*H_2Ph), 73.5 (C-4), 73.4 (O*C*H_2Ph), 72.0 (C-5), 71.0 (C-5′), 70.2, 69.4 (2C, C-6, C-6′), 68.3 (octyl OCH_2), 67.4 (C-4′), 57.1, 56.8 (2C, 2 × OMe), 31.8, 29.4, 29.3, 29.2, 26.1, 22.7 (6C, octyl CH_2), 21.7 ($ArCH_3$), 21.6 ($ArCH_3$), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{50}H_{66}O_{15}S_2$ $[M+Na]^+$ 993.3735, found 993.3736.

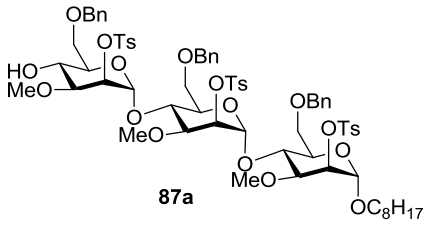
Octyl 3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (**86**): A solution of **85** (25.5 mg, 0.03 mmol) and Mg (39.9 mg, 1.66 mmol) was heated in dry methanol (1 mL) at reflux overnight. The solution was cooled to room temperature and then 1M HCl (5 mL) was added. The resulting mixture was extracted with CH_2Cl_2 and washed with brine. The organic layer was then concentrated and purified by chromatography (CH_2Cl_2–methanol 30:1) to afford a partially deprotected disaccharide (12.5 mg, 72%) as a colorless syrup. R_f 0.30 (CH_2Cl_2–methanol 20:1); ^{1}H NMR (500 MHz, CD_3OD) δ 7.36–

7.11 (m, 10H, Ar), 5.15 (d, J = 1.6 Hz, 1H, H-1′), 4.75 (d, J = 1.5 Hz, 1H, H-1), 4.54–4.38 (m, 4H, 4 × OCH_2Ph), 4.08 (dd, J = 2.9, 2.1 Hz, 1H, H-2′), 4.00 (dd, J = 3.1, 1.9 Hz, 1H, H-2), 3.85–3.78 (m, 2H, H-4, H-6), 3.78–3.65 (m, 6H, H-4′, H-5, H-5′, H-6, H-6, octyl OCH_2), 3.62 (dd, J = 10.7, 5.5 Hz, 1H, H-6), 3.48–3.44 (m, 1H, H-3), 3.45 (s, 3H, OMe), 3.44–3.39 (m, 1H, octyl OCH_2), 3.41 (s, 3H, OMe), 3.33 (dd, J = 9.1, 3.0 Hz, 1H, H-3′), 1.64–1.55 (m, 2H, octyl OCH_2CH_2), 1.41–1.24 (m, 10H, octyl CH_2), 0.89 (t, J = 6.9 Hz, 3H, octyl OCH_3); ^{13}C NMR (126 MHz, CD_3OD) δ 139.8 (Ar), 139.7 (Ar), 129.34 (2C, Ar), 129.30 (2C, Ar), 129.0 (2C, Ar), 128.9 (2C, Ar), 128.6 (Ar), 128.6 (Ar), 103.7 (C-1′), 101.4 (C-1), 83.3 (C-3), 82.2 (C-3′), 75.3 (C-4), 74.6 (C-5), 74.6 (OCH$_2$Ph), 74.4 (OCH$_2$Ph), 72.3 (C-5′), 71.4, 71.3 (2C, C-6, C-6′), 68.9 (octyl OCH_2), 68.1 (C-2′), 67.7 (C-2), 67.5 (C-4′), 57.3, 56.7 (2C, 2 × OMe), 33.0, 30.5, 30.4, 30.4, 27.4, 23.7 (6C, octyl CH_2), 14.5 (octyl CH_3); HRMS (ESI) calcd $C_{36}H_{54}O_{11}$ $[M+Na]^+$ 685.3558, found 685.3551. Hydrogenolysis of this disaccharide (9.8 mg, 0.01 mmol) was performed in with Pd–C (5 wt.%, 10 mg) in methanol (0.5 mL) under H_2 atmosphere for overnight. The catalyst was removed by filtration through Celite. The filtrate was then concentrated and the residue was purified by chromatography (CH_2Cl_2–methanol 9:1) to afford **86** (6.9 mg, 97%) as a colorless syrup. R_f 0.11 (CH_2Cl_2–methanol 9:1); $[\alpha]_D$ = +82.5 (c 0.04, methanol). The NMR spectra were identical to those previously reported in Chapter 2.[13]

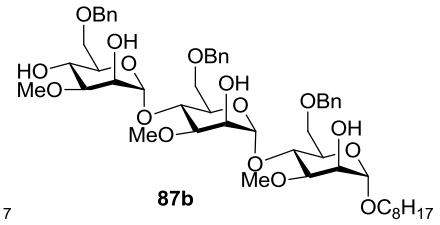
Synthesis of 87 and 88: Installation of the methyl groups was performed as described for the synthesis of **85** and the deprotection was performed as for **86**.

The NMR spectra of the products **87** and **88** were identical to those previously reported in Chapter 2.[13]

The two intermediates for the synthesis of **87** are **87a** and **87b**:



87a



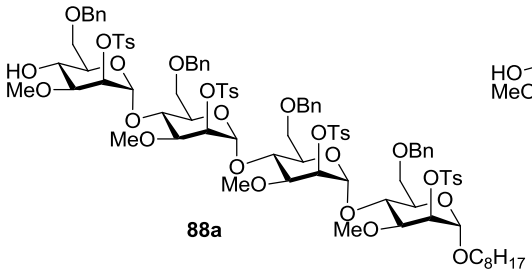
87b

Octyl 6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (87a): $[\alpha]_D$ = +9.4 (*c* 0.3, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.89–7.77 (m, 6H, 6 × Ar), 7.36–7.19 (m, 21H, Ar), 5.10 (d, *J* = 1.7 Hz, 1H, H-1″), 5.06 (d, *J* = 1.9 Hz, 1H, H-1′), 4.95–4.91 (m, 2H, H-2′, H-2″), 4.90 (d, *J* = 1.8 Hz, 1H, H-1), 4.80 (dd, *J* = 3.0, 2.0 Hz, 1H, H-2), 4.51–4.36 (m, 6H, 6 × OC*H*$_2$Ph), 3.77 (app t, *J* = 9.7 Hz, 1H, H-4″), 3.72 (app t, *J* = 9.7 Hz, 1H, H-4′), 3.71–3.61 (m, 6H, H-4, H-5, H-5′, H-5″, H-6, octyl OCH_2), 3.61–3.49 (m, 6H, H-3, 5 × H-6), 3.46 (dd, *J* = 9.3, 3.0 Hz, 1H, H-3′), 3.40 (dt, *J* = 9.8, 6.6 Hz, 1H, octyl OCH_2), 3.36 (dd, *J* = 9.5, 3.1 Hz, 1H, H-3″), 3.25 (s, 3H, OMe), 3.24 (s, 3H, OMe), 3.20 (s, 3H, OMe), 2.63 (d, *J* = 1.6 Hz, 1H, OH-4″), 2.45 (s, 3H, ArC*H*$_3$), 2.41 (s, 3H, ArC*H*$_3$), 2.41 (s, 3H, ArC*H*$_3$), 1.62–1.51 (m, 2H, octyl OCH_2CH_2), 1.35–1.23 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (126 MHz, $CDCl_3$) δ 145.0, 144.9, 144.8, 138.3, 138.3, 138.0, 134.0, 134.0, 133.6, 129.9, 129.8, 129.8, 128.4, 128.3, 128.2, 128.1, 127.9, 127.7, 127.6, 127.5, 127.5, 127.4 (30C, Ar), 99.5 (2C, C-1′, C-1″), 97.3 (C-1), 79.2 (C-3′), 79.0 (C-3), 78.4 (C-3″), 74.4 (C-4), 74.1, 74.1,

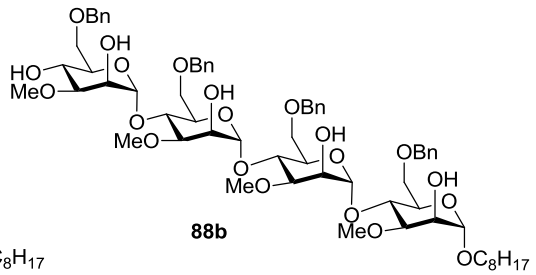
73.9 (3C, C-2, C-2′, C-2″), 73.7, 73.4, 73.4 (3C, 3 × O*C*H$_2$Ph), 73.1 (C-4′), 72.2, 71.9, 71.2 (3C, C-5, C-5′, C-5″), 70.2, 69.6, 69.3 (3C, C-6, C-6′, C-6″), 68.4 (octyl O*C*H$_2$), 67.4 (C-4″), 57.1, 56.8, 56.7 (3C, 3 × OMe), 31.8, 29.4, 29.3, 29.2, 26.0, 22.7 (6C, octyl CH$_2$), 21.7, 21.6, 21.6 (3C, 3 × Ar*C*H$_3$), 14.1 (octyl CH$_3$); HRMS (ESI) calcd $C_{71}H_{90}O_{22}S_3$ [M+Na]$^+$ 1413.4978, found 1413.4964.

Octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (87b): [α]$_D$ = +54.1 (*c* 0.4, MeOH); ^{1}H NMR (500 MHz, CD$_3$OD) δ 7.38–7.12 (m, 15H, Ar), 5.15 (m, 2H, H-1′, H-1″), 4.75 (d, *J* = 1.8 Hz, 1H, H-1), 4.52–4.34 (m, 6H, 6 × OC*H*$_2$Ph), 4.11–4.07 (m, 2H, H-2′, H-2″), 4.00 (dd, *J* = 3.1, 1.9 Hz, 1H, H-2), 3.87 (app t, *J* = 9.5 Hz, 1H, H-4′), 3.82 (app t, *J* = 9.3 Hz, 1H, H-4), 3.80–3.68 (m, 7H, H-4″, H-5, H-5′, H-5″, 2 × H-6, octyl O*C*H$_2$), 3.68–3.60 (m, 4H, 4 × H-6), 3.48–3.45 (m, 4H, H-3, OMe), 3.45–3.42 (m, 4H, H-3′, OMe), 3.43–3.39 (m, 4H, OMe, octyl O*C*H$_2$), 3.36 (dd, *J* = 9.0, 3.1 Hz, 1H, H-3″), 1.65–1.54 (m, 2H, octyl OCH$_2$C*H*$_2$), 1.40–1.22 (m, 10H, octyl CH$_2$), 0.89 (t, *J* = 7.0 Hz, 1H, octyl CH$_3$); ^{13}C NMR (126 MHz, CD$_3$OD) δ 139.8, 139.7, 139.7, 129.4, 129.3, 129.3, 129.0, 129.0, 128.9, 128.7, 128.6, 128.5 (18C, Ar), 103.7, 103.4 (2C, C-1′, C-1″), 101.4 (C-1), 83.3, 82.9 (3C, C-3, C-3′), 82.2 (C-3″), 75.3 (C-4), 75.0 (C-4′), 74.6 (O*C*H$_2$Ph), 74.6 (C-5″), 74.5, 74.3 (2C, 2 × O*C*H$_2$Ph), 73.2 (C-5′), 72.2 (C-5), 71.33, 71.31, 71.28 (3C, C-6, C-6′, C-6″), 68.9 (octyl O*C*H$_2$), 68.1 (C-2″), 67.8, 67.6 (2C, C-2′, C-4″), 67.5 (C-2), 57.4, 56.7, 56.6 (3C, 3 × OMe), 33.0, 30.5, 30.4, 30.4, 27.4, 23.7 (6C, octyl CH$_2$), 14.5 (octyl CH$_3$); HRMS (ESI) calcd $C_{50}H_{72}O_{16}$ [M+Na]$^+$ 951.4713, found 951.4706.

Two intermediates for the synthesis of **88** are **88a** and **88b**:



88a



88b

Octyl 6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside

(88a): $[\alpha]_D$ = +14.5 (*c* 0.6, $CHCl_3$); ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.88–7.77 (m, 8H,8 × Ar), 7.38–7.17 (m, 28H, Ar), 5.11 (d, *J* = 1.4 Hz, 1H, H-1‴), 5.05 (d, *J* = 1.7 Hz, 1H, H-1′), 5.02 (d, *J* = 1.5 Hz, 1H, H-1″), 4.95–4.92 (m, 3H, H-2′, H-2″, H-2‴), 4.90 (d, *J* = 1.7 Hz, 1H, H-1), 4.80 (dd, *J* = 2.9, 2.0 Hz, 1H, H-2), 4.52–4.31 (m, 8H, 8 × OC*H*$_2$Ph), 3.77 (br app t, *J* = 9.6 Hz, 1H, H-4‴), 3.73 (app t, *J* = 9.8 Hz, 1H, H-4″), 3.73–3.56 (m, 10H, H-4, H-4′, H-5, H-5′, H-5″, H-5‴, 3 × H-6, octyl OCH_2), 3.57–3.43 (m, 8H, H-3, H-3′, H-3″, 5 × H-6), 3.40 (dt, *J* = 9.8, 6.9 Hz, 1H, octyl OCH_2), 3.37 (dd, *J* = 9.5, 3.1 Hz, 1H, H-3‴), 3.27 (s, 6H, 2 × OMe), 3.22 (s, 3H,OMe), 3.20 (s, 3H, OMe), 2.63 (br s, 1H, OH-4‴), 2.45 (s, 3H,ArC*H*$_3$), 2.42 (s, 3H, ArC*H*$_3$), 2.41 (s, 6H, 2 × ArC*H*$_3$), 1.61–1.52 (m, 2H, octyl OCH_2CH_2), 1.36–1.23 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.0 Hz, 3H, octyl CH_3); ^{13}C NMR (151 MHz, $CDCl_3$) δ 145.0, 144.9, 144.8, 138.3, 138.3, 138.2, 137.9, 134.0, 134.0, 133.9, 133.6, 129.9, 129.8, 129.7, 128.3, 128.2, 128.1, 127.9, 127.6, 127.6, 127.6, 127.5, 127.4, 127.4, 127.4 (48C, Ar), 99.5 (2C, C-1″, C-1‴), 99.4 (C-1′), 97.2 (C-

1), 79.1 (C-3), 78.9 (C-3″), 78.8 (C-3′), 78.4 (C-3‴), 74.6 (C-4), 74.0, 74.0, 74.0, 73.9 (4C, C-2, C-2′, C-2″, C-2‴), 73.8 (C-4′), 73.6 (O*C*H_2Ph), 73.5 (2C, 2 × O*C*H_2Ph), 73.4 (O*C*H_2Ph), 73.1 (C-4″), 72.2 (C-5′), 72.1 (C-5″), 71.9 (C-5‴), 71.2 (C-5), 70.2, 69.7, 69.5, 69.2 (4C, C-6, C-6′, C-6″, C-6‴), 68.4 (octyl OCH_2), 67.4 (C-4‴), 57.0 (OMe), 56.7 (OMe), 56.62 (OMe), 56.61 (OMe), 31.8, 29.3, 29.3, 29.2, 26.0, 22.6 (6C, octyl CH_2), 21.7 (ArC*H*$_3$), 21.6 (3C, 3 × ArC*H*$_3$), 14.1 (octyl CH_3); HRMS (ESI) calcd $C_{92}H_{114}O_{29}S_4$ $[M+Na]^+$ 1833.6221, found 1833.6199.

Octyl 6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (88b): $[\alpha]_D$ = + 48.0 (*c* 0.3, MeOH); ^{1}H NMR (600 MHz, CD_3OD) δ 7.34–7.17 (m, 20H, Ar), 5.18 (d, *J* = 1.7 Hz, 1H, H-1‴), 5.15 (d, *J* = 1.9 Hz, 1H, H-1′), 5.14 (d, *J* = 1.9 Hz, 1H, H-1″), 4.76 (d, *J* = 1.7 Hz, 1H, H-1), 4.54–4.29 (m, 8H, 8 × OC*H*$_2$Ph), 4.11 (dd, *J* = 3.1, 2.0 Hz, 1H, H-2‴), 4.10 (dd, *J* = 2.9, 2.2 Hz, 1H, H-2″), 4.07 (dd, *J* = 2.9, 2.1 Hz, 1H, H-2′), 4.00 (dd, *J* = 3.1, 1.9 Hz, 1H, H-2), 3.90 (app t, *J* = 9.5 Hz, 1H, H-4″), 3.85 (app t, *J* = 9.5 Hz, 1H, H-4′), 3.83–3.69 (m, 7H, H-4, H-4‴, H-5, H-5″, H-5‴, H-6, octyl OCH_2), 3.69–3.62 (m, 6H, H-5′, 5 × H-6), 3.56 (dd, *J* = 10.8, 2.1 Hz, 1H, H-6a′), 3.53 (dd, *J* = 10.8, 5.0 Hz, 1H, H-6b′), 3.50 (dd, *J* = 9.2, 3.1 Hz, 1H, H-3″), 3.46 (dd, *J* = 9.1, 3.2 Hz, 1H, H-3), 3.44 (s, 3H, OMe), 3.45–3.40 (m, 2H, H-3′), 3.42 (s, 3H, OMe), 3.41 (s, 3H, OMe), 3.40 (s, 3H, OMe), 3.38 (dd, *J* = 9.2, 3.1 Hz, 1H, H-3‴), 1.65–1.54 (m, 2H, octyl OCH_2C*H*$_2$), 1.41–1.22 (m, 10H, octyl CH_2), 0.89 (t, *J* = 7.0 Hz, 1H, octyl CH_3); ^{13}C NMR

(126 MHz, CD_3OD) δ 139.8, 139.7, 129.4, 129.3, 129.3, 129.0, 129.0, 129.0, 128.9, 128.7, 128.6, 128.5 (24C, Ar), 103.8 (C-1‴), 103.4 (C-1″), 103.3 (C-1′), 101.4 (C-1), 83.3 (C-3), 82.9 (2C, C-3′, C-3″), 82.2 (C-3‴), 75.1, 75.1, 75.0 (3C, C-4, C-4′, C-4″), 74.6 (C-5‴), 74.6, 74.5, 74.4, 74.2 (4C, 4 × O*C*H_2Ph), 73.2 (C-5″), 73.0 (C-5′), 72.1 (C-5), 71.3, 71.3, 71.3, 71.2 (4C, C-6, C-6′, C-6″, C-6‴), 68.8 (octyl OCH_2), 68.2 (C-2‴), 67.7 (2C, C-2″, C-4‴), 67.6 (C-2′), 67.5 (C-2), 57.4 (OMe), 56.7 (OMe), 56.6 (2C, 2 × OMe), 33.0, 30.5, 30.4, 30.3, 27.3, 23.7 (6C, octyl CH_2), 14.6 (octyl CH_3); HRMS (ESI) calcd $C_{64}H_{90}O_{21}$ $[M+Na]^+$ 1217.5867, found 1217.5866.

Hexakis(6-*O*-*t*-butyldimethylsilyl)-α-cylcodextrin (89): To a stirring ice-cold solution of α-cyclodextrin (1.0 g, 1.03 mmol) in pyridine (20 mL) was added TBSCl (931.0 mg, 6.18 mmol) followed by 4-dimethyl-aminopyridine (76.5 mg, 0.62 mmol). After heated at 50 °C for three days, the reaction mixture was concentrated to remove pyridine. The crude residue was redissovled in CH_2Cl_2 and washed with H_2O. The organic layer was then concentrated to afford a white solid, which was recrystalized with EtOH to give a white powder **89** (100 mg, 6%). $[\alpha]_D$ = +96.8 (*c* 0.4, $CHCl_3$); R_f 0.43 (CH_2Cl_2–methanol 9:1). The NMR data match with that reported for this compound.[44] ^{1}H NMR (500 MHz, $CDCl_3$) δ 6.54 (s, 1H, OH), 5.28 (s, 1H, OH), 4.91 (d, *J* = 3.2 Hz, 1H, H-1), 4.04 (app t, *J* = 9.1 Hz, 1H, H-3), 3.95 (dd, *J* = 11.4, 3.5 Hz, 1H, H-6a), 3.88 (ddd, *J* = 9.2, 3.4, 1.2 Hz, 1H, H-5), 3.78 (d, *J* = 11.5, 1.6 Hz, 1H, H-6b), 3.68 (dd, *J* = 9.6, 3.4 Hz, 1H, H-2), 3.61 (app t, *J* = 9.0 Hz, 1H, H-4), 0.91 (s, 9H, TBS(*t*-Bu)), 0.08 (s, 3H, TBS(Me)), 0.07 (s, 3H, TBS(Me)); ^{13}C NMR (126 MHz, $CDCl_3$) δ 101.4 (C-1),

81.4 (C-4), 74.5 (C-3), 73.1 (C-2), 72.2 (C-5), 62.0 (C-6), 26.0 (TBS(*t*-Bu)),18.4 (TBS(*t*-Bu)), –5.1, –5.2 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{72}H_{144}O_{30}Si_6$ $[M+Na]^+$ 1679.8250, found 1679.8235.

Hexakis(6-*O*-*t*-butyldimethylsilyl-2-*O*-toluenesulfonyl)-α-cylcodextrin (90): To a solution of **89** (50.7 mg, 0.03 mmol) in THF (0.3 mL) was added *n*-Bu_2SnCl_2 (6.4 mg, 0.02 mmol) and *N*,*N*-diisopropylethylamine (190 μL, 1.08 mmol) followed by toluenesulfonyl chloride (177.0 mg, 0.90 mmol). The reaction mixture was stirred at room temperature for six days and then purified by chromatography (hexane–EtOAc 3:1 to 1:1) to afford **90** (60.6 mg, 77%) as a colorless film. R_f 0.77 for **90** (CH_2Cl_2–methanol 30:1); $[\alpha]_D$ = +54.0 (*c* 0.6, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.82 (d, *J* = 8.3 Hz, 2H, Ar), 7.36 (d, *J* = 8.1 Hz, 2H, Ar), 5.13 (d, *J* = 3.5 Hz, 1H, H-1), 4.28 (dd, *J* = 9.9, 3.5 Hz, 1H, H-2), 3.95 (ddd, *J* = 9.9, 8.5, 3.3 Hz, 1H, H-3), 3.91 (dd, *J* = 11.5, 2.9 Hz, 1H, H-6a), 3.71 (app t, *J* = 9.0 Hz, 1H, H-4), 3.64 (d, *J* = 11.0 Hz, 1H, H-6b), 3.56 (dd, *J* = 9.3, 2.7 Hz, 1H, H-5), 3.07 (d, *J* = 3.3 Hz, OH-3), 2.49 (s, 3H, $ArCH_3$), 0.89 (s, 9H, TBS(*t*-Bu)), 0.03 (s, 6H, 2 × TBS(Me)); ^{13}C NMR (126 MHz, $CDCl_3$) δ 145.1 (1C, Ar), 133.0 (1C, Ar), 129.6 (2C, Ar), 128.4 (2C, Ar), 99.3 (C-1), 81.2 (C-4), 79.7 (C-2), 71.7 (C-5), 70.1 (C-3), 61.8 (C-6), 25.9 (TBS(*t*-Bu)), 21.8 (Ar*C*H_3), 18.3 (TBS(*t*-Bu)), –5.1, –5.2 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{114}H_{180}O_{42}S_6Si_6$ $[M+2(NH_4)]^{2+}$ 1308.4783, found 1308.4761.

Hexakis(6-*O*-*t*-butyldimethylsilyl-3-*O*-methyl-2-*O*-toluenesulfonyl)-α-cylcodextrin (91): To a stirring ice-cold solution of alcohol **90** (30.6 mg, 0.01 mmol) and MeI (15 μL, 0.22 mmol) in THF (0.5 mL) was added NaH (60% in

mineral oil, 17 mg, 0.43 mmol). The resulting solution was stirred at 0 ℃ for 1 h before methanol (1 mL) was added. The reaction mixture was then concentrated and the residue was purified by chromatography (hexane–EtOAc 3:1) to afford **91** (34.3 mg, quantitative) as a pale yellow syrup. R_f 0.66 (hexane–EtOAc 2:1); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.86 (d, *J* = 8.3 Hz, 2H, Ar), 7.35 (d, *J* = 8.1 Hz, 2H, Ar), 4.83 (d, *J* = 3.2 Hz, 1H, H-1), 4.07 (dd, *J* = 9.6, 3.2 Hz, 1H, H-2), 3.89 (dd, *J* = 11.4, 2.5 Hz, 1H, H-6a), 3.65–3.49 (m, 4H, H-3, H-4, H-5, H-6b), 3.45 (s, 3H, OMe), 2.48 (s, 3H, ArCH_3), 0.84 (s, 9H, TBS(*t*-Bu)), –0.02 (s, 3H, TBS(Me)), –0.03 (s, 3H, TBS(Me); ^{13}C NMR (126 MHz, $CDCl_3$) δ 145.0 (Ar), 133.5 (Ar), 129.6 (2C, Ar), 128.5 (Ar), 100.1 (C-1), 80.9 (C-3), 79.2 (C-4), 78.7 (C-2), 72.8 (C-5), 61.9 (OMe), 61.8 (C-6), 25.8 (TBS(*t*-Bu)), 21.8 (ArCH_3),18.2 (TBS(*t*-Bu)), –5.0, –5.2 (2C, 2 × TBS(Me)); HRMS (ESI) calcd $C_{120}H_{192}O_{42}S_6Si_6$ $[M+NH_4]^+$ 2683.0166, found 2683.0076.

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Chapter 5: Efforts toward the identification of a gene encoding the α-(1→4)-mannosyltransferase

5.1 Introduction

Mannosyltransferases (MTs) are a type of glycosyltransferase that transfer mannose residues from either guanosine diphosphate mannose (GDP-mannose) or dolichol-phosphate-mannose (Dol-P-mannose) onto an acceptor such as a glycan, glycoprotein or glycolipid.[1] Depending on the configuration of the newly formed glycosidic bond, MTs are classified as α-MTs and β-MTs. Among the 190 putative MTs (by December 2012) listed in Carbohydrate Active Enzymes (CAZy, http://www.cazy.org/) database, only two MTs catalyze glycosylation *via* an α-(1→4)-linkage.[2-3] One is annotated as an inverting enzyme and belongs to glycosyltransferase family 50.[2] Its suggested function is to transfer mannose from Dol-P-mannose onto the O-4 position of the glucosamine unit of glycosylphosphatidylinositol (GPI) anchors. The other is a retaining enzyme from glycosyltransferase family 4.[3] This enzyme is proposed to transfer mannose from GDP-mannose onto the O-4 position of glucuronic acid of a novel glycolipid involved in lipomannan biosynthesis. In addition to these two glycolipid synthesis enzymes, a third α-(1→4)-mannosyltransferase (ManT) has been identified from mycobacteria, which is involved in the biosynthesis of cytoplasmic methylmannose polysaccharides (MMPs).[4-5] However, no structural information about this enzyme was given at that time and it is not included in the CAZy database.

In Chapter 2, we discussed studies in which this mycobacterial ManT was shown to possess an unanticipated activity. This enzyme was reported to recognize only substrates that have terminal 3-*O*-methyl-mannopyranosyl residues

(e.g., **9**, Figure 5-1).[4] However, we discovered that it is also able to recognize substrates without a methyl group on this terminal mannose residue (e.g., **4**, Figure 5-1) with a higher efficiency when compared to substrates with a terminal methylated mannose.[6] We proposed that substrates lacking any methyl groups could be the natural substrates of ManT. However, as discussed in Chapter 3, tetrasaccharide **39**, which has no methyl groups, turned out to be a poor substrate for the enzyme when compared with methylated tetrasaccharides **4** and **9** (Figure 5-1). These results suggest that ManT has an extended acceptor binding pocket that interacts with a number of mannose residues of the acceptor substrate. To further explore the activity and specificity of this enzyme it became evident that we would need access to the pure ManT enzyme. As the gene encoding for ManT is unknown, in this chapter, we described the efforts using both an affinity purification strategy and a bioinformatic approach to identify the ManT and the gene encoding for it.

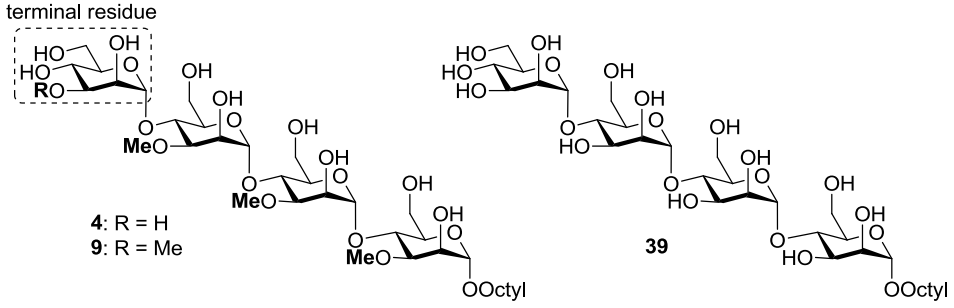


Figure 5-1. Three ManT substrates with different methylation patterns.

5.2 Affinity purification of ManT using synthetic MMP ligands

The activity of ManT was discovered in 1984 by Ballou and coworkers,[4] but the gene encoding for this enzyme has not been identified. Purification of the

enzyme from mycobacteria via an affinity approach, followed by sequencing of the protein would allow determination of the DNA sequence encoding for it. However, affinity purification of this enzyme has not been reported, likely due to limited knowledge of the ligands that could interact specifically with this enzyme. In addition, Ballou and coworkers had noted that enzymatic activity in the mycobacterial membrane was lost quickly after isolation indicating that the stability of the enzyme was low.[4] We also encountered the same problem. Ballou and coworkers identified putative substrates of ManT,[4] however, it was difficult to access large quantity of these ligands from natural sources.

Recently, we developed a synthetic approach to access these ligands. Using this approach we synthesized a panel of MMP analogs of different lengths and found the shortest substrates recognized by ManT are trisaccharides (**3** and **8**, Figure 5-2).[6] Incubating these substrates with a membrane preparation from *M. smegmatis*, which contains the ManT activity, resulted exclusively in products containing additional α-(1$\rightarrow$4)-linked mannopyranose residues. This implied that the synthetic substrates specifically interact with ManT even in the presence of other enzymes that coexist in this membrane fraction. Therefore, these trisaccharide MMP analogs appear to be suitable, and readily accessible, ligands for affinity purification of ManT. Alternatively, GDP-modified affinity resin is commercially available and could be used to bind to the nucleotide binding site of ManT. However there are multiple GDP-binding enzymes present in mycobacteria, therefore, we thought a resin modified with the acceptor substrate is expected to be more specific for ManT.

In the previous chapter we developed a short and simple methodology to install several methyl groups simultaneously onto α-(1→4)-linked mannopyranosides. Here, we employed this methodology to generate a trisaccharide MMP analog **92** containing an 8-aminooctyl group instead of an octyl group as the aglycone (Figure 5-2). This ligand was then immobilized onto Sepharose resin via the amino group, and the column assembled from these resins was used for affinity purification of ManT.

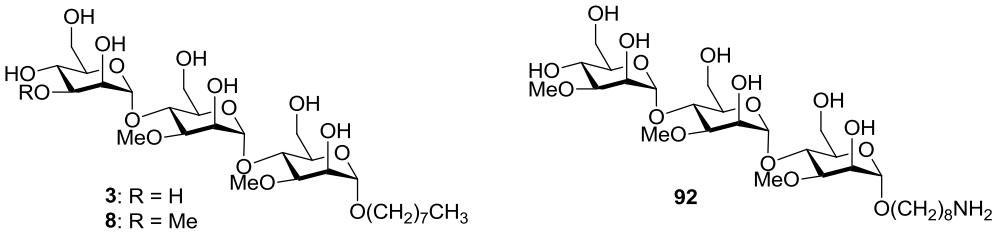
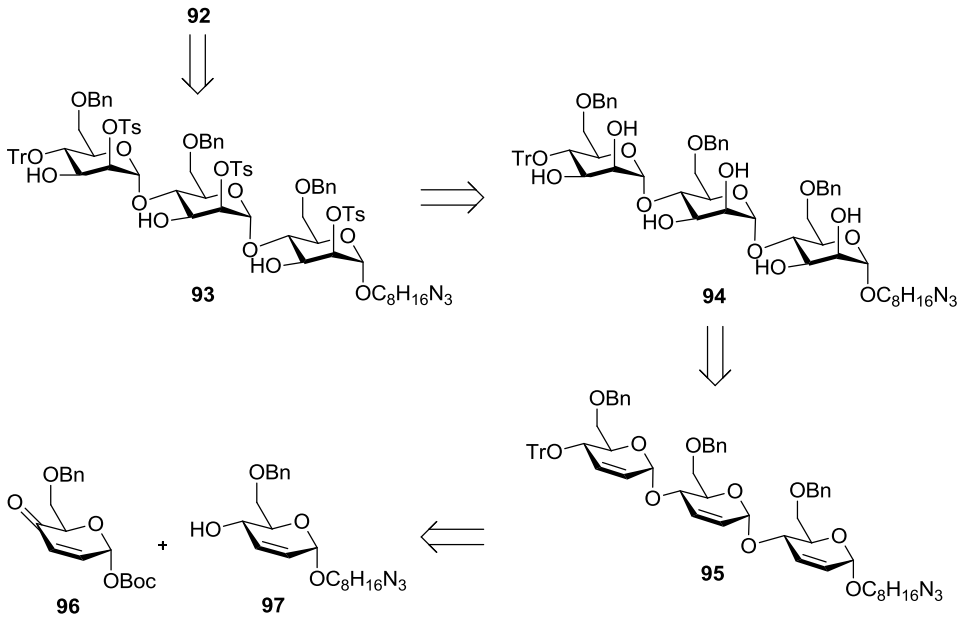


Figure 5-2. The shortest substrates recognized by ManT and the structure of the designed ligand.

5.2.1 Attempted purification with MMP-modified Sepharose

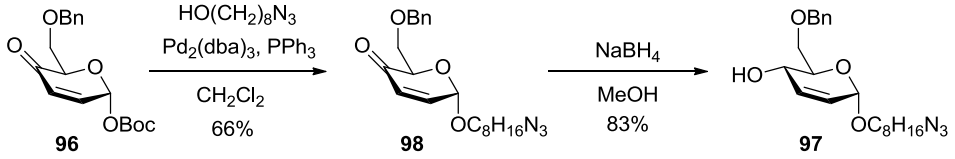
5.2.1.1 Synthesis of trisaccharide MMP ligand

The retrosynthetic analysis for the target molecule is shown Scheme 5-1. We envisioned that the amine **92** could be constructed via the methodology for regioselective multiple-alkylation reported in Chapter 4. Direct tri-*O*-methylation of **93** could give amine **92**. This per-2-*O*-tosylated trisaccharide **93** is the key intermediate in this methodology. The precursor **94** with a triphenylmethyl (trityl) substituent could be derived from triene **95**, which, in turn, could be assembled from pyranone **96** and allylic alcohol **97** using the palladium-catalyzed glycosylation method developed by O'Doherty and coworkers.[7]



Scheme 5-1. Retrosynthetic analysis of ligand **92**.

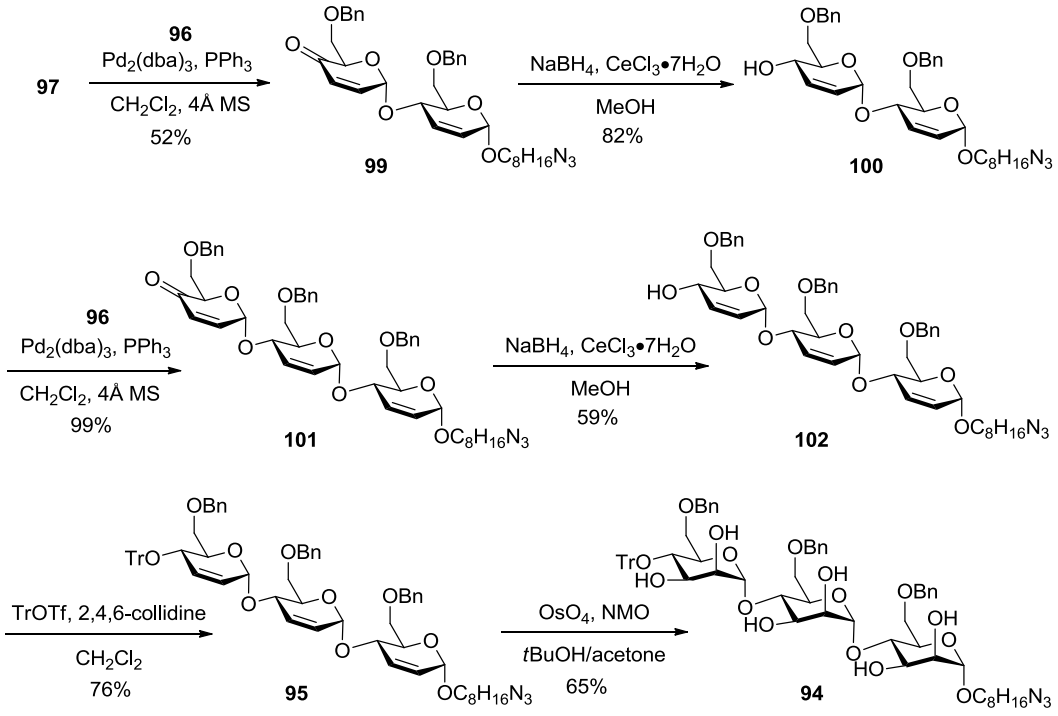
As illustrated in Scheme 5-2, the 8-azido-octyl substituent in allylic alcohol **97** was introduced by coupling pyranone **96** (from Chapter 4) with 8-azido-1-octanol[8] catalyzed by tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$). The resulting pyranone **98** was then subjected to diastereoselective reduction with sodium borohydride giving the allylic alcohol **97** in 55% overall yield over the two steps.



Scheme 5-2. Synthesis of allylic alcohol **97**.

Alcohol **97** was then coupled with a second equivalent of donor **96** to give a 52% yield of diene **99** (Scheme 5-3), which then underwent Luche reduction[9]

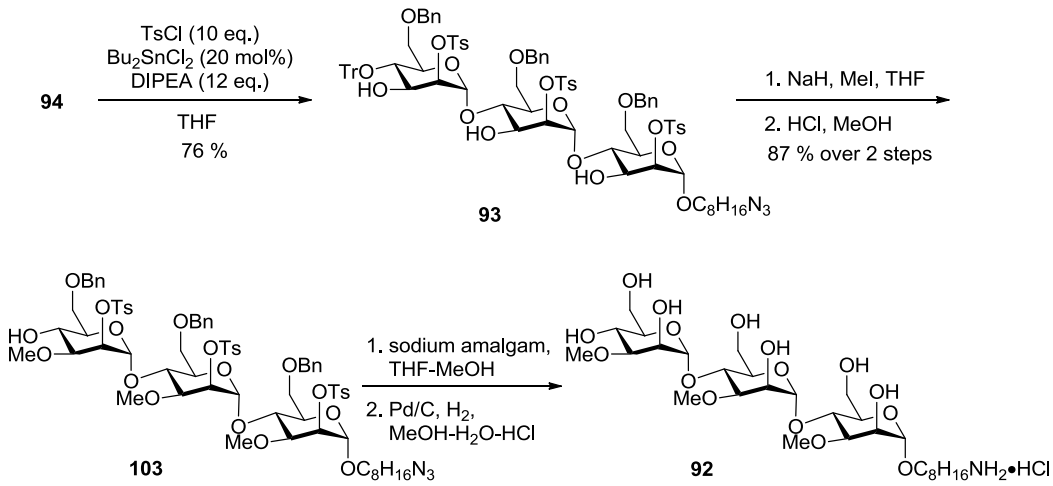
with sodium borohydride in the presence of cerium trichloride to afford alcohol **100** in 82% yield. A subsequent glycosylation with a third equivalent of **96** followed again by Luche reduction furnished the elongated triene **102** in 54% yield over the two steps. The newly formed hydroxyl group was then protected as the trityl ether **95** before being subjected to oxidation. Tandem dihydroxylation of the three alkenes from **95** with osmium tetroxide and *N*-methyl-morpholine *N*-oxide generated the trityl protected trisaccharide **94** in 49% overall yield in two steps.[10]



Scheme 5-3. Synthesis of trisaccharide **94**.

With the trisaccharide **94** in hand, we applied the methodology developed in Chapter 4 to install methyl groups onto the O-3 position of each mannopyranosyl ring (Scheme 5-4). The three pairs of *cis*-diols of **94** were

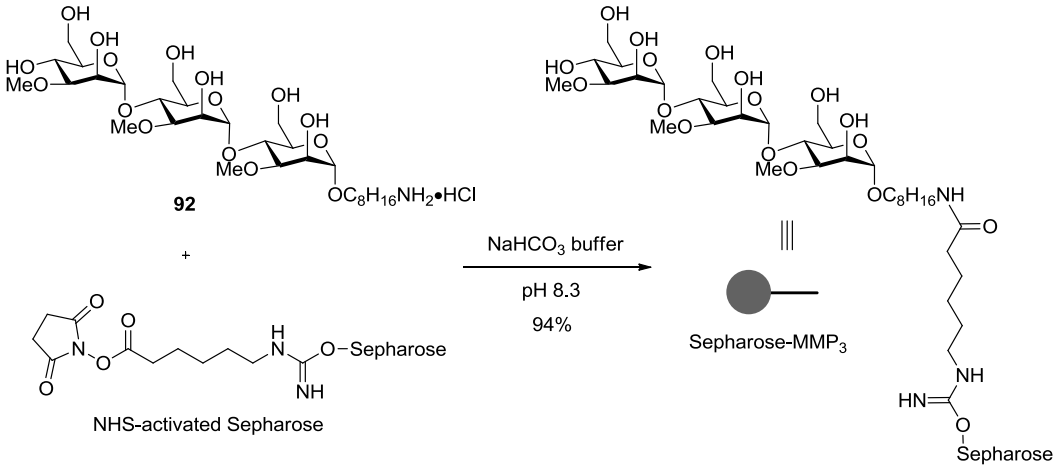
simultaneously activated, by di-*n*-butyltin dichloride (*n*-Bu_2SnCl_2), to undergo regioselective tris-tosylation to give **93** in 76% yield. As in Chapter 4, the bulky trityl substitution at the O-4 position of the non-reducing end residue resulted in the excellent regioselectivity. Subsequent methylation of **93**, followed by removal of the trityl protecting group under acidic conditions[11] furnished methylated **103** in 87% yield over the two steps. Attempts to cleave the tosyl groups with magnesium in methanol at reflux[12] failed to remove all of the tosyl groups and also partially reduced the azido group of **103**. Because sodium has been reported to both cleave toluenesulfonates (sodium naphthalene) and reduce azides (sodium amalgam),[13-14] we attempted to effect both transformations using sodium amalgam. Thankfully, under these conditions, both groups were successfully cleaved to give exclusively the free amine. However, the yield could not be determined due to difficulty in removing salt by-products from the hydrophilic product containing a free amine. Finally, removal of the benzyl groups with hydrogenation over palladium on carbon furnished the target trisaccharide MMP **92**, with an octylamine aglycone, as the hydrochloride salt. Unfortunately, the product still contained salts generated from the step involving sodium amalgam. However, further purification of **92** was not attempted, as the presence of salts proved not to interfere with the subsequent coupling with Sepharose.



Scheme 5-4. Synthesis of trisaccharide MMP ligand **92**.

5.2.1.2 Assembly of the MMP-modified Sepharose column

There are many methods are available for attaching ligands to agarose matrices to generate resins for affinity purifications.[15-16] We chose to assemble the MMP-modified affinity column by coupling amine **92** with *N*-hydroxy-succinimide (NHS)-activated Sepharose beads. The coupling was carried out in $NaHCO_3$ buffer following a protocol from GE Healthcare (Scheme 5-5). As expected, the salts present in the ligand did not affect the coupling reaction giving a 94% yield based on the amount of recovered ligand (see experimental section for details). This resin was then used to generate an affinity column to purify ManT.



Scheme 5-5. Coupling of ligand **92** with NHS-activated Sepharose.

5.2.1.3 Attempted protein purification

In Chapters 2 and 3, we used a membrane preparation from *M. smegmatis* ATCC 14468 as the source of ManT.[6] However, the genome sequence of this strain is unknown. At the onset of this project, *M. smegmatis* mc^2155 was the only strain of *M. smegmatis* species whose genome had been determined. We postulated that, if this strain also possesses ManT activity, we could purify this ManT and, in turn, identify the gene encoding for this protein. Therefore, we evaluated this strain for ManT activity. Fortunately, as shown in Figure 5-3, when incubated with tetrasaccharide **4**, both *M. smegmatis* strains exhibited ManT activity, although mc^2155 was not as active as ATCC 14468.

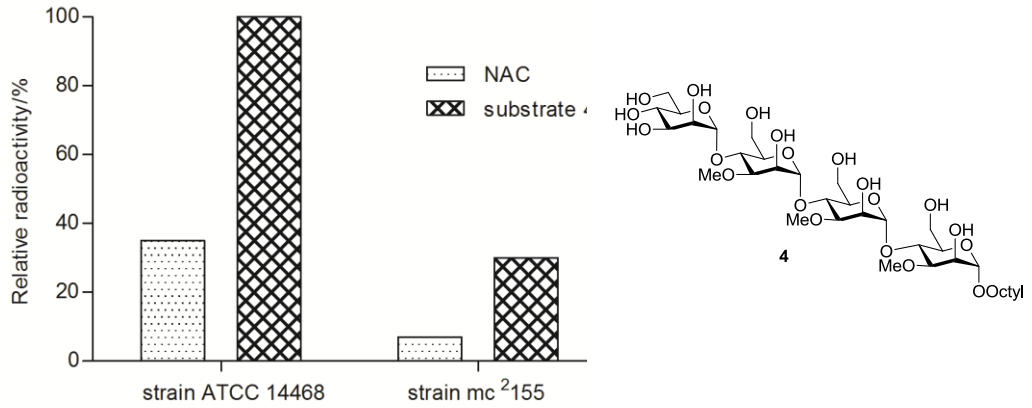


Figure 5-3. Relative activity comparison of strain ATCC 14468 and mc^{2}155 of *M. smegmatis.* The radioactivity of substrate **4** catalyzed by strain ATCC 14468 was arbitrarily set to 100%. NAC (no acceptor control) is the control assay without acceptors and the observed activities arise from endogenous acceptors present in bacteria. Incubation was carried out at 37 °C for 21 h.

Next, the crude membrane preparation from *M. smegmatis* mc^{2}155 was incubated with the MMP-functionalized Sepharose resin (Figure 5-4). We expected the ManT protein would bind to the MMP ligand. The column was then rinsed with a buffer containing methyl α-D-mannopyranoside, which we speculated would compete with the trisaccharide MMP ligand for the binding site of ManT. Thus, washing with an increasing concentration of methyl α-D-mannopyranoside was expected to elute the desired ManT from the column. The fractions collected were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE); however, no proteins were detected in any of them. In addition, no fraction showed activity when incubated with radiolabeled GDP-mannose and tetrasaccharide **4**, a known substrate of ManT.[6]

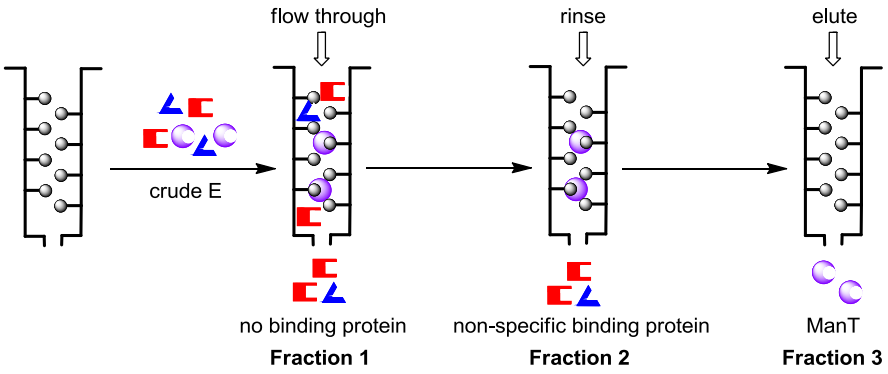


Figure 5-4. Protocol for affinity purification of ManT with MMP-functionalized Sepharose.

We postulated that our failure to isolate ManT was due, at least in part, to the low levels of enzyme expressed in mycobacteria, and the further dilution of the protein during the affinity purification. Therefore, the protein levels would be too low to be detected by the Coomassie Brilliant Blue R stain used to visualize the SDS-PAGE gel.[17] Alternatively, the binding affinity between the trisaccharide MMP ligand and ManT could be too low to retain ManT effectively on the column. Therefore, we prepared an additional affinity column with a tetrasaccharide MMP ligand, which serves as a more active substrate compared with the trisaccharide MMP analogs. Also, to avoid the enzyme being diluted during purification, we employed an alternative affinity purification procedure involving magnetic beads.[18]

5.2.2 Attempted purification with biotinylated MMP conjugates

Magnetic separation technologies have been widely applied in affinity purification of biological molecules.[18-19] Compared with conventional time-consuming methods using agarose as a matrix, separation methods employing

magnetic beads significantly reduce purification time by simplifying operational procedures.[20] Streptavidin-coated magnetic beads have been successfully applied to purify DNAs and proteins.[21-22] This strategy was attractive due to the strong interaction of the avidin–biotin complex (10^{15} M^{-1}).[23] We, therefore, decided to prepare MMP–biotin conjugates as ligands for the affinity purification of ManT. Both a trisaccharide MMP–biotin conjugate (Biotin-MMP_3, **104**) and a tetrasaccharide conjugate (Biotin-MMP_4, **105**) were prepared and used as probes for the purification of ManT (Figure 5-5).

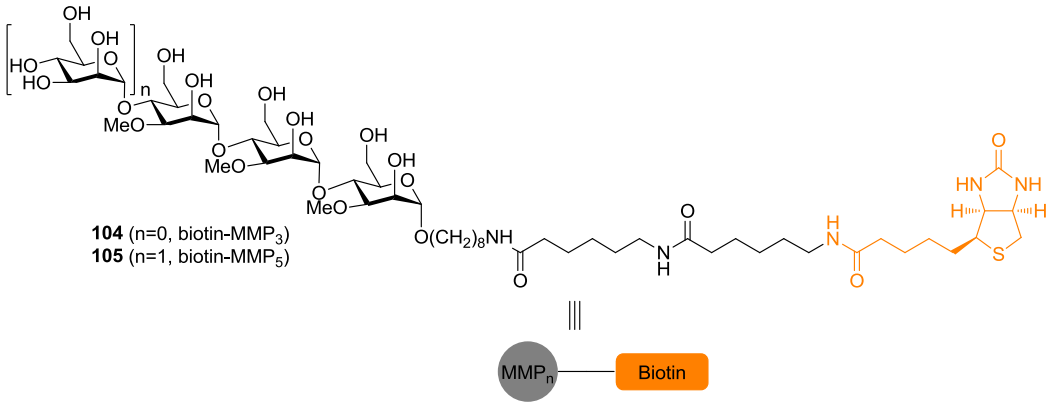
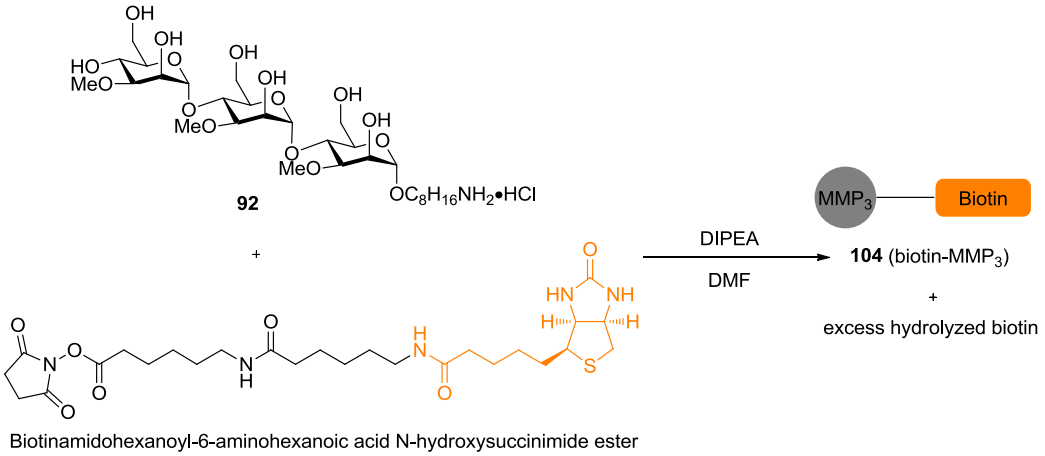


Figure 5-5. Biotinylated trisaccharide MMP **104** and tetrasaccharide MMP **105**.

5.2.2.1 Synthesis of tri- and tetrasaccharide MMP ligands

Biotinylated trisaccharide MMP **104** (biotin-MMP_3) was readily synthesized by coupling amine **92** with NHS-activated biotinamidohexanoyl-6-aminohexanoic acid in the presence of *N*,*N*-diisopropylethylamine (DIPEA) (Scheme 5-6). The crude product was then purified by chromatography on Iatrobeads (Iatron Laboratories Inc., Tokyo) followed by desalting using a C_{18} cartridge (Waters) to give Biotin-MMP_3 **104**. However, the desired product **104** was obtained as a mixture with excess biotinamidohexanoyl-6-aminohexanoic

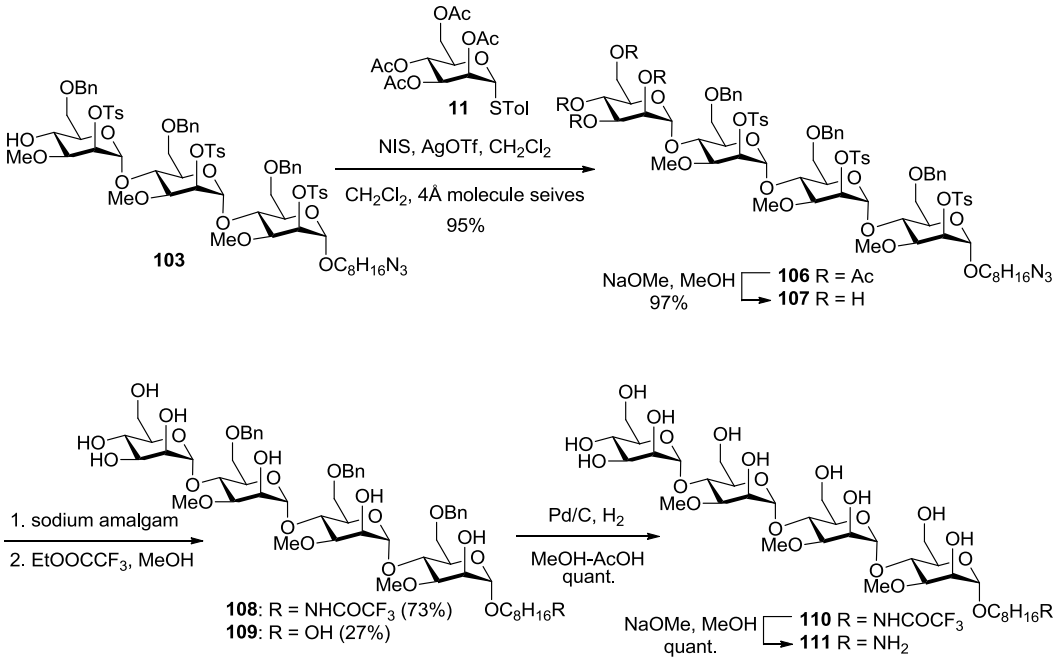
acid as the side product. We did not attempt further purification; we later found that ManT activity was not disturbed by the excess hydrolyzed biotin reagent.



Scheme 5-6. Preparation of trisaccharide conjugate Biotin-MMP_3 **104**.

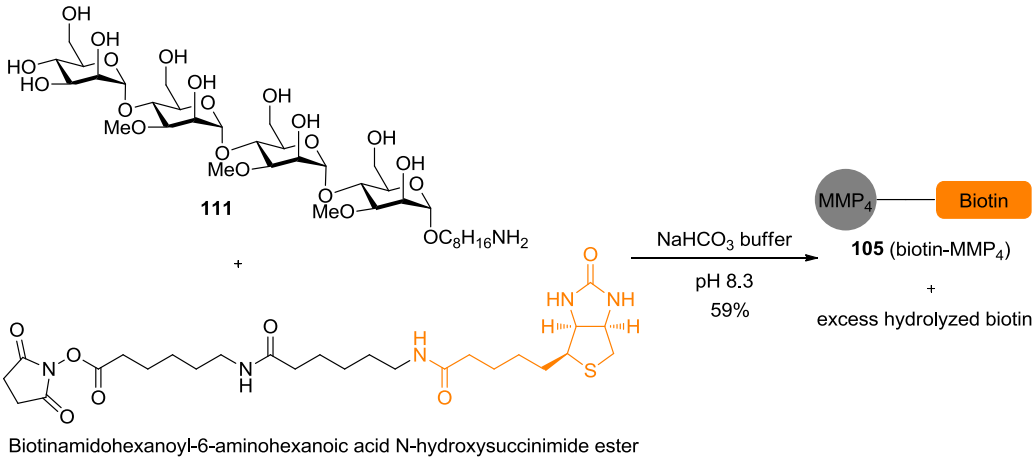
Biotinylated tetrasaccharide **105** (biotin-MMP_4) was assembled from the corresponding amine **111**, which was derived from trisaccharide **103** (Scheme 5-7). Thioglycoside **11** (from Chapter 2), activated by NIS–AgOTf,[24] was coupled with trisaccharide alcohol **103** to produce tetrasaccharide **106**, which was then deacetylated with sodium methoxide to give **107**, in 92% overall yield over the two steps. After removal of both the tosyl and azido groups of **107** with sodium amalgam, the resulting amine was protected as an *N*-trifluoroacetyl amide **108** before hydrogenolysis.[25] This extra step facilitated purification of the product. In particular, the salt produced from step with sodium amalgam was successfully removed. Unexpectedly, in addition to the desired amide **108**, which was obtained in 73% yield, we also isolated a byproduct **109**, in which the amide was replaced by a hydroxyl group. This product appears to result from cleavage of the C–N bond instead of the N–N bond of the azide moiety of **107**. However, the

mechanism of its formation is unclear. After hydrogenolysis of the benzyl groups, the amide of **110** was liberated by treating with sodium methoxide to furnish desired amine **111** quantitatively.



Scheme 5-7. Synthesis of tetrasaccharide amine **111**.

The tetrasaccharide amine **111** was then coupled with NHS-activated biotin derivative in sodium bicarbonate buffer, producing a 59% yield of the desired biotin–MMP_4 derivative **105** (Scheme 5-8). This product was again obtained as a mixture with the excess hydrolyzed biotin reagent as the side product, and as was done in the case of trisaccharide **104**, no further purification was done.



Scheme 5-8. Preparation of tetrasaccharide conjugate Biotin-MMP_4 **105**.

5.2.2.2 Evaluation of the biotinylated ligands

After successful preparation of biotin-conjugates **104** and **105**, we first evaluated them as substrates for ManT. As a comparison, the corresponding trisaccharide and tetrasaccharide analogs (**3** and **4**, respectively, Figure 5-6) were evaluated as well. Because glycosyltransferase activities usually take place at the non-reducing end of oligosaccharide substrates,[26] we assumed modification of the aglycone of the substrates should not affect their biological activities. Therefore, ManT should recognize both trisaccharide **3** and **104** with the same efficiency, and similar to tetrasacchrides **4** and **105**. As shown in Figure 5-6, both trisaccharides **104** and **3** were equally active when incubated with membrane fractions of *M. smegmatis* mc^2155. Tetrasaccharide **105** was slightly less active compare with analog **4**. However, this may be only due to the different concentrations of **105** (0.8 mM) and **4** (1 mM) being used. These results indicate that introduction of a biotin probe at the reducing end of the substrate does not affect ManT activity, which, in turn, suggests that ManT recognizes only the non-reducing end terminus of substrates.

Therefore, tetrasaccharide analogs **105** and **4**, should have the same K_m value for ManT (K_m for **4** is 20.7 ± 2.6 μM, Chapter 2). Based on this the K_d of these substrates should allow for successful purification of ManT. These studies also demonstrated that the presence of the hydrolyzed biotin reagent present in both **104** and **105** does not interfere with the enzymatic reaction.

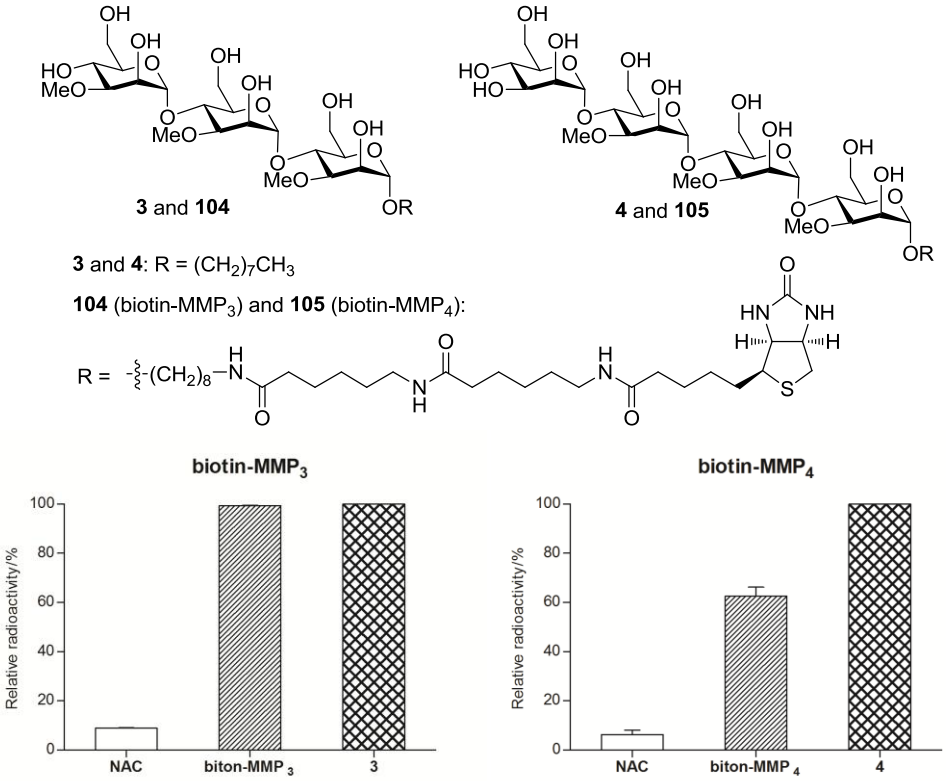
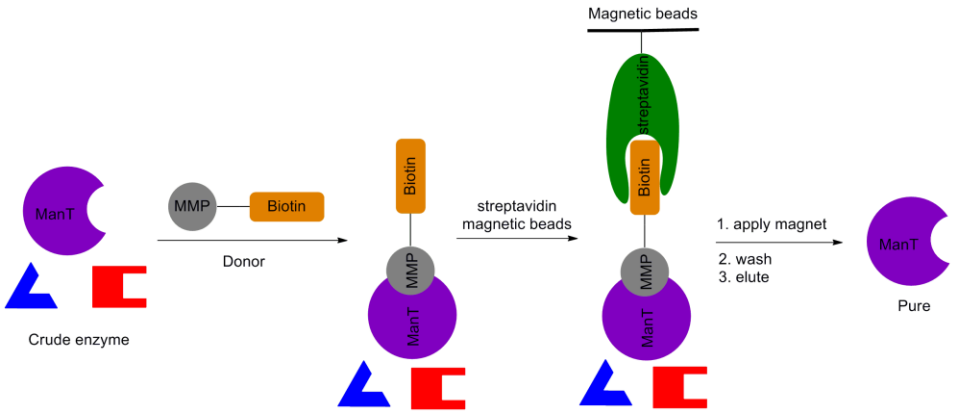


Figure 5-6. Relative activities of biotin-MMP_3 (**104**) and biotin-MMP_4 (**105**).

Left: Comparison of biotin-MMP_3 (1 mM) with trisaccharide **3** (1 mM). Incubation was carried out at 37 °C with radiolabeled GDP-mannose (1 mM) in the presence of the membrane fraction of *M. smegmatis* mc^2155 for 2.5 h. Right: Comparison of biotin-MMP_4 (0.8 mM) with tetrasaccharide **4** (1 mM). Incubation was carried out at 37 °C for 2 h, with radiolabeled GDP-mannose (1 mM) in the presence of the membrane fraction of *M. smegmatis* mc^2155. The radioactivity of **3** or **4** is arbitrarily set to 100%. NAC (no acceptor control) is the control assay without a synthetic acceptor and the observed activities likely arise from endogenous acceptors present in membrane fraction.

5.2.2.3 Attempted protein purification

Having established that both **104** and **105** function as ManT substrates, we attempted the affinity purification of ManT using streptavidin-coated magnetic beads. Freshly prepared membrane fractions of *M. smegmatis* mc^2155 were incubated with **104** and **105** (Figure 5-7) during which time the ManT protein was expected to bind with the biotinylated MMP analogs. Streptavidin-coated magnetic beads were then added to capture the biotinylated probes complexed with ManT. Pure ManT was expected to elute from the beads with methyl α-D-mannopyranoside. Again, the fractions collected were monitored by SDS-PAGE.



Conditions	Ligand	Crude enzyme	Donor[a]
1	biotin-MMP_3	membrane fraction	-
2		membrane fraction	-
3	biotin-MMP_4	membrane fraction+ detergent[b]	√
4		whole cell lysate	√

[a] √ indicated 2 equiv. donor GDP-mannose (2 equiv.) was added;
[b] Dodecyl β-D-maltoside (0.5 mM) was used as detergent;

Figure 5-7. Protocol and conditions for affinity purification of ManT with magnetic beads.

We speculated that tetrasaccharide **105**, which serves as a better substrate for ManT than trisaccharide **104**, should bind with ManT more efficiently, therefore giving more concentrated ManT by SDS-PAGE. However, no proteins

were detected in these fractions with either ligand. In addition, no protein was detected still bounding to the beads (conditions 1 and 2, Figure 5-7). We attempted various modifications of this procedure to optimize the purification conditions. These included adding detergent to stabilize membrane-associated ManT,[27] using whole cell lysate instead of membrane preparation as enzyme source, and introducing donor GDP-mannose during the binding step (condition 3-4, Figure 5-7). However, no improvement was observed.

Failure to isolate ManT using this approach was attributed to the low binding capacity of the streptavidin beads. The volume of commercial beads (1 mL, New England Biolabs) used in this experiment could capture a maximum of 200 pmol biotin, corresponding to 0.3 μg biotin-MMP_4 **105**. In other words, when 1 mg of the ligand was used, only 0.03% could be effectively captured. Therefore, this approach was abandoned and we, instead, employed a bioinformatic approach to identify the gene encoding for ManT.

5.3 Bioinformatic approach to identify genes encoding ManT and OMT

5.3.1 Bioinformatic search for putative ManT and OMT genes

Cytoplasmic 3-*O*-methyl-mannose polysaccharides (MMPs), assembled by ManT, are found only in nonpathogenic fast growing species of mycobacteria including strains of *M. smegmatis*, *M. phlei*, *M. parafortuium*, *M. cuneatum*, *M. petrophilum*, *M. chitea*, *M. vaccae*.[4,28-29] At the onset of this project, no genome sequence of mycobacterial species producing MMPs had been reported. The only

available genome sequence from these species was that of strain *M. smegmatis* mc^{2}155 (*M. smegmatis* ATCC 700084), which is closely related to the aforementioned species.[30] In the studies reported earlier in this Chapter, we found that *M. smegmatis* mc^{2}155 also possesses ManT activity, but this had not been previously reported. In contrast, MMPs are absent in pathogenic slow growing *M. tuberculosis,*[31] indicating the genes encoding the ManT would be missing in *M. tuberculosis*. Using this information as a starting point, we searched the genomes of *M. smegmatis* mc^{2}155 and all *M. tuberculosis* strains with a reported genome sequence through the NCBI (National Center for Biotechnology Information). This allowed us to identify putative glycosyltransferase genes that exist in *M. smegmatis* mc^{2}155 but are missing in all strains of *M. tuberculosis* for which genome information is currently available. In total, 104 putative proteins are predicted as glycosyltransferases (GTs) in *M. smegmatis* mc^{2}155 (by June 2012). When the sequence of each putative GT was compared with those present in *M. tuberculosis* using the basic local alignment search tool (BLAST), 64 encoded proteins that shared greater than 90% coverage and 50% amino acid sequence identity were found. Thus, they were ruled out as potential candidates for the ManT. This narrowed down our selection to only 40 putative GTs (Table 5-1).

Table 5-1. Comparison of GTs from *M. smegmatis*, *M. tuberculosis*, *M. phlei* and *S. griseus*.

Total GTs in *M. smegmatis*	GTs not similar to *M. tuberculosis*	GTs similar to *S. griseus*	GTs similar to *M. phlei*
104	40	11	5

In addition to the mycobacterial species discussed above, the bacterium *Streptomyces griseus* has also been reported to produce acetylated 3-*O*-methyl-mannose polysaccharides, which are acetylated forms of MMPs produced by mycobacteria.[32-33] We predicted that *S. griseus* would also produce an α-(1→4)-mannosyltransferse and, thus, the putative ManT in *M. smegmatis* should share high sequence identity with the same enzyme from *S. griseus.* To further narrow down the potential ManT genes we searched the genome of *S. griseus* (strain subsp. griseus NBRC 13350[34]) for homologs of the remaining 40 putative GTs from *M. smegmatis*. Protein BLASTs of the 40 GTs of *M. smegmatis* against *S. griseus* revealed 11 proteins that share sequence similarities higher than 90% (Table 5-1 and Table 5-2).

Further narrowing down of these proteins was made possible using the available genome sequence for *M. phlei* (strain RIVM601174), which was reported in June 2012.[35] Because α-(1→4)-mannosyltransferse activity has also been reported in *M. phlei*, we hypothesized it also produces a ManT homologous to that found in *M. smegmatis*. Comparing the 40 *M. smegmatis* GTs to the putative GTs of *M. phlei* resulted in only five conserved proteins (Table 5-1). Interestingly, among the 11 putative *M. smegmatis* GTs with homologs in *S. griseus* and the five GTs with homologs in *M. phlei*, only one protein (accession number: YP_890697.1) was conserved in all three bacterial species (97% and 100% similarity for *S. griseus* (YP_001826699.1) and *M. phlei* (ZP_09978075.1), respectively, (Table 5-2 and Figure 5-8). This protein, encoded by the *MSMEG_6484* gene in *M. smegmatis* mc^2155, had no demonstrated activity.

Table 5-2. List of *M. smegmatis* GTs that are homologs with GTs in *M. phlei* and *S. griseus*.[a]

M. smegmatis GTs	Homologous GTs in *S. griseus*	*M. smegmatis* GTs	Homologous GTs in *M. phlei*
YP_890697.1	YP_001826699.1, 97% (53%)	**YP_890697.1**	ZP_09978075.1, 100% (86%)
YP_886686.1	YP_001822924.1, 98% (26%)	YP_888991.1	ZP_09973166.1, 100% (59%)
ABK75015.1	YP_001822924.1, 97% (42%)	YP_889875.1	ZP_09977155.1, 100% (76%)
ABK72140.1	YP_001822924.1, 98% (26%)	YP_884976.1	ZP_09974969.1, 97% (74%)
YP_885136.1	YP_001822924.1, 97% (42%)	ABK69577.1	ZP_09974969.1, 97% (74%)
YP_885492.1	ZP_08234202.1, 93% (38%)		
ABK71398.1	ZP_08234202.1, 93% (38%)		
YP_890205.1	YP_001827653.1, 91% (26%)		
YP_890177.1	YP_001823059.1, 98% (40%)		
ABK73679.1	YP_001827653.1, 91% (26%)		
ABK72834.1	YP_001823059.1, 98% (40%)		

[a] Protein accession numbers are listed and similarity is indicated by coverage percentage (identity percentage).

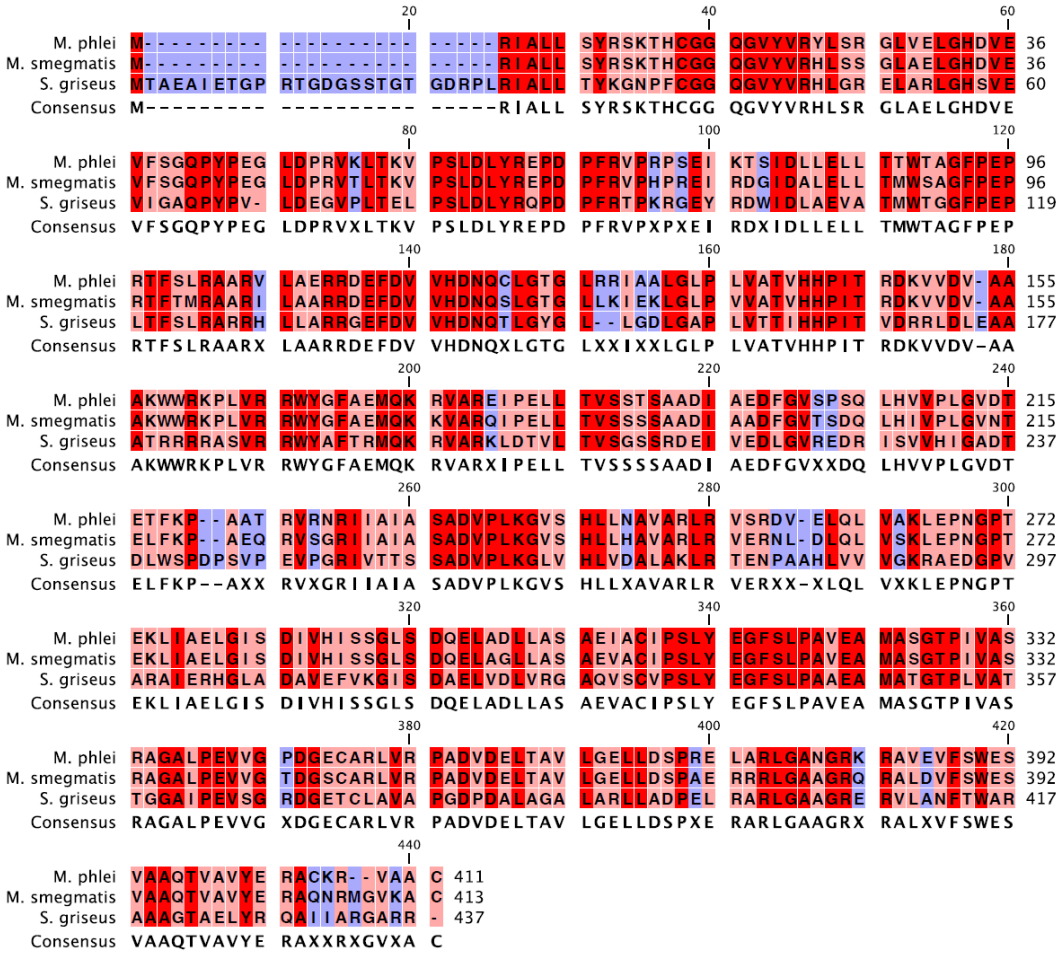


Figure 5-8. Multiple sequence alignment of ZP_09978075.1 (*M. phlei*), YP_890697.1 (*M. smegmatis*) and YP_001826699.1 (*S. griseus*). Highly conserved amino acids are shown in red, partially conserved residues are shown with pink and different residues are in blue.

Bacteria often cluster genes involved in the same biosynthetic pathway.[36] Among the genes adjacent to the putative GT gene (*MSMEG_6484*) are two genes encoding putative methyltransferases (*MSMEG_6482* and *MSMEG_6483*) (Figure 5-9). According to their annotations, *MSMEG_6482* encodes a hypothetical methyltransferase (YP_890694.1) and *MSMEG_6483* encodes an *S*-adenosyl-methionine-dependent methyltransferase (YP_890696.1). Considering that at least one methyltransferase is involved in the biosynthesis of MMPs,[5] it is highly likely that one, or two, of these putative methyltransferase genes participates in assembling these methylated polysaccharides. If this hypothesis is correct, a homologous methyltransferase gene should also be found close to the genes (*SGR_5187*) encoding the putative mannosyltransferase (YP_001826699.1) in *S. griseus*. Consistent with this prediction, a putative methyltransferase gene (*SGR_5188*) was found to be adjacent to *SGR_5187* in the *S. griseus* (Figure 5-9) genome. This gene, *SGR_5188,* shares 98% sequence similarity and 56% identity with the putative methyltransferase encoded by *MSMEG_6483* in *M. smegmatis*. Therefore, the *MSMEG_6484* gene was predicted to encode the ManT and *MSMEG_6483* gene to encode the 3-*O*-methyl-transferase (OMT) required for MMP biosynthesis. In this chapter, efforts were only focused on demonstrating the activity of the protein encoded by *MSMEG_6484*.

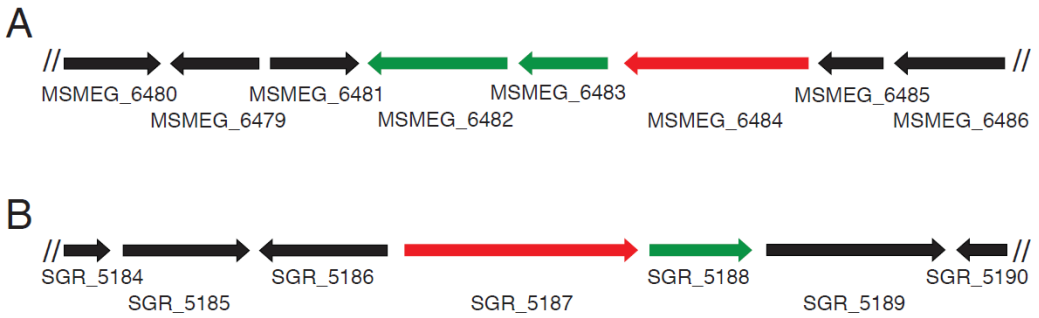


Figure 5-9. Organization of putative MMP biosynthesis genes in *M. smegmatis* mc^{2}155 (A) and *S. griseus* subsp. griseus NBRC 13350 (B). *MSMEG_6484* and *SGR_5187* (indicated in red) are predicated α-(1→4)-mannosyltransferase (ManT) genes; *MSMEG_6483* and *SGR_5187* (in green) are predicated 3-*O*-methyl-transferase (OMT) genes; *MSMEG_6482* is another putative methyltransferase gene.

5.3.2 Gene synthesis and expression of recombinant ManT (rManT)

The putative ManT gene, encoded by *MSMEG_6484* of *M. smegmatis*, was custom synthesized with codon optimization for expression in *E. coli* and cloned into vector pET-30b(+), generating the fused plasmid pET30b-*MSMEG_6484*. The recombinant protein has an additional hexahistidine (His) tag at the C-terminus for the purpose of purification.[37] The fused plasmid was then transformed into *Escherichia coli* BL21(DE3) cells, and the protein, was expressed by the induction of isopropyl 1-thio-β-D-galactopyranoside (IPTG) via the control of the lactose operon.[38] The expression led to a putative recombinant α-(1→4)-mannosyltransferase (rManT).

The activity of rManT was evaluated by an enzymatic assay using radioactive GDP-mannose as the donor and tetrasaccharide **9**, a known acceptor of the *M. smegmatis* ManT, as a substrate.[6] Because the cellular location of the

expressed protein is unknown, both the membrane fraction and the corresponding soluble cytosolic fraction of *E. coli* were examined. To our excitement, both soluble and membrane fractions exhibited activity when incubated with tetrasaccharide **9** (Table 5-3), with the activity accumulated significantly in the membrane fraction.

Table 5-3. Localization of enzymatic activity arising from *E. coli* expressed rManT.

Enzyme source	Volume of enzyme (mL)	Total activity (pmol•min^{-1}g^{-1} cell)	Specific activity pmol•min^{-1}mL^{-1}g^{-1} cell
Whole lysate (20,000 × g supernatant)	40	70.7	1.8
Soluble cytosolic fraction (200,000 × g supernatant)	40	16.1	0.4
Membrane fraction (200,000 × g pellet)	1.5	17.3	11.5

Data were taken from experiments depicted in Figure 5-10. Assays were performed in duplicate using 18 μL enzyme source incubating with 1 μL acceptor **9** (20 mM) and 1 μL radioactive GDP-mannose donor (20 mM) at 37 °C for 6 h.

In addition, assays including membrane preparations from *M. smegmatis* were performed in parallel, to compare the activity to the native ManT. The *E. coli* membrane fraction was found to be 20-fold less active than that of *M. smegmatis* (Figure 5-10). Nonetheless, this result inspired us to test another known substrate of ManT, tetrasaccharide **4**. In Chapter 2, we showed that the activity of native ManT was twice as high with **4** compared to **9**. A similar trend was expected if the rManT was, in fact, the α-(1→4)-mannosyltransferase involved in MMP biosynthesis. Indeed, the same trend in activity was observed as predicted (Figure 5-10).

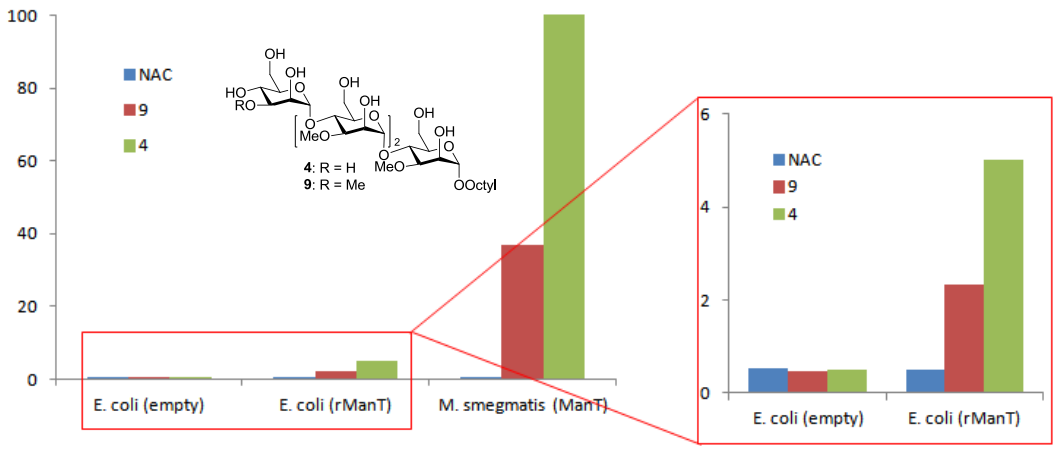


Figure 5-10. Left: Activity of *E. coli* expressed rManT. Right: Expansion of the region in box. Assays were performed using 18 μL enzyme source and incubating with 1 μL acceptor (20 mM) and 1 μL radioactive GDP-mannose donor (20 mM) at 37 °C for 6 h. The activity of substrate **4** catalyzed by the ManT in *M. smegmatis* was arbitrarily set to 100%. NAC (no acceptor control) is the control assay without added synthetic acceptor and the observed activities likely arise from endogenous acceptors present in membrane fraction.

To exclude the possibility that the observed activity arose from the function of intrinsic proteins in *E. coli*, the same *E. coli* strain containing a pET-30b(+) vector lacking the *MSMEG_6484* gene was prepared. This negative control showed no activity in either the soluble or membrane fractions (Figure 5-10). Therefore, the observed activity did, in fact, arise from the rManT encoded by the *MSMEG_6484* gene of *M. smegmatis*.

5.3.3 Efforts toward purification of *E. coli* expressed rManT

Purification of the His-tagged rManT was attempted using Ni-nitrilotriacetic acid (NTA) immobilized affinity column.[39] The hexahistidine sequence of the recombinant protein was expected to chelate with divalent Ni^{2+}

ions that are immobilized on the NTA-functionalized resin. Therefore, the His-tagged protein would be specifically retained on the resin. A buffer containing imidazole was then applied to elute the column. The imidazole competes with histidine for binding with Ni^{2+} ions, resulting in elution of the recombinant protein.

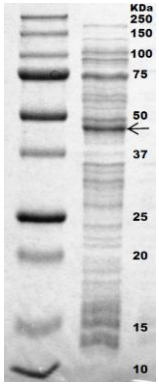


Figure 5-11. SDS-PAGE analysis of the crude whole lysate of *E. coli* containing rManT. Left lane: Protein standard ladder; Right lane: crude lysate of *E. coli*. The band indicated by arrow is close to the predicted size of rManT (45.6 kDa).

The predicted molecular weight of rManT (413 amino acids plus six added histidine residues) is 45.6 kDa. Indeed, SDS-PAGE analysis of the whole lysate of *E. coli* containing rManT showed an overexpressed protein of molecular weight between 37 and 50 KDa (Figure 5-11). The whole lysate of *E. coli* containing rManT was purified by Ni-NTA column. However, EGTA present in the cell lysis buffer (entry 1, Table 5-4) was found to be problematic, as it stripped the Ni^{2+} ion from the resin. Therefore, alternative buffers lacking EGTA were explored (entry 2–4, Table 5-4). The fractions collected were again examined by SDS-PAGE analysis. However, no protein of the expected size of rManT (45.6 kDa) was isolated. Recalling the instability of natural ManT in *M. smegmatis*,[4] the detergent

dodecyl β-D-maltoside was added into both the cell lysis buffer and the elution buffer to solubilize and stabilize the rManT protein (entry 5, Table 5-4).[27] However, no improvement was observed.

Table 5-4. Optimization of buffers used for Ni-NTA affinity purification.

Entry	Cell lysis buffer	Elute buffer
1	A: 50 mM TAPS, 10 mM $MgCl_2$, 5 mM EGTA, pH 8.2	D + NaCl + Im
2	B: 50 mM TAPS, 10 mM $MgCl_2$, pH 8.2	B + Im
3	B: 50 mM TAPS, 10 mM $MgCl_2$, pH 8.2	D + Im
4	C: 50 mM Tris, 10 mM imidazole, pH 8.0	C + NaCl + Im
5	D: 50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0	D + Im + detergent

TAPS: *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid; EGTA: Ethylene glycol tetraacetic acid; Tris: Tris(hydroxymethyl)aminomethane; Im: imidazole; Detergent: Dodecyl β-D-maltoside (0.1 mM).

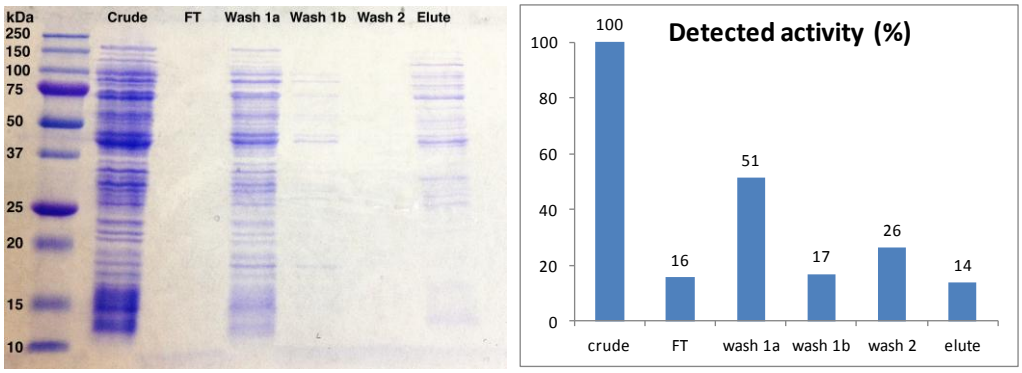


Figure 5-12. Localization of rManT after affinity purification.

Left: SDS-PAGE analysis of eluted fractions from affinity purification; Phosphate buffer D listed in Table 5-4 was used. Crude: crude cell lysate before applying to column; FT: Flow through; Wash 1a and wash 1b: phosphate buffer with no imidazole; Wash 2: 10 mM imidazole; Elute: 100 mM imidazole; Right: Detection of enzymatic activity with radioactive assay, using radioactive GDP-mannose (1 mM) as donor and tetrasaccharide **9** (1 mM) as acceptor in presence of 18 μL enzyme. Incubation was carried at 37 °C for 21 h. The activity of the crude lysate was arbitrarily set to 100%.

Failure to isolate the His-tagged rManT is likely due to the weak binding of the recombinant protein with Ni^{2+} ions immobilized on resin, as the protein band corresponding to 45.6 kDa eluted without the addition of imidazole (left, Figure 5-12). Consistent with this observation, when incubating the eluent fractions with radioactive GDP-mannose and tetrasaccharide **9**, enzyme activity was found, mainly located in the wash fraction, where no imidazole was used (right, Figure 5-12).

Immobilized metal ion affinity chromatography (IMAC) has been widely used for the purification of recombinant proteins, especially the polyhistindine-tagged proteins.[40-41] Successful purification requires that the expressed His-tags are exposed to the surface of protein so that they are accessible for coordinating with metal ions. Moreover, efficient binding requires that the His-tags are in the proper orientation to achieve the best geometry of the chelating metals.[37] We speculate that the His-tag expressed in rManT may be buried inside the protein after folding and thus is not available for metal chelation. A further modification would be to place the His-tag at the N-terminus of the expressed protein. An alternative solution is to evaluate rManT using crude preparations from *E. coli*, which is what we chose to do.

5.3.4 Evaluation of rManT using crude preparation of *E. coli*

Due to the difficulty in obtaining the rManT in a pure form, we began to characterize the protein using the crude enzyme preparations. The membrane fraction of *E. coli* was used as the enzyme source, as was done for *M. smegmatis*.

The crude enzyme was incubated with tetrasaccharide **9** and GDP-mannose as illustrated in Figure 5-13. We expected that the enzymatic products would be a series of oligomers rather than a single product, as the *M. smegmatis* ManT was shown to transfer more than one mannose residue onto the substrate (Chapters 2 and 3). The enzymatic products resulting from the incubation of **9** with *E. coli* ManT were purified using a C_{18} Sep-Pak cartridge[42] and analyzed by MALDI mass spectrometry (MALDI-MS) and NMR spectroscopy.

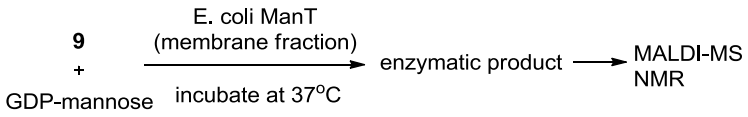


Figure 5-13. Incubation of **9** with *E. coli* ManT and analysis of enzymatic products.

5.3.4.1 MALDI-MS result reveals enzymatic products are oligomers

As expected, MALDI-MS analysis revealed the enzymatic products resulting from incubation of **9** with rManT were indeed oligomers. A series of signals differing by the mass of a hexose residue (162 Da) were observed (Figure 5-14A, $[M+Na]^+$: *m/z* = 1182 and 1344). Peaks *m/z* 1182 and 1344 correspond to products with incorporation of two and three mannose residues, respectively. This result is consistent with the enzymatic products observed from incubation of **9** with *M. smegmatis* ManT (Figure 5-14B, $[M+Na]^+$: *m/z* = 1020, 1182 and 1344), and shows that only a single enzyme is required to form extended MMP oligomers. The MALDI-MS result provided us first piece of evidence suggesting the rManT expressed in *E. coli* is the desired ManT from *M. smegmatis*.

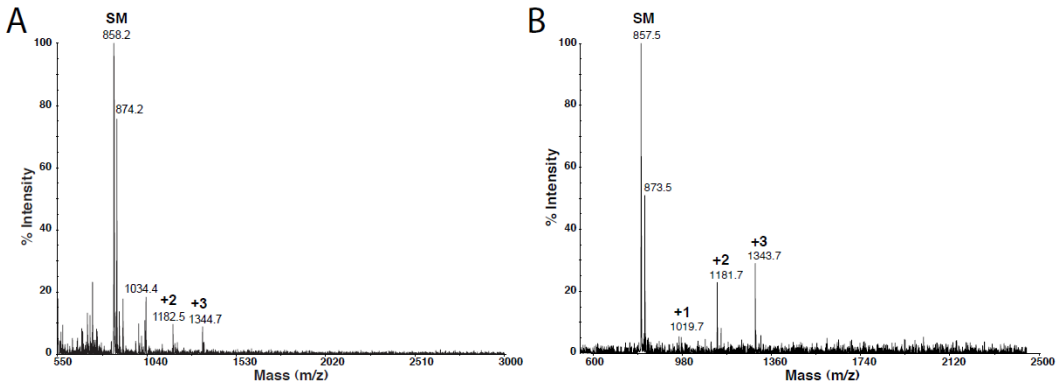


Figure 5-14. MALDI-MS analysis of the enzymatic products from *E. coli* rManT (A) and *M. smegmatis* ManT (B). SM: starting material; numbers above each peak correspond to the number of additional mannose residues incorporated into the SM.

5.3.4.2 Isolation of hexasaccharide and heptasaccharide products

Although the enzymatic oligomer products observed by MALDI-MS were as expected for products of ManT, this evidence alone was not enough to prove rManT was in fact the α-(1→4)-mannosyltransferase. The mannopyranosyl residue could be incorporated via other linkages, as *E. coli* itself also possesses mannosyltransferases.[43] Therefore, it was essential to determine unequivocally the nature of the newly formed linkages of the enzymatic products.

To address the regiochemistry of the enzymatic reaction, scale-up incubations were performed using **9** as substrate. A mixture of **9** (5 mg) and non-radiolabeled GDP-mannose was incubated in the presence of *E. coli* membrane fraction. The crude products were acetylated with acetic anhydride to facilitate purification. Thin layer chromatography (TLC) analysis revealed the formation of two major enzymatic products **112** and **113** (Figure 5-15A). High resolution ESI-MS analysis confirmed **112** was a hexasaccharide and **113** was a heptasaccharide.

As discussed in Chapter 2, incubations of the native *M. smegmatis* ManT with **9** also produced two major enzymatic products corresponding to the incorporation of two and three mannose residues, respectively (Figure 5-15B).

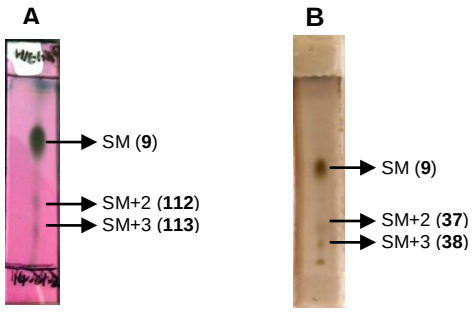


Figure 5-15. TLC analysis of the enzymatic products from *E. coli* rManT (A) and *M. smegmatis* ManT (B). Both TLC were developed in eluant system of hexane and ethyl acetate (1:2.5 for A and 1:2 for B).

The two major products were isolated by column chromatography. Both were obtained in sufficient quantities to allow structural elucidation by ^{1}H NMR spectroscopy. Resonance assignments were based on two-dimensional experiment (^{1}H–^{1}H COSY) as well as with coupling constant data characteristic of mannopyranose rings.[44] Hexasaccharide **112** (Figure 5-16A) was confirmed to be identical to the hexasaccharide product **37** (Figure 5-16C) resulting from incubation of **9** with *M. smegmatis* ManT (Chapter 2). Similarly, heptasaccharide **113** (Figure 5-16B) was identical to the other major product, **38** (Figure 5-16D), isolated from incubation of **9** with the *M. smegmatis* ManT (Chapter 2). The regiochemistry of the enzymatic reaction was indicated by the chemical shifts of H-4 of the penultimate mannose resides in both **112** and **113**. As in Chapter 2, acetylation of the reaction products helped with structure elucidation, as the

protons adjacent to the introduced acetyl groups show significant anisotropic deshielding resulting in resonances between 5.20–5.45 ppm.[45] In contrast, the chemical shifts of H-4 from the penultimate mannose residues of both **112** and **113** remained low (3.75–3.85 ppm) after acylation, indicating that the O-4 positions were glycosylated. These results provided further evidence the rManT expressed in *E. coli* is the desired α-(1→4)-mannosyltransferase.

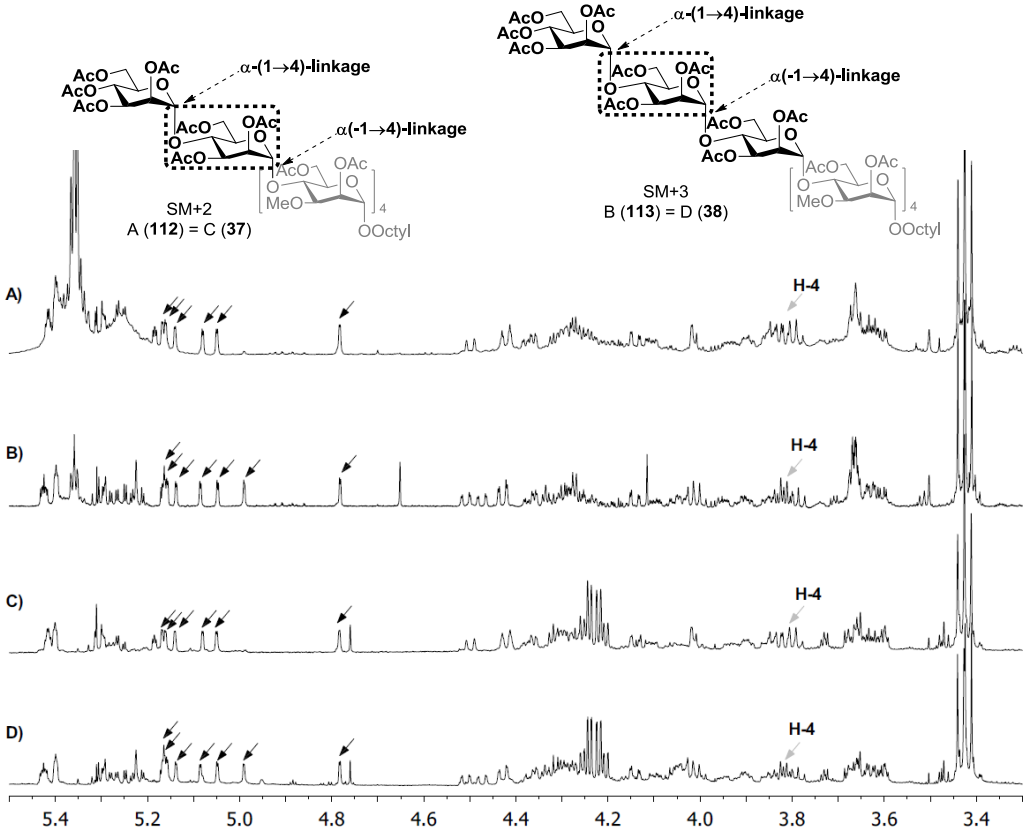


Figure 5-16. [1]H NMR analysis of the enzymatic products from *E. coli* ManT (A and B) and *M. smegmatis* ManT (C and D).

A) Hexasaccharide **112** from *E. coli* ManT; B) Hepatasaccahride **113** from *E. coli* ManT; C) Hexasaccharide **37** from *M. smegmatis* ManT; D) Heptasaccharide **38** from *M. smegmatis* ManT; Black arrows indicate anomeric protons and grey arrows indicate H-4 of the penultimate mannose reside circled with dashed box. Peaks at 5.38–5.33 ppm in A and B are unidentified impurities, as well as peaks at 4.26–4.18 ppm in C and D.

5.3.4.3 Kinetic characterization of E. coli rManT

The final evidence demonstrating the similarity of ManT and rManT was provided by kinetic analysis (Figure 5-17). A K_m value of 5.1 ± 0.6 μM was determined when using tetrasaccharide 9 as substrate of rManT. This value was closely matched the K_m value obtained from the enzymatic reaction of *M. smegmatis* ManT (K_m = 7.9 ± 1.7 μM, Table 5-5)

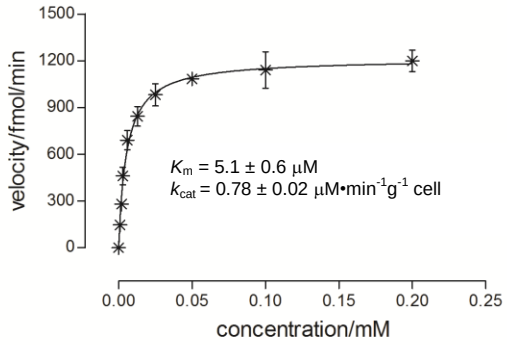


Figure 5-17. *E. coli* rManT kinetics with tetrasaccharide **9**.

Incorporation of radiolabeled GDP-mannose-[2-^{3}H] (0.5 mM) into **9** as a function of acceptor concentration. Assays were performed at 37 ℃ for 5 h, with substrate concentrations 0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, 100, 200 μ M. Control experiments without the addition of acceptors were also performed in parallel. Each experiment was carried out in duplicate. The data obtained were subjected to nonlinear regression analysis using GraphPad Prism 5.0.

Because V_{max} values rely on the concentration of the enzyme, which is, in turn, dependent on protein expression levels, we did not expect the V_{max} values for *E. coli* rManT and *M. smegmatis* ManT would be consistent. Surprisingly, both values were in the same order of magnitude (60.6 ± 1.6 nM·min^{-1} for *E. coli* and 45.3 ± 2.5 nM·min^{-1} for *M. smegmatis*, Table 5-5). Therefore, the k_{cat} and k_{cat}/K_m values, both calculated based on V_{max} value, were also consistent for the *E. coli*

and *M. smegmatis* enzymes. The close similarity of the K_m values, as well as other kinetic parameters, strongly support that the rManT expressed in *E. coli* is the same as ManT from *M. smegmatis*.

Table 5-5. Comparison of ManT form *E. coli* and *M. smegmatis*.

ManT	K_m (μ M)	V_{max} (nM·min^{-1})	k_{cat} (μ M·min^{-1}g^{-1} cell)	k_{cat}/K_m (min^{-1}g^{-1} cell)
E coli	5.1 ± 0.6	60.6 ± 1.6	0.78 ± 0.02	0.15 ± 0.03
M. smegmatis	7.9 ± 1.7	45.3 ± 2.5	0.79 ± 0.04	0.10 ± 0.02

5.4 Conclusions

In this chapter, we attempted to identify the ManT involved in MMP biosynthesis using two different approaches. The first approach used affinity purification of a crude membrane fraction with synthetic MMP ligands, which were either immobilized on a Sepharose matrix or conjugated with biotin for capture with streptavidin functionalized magnetic beads. However, purification of ManT using this approach proved unsuccessful, probably due to the low concentration of this enzyme in mycobacterial cell preparations. Therefore, we instead used a bioinformatic approach. Following this approach, a putative ManT gene from *M. smegmatis* was identified and expressed in *E. coli* as a recombinant ManT. The activity of this protein, rManT, was shown to be nearly identical to the ManT from *M. smegmatis*. Hence, it appears that we have identified the gene encoding the ManT from *M. smegmatis*. Our results reveal, for the first time, genetic information about this uncommon α-(1→4)-mannosyltransferase, and

showed that a single enzyme is capable of generating the mannose oligomers observed in previous studies of ManT in Chapters 2 and 3.

5.5 Experimental details

General methods for chemical synthesis: All reagents were purchased from commercial sources and were used without further purification unless noted. Reaction solvents were purified by successive passage through columns of alumina and copper under an argon atmosphere. All reactions were carried out under a positive pressure of argon at room temperature unless specified otherwise and were monitored by TLC on silica gel 60-F_{254} (0.25 mm, Silicycle, Quebec, Canada). Iatrobeads refer to a beaded silica gel 6RS-8060, which is manufactured by Iatron Laboratories (Tokyo). Visualization of the reaction components was achieved using UV fluorescence (254 nm) and/or by charring with acidified anisaldehyde solution in ethanol. Organic solvents were evaporated under reduced pressure and the products were purified by column chromatography on silica gel (230–400 mesh, Silicycle, Quebec, Canada). Optical rotations were measured on a Perkin–Elmer 241 polarimeter with a microcell (10 cm, 1 mL) at ambient temperature and are in units of degree·mL/(g·dm). Nuclear magnetic resonance (NMR) spectra were recorded on either Varian Inova 500 or Varian Inova 600 spectometers. ^{1}H NMR spectra were recorded at 500 MHz or 600 MHz and chemical shifts were referenced to residual $CHCl_3$ (7.26 ppm, $CDCl_3$), $CHDCl_2$ (5.32 ppm, CH_2Cl_2), or CHD_2OD (3.30 ppm, CD_3OD) and HOD (4.79 ppm, D_2O). ^{13}C NMR spectra were recorded at 125 MHz and chemical shifts were referenced

to $CDCl_3$ (77.0 ppm), $CHDCl_2$ (53.8 ppm) and CD_3OD (49.0 ppm). Reported splitting patterns are abbreviated as s = singlet, d = doublet, t = triplet, m = multiplet, br = broad, app = apparent. Assignments of NMR spectra were based on two-dimensional experiments (^1H–^1H COSY, HMQC or HSQC, and HMBC) and stereochemistry of the anomeric centers of the pyranose rings were confirmed by measuring $^1J_{C\text{-}1,H\text{-}1}$ via coupled HMQC or HSQC experiments. Electrospray mass spectra were recorded on Agilent Technologies 6220 TOF. Matrix-assisted laser desorption/ionization (MALDI) mass spectra were recorded on an AB Sciex Voyager Elite MALDI spectrometer equipped with a time-of-flight (TOF) detector, using 2,5-dihydroxylbenzoic acid (DHB) as matrix. Infrared (IR) spectra were recorded on Nicolet Magna 750 FTIR spectrometer and Nic-Plan microscope and are in units of cm^{-1}. The term "cast" refers to evaporation of a solution on a NaCl plate.

8-Aminooctyl 3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (92): Sodium amalgam was prepared by the addition of mercury (2.09 g) into sodium metal (94.4 mg). The resulting solidified sodium amalgam (3 wt.%, 1.06 g, 1.38 mmol) was added into solution of **103** (11 mg, 7.7 μmol) in THF–MeOH (1 mL, v/v 1:1) at –60 °C. The mixture was stirred overnight while warming to room temperature. The solution was then withdrawn with a pipet and concentrated. The crude intermediate was then redissolved in MeOH–H_2O–AcOH (2.6 mL, v/v/v 2:0.5:0.1). To this solution was added 5 wt.% Pd–C (10 mg) and the resulting solution was stirred under H_2 atmosphere overnight. The catalyst was removed by

filtration through Celite and the filtrate was concentrated to afford the crude trisaccharide amine **92** (10 mg) as a white solid. Further purification was not done. The amount of **92** (1.1 μmol, 14% over two steps) was determined by ^{1}H NMR in D_2O by mixing with a known amount of TsOH·H_2O. Their ratio was calculated by intergrating methyl group of TsOH·H_2O and anomeric protons of the product. R_f 0.50 (MeOH–H_2O 3:1); ^{1}H NMR (600 MHz, CD_3OD) δ 5.18 (br s, 1H, H-1″), 5.17 (br s, 1H, H-1′), 4.75 (br s, 1H, H-1), 4.13–3.37 (m, 20H, 3 × H-2, 3 × H-3, 3 × H-4, 3 × H-5, 6 × H-6, 2 × octyl OCH_2), 3.44 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.40 (s, 3H, OMe), 2.89 (br s, 2H, CH_2N), 1.80–1.42 (m, 4H, octyl CH_2), 1.48–0.96 (m, 8H, octyl CH_2); HRMS (ESI) calcd $C_{29}H_{55}NO_{16}$ $[M+Na]^+$ 696.3413, found 696.3412.

8-Azidooctyl 6-*O*-benzyl-2-*O*-toluenesulfonyl-4-*O*-triphenylmethyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**93**): The reaction was performed with **94** (65.3 mg, 0.06 mmol), *n*-Bu_2SnCl_2 (20 mol%, 5.4 mg, 0.017 mmol), DIPEA (177 μL, 1.01 mmol) and TsCl (172.0 mg, 0.91 mmol) at room temperature for 24 h. Purification of the residue by chromatography (hexane–EtOAc 2:1) gave **93** (69.2 mg, 76%) as a colorless syrup. R_f 0.46 (CH_2Cl_2–MeOH 30:1); $[\alpha]_D$ = +41.6 (*c* 0.3, CH_2Cl_2); ^{1}H NMR (600 MHz, CD_2Cl_2) δ 7.80 (d, *J* = 8.3 Hz, 2H, Ts), 7.74 (d, *J* = 8.3 Hz, 2H, Ts), 7.63 (d, *J* = 8.3 Hz, 2H, Ts), 7.39–7.08 (m, 36H, Ar), 5.14 (d, *J* = 1.7 Hz, 1H, H-1′), 5.08 (d, *J* = 3.9 Hz, 1H, H-1″), 4.76 (d, *J* = 1.7 Hz, 1H, H-1), 4.67 (dd, *J* = 3.8, 3.2 Hz, 1H, H-2″), 4.62 (dd, *J* = 3.5, 1.8 Hz, 1H, H-2), 4.59 (dd, *J* = 3.4, 1.8

Hz, 1H, H-2′), 4.55–4.38 (m, 4H, 2 × OC*H*$_2$Ph), 4.27–4.18 (m, 2H, OC*H*$_2$Ph), 4.06 (app td, *J* = 8.9, 3.4 Hz, 1H, H-3), 3.98–3.90 (m, 2H, H-3′, H-5″), 3.87 (ddd, *J* = 10.0, 6.3, 1.7 Hz, 1H, H-5′), 3.79–3.59 (m, 8H, H-3″, H-4, H-4′, H-5, H-6a, H-6b, H-6a′, octyl OCH$_2$), 3.54 (dd, *J* = 10.8, 6.3 Hz, 1H, H-6b′), 3.42–3.33 (m, 2H, H-4″, octyl OCH$_2$), 3.32 (dd, *J* = 10.2, 1.6 Hz, 1H, H-6a″), 3.24 (t, *J* = 7.0 Hz, 2H, CH$_2$N$_3$), 2.98 (d, *J* = 10.4 Hz, 1H, H-6b″), 2.97 (d, *J* = 10.3 Hz, 1H, OH-3′), 2.75 (d, *J* = 8.1 Hz, 1H, OH-3), 2.41 (s, 3H, ArC*H*$_3$), 2.40 (s, 3H, ArC*H*$_3$), 2.39 (s, 3H, ArC*H*$_3$),1.65–1.45 (m, 4H, octyl CH$_2$), 1.41–1.18 (m, 8H, octyl CH$_2$); ^{13}C NMR (126 MHz, CD$_3$OD) δ 146.6, 146.5, 146.3 (3C, Ar), 145.6 (3C, Ar), 139.8, 139.7, 139.4, 135.1, 135.0, 135.0, 131.0, 131.0, 130.9, 130.3, 129.5, 129.4, 129.4, 129.4, 129.3, 128.9, 128.9, 128.9, 128.8, 128.6, 128.6, 128.5 (48C, Ar), 100.0 (C-1), 98.6 (C-1′), 97.2 (C-1″), 89.2 (Ph$_3$*C*), 81.4 (C-2′), 81.3 (C-2″), 81.2 (C-2), 76.7 (C-4), 75.8 (C-4′), 74.4 (O*C*H$_2$Ph), 74.4 (O*C*H$_2$Ph), 74.0 (O*C*H$_2$Ph), 73.8 (C-5″), 73.3 (C-4″), 73.0 (C-5′), 72.3 (C-5), 71.1 (C-3″), 70.9 (C-6″), 70.5 (C-6′), 70.4 (C-6), 70.0 (C-3), 69.9 (C-3′), 69.3 (octyl OCH$_2$), 52.4 (CH$_2$N$_3$), 30.4, 30.3, 30.2, 29.9, 27.7, 27.2 (6C, octyl CH$_2$), 22.0 (Ar*C*H$_3$), 21.7 (2C, 2 × Ar*C*H$_3$); IR (cast film, CH$_2$Cl$_2$): 3537.4 (OH), 2095.4 (N$_3$); HRMS (ESI) calcd C$_{87}$H$_{97}$N$_3$O$_{22}$S$_3$ [M+Na]$^+$ 1654.5618, found 1654.5593.

8-Azidooctyl 6-*O*-benzyl-4-*O*-triphenylmethyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-α-D-mannopyranoside (**94**): To a solution of **95** (110.5 mg, 0.10 mmol) in *t*-butanol and acetone (2 mL, v/v 1:1) was added OsO$_4$ (2.5 wt.% in *t*-butanol, 39 μL, 0.003 mmol) and *N*-methylmorpholine *N*-oxide NMO (50% w/v in water, 0.3 mL). After stirring at room

temperature overnight, the resulting yellowish solution was treated with a saturated aqueous Na_2SO_3 solution. The mixture was concentrated to remove the *t*-butanol and then extracted with CH_2Cl_2 three times. The combined organic layers were concentrated and the resulting residue was purified by chromatography (CH_2Cl_2–MeOH 20:1) to afford **94** (78.4 mg, 65%) as a colorless syrup. R_f 0.14 (CH_2Cl_2–MeOH 15:1); $[\alpha]_D$ = +30.0 (*c* 0.1, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 7.58–7.10 (m, 30H, Ar), 5.26 (d, *J* = 1.9 Hz, 1H, H-1′), 5.02 (d, *J* = 5.8 Hz, 1H, H-1″), 4.71 (d, *J* = 1.6 Hz, 1H, H-1), 4.58–4.18 (m, 6H, 3 × OC*H*$_2$Ph), 3.95 (dd, *J* = 5.9, 3.2 Hz, 1H, H-2″), 3.93 (dd, *J* = 3.1, 2.1 Hz, 1H, H-2′), 3.90–3.85 (m, 2H, H-3′, H-5″), 3.85–3.77 (m, 7H, H-3, H-3″, H-4, H-4′, H-5, H-5′, H-6a), 3.77–3.68 (m, 5H, H-2, H-6b, H-6a′, H-6b′, octyl OCH_2), 3.55 (app t, *J* = 4.9 Hz, 1H, H-4″), 3.41 (m, 2H, H-6a″, octyl OCH_2), 3.29–3.27 (m, 1H, H-6b″), 3.25 (t, *J* = 6.9 Hz, 2H, CH_2N_3), 1.72–1.46 (m, 4H, octyl CH_2), 1.47–1.28 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, CD_3OD) δ 146.0 (3C, Ar), 139.8 (Ar), 139.7 (Ar), 139.5 (Ar), 130.4, 129.5, 129.4, 129.3, 129.1, 129.0, 129.0, 128.9, 128.8, 128.7, 128.6, 128.5, 128.4 (30C, Ar), 102.9 (C-1′, $^1J_{C\text{-}1,H\text{-}1}$ = 170.2 Hz), 101.7 (C-1″, $^1J_{C\text{-}1,H\text{-}1}$ = 172.6 Hz), 101.5 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 170.2 Hz), 89.1 (Ph$_3$*C*), 77.5 (C-4), 76.2, 76.1 (2C, C-4′, C-5″), 74.5 (OCH_2Ph), 74.4 (OCH_2Ph), 73.9 (OCH_2Ph), 73.6, 73.3, 73.0, 72.7, 72.7, 72.4, 72.3, 72.1, 72.0 (9C), 71.3 (2C, C-6, C-6′), 70.6 (C-6″), 68.7 (octyl OCH_2), 52.5 (CH_2N_3), 30.5, 30.3, 30.2, 29.9, 27.8, 27.2 (octyl CH_2); IR (cast film, MeOH): 3420.5 (OH), 2095.4 (N_3); HRMS (ESI) calcd $C_{66}H_{79}N_3O_{16}$ $[M+Na]^+$ 1192.5353, found 1192.5341.

(2*R*,3*S*,6*S*)-6-((8-azidooctyl)oxy)-2-((benzyloxy)methyl)-3-(((2*S*,5*S*,6*R*)-6-((benzyloxy)methyl)-5-(((2*S*,5*S*,6*R*)-6-((benzyloxy)methyl)-5-(trityloxy)-5,6-dihydro-2*H*-pyran-2-yl)oxy)-5,6-dihydro-2*H*-pyran-2-yl)oxy)-3,6-dihydro-2*H*-pyran (95): TrOTf was prepared *in situ* by adding a CH_2Cl_2 solution of TMSOTf (10% v/v in CH_2Cl_2, 0.78 mL, 0.34 mmol) into a stirring ice-cold solution of TfOH (116.1 mg, 0.45 mmol) in CH_2Cl_2 (7 mL). A bright yellow solution was formed after stirring at 0 °C for 5 min. This freshly-made TrOTf solution was slowly added into a stirring ice-cold solution of alcohol **102** (112.1 mg, 0.14 mmol) and 2,4,6-collidine (121.0 μL, 0.91 mmol) in CH_2Cl_2 (1.5 mL) over 3 h. MeOH (0.5 mL) was added to the reaction mixture before being concentrated. The crude residue was purified by chromatography (hexane–EtOAc 5:1) to afford trityl ether **95** (110.5 mg, 76%) as a yellow syrup. R_f 0.60 (hexane–EtOAc 2:1); $[\alpha]_D$ = +92.5 (*c* 0.4, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.53–7.02 (m, 30H, Ar), 6.06 (d, *J* = 10.4 Hz, 1H, H-3), 6.03 (d, *J* = 10.4 Hz, 1H, H-3′), 5.84 (ddd, *J* = 10.3, 2.7, 1.8 Hz, 1H, H-2), 5.76 (d, *J* = 10.5 Hz, 1H, H-3″), 5.72 (ddd, *J* = 10.3, 2.6, 2.0 Hz, 1H, H-2′), 5.45 (ddd, *J* = 10.4, 2.7, 1.9 Hz, 1H, H-2″), 5.25 (br s, 1H, H-1′), 5.09 (br s, 1H, H-1″), 5.03 (d, *J* = 2.3 Hz, 1H, H-1), 4.65–4.46 (m, 4H, 4 × OC*H*$_2$Ph), 4.43–4.36 (m, 2H, H-4, H-4′), 4.29 (s, 2H, 2 × OC*H*$_2$Ph), 4.10 (app dt, *J* = 9.0, 3.2 Hz, 1H, H-5″), 4.07–4.02 (m, 1H, H-5), 3.98–3.89 (m, 2H, H-5′, H-4″), 3.89–3.80 (m, 2H, H-6a, octyl OCH_2), 3.77 (dd, *J* = 10.7, 1.9 Hz, 1H, H-6a′), 3.72 (dd, *J* = 11.0, 6.6 Hz, 1H, H-6b), 3.68 (dd, *J* = 10.7, 5.5 Hz, 1H, H-6b′), 3.54–3.45 (m, 3H, H-6a″, H-6b″, octyl OCH_2), 3.26 (t, *J* = 7.0 Hz, 2H, CH_2N_3), 1.67–1.56 (m, 4H, octyl CH_2), 1.45–1.20 (m, 8H, octyl CH_2);

^{13}C NMR (126 MHz, $CDCl_3$) δ 144.8 (3C, Ar), 138.7 (Ar), 138.4 (Ar), 138.2 (Ar), 133.1 (C-3″), 130.1 (C-3′), 129.3 (C-3), 129.0, 128.3, 128.2, 128.2, 127.7, 127.6, 127.5, 12754, 127.4, 127.3 (28C, Ar), 127.3 (C-2), 127.2 (2C, Ar), 126.6 (C-2′), 124.7 (C-2″), 94.1 (C-1), 91.8 (C-1″), 91.3 (C-1′), 86.7 (Ph_3C), 73.5, 73.4, 73.1 (3C, 3 × O*C*H_2Ph), 70.6 (C-5″), 70.2 (C-6), 69.9 (C-5′), 69.7 (C-6′), 69.2 (C-6″), 69.2 (C-5), 68.6 (octyl OCH_2), 67.6 (C-4), 67.4 (C-4′), 66.1 (C-4″), 51.5 (CH_2N_3), 29.8, 29.3, 29.1, 28.8, 26.7, 26.2 (octyl CH_2); IR (cast film, $CHCl_3$): 2095.1 (N_3); HRMS (ESI) calcd $C_{66}H_{73}N_3O_{10}$ $[M+Na]^+$ 1090.5188, found 1090.5175.

(2*R*,3*S*,6*S*)-6-((8-azidooctyl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-ol (97): To a solution of ketone **98** (555.9 mg, 1.43 mmol) in MeOH (15 mL) at −78 °C was added $NaBH_4$ (76.4 mg, 2.02 mmol). The solution was stirred overnight while warming to room temperature. The mixture was then concentrated to remove the MeOH and then redissolved in CH_2Cl_2. After washing with water and brine, the organic layer was dried over Na_2SO_4 and concentrated and the residue was purified by chromatography (hexane–EtOAc 5:1) to afford alcohol **97** (466.0 mg, 83%) as a colorless syrup. R_f 0.43 (hexane–EtOAc 3:1); $[\alpha]_D$ = +16.3 (*c* 0.7, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.44–7.22 (m, 5H, Ar), 5.99–5.93 (br d, *J* = 10.2 Hz, 1H, H-3), 5.77 (ddd, *J* = 10.2, 2.7, 2.1 Hz, 1H, H-2), 5.03–4.95 (m, 1H, H-1), 4.66 (d, *J* = 12.0 Hz, 1H, O*C*H_2Ph), 4.62 (d, *J* = 12.0 Hz, 1H, O*C*H_2Ph), 4.33–4.20 (dddd, *J* = 8.9, 5.3, 3.5, 1.9 Hz, 1H, H-4), 3.86 (app dt, *J* = 9.1, 5.1 Hz, 1H, H-5), 3.83–3.75 (m, 2H, H-6a, octyl OCH_2), 3.72 (dd, *J* = 9.8, 5.5 Hz, 1H, H-6b), 3.50 (dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 3.27 (t, *J* = 7.0 Hz, 2H, octyl CH_2N_3), 2.30 (d, *J* = 5.3 Hz, 1H, OH-4), 1.70–1.51 (m, 4H,

octyl CH_2), 1.43–1.23 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 137.8 (Ar), 132.8 (C-3), 128.5 (2C, Ar), 127.9 (Ar), 127.7 (2C, Bn), 126.3 (C-2), 94.3 (C-1), 73.7 (OCH_2Ph), 71.0 (C-6), 69.7 (C-5), 68.7 (octyl OCH_2), 66.1 (C-4), 51.5 (CH_2N_3), 29.8, 29.2, 29.1, 28.8, 26.7, 26.1 (octyl CH_2); IR (cast film, $CHCl_3$): 3438.6 (OH), 2095.8 (N_3); HRMS (ESI) calcd $C_{21}H_{31}N_3O_4$ $[M+Na]^+$ 412.2207, found 412.2207.

(2*R*,6*S*)-6-((8-azidooctyl)oxy)-2-((benzyloxy)methyl)-2*H*-pyran-3(6*H*)-one (98): To a solution of CH_2Cl_2 (12 mL) containing **96** (740.0 mg, 2.21 mmol, from Chapter 4) and 8-azido-1-octanol (0.55 mL, 4.20 mmol) was added 2.5 mol% tris(dibenzylideneacetone)dipalladium(0) ($Pd_2(dba)_3$, 48.0 mg, 0.05 mmol) and 10 mol% triphenylphosphine (Ph_3P, 55.0 mg, 0.21 mmol). After stirring overnight, the resulting purple solution was concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 15:1) to afford **98** (564.6 mg, 66%) as a colorless syrup. R_f 0.66 (hexane–EtOAc 3:1); $[\alpha]_D$ = +11.9 (*c* 0.9, $CHCl_3$); ^1H NMR (500 MHz, $CDCl_3$) δ 7.42–7.24 (m, 5H, Bn), 6.91 (dd, *J* = 10.2, 3.5 Hz, 1H, H-2), 6.15 (d, *J* = 10.2 Hz, 1H, H-3), 5.33 (d, *J* = 3.5 Hz, 1H, H-1), 4.66–4.57 (m, 3H, H-5, 2 × OC*H*$_2$Ph), 3.97–3.91 (m, 2H, H-6a, H-6b), 3.88 (dt, *J* = 9.6, 6.8 Hz, 1H, octyl OCH_2), 3.62 (dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 3.27 (t, *J* = 6.9 Hz, 2H, octyl CH_2N_3), 1.73–1.51 (m, 4H, octyl CH_2), 1.46–1.26 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 194.4 (C=O), 144.0 (C-2), 138.0 (Ar), 128.3 (2C, Ar), 127.9 (C-3), 127.7, 127.6 (3C, Ar), 93.2 (C-1), 74.5 (C-5), 73.7 (O*C*H_2Ph), 69.6 (octyl OCH_2), 68.7 (C-6), 51.5 (octyl CH_2N_3), 29.6, 29.2, 29.1, 28.8, 26.7,

26.1 (6C, octyl CH_2); IR (cast film, $CHCl_3$): 2094.4 (N_3), 1697.8 (C=O); HRMS (ESI) calcd $C_{21}H_{29}N_3O_4$ $[M+Na]^+$ 410.2050, found 410.2045.

(2*R*,6*S*)-6-(((2*R*,3*S*,6*S*)-6-((8-azidooctyl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-2-((benzyloxy)methyl)-2*H*-pyran-3(6*H*)-one (99): A solution of CH_2Cl_2 (10 mL) containing **96** (750.0 mg, 2.24 mmol) and alcohol **97** (458.8 mg, 1.18 mmol) with 4 Å molecular sieves was stirred at room temperature for 0.5 h before 2.5 mol% $Pd_2(dba)_3$ (50.4 mg, 0.06 mmol) and 10 mol% PPh_3 (57.7 mg, 0.22 mmol) were added. After stirring at room temperature for 5 h, the resulting purple solution was concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 5:1) to afford **99** (374.0 mg, 52%) as a yellow syrup. R_f 0.37 (hexane–EtOAc 3:1); $[\alpha]_D$ = +30.8 (*c* 0.2, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49–7.15 (m, 10H, Ar), 6.81 (dd, *J* = 10.2, 3.5 Hz, 1H, H-2′), 6.16 (d, *J* = 10.4 Hz, 1H, H-3′), 6.09 (d, *J* = 10.3 Hz, 1H, H-3), 5.88 (ddd, *J* = 10.3, 2.8, 1.8 Hz, 1H, H-2), 5.57 (d, *J* = 3.6 Hz, 1H, H-1′), 5.05 (d, *J* = 2.3 Hz, 1H, H-1), 4.67–4.48 (m, 5H, H-4, 4 × OC*H*$_2$Ph), 4.46 (dd, *J* = 4.3, 2.7 Hz, 1H, H-5′), 4.00 (ddd, *J* = 9.2, 4.4, 2.2 Hz, 1H, H-5), 3.84–3.69 (m, 4H, H-6a, H-6b, H-6a′, octyl OCH_2), 3.66 (dd, *J* = 10.7, 2.7 Hz, 1H, H-6b′), 3.51 (dt, *J* = 9.6, 6.6 Hz, 1H, octyl OCH_2), 3.26 (t, *J* = 6.9 Hz, 1H, CH_2N_3), 1.70–1.50 (m, 4H, octyl CH_2), 1.44–1.19 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 193.9 (C=O), 143.6 (C-2′), 138.1 (Ar), 137.8 (Ar), 128.7 (Ar), 128.3 (2C, Ar), 128.3 (2C, Ar), 128.1 (C-3′), 127.9 (C-3), 127.7 (2C, Ar), 127.6 (Ar), 127.6 (C-2), 127.5 (2C, Ar), 94.4 (C-1), 90.2 (C-1′), 75.0 (C-5′), 73.7 (O*C*H$_2$Ph), 73.4 (O*C*H$_2$Ph), 69.5 (C-6), 69.1 (C-5), 68.8 (C-6′), 68.5 (octyl OCH_2), 68.0 (C-4), 51.5 (CH_2N_3),

29.7, 29.3, 29.1, 28.8, 26.7, 26.1 (octyl CH_2); IR (cast film, $CHCl_3$): 2094.4 (N_3), 1699.1 (C=O); HRMS (ESI) calcd $C_{34}H_{43}N_3O_7$ $[M+Na]^+$ 628.2993, found 628.2985.

(2*R*,3*S*,6*S*)-6-(((2*R*,3*S*,6*S*)-6-((8-azidooctyl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-ol (100): To a solution of ketone **99** (374.0 mg, 0.72 mmol) in MeOH (10 mL) at −78 °C was added $NaBH_4$ (37.5 mg, 0.99 mmol) and $CeCl_3•7H_2O$ (370.0 mg, 0.99 mmol). The solution was stirred overnight while warming to room temperature. The mixture was concentrated to remove the MeOH and the residue was redissolved in CH_2Cl_2. After washing with water and brine, the organic layer was over Na_2SO_4 and concentrated. The residue was purified by chromatography (hexane–EtOAc 5:1) to afford alcohol **100** (306.8 mg, 82%) as a pale yellow syrup. R_f 0.34 (hexane–EtOAc 2:1); $[\alpha]_D$ = +36.5 (*c* 0.4, $CHCl_3$); ^{1}H NMR (498 MHz, $CDCl_3$) δ 7.40–7.13 (m, 10H, Ar), 6.02 (d, *J* = 10.4 Hz, 1H, H-3), 5.94 (d, *J* = 10.2 Hz, 1H, H-3′), 5.81 (ddd, *J* = 10.3, 2.8, 1.8 Hz, 1H, H-2), 5.65 (ddd, *J* = 10.2, 2.7, 2.2 Hz, 1H, H-2′), 5.17 (app dt, *J* = 2.7, 1.5 Hz, 1H, H-1′), 5.00 (ddd, *J* = 2.7, 1.6, 1.1 Hz, 1H, H-1), 4.60–4.41 (m, 4H, 4 × OC*H*$_2$Ph), 4.40 (ddd, *J* = 9.4, 3.0, 1.6 Hz, 1H, H-4), 4.23 (dddd, *J* = 10.6, 5.2, 1.9, 1.9 Hz, 1H, H-4′), 3.98 (ddd, *J* = 9.4, 4.7, 2.6 Hz, 1H, H-5), 3.78 (dt, *J* = 9.5, 6.8 Hz, 1H, octyl OCH_2), 3.75–3.70 (m, 2H, H-6a, H-6b), 3.71–3.65 (m, 1H, H-5), 3.64 (dd, *J* = 9.6, 4.3 Hz, 1H, H-6a′), 3.52 (dd, *J* = 9.6, 5.7 Hz, 1H, H-6b′), 3.47 (dt, *J* = 9.5, 6.5 Hz, 1H, octyl OCH_2), 3.23 (t, *J* = 7.0 Hz, 2H, CH_2N_3), 2.31 (dd, *J* = 4.9, 1.3 Hz, 1H, OH-4′), 1.69–1.46 (m, 4H, octyl CH_2), 1.46–1.17 (m, 8H, octyl CH_2); ^{13}C NMR (125

MHz, $CDCl_3$) δ 138.5 (Ar), 137.6 (Ar), 133.2 (C-3′), 129.3 (Ar), 128.5 (2C, Ar), 128.3 (2C, Ar), 127.9 (C-3), 127.7 (2C, Ar), 127.4 (3C, Ar), 127.2 (C-2), 125.7 (C-2′), 94.3 (C-1), 91.1 (C-1′), 73.7 (O*C*H_2Ph), 73.2 (O*C*H_2Ph), 70.6 (C-6′), 69.9 (C-5′), 69.7 (C-6), 69.1 (C-5), 68.6 (octyl OCH_2), 67.3 (C-4), 65.9 (C-4′), 51.5 (CH_2N_3), 29.7, 29.2, 29.1, 28.8, 26.6, 26.1 (octyl CH_2); IR (cast film, $CHCl_3$): 3449.5 (OH), 2095.6 (N_3); HRMS (ESI) calcd $C_{34}H_{45}N_3O_7$ $[M+Na]^+$ 630.3150, found 630.3148.

(2*R*,6*S*)-6-(((2*R*,3*S*,6*S*)-6-(((2*R*,3*S*,6*S*)-6-((8-azidooctyl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-2-((benzyloxy)methyl)-2*H*-pyran-3(6*H*)-one (101): A solution of CH_2Cl_2 (10 mL) containing **96** (367.0 mg, 1.10 mmol) and alcohol **100** (301.2 mg, 0.49 mmol) with 4 Å molecular sieves was stirred at room temperature for 0.5 h before 2.5 mol% $Pd_2(dba)_3$ (22.6 mg, 0.025 mmol) and 10 mol% PPh_3 (27.0 mg, 0.10 mmol) were added. After stirring at room temperature overnight, the resulting purple solution was concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 7:1) to afford **101** (403.2 mg, 99%) as a yellow syrup. R_f 0.26 (toluene–EtOAc 5:1); $[\alpha]_D$ = +22.8 (*c* 0.3, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.45–7.11 (m, 15H, Ar), 6.83 (dd, *J* = 10.2, 3.5 Hz, 1H, H-2″), 6.18 (d, *J* = 10.4 Hz, 1H, H-3″), 6.11 (d, *J* = 10.2 Hz, 1H, H-3′), 6.05 (d, *J* = 10.3 Hz, 1H, H-3), 5.85 (ddd, *J* = 10.3, 2.7, 1.8 Hz, 1H, H-2), 5.79 (ddd, *J* = 10.3, 2.7, 1.9 Hz, 1H, H-2′), 5.57 (d, *J* = 3.6 Hz, 1H, H-1″), 5.27 (d, *J* = 2.6 Hz, 1H, H-1′), 5.03 (d, *J* = 2.4 Hz, 1H, H-1), 4.62 (ddd, *J* = 9.2, 3.0, 1.6 Hz, 1H, H-4′), 4.61–4.37 (m, 8H, H-4, H-5″, 6 × O*C*H_2Ph), 4.02 (ddd, *J* = 9.2, 5.7, 1.8 Hz, 1H,

H-5), 3.86–3.75 (m, 4H, H-5′, H-6a, H-6a″, octyl OCH_2), 3.71 (dd, *J* = 11.0, 5.8 Hz, 1H, H-6b), 3.64–3.57 (m, 2H, H-6a′, H-6b″), 3.55 (dd, *J* = 10.9, 1.9 Hz, 1H, H-6b′), 3.50 (dt, *J* = 9.6, 6.5 Hz, 1H, octyl OCH_2), 3.26 (t, *J* = 7.0 Hz, 2H, CH_2N_3), 1.68–1.49 (m, 4H, octyl CH_2), 1.44–1.23 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 194.0 (C=O), 143.6 (C-2″), 138.5 (Ar), 138.0 (Ar), 137.8 (Ar), 129.2 (2C, C-3, C-3′), 128.3 (4C, Ar), 128.3 (2C, Ar, 128.2 (C-3″), 127.7 (2C, Ar), 127.6 (2C, Ar), 127.6 (2C, Ar, C-2), 127.4 (Ar), 127.3 (2C, Ar), 127.3 (Ar), 127.3 (C-2′), 94.2 (C-1), 91.4 (C-1′), 90.0 (C-1″), 75.0 (C-5″), 73.7, 73.5, 73.2 (3C, 3 × O*C*H_2Ph), 69.9 (C-6), 69.7 (C-5), 69.2 (C-5′), 69.0, 68.6, 68.5 (3C, C-6′, C-6″, octyl OCH_2), 67.5, 67.4 (2C, C-4, C-4′), 51.5 (CH_2N_3), 29.8, 29.3, 29.1, 28.8, 26.7, 26.2 (6C, octyl CH_2); IR (cast film, $CHCl_3$): 2094.6(N_3), 1699.1 (C=O); HRMS (ESI) calcd $C_{47}H_{57}N_3O_{10}$ $[M+Na]^+$ 846.3936, found 846.3922.

(2*R*,3*S*,6*S*)-6-(((2*R*,3*S*,6*S*)-6-(((2*R*,3*S*,6*S*)-6-((8-azidooctyl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-yl)oxy)-2-((benzyloxy)methyl)-3,6-dihydro-2*H*-pyran-3-ol (102): To a solution of ketone **101** (194.5 mg, 0.24 mmol) in MeOH (3 mL) at −78 °C was added $NaBH_4$ (17.4 mg, 0.46 mmol) and $CeCl_3$•$7H_2O$ (100.6 mg, 0.27 mmol). The solution was stirred overnight while warming to room temperature. The mixture was concentrated to remove the MeOH and the residue was then redissolved in CH_2Cl_2. After washing with 1N HCl and brine, the organic layer was dried over Na_2SO_4, and concentrated. The resulting residue was purified by chromatography (hexane–EtOAc 3:1) to afford alcohol **102** (114.4 mg, 59%) as yellow syrup. R_f 0.31 (hexane–EtOAc 2:1); $[\alpha]_D$ = +20.9 (*c*

0.3, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.49–7.07 (m, 15H, Ar), 6.07 (d, *J* = 10.2 Hz, 1H, H-3′), 6.05 (d, *J* = 10.1 Hz, 1H, H-3), 5.99 (d, *J* = 10.2 Hz, 1H, H-3″), 5.84 (ddd, *J* = 10.3, 2.8, 1.8 Hz, 1H, H-2), 5.74 (ddd, *J* = 10.3, 2.7, 1.9 Hz, 1H, H-2′), 5.70 (ddd, *J* = 10.2, 2.6, 2.2 Hz; 1H, H-2″), 5.25 (br s, 1H, H-1′), 5.20 (br s, 1H, H-1″), 5.02 (d, *J* = 2.4 Hz, 1H, H-1), 4.59–4.40 (m, 7H, H-4′, 6 × OC*H*$_2$Ph), 4.41 (ddd, *J* = 9.5, 3.0, 1.6 Hz, 1H, H-4), 4.27 (dddd, *J* = 6.2, 3.5, 1.9, 1.9 Hz, 1H, H-4″), 4.02 (ddd, *J* = 9.3, 6.0, 1.7 Hz, 1H, H-5), 3.87–3.77 (m, 3H, H-5′, H-6a, octyl OCH_2), 3.74–3.62 (m, 4H, H-5″, H-6b, H-6a′, H-6a″), 3.60 (dd, *J* = 10.8, 2.1 Hz, 1H, H-6b′), 3.55–3.46 (m, 2H, H-6b″, octyl OCH_2), 3.26 (t, *J* = 7.0 Hz, 2H, CH_2N_3), 2.39 (d, *J* = 4.6 Hz, 1H, OH-4″), 1.65–1.57 (m, 4H, octyl CH_2), 1.43–1.15 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 138.6 (Ar), 138.4 (Ar), 137.6 (Ar), 133.3 (C-3″), 129.9 (C-3′), 129.3 (C-3), 128.5 (2C, Ar), 128.3 (2C, Ar), 128.2 (2C, Ar), 127.9 (Ar), 127.8 (2C, Ar), 127.4 (3C, Ar), 127.4 (3C, Ar), 127.3 (C-2), 126.7 (C-2′), 125.7 (C-2″), 94.2 (C-1), 91.3 (C-1′), 91.0 (C-1″), 73.7, 73.4, 73.2 (3C, 3 × O*C*H_2Ph), 70.7, 70.0 (2C, C-6′, C-6″), 69.8 (2C, C-5′, C-5″), 69.2 (C-6), 69.2 (C-5), 68.6 (octyl OCH_2), 67.5 (C-4), 66.7 (C-4′), 66.1 (C-4″), 51.5 (CH_2N_3), 29.8, 29.3, 29.1, 28.8, 26.7, 26.2 (6C, octyl CH_2); IR (cast film, $CHCl_3$): 3467.2 (OH), 2095.6(N_3); HRMS (ESI) calcd $C_{47}H_{59}N_3O_{10}$ $[M+Na]^+$ 848.4093, found 848.4076.

8-Azidooctyl 6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**103**): To a stirring ice-cold solution of alcohol **93** (65.8 mg,

0.04 mmol) and MeI (25 μL, 0.39 mmol) in THF (0.6 mL) was added NaH (60% in mineral oil, 30.0 mg, 0.75 mmol). The resulting solution was stirred at 0 ℃ for 1 h before MeOH (1 mL) was added. Then a solution of HCl in MeOH (0.1 mL, 10% v/v) was added and the reaction mixture was stirred for 0.5 h. The yellowish solution was concentrated and the residue was redissolved in CH_2Cl_2, washed with saturated aqueous $NaHCO_3$, followed by saturated aqueous Na_2SO_3 and brine. The separated organic layer was dried over Na_2SO_4, concentrated and the resulting residue was purified by chromatography (hexane–EtOAc 2:1) to afford **103** (34.5 mg, 60% over two steps) as a colorless syrup. R_f 0.20 (hexane–EtOAc 2:1); $[\alpha]_D$ = +6.7 (*c* 0.3, $CHCl_3$); ^{1}H NMR (498 MHz, $CDCl_3$) δ 7.98–7.68 (m, 6H, Ar), 7.45–7.15 (m, 21H, Ar), 5.10 (d, *J* = 1.4 Hz, 1H, H-1″), 5.06 (d, *J* = 1.5 Hz, 1H, H-1′), 4.94–4.88 (m, 3H, H-1, H-2′, H-2″), 4.79 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2), 4.51–4.32 (m, 6H, 6 × OC*H*$_2$Ph), 3.81–3.47 (m, 14H, H-3, H-4, H-4′, H-4″, H-5, H-5′, H-5″, 2 × H-6, 2 × H-6′, 2 × H-6″, octyl OCH_2), 3.45 (dd, *J* = 9.3, 2.9 Hz, 1H, H-3′), 3.40 (dt, *J* = 9.9, 6.8 Hz, 1H, octyl OCH_2), 3.35 (dd, *J* = 9.6, 3.0 Hz, 1H, H-3″), 3.25 (t, *J* = 6.9 Hz, 2H, CH_2N_3), 3.24 (s, 3H, OMe), 3.23 (s, 3H, OMe), 3.19 (s, 3H, OMe), 2.62 (d, *J* = 1.9 Hz, 1H, OH-4″), 2.44 (s, 3H, ArC*H*$_3$), 2.41 (s, 3H, ArC*H*$_3$), 2.40 (s, 3H, ArC*H*$_3$), 1.67–1.46 (m, 4H, octyl CH_2), 1.45–1.20 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 145.0, 144.9, 144.8, 138.3, 138.0, 134.1, 134.0, 133.6, 129.8, 129.8, 129.7, 128.4, 128.3, 128.2, 128.1, 128.1, 127.9, 127.7, 127.6, 127.5, 127.5, 127.4 (36C, Ar), 99.5 (2C, C-1′, $^1J_{C\text{-}1,H\text{-}1}$ = 174.6 Hz, C-1″, $^1J_{C\text{-}1,H\text{-}1}$ = 174.6 Hz), 97.3 (C-1, $^1J_{C\text{-}1,H\text{-}1}$ = 169.3 Hz), 79.2 (C-3), 79.0 (C-3′), 78.4 (C-3″), 74.4 (C-2′), 74.0 (2C, C-2, C-4), 73.9 (C-2″), 73.7 (OCH_2Ph), 73.4

(2C, 2 × OC*H*$_2$Ph), 73.1, 72.2, 71.9, 71.2 (4C, C-4′, C-5, C-5′, C-5″), 70.3 (C-6″), 69.6 (C-6), 69.3 (C-6′), 68.3 (octyl OCH_2), 67.5 (C-4″), 57.1 (OMe), 56.8 (OMe), 56.7 (OMe), 51.5 (CH_2N_3), 29.3, 29.2, 29.0, 28.8, 26.7, 25.9 (6C, octyl CH_2), 21.7 (Ar*C*H_3), 21.6 (Ar*C*H_3) 21.1 (Ar*C*H_3); IR (cast film, $CHCl_3$): 2095.5 (N_3); HRMS (ESI) calcd $C_{71}H_{89}N_3O_{22}S_3$ $[M+Na]^+$ 1454.4992, found 1454.4979.

Biotin-MMP$_3$ (104): The crude trisaccharide amine **92** (1.6 mg) was dissolved in DMF (0.1 mL). To this solution was added *N*,*N*-diisopropylethylamine (25 μL, 144 μmol) and biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester (0.9 mg, 1.6 μmol). The resulting solution was stirred at room temperature for 2 h. The crude product was then purified by chromatography on Iatrobeads followed by desalting using a C_{18} cartridge (Waters) to give a mixture of Biotin-MMP$_3$ **104** (0.3 mg, quantitative, by NMR) and hydrolyzed biotinylating reagent (0.14 mg). The ratio was determined by ^{1}H NMR spectroscopy. R_f 0.11 (EtOAc–MeOH–H_2O 7:2:1); ^{1}H NMR (600 MHz, D_2O) δ 5.21 (d, *J* = 1.9 Hz, 1H, H-1′), 5.18 (d, *J* = 1.9 Hz, 1H, H-1″), 4.89 (d, *J* = 1.6 Hz, 1H, H-1), 4.60 (dd, *J* = 7.8, 5.1 Hz, 1H, biotin), 4.41 (dd, *J* = 7.9, 4.5 Hz, 1H, biotin), 4.23–4.17 (m, 2H, H-2′, H-2″), 4.15 (dd, *J* = 3.0, 1.7 Hz, 1H, H-2), 3.91–3.51 (m, 16H, H-3, H-3′, H-3″, H-4, H-4′, H-4″, H-5, H-5′, H-5″, 2 × H-6, 2 × H-6′, 2 × H-6″, octyl OCH_2), 3.48–3.40 (m, 1H, octyl OCH_2), 3.45 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.44 (s, 3H, OMe), 3.36–3.28 (m, 1H, biotin), 3.22–3.12 (m, 6H, biotin), 2.98 (dd, *J* = 13.0, 5.0 Hz, 1H, biotin), 2.77 (d, *J* = 13.1 Hz, 1H, biotin), 2.29–2.14 (m, 6H, biotin), 1.81–1.22 (m, 30H, biotin); LRMS (MALDI) calcd $C_{51}H_{91}N_5O_{20}S$ $[M+Na]^+$ 1148.5875, found 1149.70.

Biotin-MMP$_4$ (105): The crude amine **111** (2.7 mg, 2.2 μmol) was dissolved in $NaHCO_3$ coupling buffer (0.5 mL, 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3). Biotinamidohexanoyl-6-aminohexanoic acid *N*-hydroxysuccinimide ester (2.0 mg, 3.5 μmol) was added and the resulting solution was stirred at room temperature for 2 h. The crude product was purified using a C_{18} cartridge (Waters) to give a mixture of Biotin-MMP$_4$ **105** (1.7 mg, 87%) and excess biotinylating reagent (0.8 mg). The ratio was determined by ^{1}H NMR spectroscopy. R_f 0.34 (EtOAc–MeOH–H_2O 7:2:1); ^{1}H NMR (600 MHz, CD_3OD) δ 5.24–5.20 (m, 2H, H-1′, H-1″), 5.17 (d, *J* = 1.5 Hz, 1H, H-1‴), 4.77 (d, *J* = 1.6 Hz, 1H, H-1), 4.48 (dd, *J* = 7.9, 5.1 Hz, 1H, biotin), 4.29 (dd, *J* = 7.8, 4.5 Hz, 1H, biotin), 4.09 (m, 2H, H-2′, H-2″), 4.01 (dd, *J* = 3.2, 1.8 Hz, 1H, H-2), 3.95–3.52 (m, 19H, H-2‴, H-3‴, H-4, H-4′, H-4″, H-4‴, H-5, H-5′, H-5″, H-5‴, 2 × H-6, 2 × H-6′, 2 × H-6″, 2 × H-6‴, octyl OCH_2), 3.52–3.44 (m, 4H, H-3, H-3′, H-3″, octyl OCH_2), 3.43 (s, 3H, OMe), 3.43 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.24–3.11 (m, 7H, biotin), 2.92 (dd, *J* = 12.7, 5.0 Hz, 1H, biotin), 2.70 (dd, *J* = 12.6, 2.6 Hz, 1H, biotin), 2.23–2.05 (m, 6H, biotin), 1.79–1.19 (m, 30H, biotin).

8-Azidooctyl 2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**106**): A mixture of thioglycoside **11** (20.0 mg, 0.015 mmol, from Chapter 2), alcohol **103** (22.2 mg, 0.015 mmol) and powdered 4Å molecular sieves were dissolved in CH_2Cl_2 (1 mL) and stirred at 0 ℃ for 0.5 h. Then *N*-iodosuccinimide (15.0 mg, 0.067 mmol) and

silver trifluoromethanesulfonate (1.1 mg, 0.004 mmol) were added. After stirring overnight while warming to room temperature, triethylamine (1 mL) was added and the reaction mixture was filtered through Celite. The filtrate was concentrated and the resulting crude residue was purified by chromatography (hexane–EtOAc 2:1 to 3:2) to afford tetrasaccharide **106** (25.9 mg, 95%) as a colorless syrup. R_f 0.35 (hexane–EtOAc 3:2); $[\alpha]_D$ = +26.6 (*c* 0.1, $CHCl_3$); ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.92–7.76 (m, 6H, Ar), 7.41–7.19 (m, 21H, Ar), 5.32 (dd, *J* = 2.6, 1.9 Hz, 1H, H-2‴), 5.28–5.23 (m, 2H, H-3‴, H-4‴), 5.19 (d, *J* = 1.7 Hz, 1H, H-1‴), 5.03 (d, *J* = 2.0 Hz, 1H, H-1′), 5.01 (d, *J* = 1.9 Hz, 1H, H-1″), 4.99 (dd, *J* = 2.9, 2.2 Hz, 1H, H-2′), 4.97–4.92 (m, 2H, H-1, H-2″), 4.84 (dd, *J* = 3.0, 1.9 Hz, 1H, H-2), 4.54–4.40 (m, 6H, 6 × OC*H*$_2$Ph), 4.13 (dd, *J* = 12.3, 4.8 Hz, 1H, H-6‴), 3.91 (ddd, *J* = 9.7, 4.9, 2.4 Hz, 1H, H-5‴), 3.87 (app t, *J* = 9.6 Hz, 1H, H-4″), 3.81 (dd, *J* = 12.3, 2.3 Hz, 1H, H-6a‴), 3.75–3.62 (m, 8H, H-4, H-4′, H-5, H-5′, H-5″, 2 × H-6, octyl OCH_2), 3.60–3.47 (m, 7H, H-3, H-3′, H-3″, 4 × H-6), 3.42 (dt, *J* = 9.8, 6.8 Hz, 1H, octyl OCH_2), 3.30 (s, 3H, OMe), 3.29 (t, *J* = 6.9 Hz, 2H, CH_2N_3), 3.24 (s, 3H, OMe), 3.22 (s, 3H, OMe), 2.47 (s, 3H, ArC*H*$_3$), 2.43 (s, 6H, 2 × ArC*H*$_3$), 2.17 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.01 (s, 3H, Ac), 1.69–1.52 (m, 4H, octyl CH_2), 1.45–1.30 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, $CDCl_3$) δ 170.6 (C=O), 170.0 (C=O), 169.9 (C=O), 169.5 (C=O), 145.0, 145.0, 144.9, 138.4, 138.3, 138.2, 134.0, 133.9, 133.7, 129.9, 129.8, 129.8, 128.3, 128.2, 128.1, 127.9, 127.9, 127.6, 127.5, 127.4, 127.4 (36C, Ar), 99.6 (C-1′), 99.4 (C-1″), 99.1 (C-1‴, $^1J_{C\text{-}1,H\text{-}1}$ = 177.5 Hz), 97.3 (C-1), 79.1 (C-3″), 79.1 (C-3), 78.7 (C-3′), 74.6 (C-4), 74.1, 74.0, 74.0 (3C, C-2, C-2′, C-2″), 73.9 (C-4′), 73.5, 73.4,

73.4 (3C, 3 × O*C*H_2Ph), 73.0 (C-4″), 72.2 (C-5′), 72.0 (C-5″), 71.1 (C-5), 69.4, 69.3, 69.2, 69.2, 69.2, 69.1 (6C, C-2‴, C-3‴, C-5‴, C-6, C-6′, C-6″), 68.3 (octyl OCH_2), 65.9 (C-4‴), 62.3 (C-6‴), 56.8, 56.6, 56.6 (3C, 3 × OMe), 51.4 (CH_2N_3), 29.3, 29.2, 29.0, 28.8, 26.7, 25.9 (6C, octyl CH_2), 21.7 (Ar*C*H_3), 21.6 (Ar*C*H_3), 21.6 (Ar*C*H_3), 20.9 (Ac), 20.7 (3C, 3 × Ac); IR (cast film, $CHCl_3$): 2096.5 (N_3), 1751.1 (C=O); HRMS (ESI) calcd $C_{85}H_{107}N_3O_{31}S_3$ $[M+Na]^+$ 1784.5943, found 1784.5938.

8-Azidooctyl α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-2-*O*-toluenesulfonyl-α-D-mannopyranoside (**107**): Tetrasaccharide **106** (10.6 mg, 0.006 mmol) was dissolved in MeOH (1 mL) and NaOMe (1.0 M) was added until the pH of the solution was 9. After stirring at room temperature overnight, the mixture was neutralized with Amberlite IR120 H^+ ion exchange resin and then concentrated. The crude residue was purified by chromatography (CH_2Cl_2–MeOH 15:1) to afford **107** (9.3 mg, 97%) as a white solid. R_f 0.23 (CH_2Cl_2–MeOH 15:1); $[\alpha]_D$ = +36.0 (*c* 0.5, MeOH); ^{1}H NMR (500 MHz, CD_3OD) δ 7.97–7.80 (m, 6H, Ar), 7.53–7.37 (m, 6H, Ar), 7.36–7.11 (m, 15H, Ar), 5.09 (d, *J* = 1.7 Hz, 1H, H-1‴), 4.95 (d, *J* = 2.0 Hz, 1H, H-1′), 4.92 (dd, *J* = 2.9, 2.1 Hz, 1H, H-2′), 4.91–4.88 (m, 2H, H-1″, H-2″), 4.81–4.76 (m, 2H, H-1, H-2), 4.53–4.25 (m, 6H, 6 × O*CH*$_2$Ph), 3.83 (dd, *J* = 2.8, 1.9 Hz, 1H, H-2‴), 3.82 (app t, *J* = 9.4 Hz, 1H, H-4″), 3.71–3.56 (m, 14H, H-3‴, H-4, H-4′, H-4‴, H-5′, H-5″, H-5‴, 6 × H-6, octyl OCH_2), 3.56–3.51 (m, 2H, H-3, H-5), 3.49 (dd, *J* = 9.1, 2.9 Hz, 2H, H-3′, H-3″),

3.48–3.36 (m, 3H, 2 × H-6, octyl OCH_2), 3.26 (t, J = 6.8 Hz, 2H, CH_2N_3), 3.20 (s, 3H, OMe), 3.16 (s, 3H, OMe), 3.14 (s, 3H, OMe), 2.46 (s, 3H, ArCH_3), 2.41 (s, 3H, ArCH_3), 2.40 (s, 3H, ArCH_3), 1.65–1.47 (m, 4H, octyl CH_2), 1.47–1.23 (m, 8H, octyl CH_2); ^{13}C NMR (126 MHz, CD_3OD) δ 145.4, 145.4, 138.3, 138.2, 138.2, 133.7, 133.7, 133.5, 129.8, 129.8, 129.7, 128.0, 128.0, 127.9, 127.8, 127.7, 127.5, 127.4, 127.3, 127.2, 127.1 (36C, Ar), 102.0 (C-1‴), 99.2 (2C, C-1′, C-1″), 97.3 (C-1), 79.3, 79.0, 78.5 (3C, C-3, C-3′, C-3″), 74.5, 74.4, 74.3, 74.1, 73.8, 73.7 (6C, C-2, C-2′, C-2″, H-4, H-4′, H-5), 73.2, 73.0, 73.0 (3C, 3 × OCH_2Ph), 72.3, 72.1, 71.9 (3C, C-4″, C-5′, C-5″), 71.0, 70.9, 70.8 (3C, C-2‴, C-3‴, C-5‴), 69.2, 68.8, 68.7 (3C, C-6, C-6′, C-6″), 67.9 (octyl OCH_2), 66.8 (C-4‴), 61.3 (C-6‴), 55.9 (OMe), 55.9 (OMe), 55.8 (OMe), 51.0 (CH_2N_3), 28.8, 28.8, 28.7, 28.5, 26.3, 25.6 (6C, octyl CH2), 20.3 (2C, ArCH_3), 20.2 (ArCH_3); IR (cast film, MeOH): 3419.8 (OH), 2095.8 (N_3); HRMS (ESI) calcd $C_{77}H_{99}N_3O_{27}S_3$ $[M+Na]^+$ 1616.5520, found 1616.5504.

8-*N*-(2,2,2-trifluoroacetamido)octyl α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-6-*O*-benzyl-3-*O*-methyl-α-D-mannopyranoside (**108**): Sodium amalgam was prepared by the addition of mercury (2.09 g) to sodium metal (94.4 mg). The resulting solidified sodium amalgam (3 wt.%, 0.56 g, 0.73 mmol) was added to solution of **107** (10.5 mg, 0.006 mmol) in MeOH (1 mL) at 0 °C. The mixture was stirred for 2 h until TLC indicated disappearance of the starting material. The solution was then withdrawn with a pipet and transferred into a new flask. After cooling to 0 °C, ethyl trifluoroacetate (25 μL, 0.06 mmol)

was added and the mixture was stirred for 1 h. The resulting mixture was concentrated and purified by chromatography (CH_2Cl_2–MeOH 7:1 to 4:1) to afford amide **108** (5.8 mg, 73%) as a colorless film, as well as a byproduct **109** (2.0 mg, 27%). R_f 0.49 for **108** and 0.26 for **109** (CH_2Cl_2–MeOH 7:1); Data for **108**: ^{1}H NMR (600 MHz, CD_3OD) δ 7.41–7.10 (m, 15H, Ar), 5.18 (d, *J* = 1.8 Hz, 1H, H-1‴), 5.17 (d, *J* = 2.0 Hz, 1H, H-1″), 5.15 (d, *J* = 1.9 Hz, 1H, H-1′), 4.76 (d, *J* = 1.8 Hz, 1H, H-1), 4.56–4.30 (m, 6H, 6 × OC*H*$_2$Ph), 4.10 (dd, *J* = 2.8, 2.2 Hz, 1H, H-2′), 4.07 (dd, *J* = 3.0, 2.1 Hz, 1H, H-2″), 4.00 (dd, *J* = 3.1, 1.9 Hz, 1H, H-2), 3.92 (dd, *J* = 3.0, 1.9 Hz, 1H, H-2‴), 3.90–3.52 (m, 18H, H-3′, H-4, H-4′, H-4″, H-4‴, H-5, H-5′, H-5″, H-5‴, 8 × H-6, octyl OCH_2), 3.49–3.42 (m, 4H, H-3, H-3″, H-3‴, octyl OCH_2), 3.42 (s, 6H, 2 × OMe), 3.41 (s, 3H, OMe), 3.25 (t, *J* = 7.1 Hz, 2H, CH_2N), 1.70–1.48 (m, 4H, octyl CH_2), 1.48–1.15 (m, 8H, octyl OCH_2); ^{13}C NMR (126 MHz, CD_3OD) δ 139.7, 129.4, 129.3, 129.3, 129.1, 129.0, 128.9, 128.7, 128.6, 128.6 (18C, Ar), 103.5, 103.4, 103.2 (3C, C-1′, C-1″, C-1‴), 101.4 (C-1), 83.3, 83.1, 82.9 (3C, C-3, C-3′, C-3′), 75.4, 74.9, 74.9 (3C, C-4, C-4′, C-5), 74.6, 74.4, 74.3 (3C, 3 × O*C*H$_2$Ph), 74.2 (C-4′), 73.2, 73.0, 72.5, 72.4, 72.1 (5C, C-2′, C-3‴, C-5′, C-5″, C-5‴), 71.3, 71.2, 71.2 (3C, C-6, C-6′, C-6″), 68.9 (octyl OCH_2), 68.3 (C-4‴), 67.8, 67.7, 67.6 (3C, C-2, C-2′, C-2′), 62.8 (C-6‴), 56.6 (3C, 3 × OMe), 40.7 (CH_2N), 30.5, 30.3, 30.2, 29.8, 27.7, 27.2 (6C, octyl CH_2), Carbon signals from the trifluroacetamido group were not visible due to coupling with fluorine, which split the signal into a quartet and reduced the intensity of the signal; LRMS (MALDI) calcd $C_{58}H_{82}F_3NO_{22}$ $[M+Na]^+$ 1224.5178, found 1225.57 (instrument not calibrated); Data for **109**: ^{1}H NMR (600 MHz, CD_3OD) δ 7.35–

7.18 (m, 15H, Ar), 5.23–5.14 (m, 3H, H-1′, H-1′, H-1″), 4.78 (d, *J* = 1.9 Hz, 1H, H-1), 4.58–4.30 (m, 6H, 3 × OCH_2Ph), 4.12–4.07 (m, 2H, H-2′, H-2″), 4.01 (dd, *J* = 3.4, 1.5 Hz, 1H, H-2), 3.93 (dd, *J* = 2.7, 1.9 Hz, 1H, H-2‴), 3.92–3.50 (m, 18H, H-3′, H-4, H-4′, H-4″, H-4‴, H-5, H-5′, H-5″, H-5‴, 8 × H-6, octyl OCH_2), 3.50–3.39 (m, 4H; H-3, H-3″, H-3‴, octyl OCH_2), 3.43 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.41 (s, 3H, OMe), 3.25 (t, *J* = 6.8 Hz, 1H, CH_2N), 1.66–1.45 (m, 4H, octyl CH_2), 1.43–1.15 (m, 8H, octyl CH_2); LRMS (MALDI) calcd $C_{56}H_{82}O_{22}$ $[M+Na]^+$ 1129.5196, found 1130.51 (instrument not calibrated).

8-*N*-(2,2,2-trifluoroacetamido)octyl α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (**110**): To a solution of amide **108** (2.7 mg, 2.2 μmol) in MeOH (1 mL) was added 5 wt. % Pd–C (10 mg). The reaction mixture was stirred overnight under a H_2 atmosphere, and then the catalyst was removed by filtration through Celite. The filtrate was concentrated and the crude product **110** (2.7 mg) was obtained as a pale colorless film. R_f 0.34 (EtOAc–MeOH–H_2O 7:2:1); ^{1}H NMR (600 MHz, CD_3OD) δ 5.21 (d, *J* = 2.0 Hz, 1H, H-1′), 5.21 (d, *J* = 1.9 Hz, 1H, H-1″), 5.17 (d, *J* = 1.8 Hz, 1H, H-1‴), 4.77 (d, *J* = 1.6 Hz, 1H, H-1), 4.12–4.06 (m, 2H, H-2′, H-2″), 4.01 (dd, *J* = 3.1, 1.8 Hz, 1H, H-2), 3.94–3.53 (m, 19H, H-2, H-2‴, H-4, H-4′, H-4″, H-4‴, H-5, H-5′, H-5″, H-5‴, 2 × H-6, 2 × H-6′, 2 × H-6″, 2 × H-6‴, octyl OCH_2), 3.52–3.38 (m, 4H, H-3, H-3′, H-3″, octyl OCH_2), 3.43 (s, 3H, OMe), 3.43 (s, 3H, OMe), 3.42 (s, 3H, OMe), 3.27 (t, *J* = 7.2 Hz, 2H, CH_2N), 1.67–1.48 (m, 4H, octyl CH_2), 1.49–1.20 (m, 8H, octyl CH_2).

8-Aminooctyl α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranosyl-(1→4)-3-*O*-methyl-α-D-mannopyranoside (**111**): Amide **110** (2.7 mg, 2.2 μmol) was dissolved in MeOH (1 mL) and NaOMe (1.0 M) was added until the pH of the solution was 9. After stirring at room temperature overnight, the mixture was concentrated without neutralization to obtain amine **111** (~ 10 mg crude). ^{1}H NMR (600 MHz, CD_3OD) δ 5.21 (d, *J* = 1.4 Hz, 1H, H-1‴), 5.20 (d, *J* = 2.0 Hz, 1H, H-1″), 5.16 (d, *J* = 1.5 Hz, 1H, H-1′), 4.77 (d, *J* = 1.4 Hz, 1H, H-1), 4.12–4.07 (m, 2H, H-2′, H-2″), 4.01 (dd, *J* = 2.7, 2.2 Hz, 1H, H-2), 3.94–3.39 (m, 22H; H-3, H-3′, H-3″, H-3‴, H-4, H-4′, H-4″, H-4‴, H-5, H-5′, H-5″, H-5‴, 8 × H-6, octyl OCH_2), 3.44 (s, 3H, OMe), 3.43 (s, 3H, OMe), 3.42 (s, 3H, OMe), 2.61 (t, *J* = 7.2 Hz, 2H, CH_2N), 1.67–1.56 (m, 4H, octyl CH_2), 1.44–1.19 (m, 8H, octyl CH_2).

Coupling of 92 with NHS-activated Sepharose: Preparation of the MMP-modified Sepharose resin followed a protocol from GE Healthcare. Briefly, NHS-activated Sepharose (*N*-hydroxysuccinimidyl-Sepharose 4 fast flow, Sigma, 1.2 mL suspended in isopropanol) was washed with ice-cold acidification buffer (1 mM HCl) and then mixed with ligand **92** (3.1 mg) in coupling buffer (0.5 mL, 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) at room temperature for 2 h. The mixture was then transferred into a column cartridge (BioRad) and the resin was washed with Milli-Q water (10 mL). This eluent was collected and concentrated for the purpose of calculating the amount of recovered ligand. The resin was then deactivated with blocking buffer (0.5 M ethanolamine, 0.2 M $NaHCO_3$, 0.5 M NaCl, pH 8.3) and rinsed with wash buffer (0.1 M NaOAc, 0.5 M NaCl, pH 4.0).

After washing with alternate blocking and wash buffer, the column was stored at 4 °C until use.

The amount of recovered ligand was determined by ^{1}H NMR spectroscopy in D_2O using accurately weighted pTsOH·H_2O as an internal standard. Using this method, the amount of amine in **92** (3.1 mg) was calculated as 3.4 μmol and the recovered amine was 0.2 μmol. Therefore, the coupling efficiency was determined as 94%.

Growth of *M. smegmatis* ATCC 14468 and mc^{2}155: Both *M. smegmatis* strains were grown at 37 ℃ to mid-log phase (48 h) in a medium containing yeast extract (0.3 g), peptone (0.5 g), glycerol (2 g) and Tween 80 (0.2 mL) in Milli-Q water (100 mL). The culture (50 mL) was then transferred to 2 × 1 L of fresh media and cultured for an additional 48 h. Cells were harvested by centrifugation and stored at –20 ℃ until use.

Preparation of ManT fraction from *M. smegmatis*: All of the following steps were carried out at 0 ℃ on ice. Wet cell pellets (from 1 L culture) of *M. smegmatis* were resuspended in Tris(hydroxymethyl)methyl]-3-aminopropanesulfonic acid (TAPS) buffer (4 mL/g, 50 mM TAPS, 10 mM $MgCl_2$, 5 mM EGTA, pH 8.2). Cells were lysed by successive passage through a benchtop cell disruptor (Constant systems, Inc.), three times at 35 Kpsi. The cell debris was then removed by centrifugation at 20,000 × g for 30 min. The supernatant whole cell lysate was next centrifuged at 200,000 × g for 1 h and the supernatant was discarded. The resulting bottom brown pellet was resuspended in TAPS buffer (0.5 mL, 50 mM TAPS, 10 mM $MgCl_2$, 5 mM EGTA, pH 8.2) and

was used as the source of ManT. All enzyme preparations were made fresh and used immediately after preparation.

Evaluation of biotin–MMP conjugate with radioactive enzymatic assay: The incubation protocols were established based on earlier work by Ballou and Weisman.[4] Trisaccharide **3**, tetrasaccharide **4**, biotin–MMP_3 **104** or biotin–MMP_4 **105** were used as acceptors in separate assays. Each assay contained GDP-mannose (20 mM, 1μL) with guanosine diphosphate mannose-[2-^{3}H] (0.04 μCi, American Radiolabeled Chemicals, Inc.), acceptor (20 mM, 1 μL) and the ManT source (18 μL), in a total volume of 20 μL. All assays were performed in duplicate and control assays lacing acceptor substrates were performed in parallel to correct for any background activity arising from endogenous acceptors present in the membrane preparations. Enzymatic products were purified from each assay using a C_{18} Sep-Pak cartridge (Waters).[42] After incubation at 37 ℃ for a period as indicated, the assays were stopped by dilution with Milli-Q water (0.6 mL) before loading onto a C_{18} cartridge (prewashed with MeOH followed by H_2O). Any unreacted radioactive guanosine diphosphate mannose-[2-^{3}H] donor was removed by washing the cartridge with Milli-Q water (50 mL). The enzymatic products containing a hydrophobic octyl or azidooctyl chain were then eluted with MeOH (4 mL), mixed with Ecolite cocktail (10 mL) and counted on a scintillation counter (Beckman). The raw dpm (decay per minute) values obtained from scintillation counter were used to calculate the enzymatic activity.

Protein purification with MMP-modified Sepharose: The membrane fractions suspended in TAPS buffer (0.5 mL) were incubated with MMP-modified

Sepharose resin at 37 °C. Gentle shaking was applied. Incubation time was varied from 2 h to 5 h. The mixture was then transferred to a column cartridge (BioRad). After collecting the flow through fraction, the resin was washed with TAPS buffer containing 200 mM NaCl and then eluted with increased concentration of methyl α-D-mannopyranoside (1–50 mM). The fractions collected were monitored by SDS-PAGE. The purification protocol is illustrated in Figure 5-4.

Protein purification with biotinylated MMP: The membrane fractions suspended in TAPS buffer were incubated with biotin-MMP_3 or biotin-MMP_4 (0.4–0.8 mg) at 37 °C in an Eppendorf tube while shaking gently. Incubation time was varied from 3 h to 21 h. Streptavidin magnetic beads (1 mL, New England Biolabs) were then added to the incubation mixture. After 15 min, the solution was removed and the magnetic beads were washed with TAPS buffer containing 200 mM NaCl and an increasing amount of methyl α-D-mannopyranoside. Fractions were collected and monitored by SDS-PAGE. To examine any protein retained on the beads, a small amount of the beads was resuspended in water and the proteins were denatured by heating in SDS at 95 ℃ and monitored by SDS-PAGE. Condition optimizations are illustrated in Figure 5-7.

Construction of plasmid containing *MSMEG_6484*: The *MSMEG_6484* gene was custom synthesized with codon optimization for expression in *E. coli* and cloned into vector pET-30b(+) by GenScript USA Inc. (Piscataway, NJ, USA), generating pET30b-*MSMEG_6484*. The resulting recombinant ManT (rManT) contains an additional hexahistidine (His) tag at the C-terminus.

***E. coli* transformation with *MSMEG_6484* gene:** Gene pET30b-*MSMEG_6484* was transformed into *E. coli* BL21(DE3) cells following a protocol from NEB (New England Biolabs Inc.). The lyophilized gene pET30b-*MSMEG_6484* (4 μg) was thawed on ice before being mixed with 20 μL Milli-Q water (200 ng/μL). The gene (150 ng, 0.75 μL) was added into *E. coli* BL21(DE3) cells (50 μL) and mixed by flicking the tube (no vortex). The mixture was incubated on ice for 15 min before a heat shock was applied by placing it into a water bath at 42 °C for 30 sec. Luria-Bertani (LB) broth (10 g tryptone, 5 g yeast extract, 5 g NaCl in 1 L, 1 mL,) was then added and the transformed bacteria were incubated at 37 °C for 1 h with vigorous shaking (200 rpm). The resulting cells (50–100 μL) were spread on a pre-warmed LB agar plate supplemented with kanamycin. The plate was in incubated at 37 ℃ for 16 h until colonies were formed. A single colony from the above antibiotic selection plate was resuspended in 3.5 mL LB media containing kanamycin (25 μg/mL). After incubation at 37 ℃ for 6 h, the cells were used to prepare a glycerol stock (10% glycerol) that was stored at –80 ℃.

Expression of rManT in *E. coli* BL21(DE3): Protein expression was performed following a protocol from NEB (New England Biolabs Inc.). The *E. coli* BL21(DE3) cells containing the pET30b(+)-MSMEG_6484 plasmid from the stock solution were grown in kanamycin (25 μg/mL) supplemented LB media (50 mL, adjust pH 7.4) at 37 °C for 16 h. The solution was then transferred into 950 mL LB media with kanamycin and incubated at 37 °C for 1.5–2 h until the OD_{600} reached 0.4–0.8. Expression of *MSMEG_6484* was induced by the addition of

isopropyl β-D-thiogalactopyranoside (IPTG, 250 μM). After 5 h at 30 °C, the cells were harvested giving 5–6 g wet cells from a 1 L culture.

***E. coli* empty vector control:** *E. coli* BL21(DE3) cells containing an empty pET-30b(+) vector lacking the *MSMEG_6484* gene were prepared following the procedures described above. The resulting cells were grown and induced exactly as the transformed *E. coli* BL21(DE3) cells. This strain served as a negative control and was evaluated simultaneously with the gene *MSMEG_6484* transformed *E. coli* cells.

Evaluation of *E. coli* rManT using radioactive assay: Wet cells (4 mL/g) of transformed *E. coli* BL21(DE3) were resuspended in TAPS buffer (50 mM TAPS, 10 mM $MgCl_2$, 5 mM EGTA, pH 8.2). Cell lysis was performed by passing through a benchtop cell disruptor (Constant Systems, Inc.) at 20 Kpsi. The cell debris was then removed by centrifugation at 20,000 × g for 30 min and the obtained supernatant whole cell lysate was next centrifuged at 200,000 × g for 1 h. The resulting bottom brown pellet was resuspended in 1 mL TAPS buffer as membrane preparations. The remaining top supernatant fraction was also evaluated and it was used directly without further purification. Membrane fractions of *M. smegmatis* and the *E. coli* strain with an empty vector were prepared similarly as above. All enzyme preparations were made fresh and used immediately after preparation.

Both membrane pellet and corresponding soluble preparations obtained from transformed *E. coli* BL21(DE3) were evaluated as sources of rManT, using tetrasaccharide **4** or **9** individually as substrates. Each assay contained GDP-

mannose (20 mM, 1μL) with guanosine diphosphate mannose-[2-^{3}H] (0.04 μCi, American Radiolabeled Chemicals, Inc.), acceptor (20 mM, 1 μL) and the ManT source (18 μL), in a total volume of 20 μL. All assays were performed in duplicate and control assays without acceptors were performed in parallel to correct for any background activity. After incubation at 37 ℃ for the period indicated, the assays were stopped by dilution with Milli-Q water (0.6 mL) before the mixture was loaded onto a C_{18} cartridge (prewashed with MeOH followed by H_2O). Any unreacted radioactive guanosine diphosphate mannose-[2-^{3}H] was removed by washing the cartridge with Milli-Q water (50 mL). The enzymatic products containing a hydrophobic octyl chain were then eluted with MeOH (4 mL), mixed with Ecolite cocktail (10 mL) and counted on a scintillation counter (Beckman). The raw dpm (decay per minute) values obtained from scintillation counter were used to calculate the enzymatic activity of rManT. In parallel, membrane fractions of *M. smegmatis* and the *E. coli* strain with empty vector were evaluated as positive and negative controls, respectively.

Attempts toward the purification of *E. coli* rManT with Ni–NTA affinity column: Ni–NTA affinity purification was attempted using the whole cell lysate rather than the membrane preparations of transformed *E. coli* BL21(DE3) expressing rManT. The cells were lysed in three different buffers: TAPS buffer (A: 50 mM TAPS, 10 mM $MgCl_2$, 5 mM EGTA, pH 8.2); TAPS buffer without EGTA (B);Ttris(hydroxymethyl)aminomethane (Tris) buffer (C: 50 mM Tris, 500 mM NaCl, 10 mM imidazole, pH 8.0). Ni–NTA affinity column (1.5 mL resin, Qiagen) was equilibrated with the same buffer as for used for cell lysis before

loaded with the cell lysate. The flow through fraction was collected and the resins were eluted with the same buffer with increasing amounts of imidazole (10–250 mM). The fractions collected were monitored by UV absorbance at 280 nm and SDS-PAGE analysis.

Large scale incubation of *E. coli* rManT with 9 and isolation of enzymatic products: Tetrasaccharide **9** (5.1 mg, 6.1 μmol), non-radiolabeled GDP-mannose (6.7 mg, 10.0 μmol) and alkaline phosphatase (2 μL) were incubated in the presence of the membrane fraction of transformed *E. coli* BL21(DE3) (0.7 mL, a total of 1.5 mL was obtained from 9.7 g wet pellet resulting from a single 1 L cell culture). After incubation at 37 ℃ for 24 h, the assay reaction was stopped by centrifugation at 14,000 rpm for 20 min to remove the enzyme. The supernatant was applied to a C_{18} Sep-Pak cartridge (Waters, Milford, MA, USA) and washed with Milli-Q water (10 mL). The crude enzymatic products were eluted with MeOH (5 mL), concentrated and examined by MALDI-MS (Figure 5-14). The crude products were then acylated with acetic anhydride (200 μL) in pyridine (0.7 mL). After stirring at room temperature overnight, the pyridine was removed by co-evaporating with toluene and the resulting mixture was diluted with EtOAc. This solution was washed with saturated aqueous $NaHCO_3$, 1M HCl and brine. The organic layer containing per-acetylated products was dried over Na_2SO_4 and concentrated. The crude product was then dissolved in 0.1 mL EtOAc and spotted on a preparative thin layer chromatography plate. The plate was developed in a elute system of hexane–EtOAc (2:5). Two major products, **112** and **113**, were visualized by charring with acidic *p*-anisaldehyde stain in sulfuric acid (Figure 5-

15). The bands corresponding to the two products were scraped from the plate and re-dissolved in MeOH. After filtering the silica gel, the filtrate was concentrated and re-purified by passing through a short silica gel cartridge and eluting with CH_2Cl_2–MeOH. The eluate was concentrated and then lyophilized to afford **112** and **113**, both as colorless films and both in less than 0.1 mg quantities. They were characterized by ^{1}H NMR spectroscopy (700 MHz in $CDCl_3$) and the data were shown in Figure 5-16. For **112**: HRMS (ESI) calcd $C_{78}H_{116}O_{46}$ $[M+Na]^+$ 1811.6630, found 1811.6668. For **113**: HRMS (ESI) calcd $C_{90}H_{132}O_{54}$ $[M+Na]^+$ 2099.7475, found 2099.7473.

Kinetics of *E. coli* rManT: 1.5 mL fresh membrane fraction obtained from 9.7 g wet cell pellets of transformed *E. coli* BL21(DE3) in 1 L culture was used (the same enzyme fraction as used in scale-up incubation). Enzymatic activities were determined using a fixed donor concentration (0.5 mM GDP-mannose) and various acceptor concentrations (0.8, 1.6, 3.1, 6.3, 12.5, 25, 50, 100, 200 μM). Each assay had donor (4 μL, 20 mM, contain 0.04 μL radioactive GDP-mannose-[2-^{3}H]), acceptor **4** or **9** (4 μL) and enzyme (12 μL) in a total volume of 20 μL. Assays were incubated 5 h at 37 ℃. A control experiment without the addition of the acceptor was also performed in parallel. Each experiment was done in duplicate. The kinetic parameters K_M and V_{max} were obtained using the Michaelis–Menten equation, by nonlinear regression analysis with GraphPad Prism 5.0 software (Figure 5-17).

Calculation of V_{max} and k_{cat}/K_M for *E. coli* rManT with tetrasaccharide 9:

$K_m = 5.1 \pm 0.6$ μM

V_{max} = (1212.0 ±32.6 fmol·min^{-1})/ 20 μL total volume = 60.6 ± 1.6 nM·min^{-1}

[enzyme amount] = 9.7g cell/1.5mL buffer × 12 μL = 77.6mg cell in each assay

$k_{cat} = V_{max}/[E]$ = 0.78 ± 0.02 μM·min^{-1}g^{-1} cell

k_{cat}/K_m = 0.15 ± 0.03 min^{-1}g^{-1} cell

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Chapter 6: Summary and future work

6.1 Summary

Mycobacteria are known to produce a number of unique polysaccharides, including two groups of intracellular polymethylated polysaccharides (PMPS) found only in mycobacteria and related bacterial species.[1-2] These polysaccharides, which include the 6-*O*-methylglucose lipopolysaccharides (MGLPs)[3] and 3-*O*-methylmannose polysaccharides (MMPs),[4] are believed to play a role in lipid metabolism in mycobacterial species.[5-6] However further studies are required to confirm this function. In this thesis, I have described our studies of the biosynthesis of MMPs, focusing on the activity of a mycobacterial α-(1→4)-mannosyltrasferase (ManT)[7] involved in their biosynthesis.

This thesis studies contributed to understanding of ManT by, firstly, studying the substrate scope of this enzyme, which resulted in a discovery of an unexpected activity of ManT (Chapter 2 and 3); secondly, revealing the importance of methyl groups on ManT substrates (Chapter 3); and finally, identifying the gene encoding for ManT (Chapter 5), which was partially facilitated by synthetic methodology I developed to construct substrates of ManT (Chapter 4).

6.1.1 Exploring the substrate specificity of ManT

There are at least two enzymes involved in the biosynthesis of MMPs in mycobacteria, an α-(1→4)-mannosyltransferase (ManT)[7] and a 3-*O*-methyltransferase (OMT).[8] The activity of both enzymes was first described by Ballou and coworkers in 1984;[7-8] however, little has been done since then and

neither enzyme had been successfully isolated. In Chapter 2, we sought to expand upon the initial studies of ManT activity and specificity, using a panel of chemically synthesized MMP analogs with lengths varying from mono- to pentasaccharides. Two groups of analogs were prepared, possessing either a mannose or 3-*O*-methyl-mannose residue at the non-reducing end. ManT was previously reported to recognize only tetrasaccharide or larger substrates containing a terminal methylated residue (*e.g.*, **9**, Figure 6-1). However, contrary to those reports, I found ManT was able to recognize substrates both containing and lacking a terminal methyl group (*e.g.*, **9** and **4**, respectively, Figure 6-1). The trisaccharide and larger substrates were all found to be substrates for ManT, with the substrates lacking a methyl group on the terminal mannose (*e.g.*, **4**, Figure 6-1) showing higher activity. Moreover, glycosylation catalyzed by ManT resulted in not a single product, but a homologous series of oligomers corresponding to the incorporation of multiple mannose residues. These results indicate that ManT acts as a polymerizing glycosyltransferase. These results suggest MMP biosynthesis does not necessarily proceed through alternating methylation and mannosyl transfer reactions.[8] Instead, the entire α-(1$\rightarrow$4)-linked mannose core could be assembled before the addition of methyl groups, and hence, ManT would recognize mannose substrates lacking any methyl groups (e.g., **39**, Figure 6-1).

In Chapter 3, I set out to test this hypothesis by evaluating the activity of ManT with a substrate lacking methyl groups. Using tetrasaccharide **39** (Figure 6-1), I demonstrated that ManT could indeed recognize a tetrasaccharide without

methyl groups, although with a significantly lower activity compared with tetrasaccharides **4** and **9** used in Chapter 2.

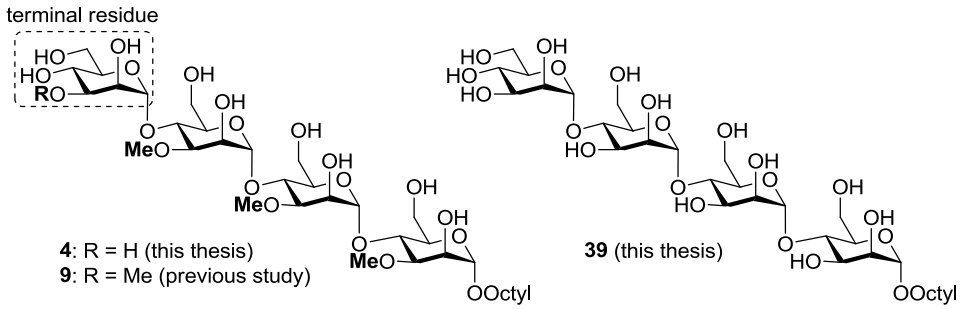


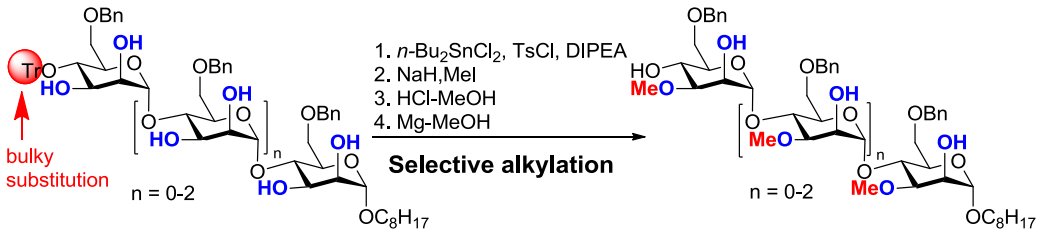
Figure 6-1. Three types of ManT substrates with variation of degree of methylation.

6.1.2 The importance of methylation for substrate recognition by ManT

By carrying out kinetic studies of three representative ManT substrates (Figure 6-1), I was able to determine the influence of methylation pattern on substrate recognition by ManT. The substrate methyl groups influence activity in different ways depending on their locations. The methyl group at the non-reducing end does not contribute substantially to substrate binding with ManT, and its presence actually results in a slight decrease in the maximum catalytic activity of the reaction. In contrast, methyl groups located on the other non-reducing mannose residues appear to contribute to substrate binding, as removing these groups resulted in a dramatic decrease in binding affinity. Based on the results from both Chapters 2 and 3, we propose that, ManT recognizes not only the non-reducing end terminus of substrate but has an extended binding pocket that interacts with some of the remaining methylated mannose residues of the acceptor substrate.

6.1.3 A methodology for rapid construction of ManT substrates

The results described in Chapters 2 and 3 suggested that ManT has a relaxed substrate binding pocket; thus, further mapping the specificity of ManT would require access to a panel of structurally diverse analogs of MMPs. During the course of synthesizing these analogs, I developed a four-step methodology to quickly install several methyl groups directly onto oligosaccharides (Scheme 6-1). The key reaction involves *n*-Bu_2SnCl_2-mediated simultaneous activation of *cis*-diols. By tuning protecting groups on the substrates, I was able to functionalize multiple pairs of *cis*-diols in a consistent and highly regioselective manner. This methodology was then used, in Chapter 5, to access MMP-functionalized ligands for the attempted affinity purification of ManT.



Scheme 6-1. Methodology of regioselective functionalization of oligosaccharides.

6.1.4 Identifying a gene encoding for ManT

All the studies in Chapters 2 and 3 were performed using crude extract of mycobacteria as ManT source. In Chapter 5, efforts were made to purify the ManT using affinity chromatography. The MMP ligands synthesized following the methodology in Chapter 4 were either immobilized on a Sepharose matrix or conjugated with biotin for capture with streptavidin-functionalized magnetic beads. However, the purification of ManT using this approach proved

unsuccessful, probably due to the low concentration of the enzyme in mycobacterial cell preparations, as well as its instability during purification.[7]

As an alternative, I used a bioinformatic approach. Since the onset of this project, a number of genomes of organisms producing MMPs (including *Mycobacterium smegmatis*,[9] *M. phlei*,[10] and *Streptomyces griseus*[11]) were reported. Thus, by rationally screening the unassigned glycosyltranferases from *M. smegmatis* with those of the *M. phlei* and *S. griseus*, a putative ManT gene was identified. The gene was expressed recombinantly in *E. coli* and the activity was found to be nearly identical to that of the native ManT in *M. smegmatis* cell wall preparations. The similarity between the native ManT from *M. smegmatis* and the recombinant ManT (rManT) was demonstrated by three experiments. First, Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS) was used to show that the enzymatic products from both ManT and rManT are the same homologous series of oligomers. Nuclear Magnetic Resonance (NMR) analysis of the products isolated from rManT reactions then showed that they were structurally identical to those from ManT. Finally, the kinetic parameters were nearly identical for both rManT and ManT. Hence, the gene encoding the ManT was successfully identified as *MSMEG_6484* from *M. smegmatis*. This study also showed that a single protein demonstrated the activity that was observed when using *M. smegmatis* cell preparations. Thus, the products observed in Chapter 2 could in fact result from the activity of a single protein.

6.2 Future work

The ultimate goal of this thesis project is to understand the mechanism for the biosynthesis of MMPs produced by mycobacteria.[7-8] The work described in this thesis has focused on only one of the two enzymes, ManT,[7] involved in MMP biosynthesis. So far, we have successfully identified the gene encoding for ManT, however, additional conditions will need to be explored to optimize the expression and purification of rManT in *E. coli*. Also, to further confirm the role of the ManT gene in MMP biosynthesis, a knockout strain of *M. smegmatis* lacking this enzyme will be prepared to examine the effect on MMP production.

In addition, the other MMP biosynthesis enzyme, 3-*O*-methyltransferase (OMT),[8] has not been fully characterized thus far. Our preliminary studies to examine this enzyme using synthetic MMP analogs were not successful (Chapter 2). Fortunately, in the course of identifying the gene encoding for ManT, we discovered two putative methyltransferase genes located in close proximity to the verified ManT gene (Chapter 5). Our future work will focus on exploring the proteins encoded by these two genes as potential candidates for OMT. With the information gathered from both ManT and OMT, we expect to elucidate a mechanism for MMP biosynthesis. In particular, we are interested in understanding how the initiation and termination steps occur during MMP biosynthesis. Once the proteins responsible for all steps in MMP biosynthesis are identified, it will be possible to perform knock out studies of each protein in this process in order to further explore their physiological role in mycobacteria.

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