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Developments in the Synthesis of Mycobacterial Phenolic Glycolipids

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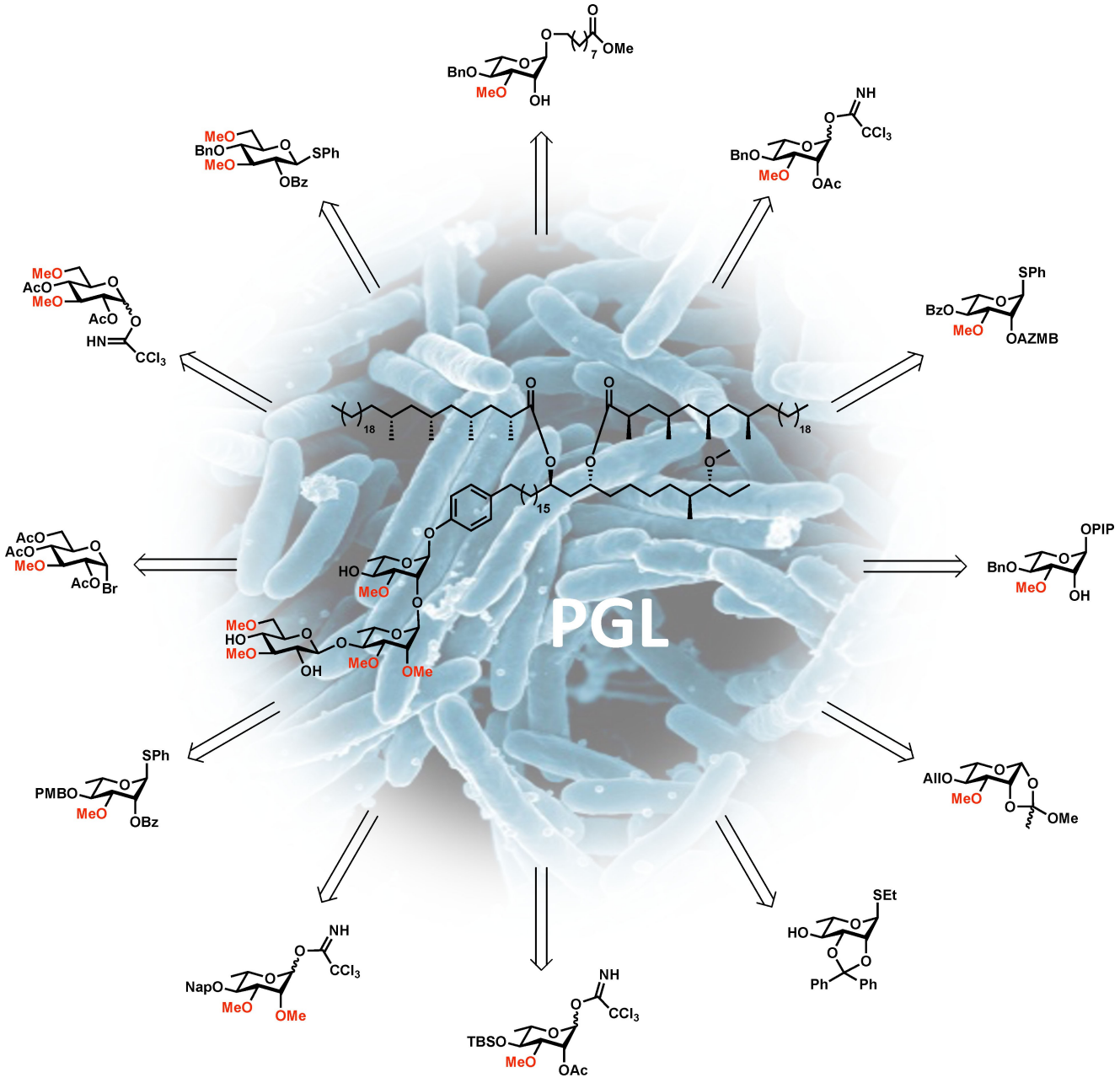

Abstract: The highly lipophilic outer barrier of mycobacteria, such as *M. tuberculosis* and *M. leprae*, is key to their virulence and intrinsic antibiotic resistance. Various components of this mycomembrane interact with the host immune system but many of these interactions remain ill-understood. This review covers several chemical syntheses of one of these components, mycobacterial phenolic glycolipids (PGLs), and outlines the interaction of these PGLs with the human immune system, as established using these well-defined pure compounds.

Keywords:

1. Introduction

Mycobacteria, such as the *Mycobacterium tuberculosis* complex (MTBC) and *Mycobacterium leprae*, are arguably the most successful of all microorganisms in invading and parasitically inhabiting both animals and humans.[1] MTBC causes tuberculosis, an infectious pulmonary disease which has plagued humanity for many centuries.[2–5] Tuberculosis (TB) claims >1.5 million lives yearly, which makes it the most deadly infectious disease globally. It is estimated that 25% of the world population has a latent infection of TB, of which 10% will progress to active disease. *Mycobacterium leprae* causes leprosy, a disease associated with loss of sensation, blindness, other lifelong handicaps and irreversible deformities.[6] Just like tuberculosis, leprosy may remain dormant in the host for years before the disease becomes active.

Mycobacteria belong to a subgroup of Gram-positive bacteria named the *Corynebacterineae*, which have an outer permeability barrier, also called the mycomembrane, which is analogous to the outer membrane of Gram-negative bacteria in an organizational manner[7] but is of a much more lipophilic character (Figure 1).[8] This highly lipophilic outer barrier is thought to be the key to the virulence and intrinsic antibiotic resistance of mycobacteria. The mycomembrane is covalently attached to arabinogalactan, a branched polysaccharide of galacto- and arabinofuranosides, which in turn is attached to peptidoglycan.

The inner leaflet of the mycomembrane consists of mycolic acids, which are long-chain (C_{22}–C_{100}) fatty acids specific to *Coryinebacterineae*.[9] The outer leaflet is composed of a variety of species-specific lipids, glycolipids (See Figure 2) and proteins, which include, but are not limited to: phosphatidyl *myo*-inositol mannosides (PIMs), trehalose containing glycolipids such as lipooligosaccharides (LOS), sulfoglycolipids (SGL), trehalose monomycolate (TMM), trehalose dimycolate (TDM), diacyl-, triacyl- and pentaacyltrehalose (DAT, TAT and PAT, respectively), phthiocerol dimycocerosates (PDIM) and phenolic glycolipids (PGLs), which are phenolphthiocerol based glycolipids with a fatty acid backbone which greatly resembles PDIM.[1,10–12] The lipid content and composition of the cell wall of Mtb are important for infectivity of the bacterium and it varies during different stages of infection.[13] When the bacterium enters the lungs of the host, TLR-2, TLR-4 and Mincle receptors of alveolar macrophages and dendritic cells recognize TMM and TDM and this initiates an immune response.[14,15] Later in the infection cycle mycolic acid is released from TMM and TDM,[16] which dampens the immune response, as free mycolic acid can inhibit TLR-2 mediated pro-inflammatory pathways.[17] During the transition to the chronic phase of infection the host-imposed stress induces Mtb to produce more immune dampening lipids such as DAT, PAT, SGLs, PDIM and PGLs.[18–20]

PGLs and related compounds (PDIM and pHBAD, Figure 3) play a major role in the virulence of mycobacterial strains.[21–24] PGLs have been shown to inhibit the Toll-Like Receptor 2 (TLR2) mediated immune response, thereby reducing the production of multiple pro-inflammatory cytokines, such as TNF-α, IL-6, CCL2 and NF-κ_B.[20,25] PDIM masks other pathogen associated molecular patterns (PAMPs) on the cell wall, thereby inhibiting other pattern recognition receptor (PRR) mediated responses.[26,27] PDIM is also thought to play a role in the membrane disruption of the phagosome membrane of human lymphatic endothelial cells, allowing the bacterium to remain in the cytosol, where it can grow more rapidly.[28] Furthermore, PGLs are able to recruit permissive macrophages through chemokine receptor 2 (CCR2), enabling the bacterium to travel to the lower respiratory tract.[26,29,30] If a strain of Mtb is unable to produce PGLs it will secrete an increased amount of pHBADs, biosynthetically closely related glycans, which inhibit the production of IFN-γ, thereby also dampening the immune response.[31–33]

PGLs first occurred in the scientific literature when a lipid was found during a study of waxes of *M. bovis*,[34] characterized by an infrared spectrum, identical to one from a lipid of *M. kansasii*[35] and these were later called mycoside B and myco-

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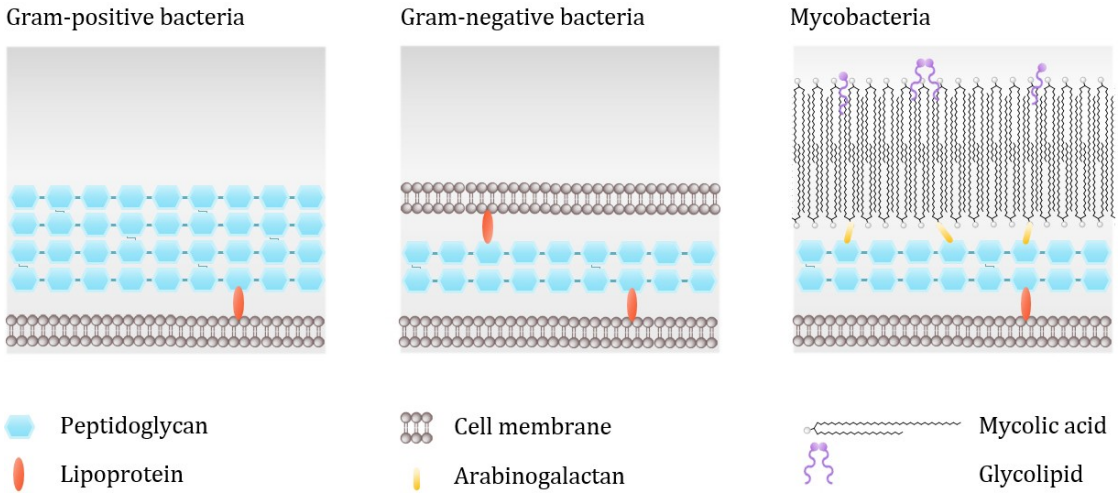


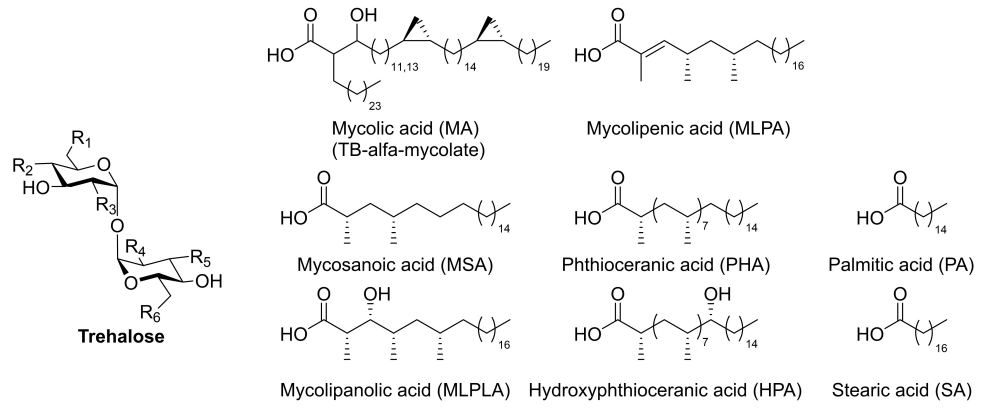
Figure 1. Different types of bacterial cell wall.

side A, respectively.[36] In 1981 Brennan and coworkers discovered a glycolipid with the same aglycone as mycoside A and B in the liver of an *M. leprae* infected armadillo in surprisingly large quantities.[37] Using gas-liquid chromatography-mass spectroscopy (GLC-MS), and later NMR studies,[38] they determined the structure to be as shown in Figure 3. The structure appeared to be unique to *M. leprae*, which makes it useful for the serological differentiation from other mycobacteria. It was at this point that they coined the name "phenolic glycolipid" (PGL) and this particular PGL was called PGL-I (Figure 3). Two more glycoforms were found shortly thereafter which seemed to differ in the methylation pattern.[39]

This review describes the synthetic chemistry developed to date to access *M. tuberculosis* and *M. leprae* PGL structures, with a focus on the assembly of the glycan part. Given the challenges associated with the complex structure of the PGLs the complete total synthesis of only a single PGL has been achieved so far. The first part will deal with the synthesis of *M. leprae* PGL glycans, while the second part is devoted to *M. tuberculosis* PGLs. Applications of the described synthetic glycans will be introduced.

2. *M. Leprae* PGL Glycans

The structure of the three PGL glycoforms (PGL-I, PGL-II and PGL-III) of *M. leprae* are shown in Figure 3. The general structure of *M. leprae* PGL consists of two α (1→2) linked rhamnoses which are β (1→4) linked to glucose on the non-reducing end. The reducing end rhamnoside is functionalized with a characteristic phenol, which is extended with a phthiocerol lipid, carrying two mycocerosic acids. To confirm the structures and explore their potential in serodiagnosis, Brennan and coworkers set out to synthesize the glycoforms of *M. leprae* PGLs.[40] Key in the syntheses of PGL glycans is the timing of the introduction of the multiple methyl ethers. Different strategies have been developed for the regioselective introduction of these groups both in the monosaccharide building block stage as well as in later stages of the synthesis after assembly of di- and trisaccharides. In the first PGL glycan synthesis, Brennan and co-workers[40] used building blocks **1**, **3** and **5** (Scheme 1). The regioselective methylation of the glucose building block was achieved using 1,2,5,6-di-O-isopropylideneglucofuranose as starting material, a tactic that has found wide application since. The regioselective methylation of the reducing end rhamnoside building block was accomplished by first selectively protecting the C-2-hydroxy group in **2** with an allyl ether, using phase-transfer catalysis conditions.[41] Next the remaining free alcohol could be methylated after which the allyl group was removed to provide the desired building block **3**. To assemble the trisaccharide, glucosyl bromide **5** and rhamnose **1** were connected under Helferich conditions using mercury cyanide.[42] Next the acid labile trityl and isopropylidene groups were removed and the liberated alcohols were methylated. The anomeric benzyl ether was then reductively removed after which acetylation and bromination provide the disaccharide donor for the next Helferich glycosylation. After connecting the disaccharide and rhamnose acceptor **3** and separating the 2 : 1 anomeric mixture, the acetyl groups were cleaved and the anomeric benzyl removed to provide the trisaccharide lactol **8**. As this synthesis was performed to deliver material for structure confirmation, no conjugation handle was attached to the reducing end. Nevertheless, the trisaccharide was conjugated to free lysines of BSA by means of a reductive amination and this neo-glycoconjugate was used for the detection of anti-PGL-I antibodies. While the reducing end of the trisaccharide



Glycolipid	R_1	R_2	R_3	R_4	R_5	R_6
TMM	MA	OH	OH	OH	OH	OH
TDM	MA	OH	OH	OH	OH	MA
DAT-I	OH	OH	OH	SA	MSA	OH
DAT-II	OH	OH	OH	SA	MLPLA	OH
TAT	OH	OH	OH	SA	MLPA	PA
PAT	OH	MLPA	MLPA	SA	MLPA	MLPA
SL-I	HPA	OH	SO_3^-	SA/PA	PHA	HPA
SL-II	HPA	OH	SO_3^-	SA/PA	HPA	HPA
SL-III	OH	OH	SO_3^-	SA/PA	HPA	HPA
SL-IV	OH	OH	SO_3^-	SA/PA	HPA	OH

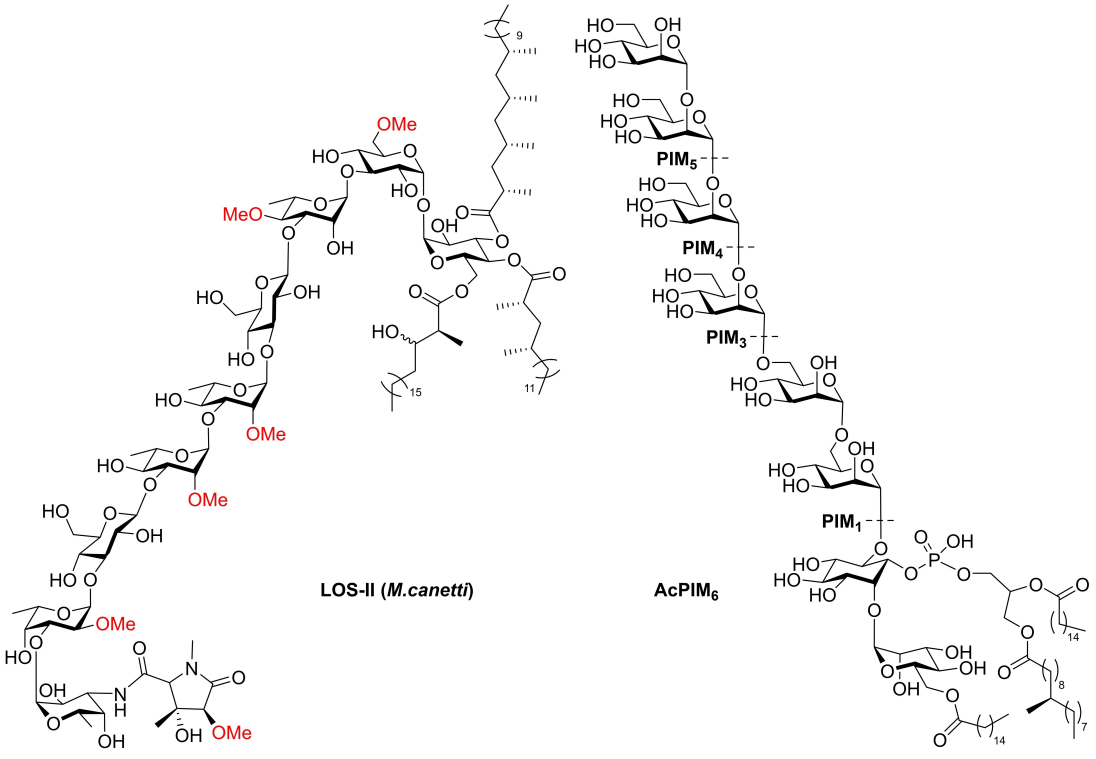


Figure 2. Schematic representation of mycobacterial glycolipids. TMM = trehalose monomycolate, TDM = trehalose dimycolate, DAT = diacyl trehalose, TAT = triacyl trehalose, PAT = pentaacyl trehalose, SL = sulfoglycolipid, LOS = lipooligosaccharide, PIM = phosphatidyl myo-inositol mannoside.

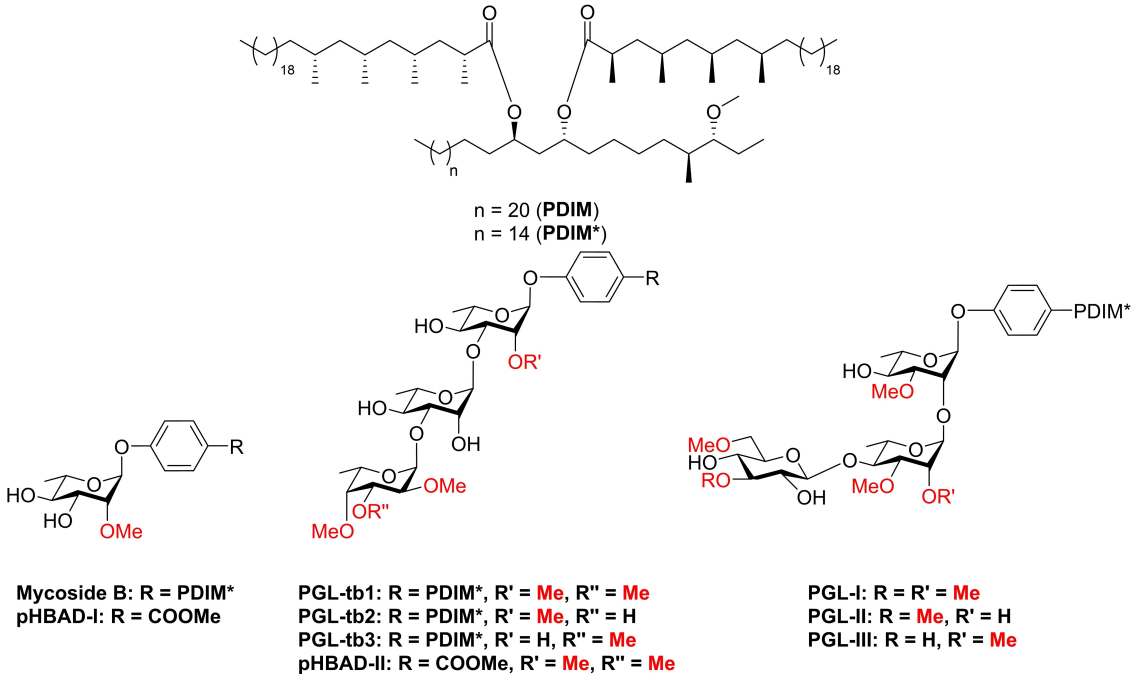
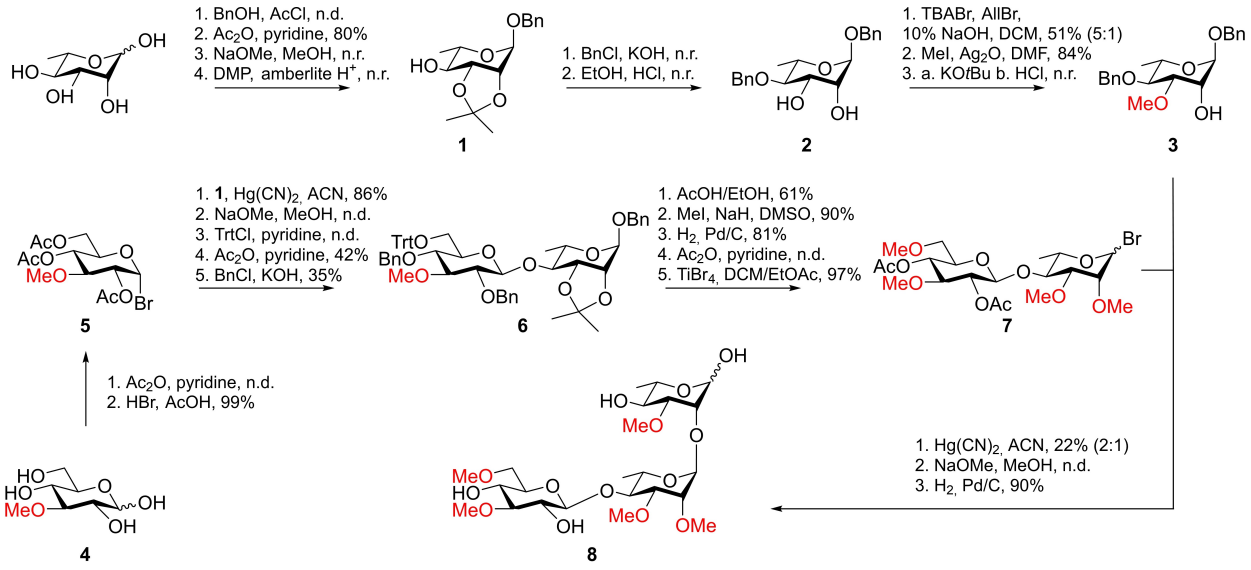


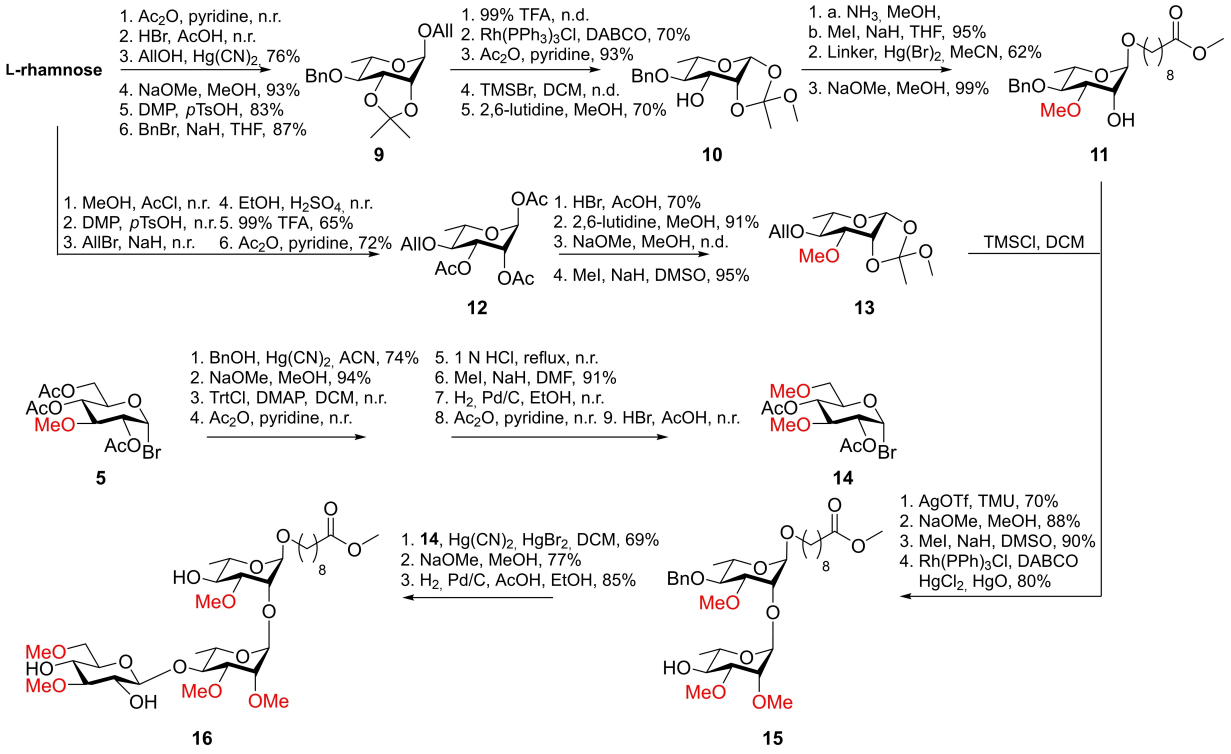
Figure 3. Phthiocerol dimycoceroserate (PDIM) and related compounds: phenolic glycolipids (PGLs) and para-hydroxybenzoic acid derivatives (pHBADs).



Scheme 1. First reported synthesis of the PGL-I glycan by Brennan and coworkers in 1984.[40] (n.r. = yield not reported, n.d. = yield not determined).

structure was destroyed in this process, the conjugate could be used to detect anti-PGL-I antibodies.

In 1988 Brennan and co-workers reported another synthesis of the PGL-I glycan.[43] In this synthesis they incorporated a nonyl linker on the reducing end for conjugation to BSA (Scheme 2). The regioselective methylation of the reducing end rhamnose was achieved using 1,2-orthoester building block **10**. The orthoester was then used to stereoselectively introduce the nonyl linker.[44] A similar orthoester building block (**13**, carrying a C-4-O-allyl ether) was transformed to the anomeric chloride by solvation in TMSCl, after which Koenigs Knorr conditions[45] were used to connect the two rhamnosides. Deacetylation then set the stage to introduce the C-2'-OMe. Deallylation and glucosylation with glucosyl



Scheme 2. Synthesis of PGL-I glycan as reported by Brennan and coworkers in 1988.[43](n.r. = yield not reported, n.d. = yield not determined, TMU = N,N,N,N-tetramethylurea)

donor **14**[46] delivered the fully protected trimer, which was deprotected to provide the target PGL-I trisaccharide. The acyl azide procedure, originally developed by Lemieux and co-workers[47,48] was used for conjugation to BSA, keeping the structure of the reducing end rhamnose intact. In 1987 Fujiwara and Izumi also synthesized the PGL-I glycan using very similar chemistry. They incorporated a 3-(4-hydroxyphenyl)propionyl linker on the reducing end, more closely resembling the native structure of PGL-I.[49]

Brennan and coworkers also investigated the use of truncated PGL-I glycans for diagnostic purposes, in order to reduce the synthetic complexity and to determine structure-activity relationship with regards to antibody binding.[40,43,44,46,50] It was found that the methyl ether on the C-3 position of the terminal glucose was the most important structural determinant for antibody binding. According to their results the disaccharide based conjugate which contained the terminal glucose and central rhamnose (Natural Disaccharide – Octylcarbonyl – bovine serum albumin (ND–O-BSA, Figure 4)) was just as effective as the trisaccharide based conjugates for the detection of antibodies. Due to this ability to bind to anti-PGL-I antibodies and its synthetic simplicity this conjugate (as well as the human serum albumin version) has been the standard for leprosy diagnosis ever since.[50–53]

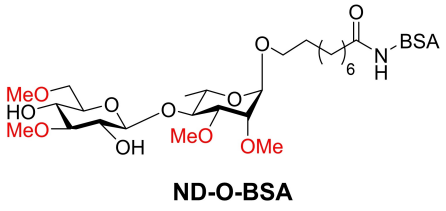
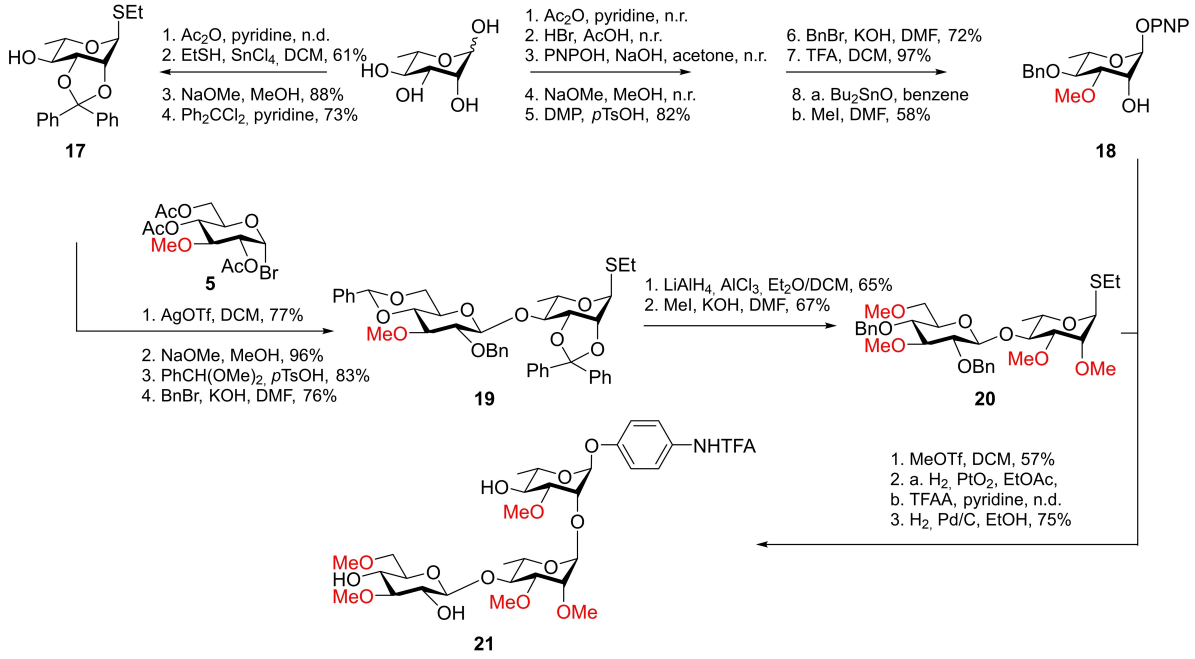


Figure 4. Natural Disaccharide – Octylcarbonyl – Bovine Serum Albumin (ND–O-BSA), the most commonly used conjugate for the detection of anti-PGL-I antibodies.

Since the reported synthesis in 1988 multiple groups have synthesized one or multiple *M. leprae* PGL glycans.[25,54–59] Líptak and coworkers investigated a new approach towards PGL-I in 1993.[59] They set out to reduce the total amount of steps required by concurrently installing multiple methyl ethers in a single step (Scheme 3). The reducing end rhamnose was selectively methylated using Bu_2SnO in refluxing benzene, and subsequent treatment of the resulting stannylidene acetal with MeI in DMF.[60] The central thiorhamnose (**17**) was to be protected with a diphenylmethylene acetal,[61] which left the C-4 alcohol free to be coupled to glucosyl bromide **5**. After the coupling and subsequent deacetylation, a benzylidene acetal was installed on the 4,6-diol of the terminal glucose.



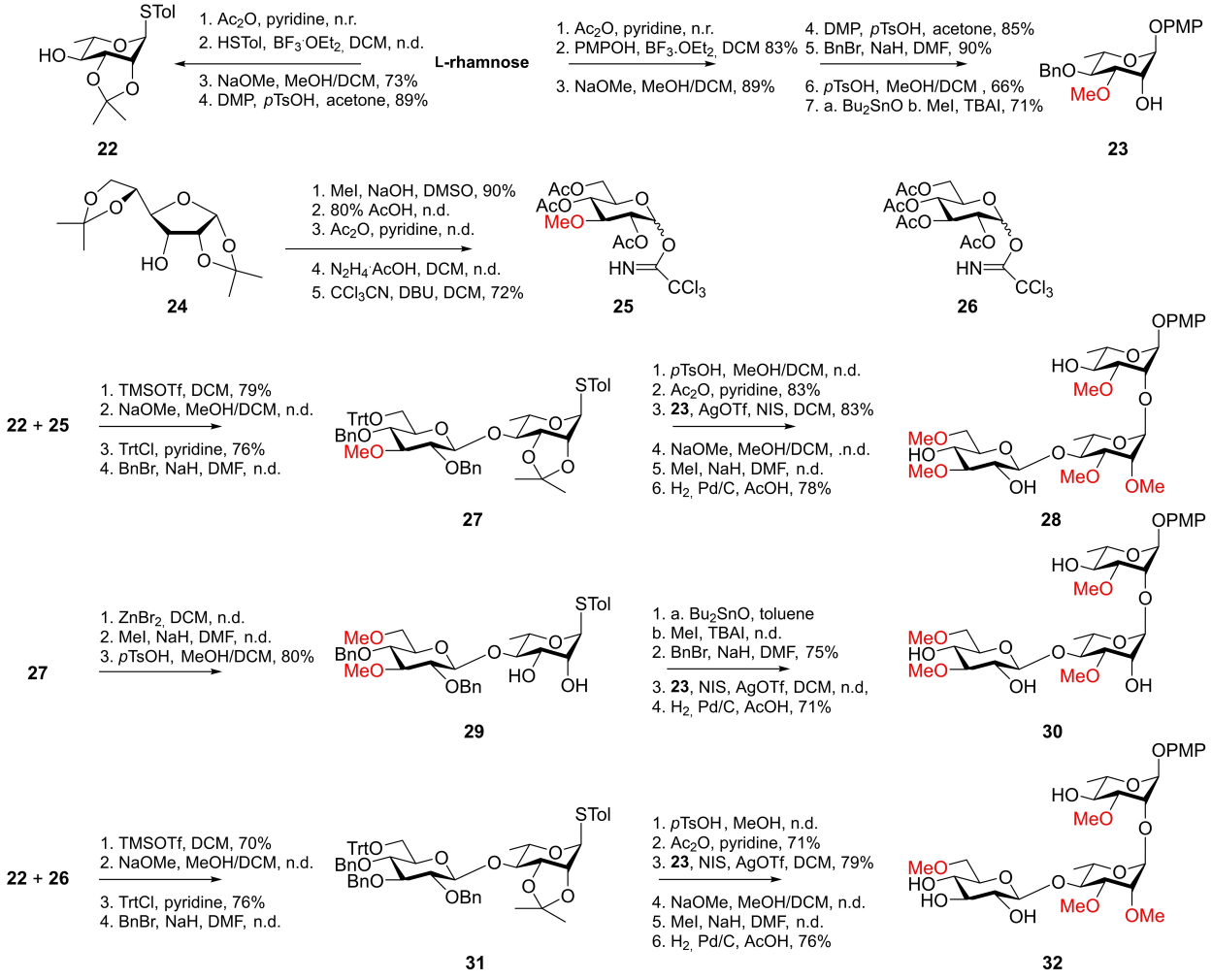
Scheme 3. Synthesis of PGL-I glycan as reported by Liptak and coworkers in 1993.[59] (n.r. = yield not reported, n.d. = yield not determined)

Benzylation of the remaining C-2 alcohol gave intermediate **19** which was subjected to dichloroalane, a 1 : 3 mixture of $LiAlH_4$ and $AlCl_3$. This mixture simultaneously liberated the primary alcohol of glucose by means of a reductive opening, while removing the diphenylmethylene acetal altogether. The resulting three alcohols were then methylated in a single step to give thiodisaccharide **20**, which was activated with MeOTf[62] and coupling to rhamnose **18** proceeded in a stereoselective manner to give the requisite trans glycosidic linkage. The original plan was to simultaneously remove all benzyl ethers and reduce the aryl nitro group in the obtained trisaccharide with a single hydrogenation step. This proved to be more difficult than expected and a complex mixture was obtained. Therefore, they chose to first reduce the nitro moiety with Adam's catalyst (PtO_2) and acylate the resulting free amine with trifluoroacetic anhydride. The final deprotection then gave trisaccharide **21** in 25 overall steps, a big improvement over the previously reported syntheses. No further application of the obtained trisaccharide has been reported.

The group of Lowary synthesized all three known *M. leprae* trisaccharides in 2013 (Scheme 4).[55] In their syntheses they made use of organotin chemistry to regioselectively introduce the methyl ethers on the rhamnosides. The reducing end was capped with a *para*-methoxyphenol as an approximation of the *para*-substituted aglycone of the natural product. In a chemoselective glycosylation strategy the central rhamnose thioglycoside was glycosylated with glucosyl building block **25** or **26** to provide the thiodisaccharide. At the disaccharide stage different methylation patterns were installed, before the trisaccharides were generated in *N*-Iodosuccinimide (NIS) mediated glycosylation reactions.

With these compounds in hand, they performed cytokine stimulation assays using THP-1 cells to find that PGL-I and the synthesized compounds could not induce cytokine production to a measurable degree. It was observed however, that they inhibited TLR2-mediated cytokine release in a concentration dependent manner. It was found that the size of the glycans and the methylation pattern were crucial for this inhibition, with the full PGL-I being the most potent. The inhibitory activity was higher when a glycan with a hydrophobic tail (**37**) was tested (scheme 5A), which may explain why native PGL-I was the most potent inhibitor. A similar route was used by the same group to make squaramide based glycoconjugates for an array to probe the interactions of mycobacterial glycans with the innate immune system (scheme 5B).[63]

The group of Astarie-Dequeker synthesized the *M. leprae* PGL-I glycan, as well as those of Mtb and *M. bovis* in 2016 (Scheme 6).[25] The building blocks were made using a combination of orthoester and organotin chemistry. The oligosaccharides were assembled using trichloroacetimidates **40**, **41** and **43**[54] and the reducing end was capped with a *para*-cresol. The compound was used to determine possible binding to chemokine receptor 3 (CR3). It was found that CR3 could bind the glycans, but the interaction was lower than that with native PGL-I, indicating that the lipophilic

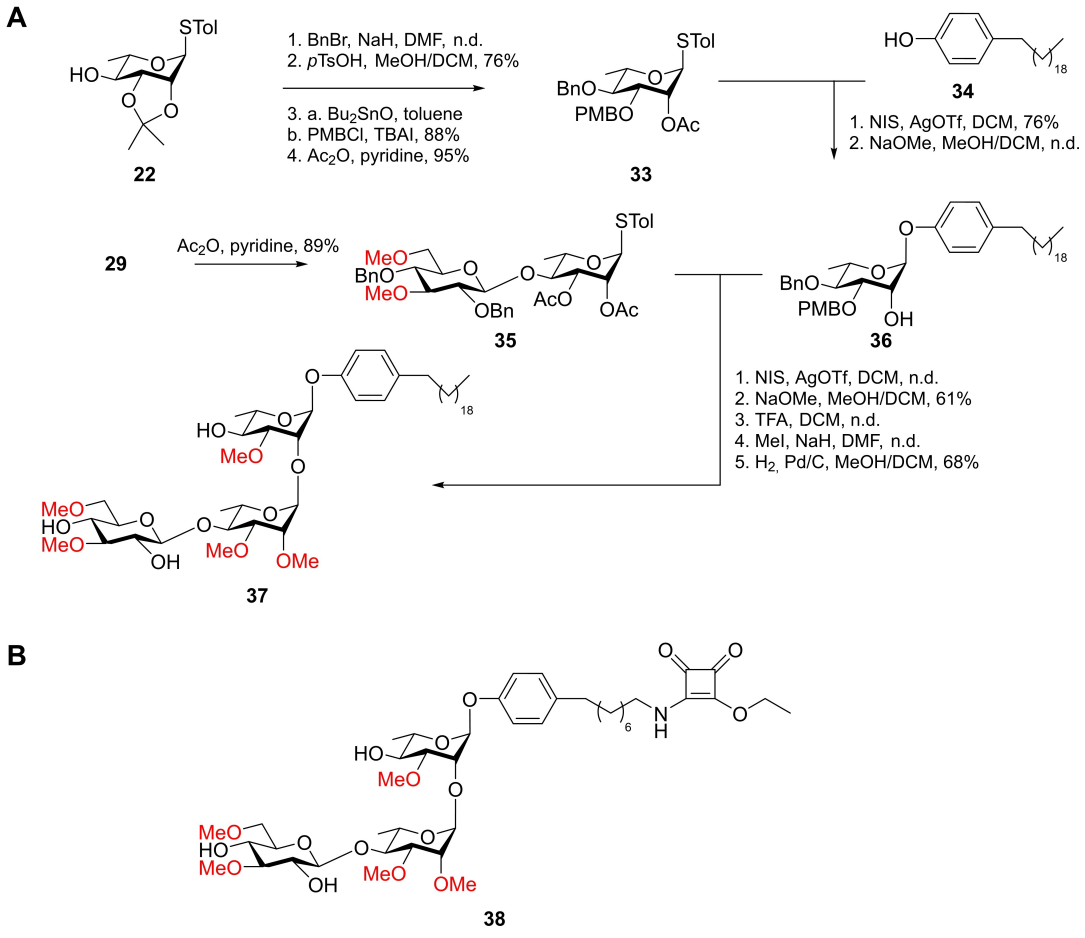


Scheme 4. Synthesis of M.leprae PGL glycans as reported by Lowary and coworkers in 2013.[55] (n.r. = yield not reported, n.d. = yield not determined)

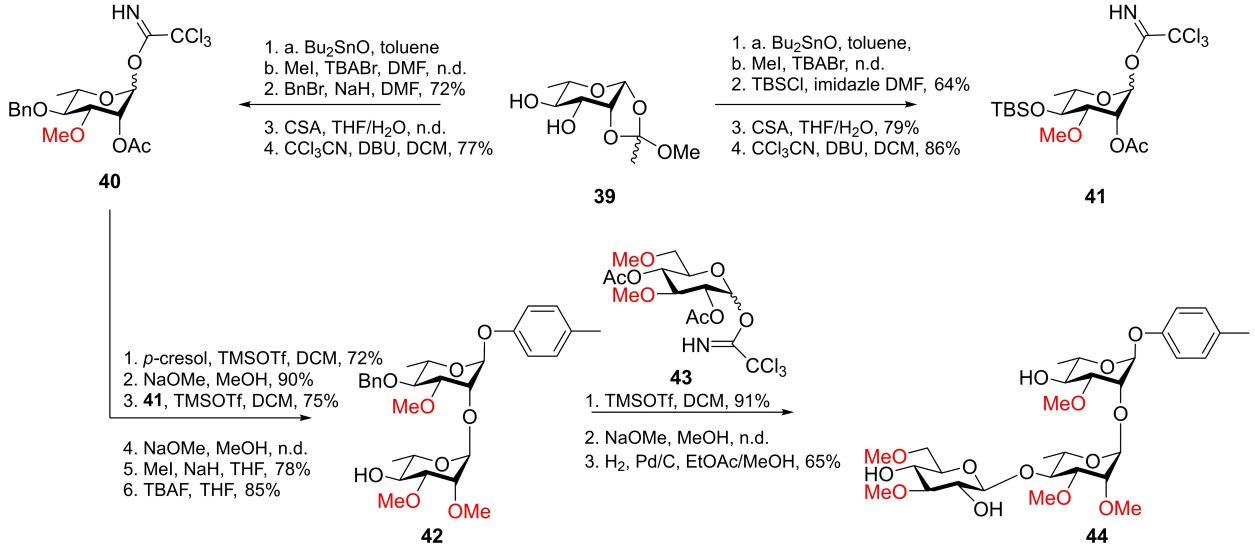
aglycone enhances binding affinity. The binding of the sugar moieties is thought to be mediated by the lectin domain of CR3, which is also known to be capable of selectively binding to yeast β(1→3) glucans.[64] A similar route was described by Luo *et al.*, who used a *p*-aminoethylphenol on the reducing end to make a biotinylated PGL-I antigen for diagnostic purposes.[58]

The group of Tanaka synthesized the PGL-I and PGL-tb1 glycans in 2017 (Scheme 7).[56] The reducing end rhamnose **46**[65] was selectively methylated using organotin chemistry. The 2-O position was protected with an 2-azidomethylbenzoate,[66] which could offer anchimeric assistance during the ensuing glycosylation reactions and it could be orthogonally removed in the presence of the benzoyl on the 4-O position. The thioglucose donor **50** was prepared from intermediate **49**.[67] In contrast to other syntheses discussed above, Tanaka and coworkers opted for a pre-glycosylation methylation approach for the central rhamnose moiety. The glycosylations of the 2,3-di-*O*-methyl rhamose donor were affected under halide ion catalyzed conditions, using I_2 and Bu_4NOTf.[68] As previously reported, these conditions could be used to install the desired α-rhamnosyl linkage with good stereoselectivity, in the absence of a C-2 neighboring group.[69]

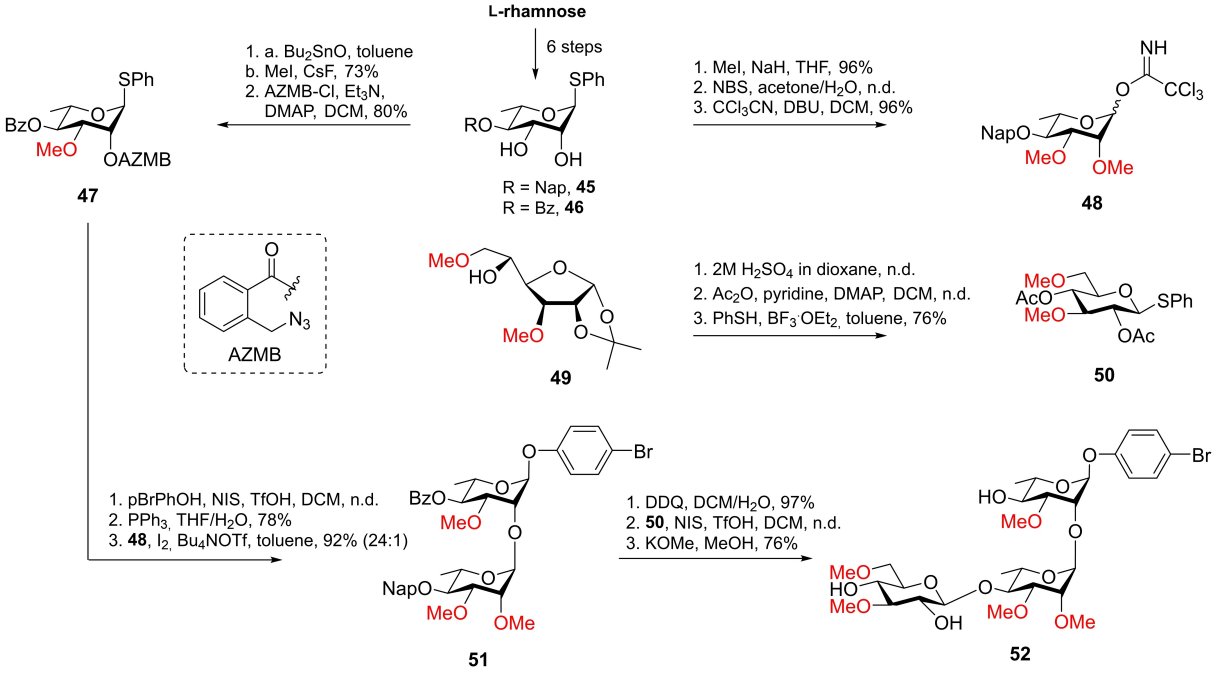
The assembled glycans were capped the with a *para*-bromophenol on the reducing end which could be used for a sequential Suzuki-Miyaura coupling with boracyclane[70,71] to attach a hydrophobic anchor or fluorophore (Scheme 8). Direct coupling with brominated TokyoGreen® did not proceed well, possibly due to the lack of solubility and reduced reactivity of the deprotonated fluorophore. Temporary protection of the phenolic alcohol in TokyoGreen® with a methoxymethyl (MOM) ether circumvented this problem and delivered the desired compounds after acidic hydrolysis. The finished compounds were tested for their immunomodulatory



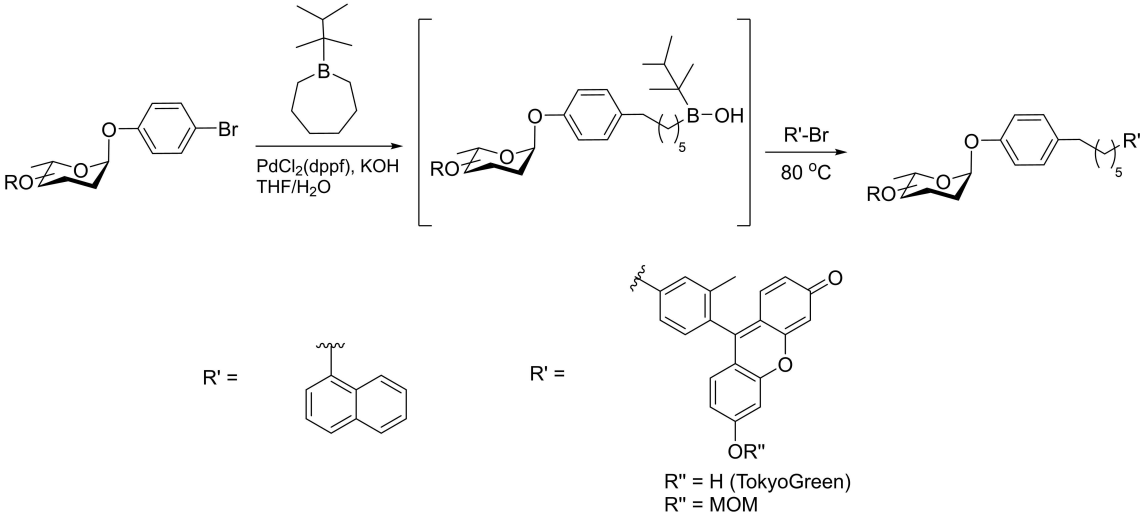
Scheme 5. A: Synthesis of PGL-I glycan with a hydrophobic anchor.[55] B: PGL-I glycan with a squaramide as a conjugation handle.[63] (n.r. = yield not reported, n.d. = yield not determine d)



Scheme 6. Synthesis of PGL-I glycan as reported by Astarie-Dequeker and coworkers in 2016.[25] (n.r. = yield not reported, n.d. = yield not determine d)



Scheme 7. Synthesis of PGL-I glycan as reported by Tanaka and coworkers in 2017. (n.r. = yield not reported, n.d. = yield not determined, TMU = N,N,N,N-tetramethylurea)



Scheme 8. Sequential Suzuki-Miyaura coupling as reported by Tanaka et al.[56]

capabilities using bone-marrow-derived macrophages which were activated with TDM. It was observed that compounds containing just the aryl bromide on the reducing end did not inhibit TNF-α secretion. The compounds which had a naphthyl as a lipid mimic on the other hand, did inhibit cytokine secretion in a dose-dependent manner.

Recently Van Dijk *et al.*[72] reported the synthesis of PGL-I, II and III glycoconjugates based on a strategy developed by Barroso *et al.*[73] for the synthesis of PGL-tb1 (*vide infra*). The conjugates were used for the lab-free diagnosis of leprosy by incorporating them in an Up-Converting Particle Lateral Flow Assay (UCP-LFA).[74–76] When the trisaccharide based conjugates were evaluated for their diagnostic potential the results obtained reaffirmed previous findings which determined the C-3-*O*-methyl of the terminal glucose to be the most important structural determinant for antibody binding.[40] The

syntheses of PGL-I described above are summarized in Table 1.

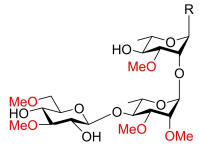
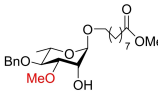
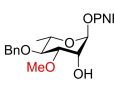
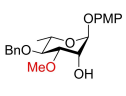
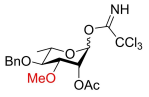
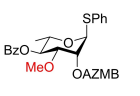
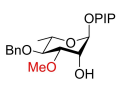
3. M. Tuberculosis PGL Glycans

To explore the use of *M. tuberculosis* PGLs in diagnostics, vaccine purposes and enable interaction studies at the molecular level, various syntheses of the MTb PGLs and pHBADs have been developed as well.[25,31,56,73,77–79] The structures of the Mtb PGL glycoforms are depicted in Figure 3. The general structure consists of two α (1→3) linked rhamnoses which are α (1→3) linked to an L-fucose residue on the non-reducing end. The terminal fucose is 1,2-*cis* linked, which necessitates the development of effective glycosylation chemistry that cannot be built on neighboring group participation.

The group of Van Boom was the first to synthesize the PGL-tb1 trisaccharide (Scheme 9).[77] Both rhamnose building blocks were generated from the same diol intermediate, which was either diacetylated or selectively acetylated on the 2-O position using trimethylorthoformate.[80] After a linker was coupled to the diacetylated donor **54** and the product was deacetylated and the C-2-alcohol was selectively methylated using phase-transfer catalysis.[81] Monoacetylated rhamnose **55** was chemoselectively and stereoselectively coupled to fucose donor **56** using iodonium dicollidine triflate (IDCT), which gave better results than the corresponding perchlorate salt (IDCP). This method gave a disaccharide which could be directly coupled to monosaccharide acceptor **57**. After deprotection this route gave the desired trisaccharide **59** in only 19 steps.

The first reported synthesis of the PGL-tb1 with a phenol on the reducing end was performed by Fujiwara (Scheme 10).[78] A 1,4-di-*O*-benzyl rhamnose intermediate, previously reported by Brennan and co-workers in 1984 for the assembly of *M. leprae* PGL-I (**2**, See Scheme 1),[40] was used as a starting point. From this intermediate phase transfer catalysis conditions were used to either install a methyl ether or a benzyl group on the 2-*O* position. The building blocks were glycosylated using Koenigs Knorr conditions.[45] Unfortu-

Table 1. Overview of PGL-I glycan syntheses described above.

		Brennan 1984	Brennan 1988	Liptak 1993	Lowary 2013	Astarie-Dequeker 2016	Tanaka 2017	van Dijk 2021
	1							
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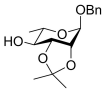
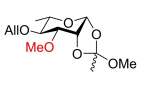
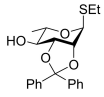
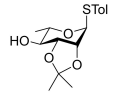
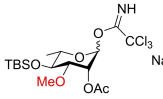
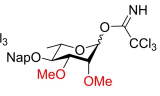
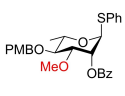
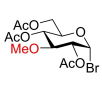
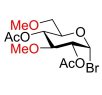
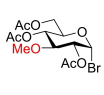
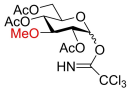
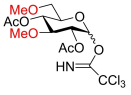
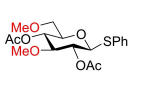
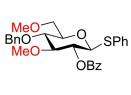
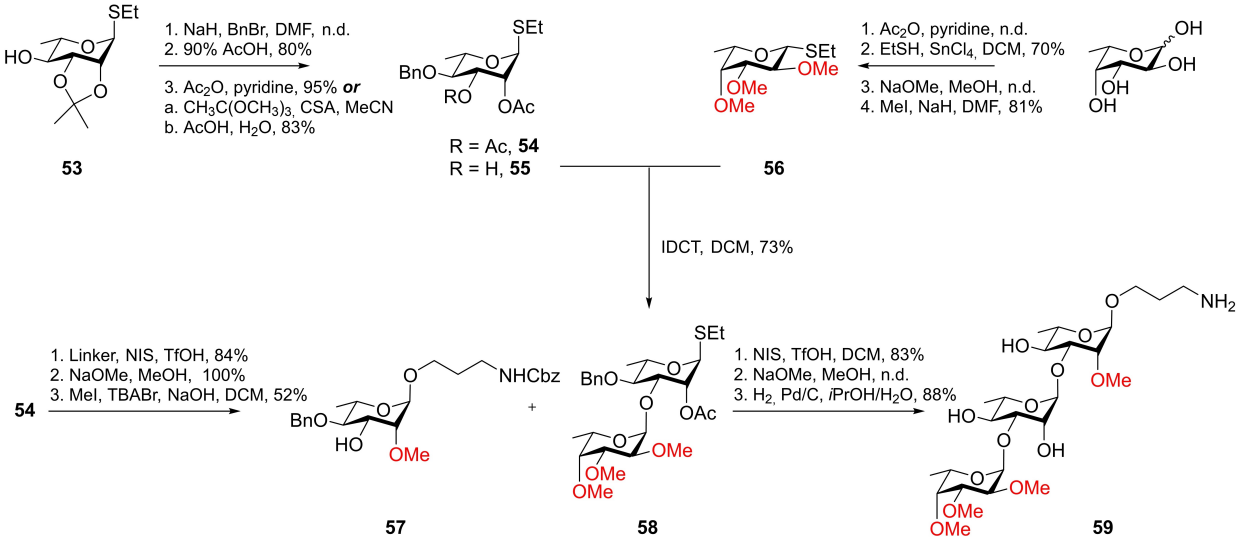
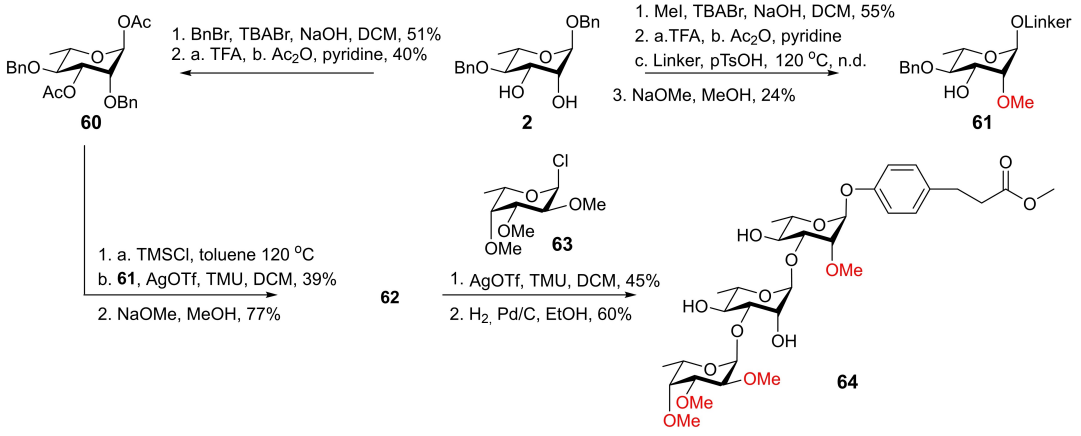
Building blocks	2							
		4 Steps	10 Steps	4 Steps	4 Steps	7 Steps	9 Steps	8 Steps
	3							
		5 Steps	14 Steps	5 Steps	6 Steps	8 Steps	8 Steps	8 Steps
Pre- or post-glycosylation methylation	Pre:	-	1	1	-	1,3	1,2,3	1,3
	Post:	1,2,3	2,3	2,3	1,2,3	2	-	2
Glycosylation method		$Hg(CN)_2$	Orthoester / $Hg(CN)_2$	AgOTf / MeOTf	TMSOTf / NIS, AgOTf	TMSOTf	NIS, TfOH / I_2, Bu_4NOTf	Ph_2SO, Tf_2O
Glycosylation order		1 + [2 + 3]	[1 + 2] + 3	1 + [2 + 3]	1 + [2 + 3]	[1 + 2] + 3	[1 + 2] + 3	[1 + 2] + 3
Regioselective methylation		Phase transfer catalysis	Orthoester	Bu_2SnO, MeI	Bu_2SnO MeI, TBABr	Bu_2SnO MeI, TBAI	Bu_2SnO MeI, CsF	Bu_2SnO MeI, CsF
Reducing end		Lactol	Nonyl linker	*p*-trifluoroacet-amidophenol	*p*-methoxyphenol	*p*-cresol	*p*-bromophenol	*p*-iodophenol
Total number of steps		28	46	25	27	27	25	29
Application		Structure confirmation	Detection of anti-PGL-I antibodies	None reported	THP-1 cytokine secretion assays	Tested for binding to CR3	BMM cytokine secretion assays	UCP-LFA based diagnostics



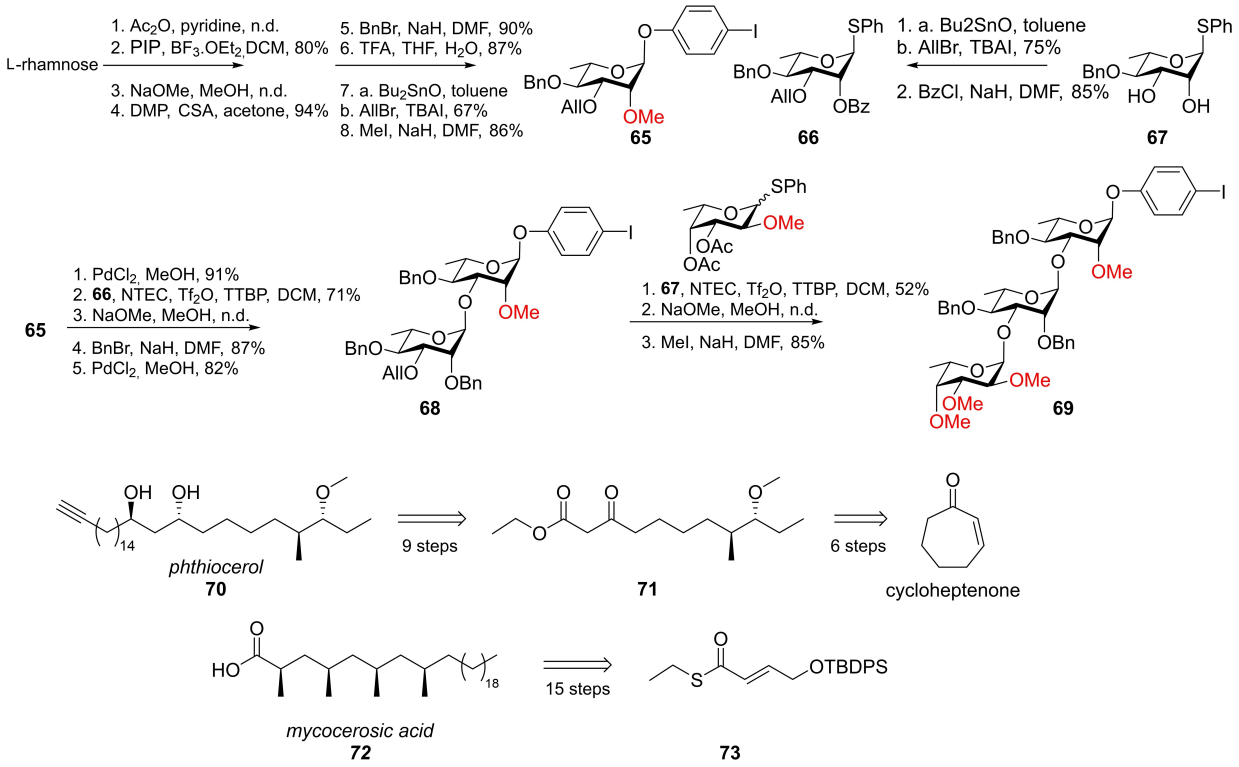
Scheme 9. Synthesis of the PGL-tb1 glycan as reported by van Boom and coworkers in 1990.[77] (n.r. = yield not reported, n.d. = yield not determine d)



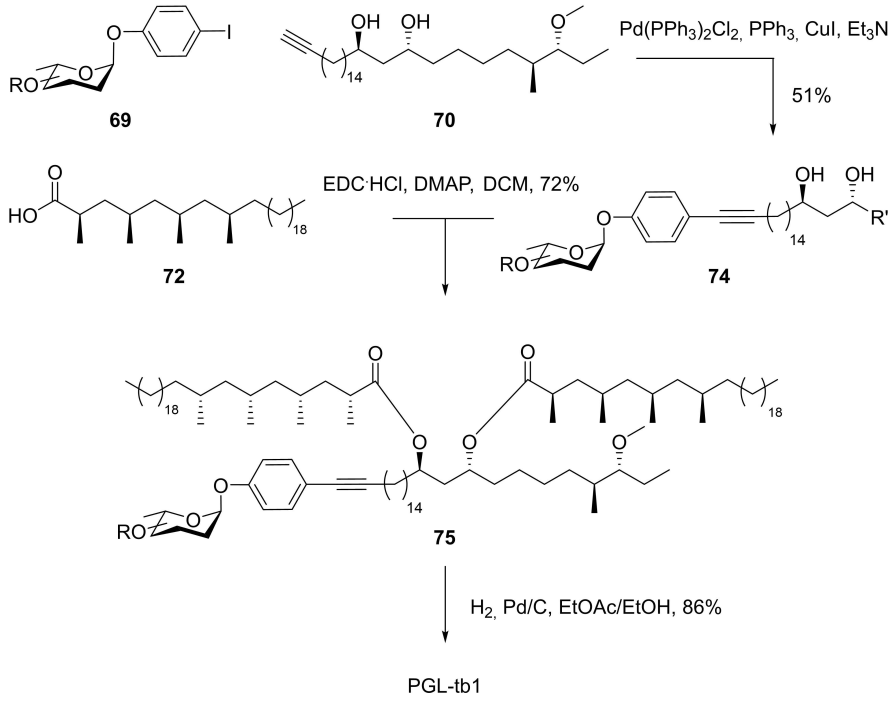
Scheme 10. Synthesis of the PGL-tb1 glycan as reported by Fujiwara in 1991.[78] TMU = N,N,N,N-tetramethylurea. (n.r. = yield not reported, n.d. = yield not determine d)

nately both the phase transfer catalysis and glycosylation reactions provided the products with relatively low yield and selectivity. The target products could be obtained nonetheless to generate BSA conjugates in analogy to the *M. leprae* PGL conjugates. The Mtb PGL conjugates however were not as sensitive or specific and thus not as useful for diagnostics.

In 2012 Barroso *et al.* set out to synthesize the complete native PGL-tb1, including the complex phthiocerol and mycocerosic acids (Scheme 11 and 12).[73] The regioselective protection of the 3-*O* position of the required rhamnose building blocks was achieved using organotin chemistry.[82] The reducing end was capped with a iodophenol as this allowed for a Sonogashira coupling to attach the phthiocerol chain later in the synthesis. The glycosylation reactions were performed with a combination of *N*-thiophenyl-ε-caprolactam and Tf_2O as a thiophilic promoter.[83] A strategy was chosen to increase the stereoselectivity of the fucose donor during glycosylations by installing acetyl groups on the C-3 and C-4-alcohols.[84] This did however increase the number of steps required for the building block and in the trisaccharide phase of the synthesis. Key steps of the synthesis of phthiocerol include a tandem copper/phosphoramidite-catalyzed asymmetric conjugation addition to cycloheptenone to introduce the *anti*-methoxy methyl unit.[85–87] The 1,3-*anti* diol was introduced by means of an asymmetric hydrogenation of β-keto ester **71**,[88,89] followed by an Evans-Saksena reduction.[90] Mycocerosic acid was synthesized by the iterative process of



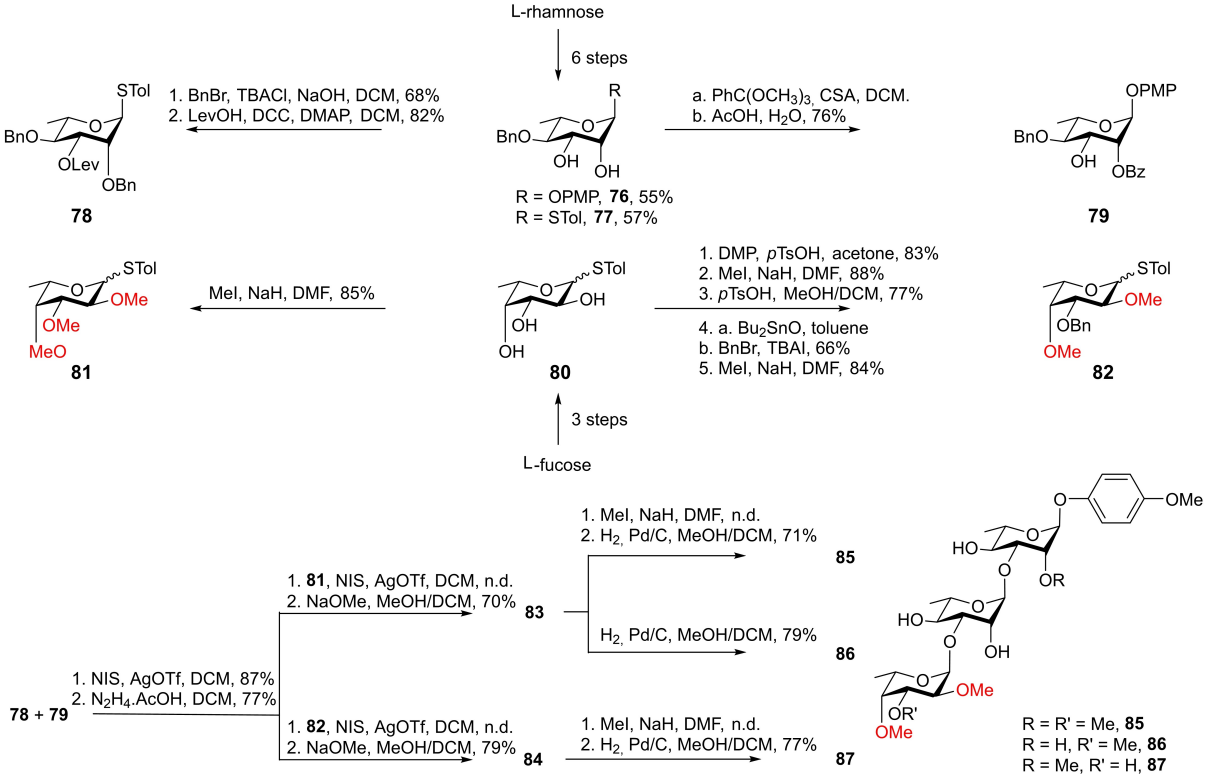
Scheme 11. PGL-tb1 glycan assembly and retrosynthesis of phthiocerol and mycocerosic acid as reported by Barroso et al.[73] NTEC = N-thiophenyl-ε-caprolactam. (n.r. = yield not reported, n.d. = yield not determine d)



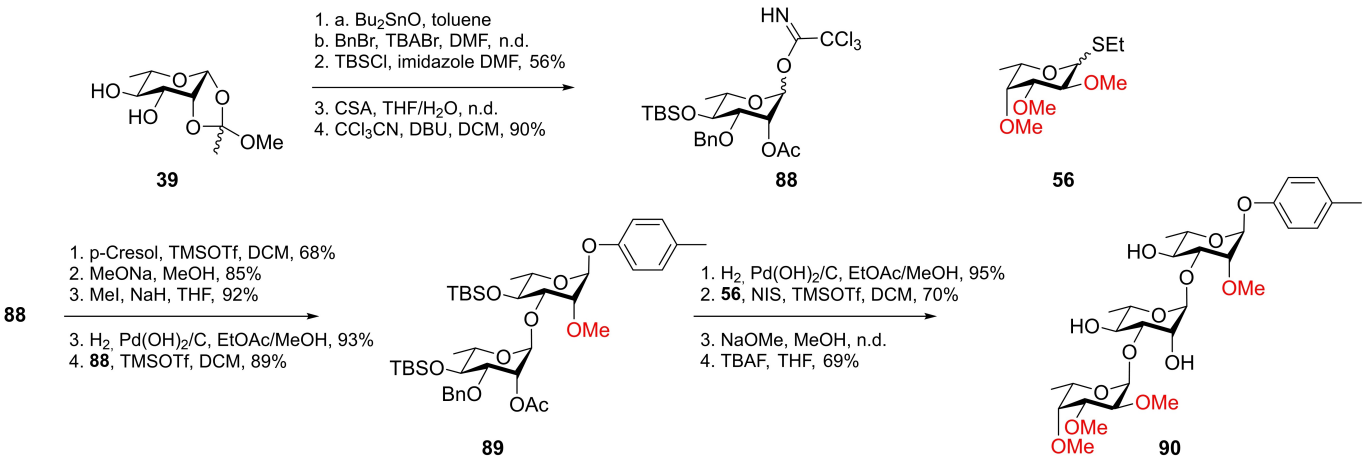
Scheme 12. Final stage of assembly of PGL-tb1 as reported by Barroso et al.[73]

copper-catalyzed conjugate addition of Grignard reagents to α,β-unsaturated thioesters.[91–94]

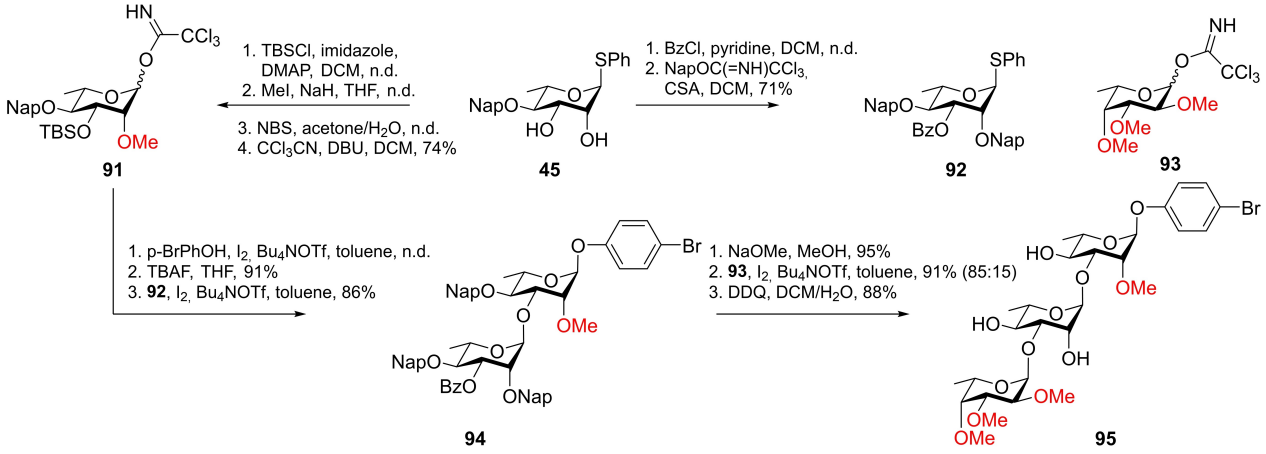
The esters present in the target PGL-tb1 necessitated a strategy which involved the use of protecting groups that do not require acidic or basic conditions for their removal. This meant that the benzoyl group in donor **66** that was used for participation had to be swapped for a benzyl ether in the stage of disaccharide (**68**) during the assembly of the trisaccharide (Scheme 11). This increased the total amount of steps required for the trisaccharide but made it possible to attach phthiocerol with a Sonogashira cross coupling after which the mycocerosic acids could be attached using EDC and DMAP. During the global deprotection both the benzyl ethers and the internal alkyne, which was formed after the cross coupling, were



Scheme 13. Synthesis of Mtb PGL glycans as reported by Lowary and coworkers.[95] (n.r. = yield not reported, n.d. = yield not determined)



Scheme 14. Synthesis of the PGL-tb1 glycan as reported by Astarie-Dequeker and coworkers.[25] (n.r. = yield not reported, n.d. = yield not determined)



Scheme 15. Synthesis of the PGL-tb1 glycan as reported by Tanaka and coworkers.[56] (n.r. = yield not reported, n.d. = yield not determined)

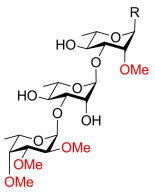
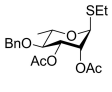
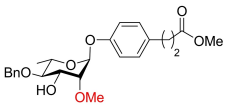
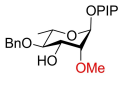
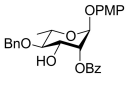
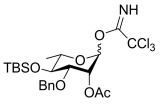
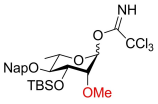
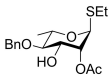
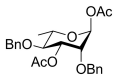
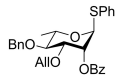
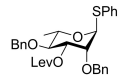
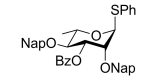
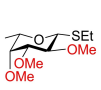
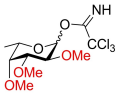
reduced completing the highly convergent synthesis (Scheme 12). To date this has been the only PGL that has been synthesized to include the complete complex phthiocerol lipid. A similar approach was adopted by Meng *et al.*[79] in the generation of neo-glycoconjugates, in which the PGL-tb1 glycan, carrying a simple phenol lipid, was conjugated, through squarate chemistry, to a CRM_{197} carrier protein. It was shown that this model vaccine was capable of eliciting a high titer of anti-PGL-tb1 IgG antibodies.

In 2014, the group of Lowary synthesized all three Mtb PGL glycans.[95] As depicted in Scheme 13, the regioselective protection of the rhamnose and fucose building blocks was achieved using orthoester chemistry, phase-transfer catalysis conditions and organotin chemistry. The reducing end was capped with a *para*-methoxyphenol and the thioglycoside donors were activated using NIS/AgOTf.[96] The selectivity of the fucosylation with the trimethylated donor was greatly enhanced by using an "inverse glycosylation procedure", in which a solution of the donor was slowly added to a mixture of the acceptor, NIS and AgOTf.[97,98] The assembled compounds were evaluated for their immunomodulatory capabilities and the structures showed a concentration-dependent inhibitory effect on the release of pro-inflammatory cytokines. PGL-tb1 turned out to be the most potent, in line with results previously obtained with *M. leprae* PGL glycans, where it was found that the structure with most methyl ethers showed the greatest inhibitory activity.[55] A similar route of synthesis was used by the same group to make squaramide based glycoconjugates, which were used to generate an array to probe the interactions of the mycobacterial glycans with lectins that play a role in the innate immune system.[63]

The group of Astarie-Dequeker synthesized the PGL-tb1 glycan in 2016[25] using a single rhamnose building block (**88**) that was assembled through a combination of orthoester and organotin chemistry (Scheme 14). A trichloroacetimidate donor was used, carrying an acetyl as a participating group, which was replaced for the required methyl ether after the first glycosylation. The reducing end was capped with a *para*-cresol moiety to mimic the natural product. Benzyl ethers were used for temporary protection and the fucosylation was performed with NIS and TMSOTf. Just two building blocks were required for this assembly and the synthesis therefore required only 19 steps in total. The PGL-tb1 glycan was able to inhibit TLR2-dependent NF-κB activation of THP-1 cells triggered by PAM3CSK4, which indicated that the glycans of both *M.tuberculosis* and *M.leprae* are able to antagonize TLR-2. PDIM alone showed no inhibitory activity but native PGLs showed a 10 fold increase over the glycans lacking the lipid tail. It was therefore hypothesized that the trisaccharides are responsible for the specificity of binding to TLR2, but that the common lipid core enhances the affinity, possibly by improving presentation. Further investigations with a library of aglycon analogs in combination with docking studies may shed light on the interaction between PGLs and TLR2.

In 2017 Tanaka *et al.* reported the MTb PGL synthesis depicted in Scheme 15.[56] They opted for a pre-glycosylation methylation approach for the methylated rhamnose and fucose building blocks, making use of their halide ion catalyzed glycosylation chemistry using I_2 and Bu_4NOTf.[68] When they performed the synthesis of the trisaccharide with a rhamnose building block starting from intermediate **45**,[65] carrying a C-2-*O*- benzoyl, the final fucosylation produced an inseparable 1 : 1 anomeric mixture. To improve this glycosylation, they chose to rely on their halide ion based glycosylation for the disaccharide coupling. Therefore, rhamnose donor **92** was synthesized, which was selectively benzoylated on the C-3-alcohol, after which the 2-*O* position was naphthylated using naphthyl imidate to prevent migration of the benzoate under

Table 2. Overview of PGL-tb1 glycan syntheses described above. (n.a. = not applicable)

		van Boom 1990	Fujiwara 1991	Barroso 2012	Lowary 2014	Astarie-Dequeker 2016	Tanaka 2017
	1						
		7 Steps	11 Steps	8 Steps	7 Steps	7 Steps	10 Steps
Building blocks	2						
		7 Steps	8 Steps	8 Steps	8 Steps		8 Steps
	3						
		4 Steps	5 Steps	7 Steps	4 Steps	4 Steps	6 Steps
Pre- or post-glycosylation methylation	Pre:	3	1,3	-	3	3	1,3
	Post:	1	-	1,3	1	1	-
Glycosylation method		IDCT / NIS, TfOH	AgOTf, TMU	NTEC, Tf_2O, TTBP	NIS, AgOTf	TMSOTf / NIS, TMSOTf	I_2, Bu_4NOTf
Glycosylation order		1 + [2 +3]	[1 + 2] + 3	[1 + 2] + 3	[1 +2] + 3	[1 +2] + 3	[1 +2] + 3
Regioselective methylation		Phase transfer catalysis	Phase transfer catalysis	Bu_2SnO AllBr, TBAI	n.a.	n.a.	Steric bulk of TBS
Reducing end		Propylamine linker	*p*-propionylphenol linker	**PDIM***	*p*-methoxyphenol	*p*-cresol	*p*-bromophenol
Total number of steps		19	21	30	24	19	24
Application		None reported	Detection of anti-PGL-tb1 antibodies	Structure confirmation	THP-1 cytokine secretion assays	THP-1 cytokine secretion assays	BMM cytokine secretion assays

basic conditions. The disaccharide coupling proceeded with a higher yield and only the α product was formed, which may be accounted for by long range participation of the C-3-*O*-benzoyl.[99] When the fucosylation was attempted with the C-2'-*O*-naphthyl disaccharide acceptor the selectivity of the glycosylation was increased to a 85 : 15 (α/β), highlighting the importance of acceptor reactivity in the glycosylation reaction.[100,101]

In contrast to results obtained by Astarie-Dequeker and coworkers, a glycan lacking the terminal trimethylated fucose showed a greater inhibition of TNF-α secretion than the corresponding trisaccharide. The syntheses of PGL-tb1 described above are summarized in Table 2.

4. Conclusion

Progress in carbohydrate chemistry has enabled the efficient synthesis of the PGL glycans. The assembly of the necessary building blocks requires regioselective manipulations, which are nowadays well established. A multitude of glycosylation conditions have allowed for the stereoselective condensation of these building blocks to form the complex trisaccharides, that can be used to investigate the interactions between the human immune system and the bacterial glycans. Insights acquired by these investigations has spurred the development of new ways to diagnose and treat the diseases caused by these pathogens. Although much progress has been made in synthetic carbohydrate chemistry, allowing access to the PGL trisaccharides, the synthesis of a complete PGL, including the phthiocerol and mycocerosic acids, has only once been reported. No immunological evaluation of the assembled PGL has been reported. The synthetic PGL glycans have been used to establish how the different glycosylation and methylation patterns shape the immune response. As PGLs have been reported to interact with TLR2, a PRR that binds lipopeptides, it is expected that the attaching the complete phthiocerol chain and mycocerosic acids will significantly impact the activity of the PGL. A library of complete PGLs will be required to fully understand the interaction of these molecules with the host immune system.

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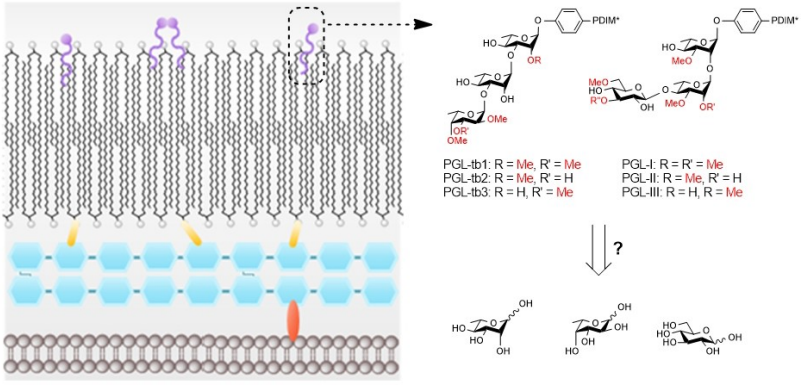

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Developments in the Synthesis of Mycobacterial Phenolic Glycolipids

Phenolic glycolipids are a cell wall component of mycobacteria which are thought to be virulence factors. This review summarizes the development of synthetic phenolic glycolipids and the application thereof in immunological research.