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Synthesis of an Immunologically Active Heptamannoside of *Mycobacterium tuberculosis* by the [Au]/[Ag]-catalyzed Activation of Ethynylcyclohexyl Glycosyl Carbonate Donor

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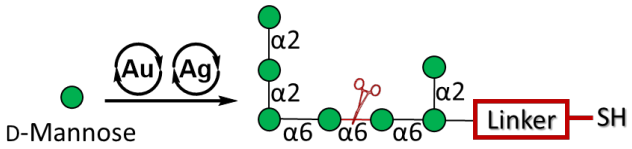
Supporting Information Placeholder



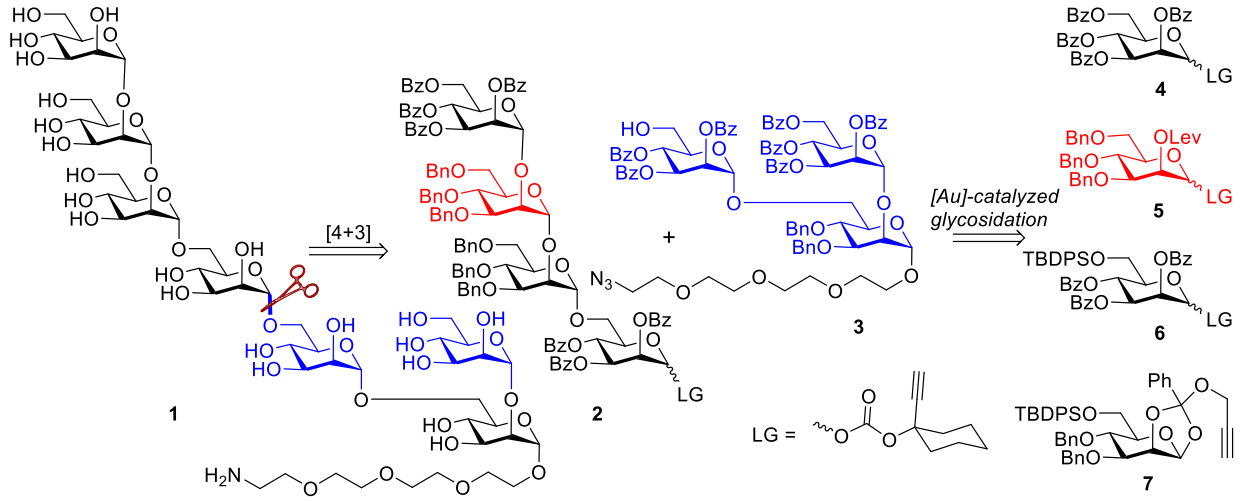
ABSTRACT: Tuberculosis (TB) is one of the most dreadful diseases killing more than 3 million humans annually. *M. tuberculosis* (MTb) is the causative agent for TB and it has a thick and waxy cell wall, an attractive target for immunological studies. In this study, a heptamannopyranoside containing 1→2 and 1→6 α-mannopyranosidic linkages has been explored for the immunological evaluations. The conjugation-ready heptamannopyranoside was synthesized by exploiting the salient features of recently discovered [Au]/[Ag]-glycosidation of ethynylcyclohexyl glycosyl carbonate donors. The glycan was conjugated to the ESAT6, an early secreted protein of MTb for further characterisation as a potential subunit vaccine candidate.

Tuberculosis is one of the diseases that has the longest history of burden to mankind with no sigh of relief.[1] *Mycobacterium tuberculosis* (MTb) is the causative agent for tuberculosis (TB) that is known to be dormant in the host until the immunity of the host is compromised and becomes active when the host immunity is compromised (e.g. HIV-AIDS).[1b,1c] The current prognosis for TB includes multi-drug therapy over a prolonged time. According to the World Health Organization vaccination is considered to be the most cost-effective strategy for controlling infectious disease.[1d] Unfortunately, today the only vaccine available against TB is the *Mycobacterium bovis* bacillus Calmette-Guerin (BCG).[1e] Although BCG has been shown to provide good protection against disseminated TB in children, its protective efficacy often fades in adolescence and fails to protect against pulmonary TB. The reported efficacy of BCG[1e] against adult pulmonary TB varies hugely (0–80%). Several mycobacterial protein antigens have been employed alone, together with BCG or as booster vaccines but without much success.[1e] A better understanding of the immunology of carbohydrates has paved the way for the development of more safe and efficient carbohydrate-based vaccines.[2] Indeed, many vaccines developed against bacterial infections are carbohydrate vaccines. Unfortunately, carbohydrate antigens are T-cell independent and provide poor immunogenicity unless they are converted to T-cell dependent through conjugation to a carrier protein.[2] *Mycobacterium tuberculosis* cell wall associated glycolipids LAM, LM, and PIM have been shown to modulate host immune responses through interaction with several pivotal immune cells.[3a] While LAM of TB has been suggested in many studies as a vaccine candidate against mycobacterial infection,[3] the protective efficacy of mycobacterium PIM is not yet explored. This is very interesting since, in contrast to LAM, PIM has been shown to exert potent anti-inflammatory activities.[3d]

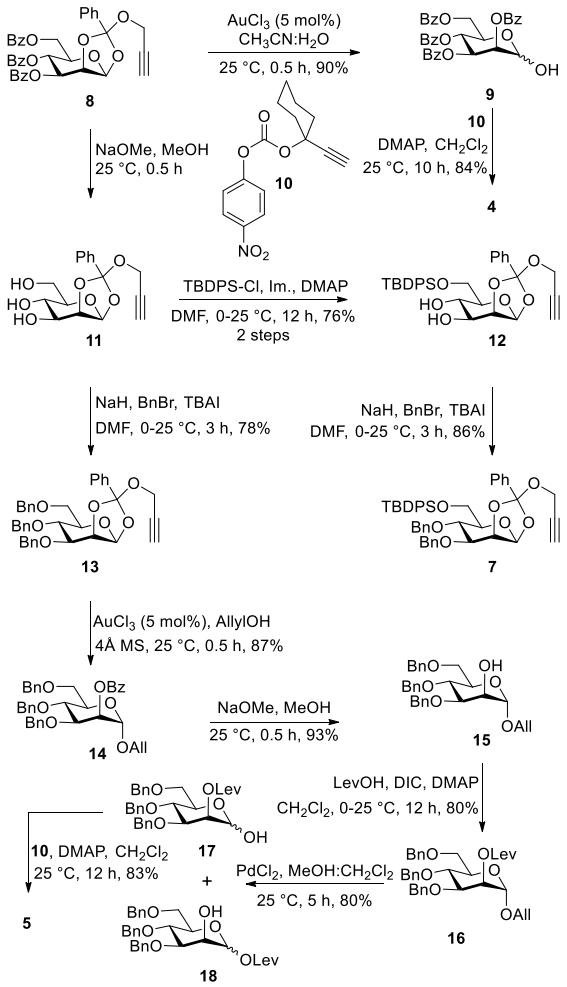
The chemical structure of the highly conserved LAM revealed that it comprises α-1,6 and α-1,2 mannopyranosyl linkages which are further attached to a phosphatidylinositol moiety.[4] Our long-standing interest in the synthesis of oligosaccharides coupled with their biological evaluation enticed us to hypothesize a branched heptamannopyranoside (**1**), equipped with α-1,6 and α-1,2 mannopyranosides, could become a potential vaccine candidate against TB after conjugating with ESAT-6,[5] an early secreted protein of MTb.

Heptamannopyranoside (**1**) can be synthesized from the glycosyl donor **2** and the aglycone **3** in [4+3] fashion. The presence of the benzoate at the C2-position ensues the formation of α-linkage through the neighboring group participation. Azide is useful as it can be reduced to an amine while performing hydrogenolysis at the last stages of the synthesis. Saccharides **2** and **3** can be synthesized from the four monosaccharide building blocks **4**-**7** (Scheme 1). The synthesis of compound **1** has been envisioned by the presence of three independent protecting groups (benzoyl, levulinoyl, and silyl ether). Identification of mild glycosidation conditions is very essential for the synthesis

Scheme 1. Retrosynthesis of the heptamannopyranoside



Scheme 2. Synthesis of monosaccharide building blocks

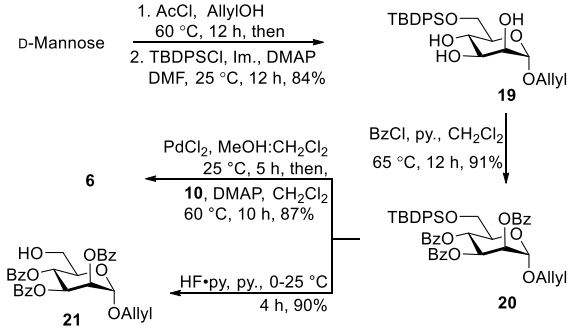


of any oligosaccharide. In the absence of a universal glycosylation method for the synthesis of any oligosaccharide, several groups have advanced their syntheses by deploying different classes of glycosyl donors. Many glycoconjugates were synthesized by invoking thioglycosyl,[6a] phosphate,[6b] pentenyl,[6c] alkynyl esters[6d] and trichloroacetimidyl donors.[6e] The recently discovered ethynylcyclohexyl glycosyl donors[6f] is quite promising as it offers a unique advantage of mild reaction conditions, catalytic activation using gold salts, enhanced performance with the synergistic action of silver salts, faster reaction kinetics, and high functional group tolerance.[6f-6j] Hence, assembly of oligosaccharide **1** was envisioned by the use of gold-catalyzed glycosidations. The Benzoate at the C2 position shall be installed using gold-catalyzed activation of propargyl 1,2-*O*-orthoester and all other glycosidations can be performed by the use of ethynylcyclohexyl carbonate glycosyl donor chemistry, which our group discovered.[6f]

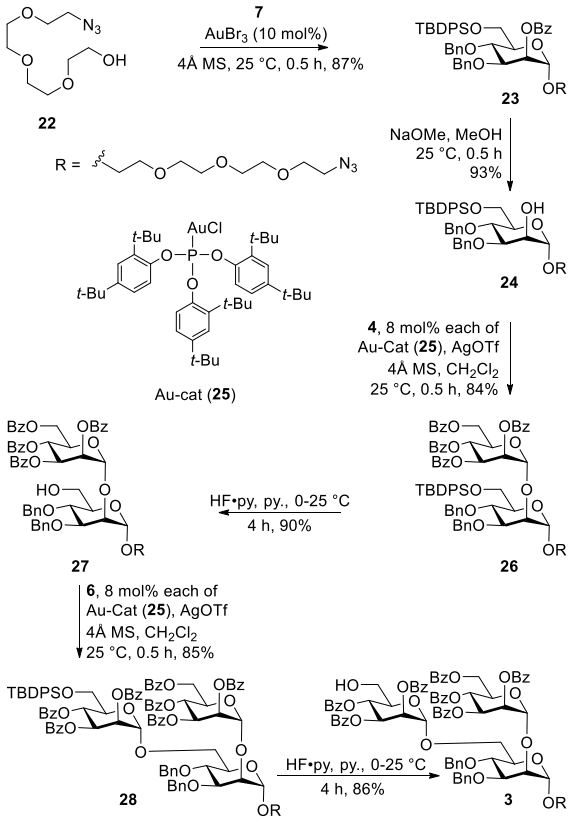
The journey commenced with the synthesis of per-*O*-benzoyl carbonate donor (**4**) starting from the easily accessible mannopyranosyl orthobenzoate **8**. Orthobezoate **8**[6e] was hydrolyzed catalytically by treating it with 5 mol% of $AuCl_3$ in 3:1 ratio of acetonitrile and water to obtain hemiacetals **9** in 90% yield which was treated with the carbonate reagent **10** and DMAP in CH_2Cl_2 for 10 h to isolate the desired mannopyranosyl donor **4** in 84% yield. In parallel, the propargyl orthobenzoate **8** was subjected to Zemplén conditions (NaOMe/MeOH) to obtain the known triol **11** that was split into portions. The first portion of triol **11** was treated with TBDPSCl, Imidazole, and DMAP in DMF to obtain the silyl ether **12** in 76% yield over two steps. Remaining two secondary -OH groups were blocked as benzyl ethers using NaH and BnBr to afford the required monosaccharide **7** (Scheme 2). The three hydroxyl moieties present in the second portion of triol **11** were protected as benzyl ethers using NaH, BnBr, and TBAI in DMF to get the tri-*O*-benzyl mannopyranosyl orthobenzoate **13** in 78% yield. The first gold-catalyzed glycosidation of donor **13** in the presence of allyl alcohol gave the allyl mannopyranoside **14** in 87% yield. The assembly of heptasaccharide requires the deprotection of different protecting groups. Therefore, levulinoates are identified as suitable ones since they can play dual roles *viz.* firstly, they will impart the neighboring group participation to give α-mannopyranosides and secondly, they can be deprotected in the presence of benzoates using hydrazine hydrate. Hence, the C2-benzoate of compound **14** was saponified using NaOMe/MeOH to obtain an alcohol **15** that was converted to the levulinoate **16** by treating it with levulinic acid under DIC/DMAP conditions in 80% yield within 12 h. Surprisingly, cleavage of the allyl group of levulinoate **16** using the $PdCl_2$ in methanol and dichloromethane resulted in a mixture of desired compound **17** along with the anomeric levulinoate **18**. However, the hurdle was quickly circumvented by the use of a ternary solvent system[6c]

to obtain the desired hemiacetals **17** which were quickly converted to the carbonate mannopyranosyl donor **5** using the carbonate reagent **10** under the aforementioned conditions. In parallel, the synthesis of the donor **6** was initiated from D-mannose by transforming it to the triol **19** in two steps involving modified Fischer conditions to get the allyl mannopyranoside whose C6-OH was protected as its TBDPS-ether under standard conditions. The three hydroxyl moieties were protected as benzoates utilizing BzCl/py. to obtain compound **20** in 91% yield. Compound **20** was split into portions; the first portion was converted to the desired manno-

Scheme 3. Synthesis of donor **6** and the aglycon **21**



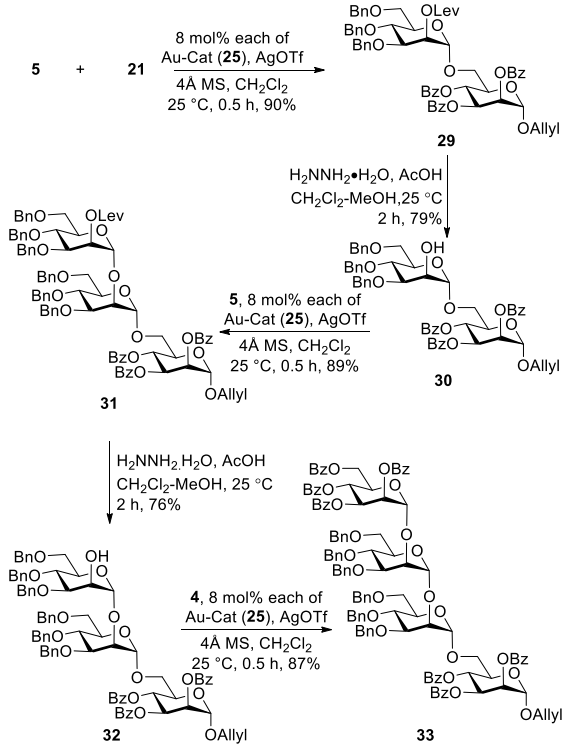
Scheme 4. Synthesis of trisaccharide **3**



pyranosyl donor **6** in 87% over two steps under the conditions mentioned above and the second portion was treated with HF•py in pyridine to afford the aglycone **21** in 90% yield within 4 h (Scheme 3). Synthesis of all four glycosyl donors **4**-**7** and the aglycon **21** set the stage for the assembly of the heptamannopyranoside **1**.

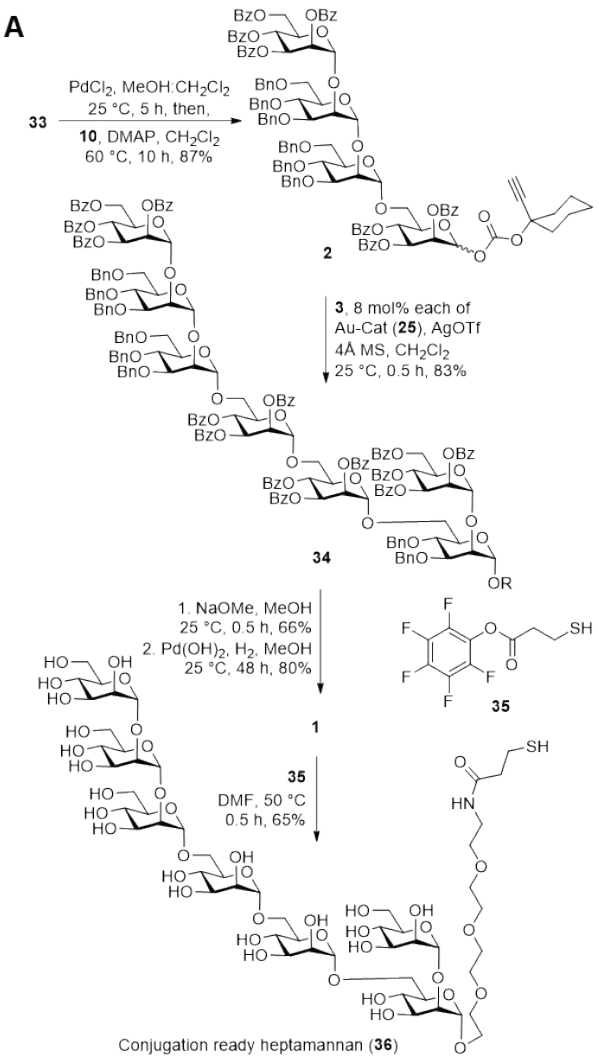
The non-reducing end of the heptasaccharide **1** has an amino functionality that has been envisioned from the corresponding azide. Accordingly, the easily accessible tetraethylene glycol azide **22** (OH-TEG-N_3) was treated with the above-synthesized glycosyl donor **7** in the presence of the catalytic amount of $AuBr_3$ and 4Å molecular sieves powder at room temperature for 30 min to afford the TEG-N_3 glycosidated mannopyranoside **23** in 87% yield. The presence of the azido functionality from the IR spectrum (2100 cm^{-1}) and the anomeric carbon at δ 99.2 ppm confirmed the α-mannopyranosidic linkage of monosaccharide **23**. Zemplén conditions afforded the C2-OH **24** that was glycosylated with the donor **4** under standard conditions of 8 mol% each of Au-phosphite catalyst **25** and AgOTf in the presence of 4Å MS powder in CH_2Cl_2 to afford the disaccharide **26** in 84% yield.[6a] Further, the hydrolysis of the silyl ether of compound **26** using HF•py in py resulted in the formation of an alcohol **27**. Further, another glycosylation of aglycon **27** with the donor **6** utilizing 8 mol% each of Au-catalyst **25** and AgOTf afforded the trisaccharide **28** that was subjected to the F^- based cleavage of the silyl ether to get the desired trisaccharide **3** in 86% yield (Scheme 4).

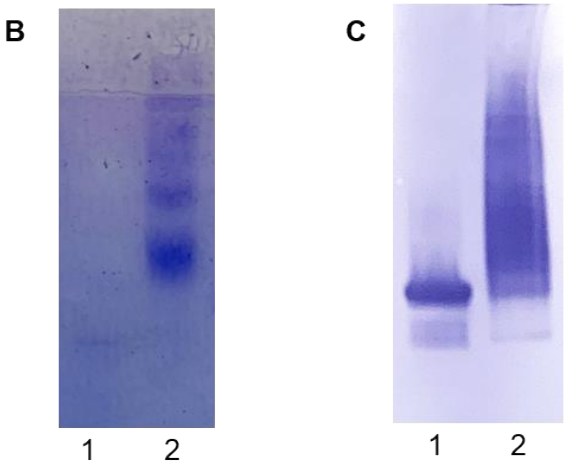
Scheme 5. Synthesis of the tetrasaccharide



In another set of experiments, glycosyl donor **5** and the aglycone **21** were treated with 8 mol% each of the catalyst **25** and the AgOTf to obtain the disaccharide **29**. Further, the disaccharide **29** was subjected to the deprotection of levulinoate group using hydrazine hydrate at 25 °C to afford an alcohol **30** that was glycosylated with a molar equivalent of donor **5** under [Au]/[Ag]-conditions to afford the trisaccharide **31** that was transformed to **32** using hydrazine. Silver-assisted gold-catalyzed glycosylation between the glycosyl donor **4** and the trisaccharide **32** underwent smoothly to give the tetrasaccharide **33** in 87% yield (Scheme 5). In the ^{13}C NMR spectrum of compound **33**, the four anomeric carbons were identified at δ 100.5, 99.3, 99.0, and 96.4 ppm and the HRMS spectrum also confirmed the molecular ion [*m/z* Calcd for $C_{118}H_{110}O_{28}Na$ = 1998.7115; Found 1998.7109], thereby confirming the structural homogeneity of the tetrasaccharide **33**. $PdCl_2$-catalyzed hydrolysis of the allyl glycoside in the binary solvent system containing 1:1 MeOH:CH_2Cl_2 for 5 h resulted in hemiacetals

Scheme 6. Synthesis of heptamannan and immunological characterization





Synthesis of the conjugation ready heptamannan **36** (A) and immunological characterization of **36**-ESAT6 conjugate (B and C). A. Synthesis route for Gel electrophoresis (4-12%), lane 1: synthetic **36**; lane 2: **36**-ESAT6 conjugate; B. Western blot of ESAT6 (lane 1), and **36**-ESAT6 conjugate developed with monoclonal antibodies directed against purified native PIM6.

which were quickly converted to corresponding glycosyl carbonate using the carbonate reagent **10** in the presence of DMAP to afford the tetrasaccharide donor **2** in 87% yield over two steps. In continuation, the final [4+3]-glycosylation between the donor **2** and the aglycon **3** in the presence of 8 mol% each of Au-catalyst **25** and AgOTf underwent effortlessly to give the fully protected heptasaccharide **34** in 83%. Formation of the heptasaccharide **34** was confirmed with the presence of seven anomeric carbons at δ 100.3, 99.8, 99.3, 99.2, 99.0, 98.2, 98.1 ppm and in addition, the compound also showed matching molecular ion in the MALDI-TOF (ESI) m/z: $[M+Na]^+$ Calcd for $C_{204}H_{191}N_3O_{53}SiNa$ 3555.2308; Found 3555.2304. Benzoates of compound **34** were deprotected under Zemplén conditions followed by the hydrogenation using $Pd(OH)_2$, H_2 in MeOH resulting in the deprotection of all the benzyl groups and the reduction of the terminal $N_3 \rightarrow NH_2$ to afford the target molecule **1** that was carried forward. The amidation between the amine **1** and the commercially available active ester **35** in DMF at 50 °C afforded the conjugation-ready Heptamannoside **36** in 65% yield (Scheme 6A).

The oligosaccharide **36** is synthesized with a sulfhydryl spacer-arm to facilitate its covalent conjugation to bromoalkylated ESAT6.[7] Physicochemical characterization of the neoglycoconjugate was assessed by gel electrophoresis, and Western blotting as described earlier[3a] using monoclonal antibodies raised against purified native PIM6 of H37Rv developed in our laboratory (Scheme 6B, 6C). Gratifyingly, the neoglycoconjugate was recognized by those antibodies.

In summary, a heptamannopyranoside resembling mycobacterial PIM6 without acyl moiety was synthesized in high quantity and purity in a [4+3]-fashion by employing [Au]/[Ag]-catalyzed glycosidation methodology. The glycan was treated with activated propionate to afford a neoglycoconjugate with the -SH moiety at the terminal position to facilitate covalent conjugation to ESAT-6, an early secreted protein of MTb. In this study, ESAT6 is selected as a carrier protein since it contains numerous epitopes recognized by a very high percentage of individuals.[5] The data here suggests the **36**-ESAT6 conjugate can be investigated for immunological response with an overarching goal of developing a TB vaccine in future.

ASSOCIATED CONTENT

Data Availability Statement

The data underlying this study are available in the published article and its Supporting Information

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

Experimental details, characterization data and spectra charts are available as PDF files.

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Author Contributions

The manuscript was written through the contributions of all authors and all authors have approved the final version of the manuscript. All the chemistry work is carried out at IISER Pune. Immunological studies were performed at Biopromic AB in Sweden.

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