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Chemoenzymatic Synthesis of Arabinomannan (AM) Glycoconjugates as Potential Vaccines for Tuberculosis

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Abstract

Mycobacteria infection resulting in tuberculosis (TB) is one of the top ten leading causes of death worldwide in 2018, and lipoarabinomannan (LAM) has been confirmed to be the most important antigenic oligosaccharide on the TB cell surface. In this study, a convenient synthetic method has been developed for synthesizing three branched oligosaccharides derived from LAM, in which a core building block was prepared by enzymatic hydrolysis in flow chemistry with excellent yield. After a series of steps of glycosylations, the obtained oligosaccharides were conjugated with recombinant human serum albumin (rHSA) and the *ex-vivo* ELISA tests were performed using serum obtained from several TB-infected patients, in order to evaluate the affinity of the glycoconjugate products for the human LAM-antibodies. The evaluation results are positive, especially compound **21** that exhibited excellent activity which could be considered as a lead compound for the future development of a new glycoconjugated vaccine against TB.

Keywords: lipoarabinomannan; glycoconjugate; chemoenzymatic synthesis; tuberculosis; vaccine

1. Introduction

Ranking above HIV in 2018, around 1.5 million people died from tuberculosis (TB), which is among the top 10 causes of death. Moreover, 25% of the world's population has latent TB, meaning civilians have been infected by TB bacteria without showing signs of illness. Due to the serious situation, ending the TB epidemic becomes one of the health targets of the Sustainable Development Goals by 2030[1]. Although tuberculosis is curable by administering medicine, the efficacy of drugs is limited by multiple drug resistance (MDR) and extensively-drug resistance (XDR)[2]. As an example, it is estimated that during 2018 there were 484000 new cases resistant to the most effective first-line drug rifampicin, leading to vaccination as still being the ideal method of fighting against TB. However, BCG (Bacillus Calmette-Guérin) is the only licensed vaccine against TB and it is only effective for children[3]. Thus, there remains an urgency to develop novel vaccines for TB prevention.

Carbohydrates are commonly found in nature as one of most complicated and diverse classes of biomolecules. They play important roles in fundamental biological processes with the outcome that many pharmaceuticals and vaccines use them as building blocks[4], and today glycoconjugate vaccines are being regarded as one of the safest and most effective anti-infective vaccines being manufactured in the last few decades[5, 6]. Commercial glycoconjugate vaccines are commonly obtained from antigenic sugar isolated from pathogens[4, 7, 8]. For *Mycobacterium tuberculosis* (M.TB) the most important sugar-antigen is the glycolipid lipoarabinomannan (LAM) that is also common to other *Mycobacterium species.*

Now the complex structure of the M.TB membrane oligosaccharides has been well established[9]. In the case of LAM, the arabinomannane (AM) is the antigenic structure composed by an internal mannan backbone, made of α(1→6) and α(1→2) D-mannose units and covered by a long and branched arabinan domain. The non-reducing terminus of AM is composed by branched motifs including D-Ara-β(1→2) disaccharide units linked with D-arabinose by α(1→5) and α(1→3) bonds (**Figure 1 A**). This motif can also be end capped with D-mannose or D-Man-α(1→2)D-Man units in LAM[10]. Branched motifs of D-arabinose by α(1→5) and α(1→3) bonds are also involved in different parts of the arabinan domain of LAM (**Figure 1 B**). Moreover, these D-Ara motifs of LAM are common with arabinogalactane (AG), another important oligosaccharide in M.TB membrane which is crucial for cell growth, viability, and virulence[11, 12].

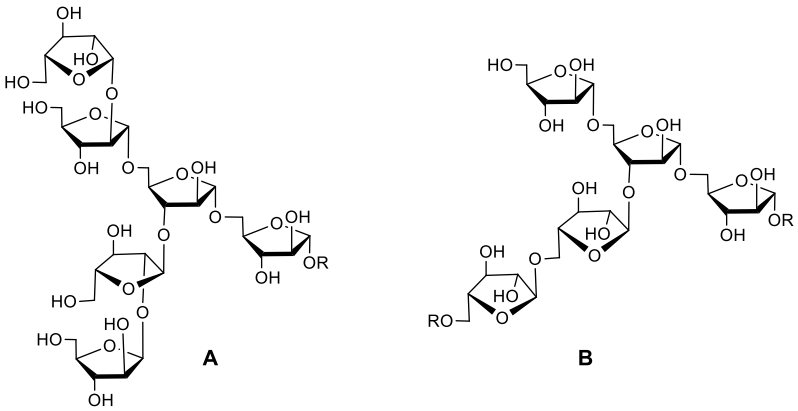


Figure 1. Structure of the branched motifs in AM

Since different glycoforms of LAM exposed outside the M.TB membrane were proven to stimulate the production of specific antibodies[13] and this antigen is one of the most important in the BCG vaccine, the conjugation of the AM part with protein has attracted the interests of many researchers and has been proposed as a strategy for the development of new and efficient TB-vaccines[14, 15] targeting the LAM antigen, with the advantage that these products could be active against different species of virulent mycobacteria in humans (such as *Mycobacterium avium* Spp.). Moreover, for the complexity of LAM structure, scientists also studied different types of synthetic AM-analogues and interestingly these analogues became antigenic after conjugating with suitable carrier proteins or lipids[16, 17]. However, until now there is no evidence to show which are the epitopes for this immunological activity. Thus, more fundamental study of AM based vaccine is crucial for the development of efficient sub-unit glycoconjugated vaccines.

In carbohydrate chemistry, the synthesis of oligosaccharides can be strongly limited by the complex chemical procedures involving protection/deprotection strategies by different protecting groups. As one of the most widely used protecting groups, acetyl is widely used in sugar chemistry and different chemical approaches have been reported to deprotect it. Compared to the chemical procedure, biocatalytic deacetylation strategies can selectively deprotect acetyl groups in peracetylated sugars in the desired position, using mild condition and aqueous medium[18, 19]. Moreover, the development of flow chemistry using immobilized enzyme on packed bed reactors provides wider applications in material discovery, future medicine and compound synthesis[20, 21], which saves time and labor to a significant degree. In addition, for the development of glycoconjugated vaccines, the selection of an adequate chemical linker in the anomeric sugar for

protein glycosylation should be considered and the synthetic strategy designed accordingly[7, 22, 23].

In the present work, acetyl was used as the only protecting group to avoid the redundant procedures and enzymatic regioselective hydrolysis was conducted to furnish arabinose building blocks with free hydroxy groups in one or two desired positions starting from peracetylated arabinose thioglycosides. Furthermore, in order to improve the performances of the enzymatic reactions, the use of flow-based bioreactors has been also considered. After the required steps of glycosylations, the motif containing D-arabinose branched with α(1→5) and α(1→3) D-Ara and some analogues were prepared considering the combination of different arabinose and mannose units, and then glycosylated with a mannose building block possessing a terminal thiocyanomethyl group at the anomeric position[24]. In fact, after *one-pot* deprotection and activation of the anomeric linker, the glycans obtained were combined with recombinant human serum albumin (rHSA) protein. Finally, the three obtained glycoconjugates (in **Figure 2**) were submitted to biological evaluation by *ex-vivo* tests performed on serum obtained from several infected patients in order to evaluate their affinity for the human polyclonal antibodies against TB.

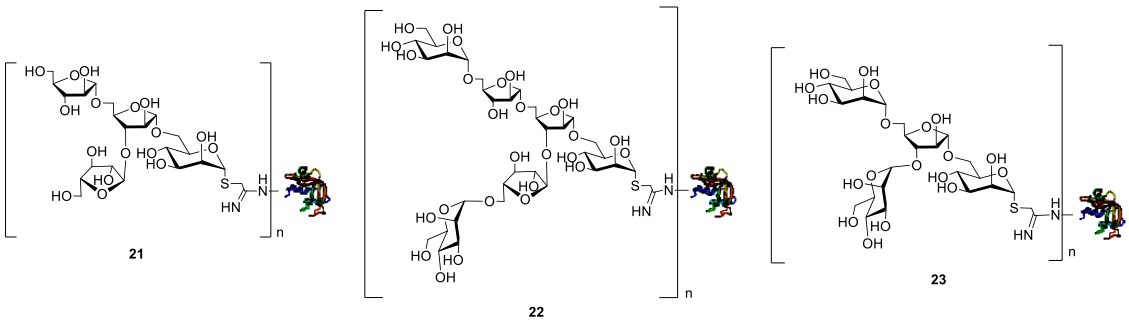


Figure 2. Glyconjugated products as AM analogues

2. Results and discussion

2.1 Synthesis of the oligosaccharide

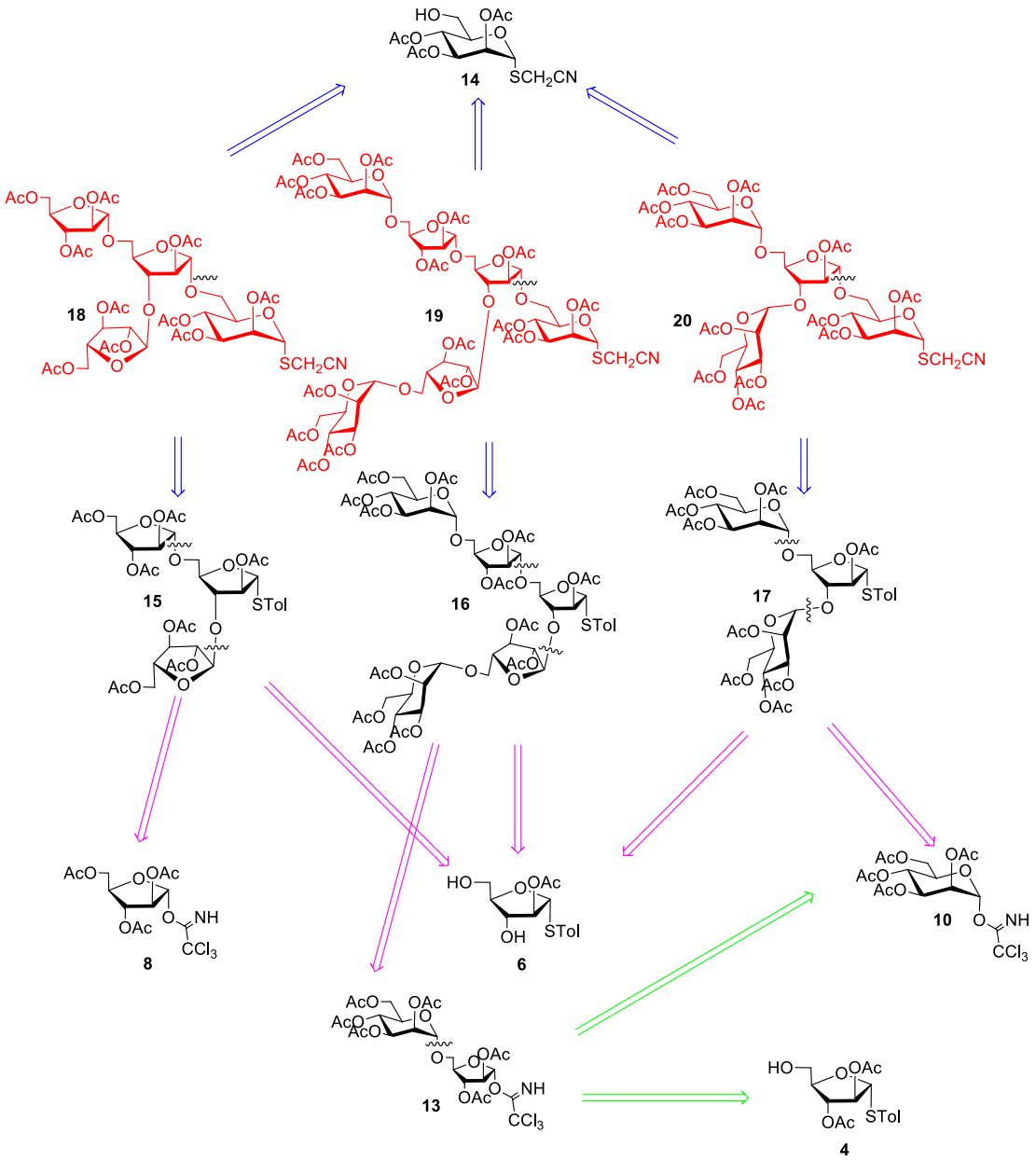
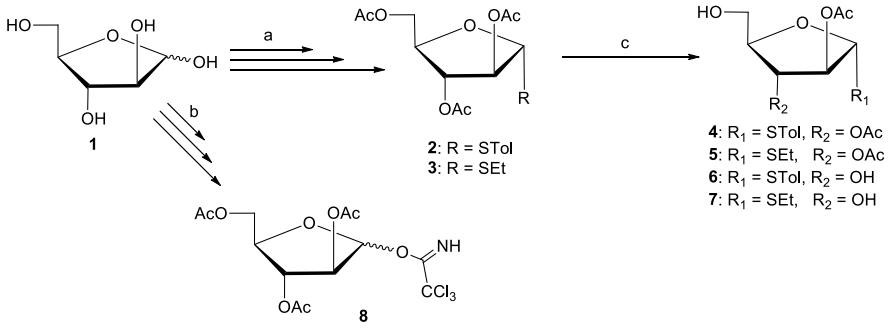


Figure 3. Retrosynthetic analysis for protected AM compounds **18**, **19** and **20**.

For the synthesis of three acetylated oligosaccharides, intermediates needed are shown in **Figure 3** based on the retrosynthetic analysis. First, synthesis of tetrasaccharide **18** would start with trisaccharide **15** and monosaccharide **14**, while the trisaccharide **15** is constructed from monosaccharides **6** and **8**. Then for compound **19**, using the same monosaccharide **14**, a pentasaccharide **16** would be used which can be constructed by disaccharide **13** and monosaccharide **6**, where **13** is synthesized from the two monosaccharides **4** and **10.** Finally, trisaccharide **17** and monosaccharide **14** are required for preparing **20,** in which building block **17** is constructed by compound **6** and **10**.



Scheme 1. Reagents and conditions: Transformations (a) and (b) have been conducted using reported protocols[25,27]; (c) CALB (Nvozyme 435), *t*-BuOH (4% water) (**4** and **5**) / *t*-BuOH (8% water) (**6** and **7**), 60 °C, 1000 rpm, 15 h (**4** and **5**) / 80 h (**6** and **7**), 65% (**4**), 43% (**5**), 65% (**6**), 48% (**7**), respectively.

As shown in S**cheme 1**, the synthesis of monosaccharides **2** and **3** started with commercially available D-arabinose **1**. Since both furanose and pyranose forms can be obtained during the one step acetylation of D-arabinose, hydroxy group at C-1 or C-5 position needs to be protected first. Thus, peracetylated arabinofuranose was synthesized by three steps which are anomeric methoxy introduction, acetylization and anomeric acetylization[25]. Then, Lewis acid was used to activate the anomeric position introducing the sulfur group to afford compounds **2** and **3** at low temperature. After that, enzymatic reactions catalyzed by immobilized CALB (Nvozyme 435) were performed to selectively remove the acetyl group only in C-5 (products **4** and **5**) and in both C-3 and C-5 (products **6** and **7**). From ^{1}H NMR and ^{1}H-^{1}H COSY spectra (see supporting information), the acetyl on C-5 position was first deprotected before the removal of C-3 acetyl. And compared to taking monosaccharide **3** as raw material, compound **2** can remove both acetyls on C-3 and C-5 positions with higher yield implying that building block **2** is more suitable as an intermediate for further synthesis. Afterwards, the conditions for the optimization of the selective hydrolysis of substrate **2** in C-5 position to obtain products **4**, and in C-5 and C-3 for synthesis of intermediate **6**, were tested and reported in the supporting information (**table S1 and S2**). From results obtained by adjusting water and enzyme amounts, as well as controlling the reaction time, it was deduced that larger amounts of water and enzyme along with longer reaction time promoted the deprotection of two acetyls.

A flow chemistry approach was also considered for the enzymatic deprotection of thioglycosyl arabinose **2** since immobilized CALB was reported to dramatically improve performance in the

regioselective hydrolysis of peracetylated arabinose[26]. Thus, immobilized CALB was packed into a glass column and a solution of **2** in *t*-BuOH/water flowed through it. Reaction parameters (i.e., concentration, amount of water, temperature, pressure and residence time) have been optimized for the preparation of intermediates **4** and **6** according to the experiment reported in the additional information (**Table S3 and S4**). All the optimization studies were performed with the same bioreactor without observing any relevant loss of activity. Thus the column reactor can be used to perform in continuous flow reaction for at least 100 h in the condition used (working in *t*-BuOH with water between 2% and 10%; 60°C and atmospheric pressure). In the hydrolysis of thiglycosyl arabinose **2,** the reaction outcome improved compared with the results obtained by batch (**Table 1**). Thus, 67% yield of product **4** was obtained in only one half hour, while substrate **2** was completely converted into product **6** (98% by HPLC analysis of the reaction) after 18 hours of recirculation in flow condition. The system was then tested in the synthesis of intermediate **6** on the preparative gram scale. Recirculating the substrate solution for 48 h (**Table 1**), less than 350 mg of biocatalyst was able to efficiently hydrolyse 880 mg of **2** allowing the production of **6** in 80% yield after purification.

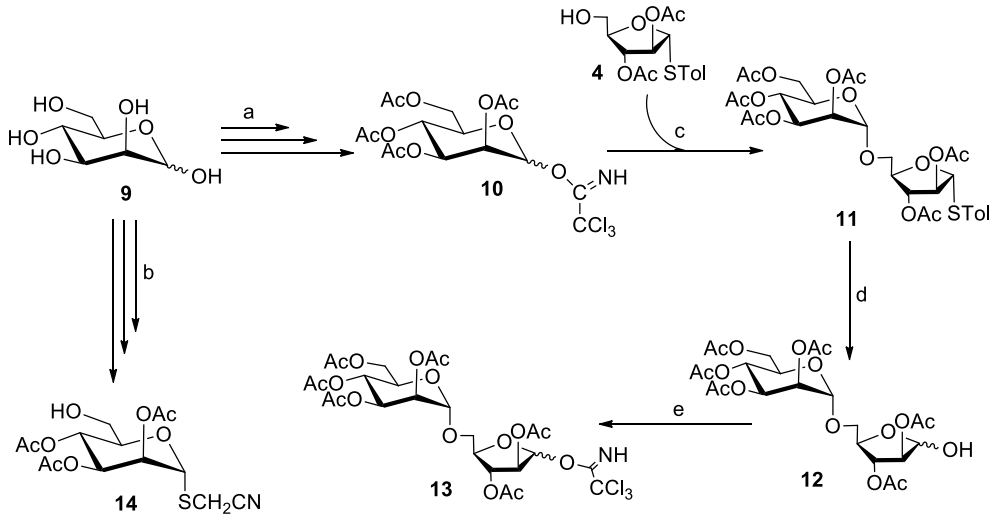
Table 1. Performances obtained in the hydrolysis of substrate **2** catalyzed by immobilized CALB

Entry	Substrate **2**	Enzyme	Product	Condition	Time	Yields
1	30 mg	150 mg	**4**	Batch [(a)]	15 h	65%
2	90 mg	345 mg	**4**	Flow [(b)]	0.5 h	67%
3	36 mg	150 mg	**6**	Batch [(c)]	80 h	65%
4	75 mg	345 mg	**6**	Flow [(d)]	18 h	98% (HPLC)
5	880 mg	345 mg	**6**	Flow [(e)]	48 h	80%

Batch: (a) *t*-BuOH (water 8%), volume: 1.2 mL, 1000 rpm, 60 °C; (c) *t*-BuOH (water 8%), volume: 1.2 mL, 1000 rpm, 60 °C; Flow: All the conditions use bioreactor volume: 1.54 mL. 60°C; substrate **2** concentration: 25 mg/mL: (b) *t*-BuOH (water 4%), flow rate 52 µL/min, pressure: Atm. (d) *t*-BuOH (water 4%); Recirculation: flow rate 52 µL/min, pressure: Atm. Conversion calculated by HPLC analysis. (e) t-BuOH (water 2%); Recirculation: flow rate 104 µL/min ; pressure: 75 psi.

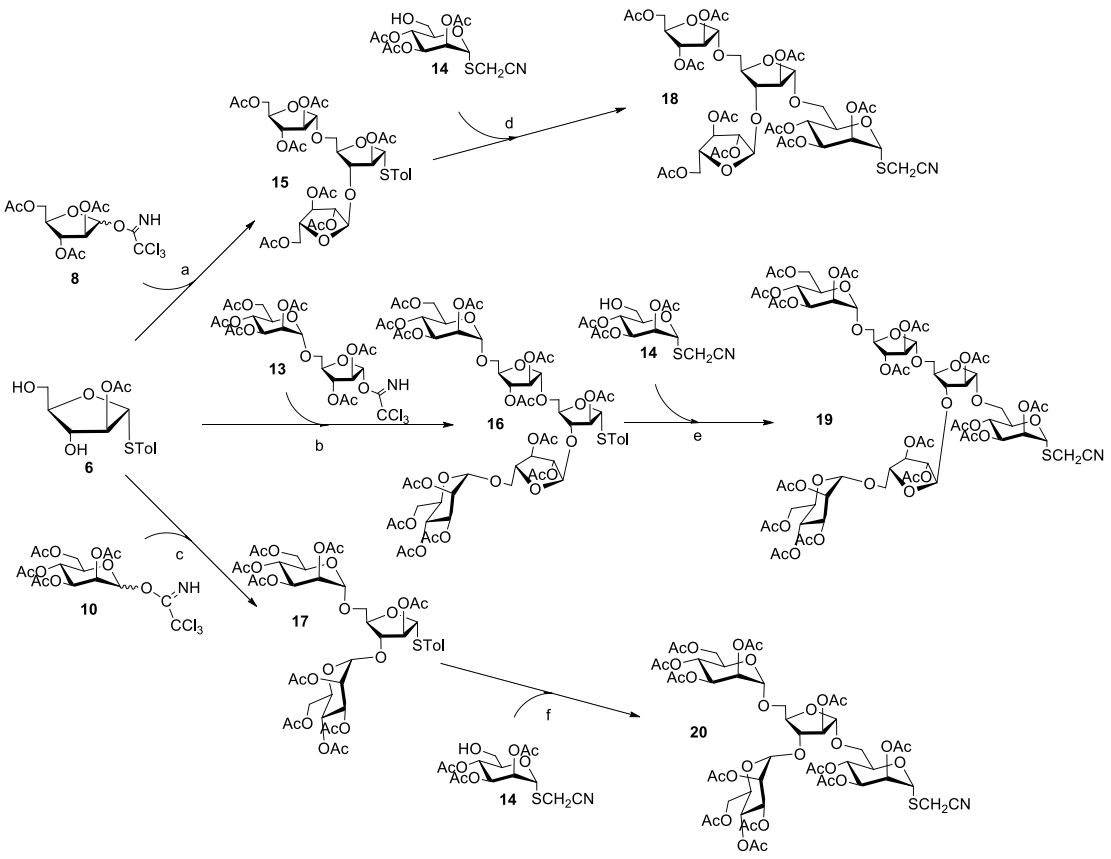
On the other hand, metal catalyst was applied to remove the anomeric acetyl in peracetylated arabinofuranose followed by imidate introduction to prepare monosaccharide **8** according to the

known approaches[27]**.**



Scheme 2. Reagents and conditions: Transformations (a) and (b) have been conducted using reported protocols[23,28]; (c) $BF_3{\cdot}Et_2O$, 4Å MS, CH_2Cl_2, -60 °C, 30 min, 55%; (d) NIS, AgOTf, TTBP, wet CH_2Cl_2, 0 °C - RT, 1 h, 90%; (e) CCl_3CN, DBU, CH_2Cl_2, 0 °C, 30 min, 90%.

In **Scheme 2**, D-mannose was used as starting material to synthesize monosaccharide trichloroacetimidate **10** as intermediate by the reported methods[28]. After that, glycosylation was performed using donor **10** and acceptor **4** catalyzed by $BF_3{\cdot}Et_2O$ at low temperature. In this step, we found that reaction temperature has an effect on the preferred reaction pathway, and low temperature can avoid the byproduct to some degree. When reaction was carried out at 0 °C, almost no targeting product was obtained because the Lewis acid activated the STol group in compound **4**, and when the temperature decreased to -30 °C and -60 °C, the yield came to 40% and 55%, respectively. However, no increase of the output was observed when temperature continued declining implying that -60 °C is the proper temperature balancing the yield and economic cost. Afterwards, the sulfur group in compound **11** was removed by NIS/AgOTf and 2,4,6-Tri-*tert*-butylpyrimidine (TTBP) in wet CH_2Cl_2 obtaining compound **12** with free hydroxy at the anomeric center, and finally intermediate **12** was transformed into trichloroacetimidate **13** at 0 °C with CCl_3CN and DBU for further use. On the other hand, the known compound **14** was synthesized by several steps, including a final enzymatic hydrolysis catalyzed by *Candida rugose* lipase for deprotection in C-6, according to the literature[23].



Scheme 3. Reagents and conditions: (a) $BF_3{\cdot}Et_2O$, 4Å MS, CH_2Cl_2, 0 °C, 30 min, 40%; (b) $BF_3{\cdot}Et_2O$, 4Å MS, CH_2Cl_2, 0 °C, 1 h, 32%; (c) $BF_3{\cdot}Et_2O$, 4Å MS, CH_2Cl_2, 0 °C, 1 h, 37%; (d) NIS, AgOTf, 4Å MS, CH_2Cl_2, -30 °C, 50 min, 60%; (e) NIS, AgOTf, 4Å MS, CH_2Cl_2, -30 °C, 50 min, 49%; (f) NIS, AgOTf, 4Å MS, CH_2Cl_2, -30 °C, 50 min, 55%

With the three donors prepared (**8**, **10** and **13**), glycosylations were carried out with acceptor **6**, (**Scheme 3).** Since catalyst and temperature may affect the reaction, different conditions were studied. For the reason that both $BF_3{\cdot}Et_2O$ and TMSOTf were taken as promoters for the glycosylation, with $BF_3{\cdot}Et_2O$ providing more products than TMSOTf, only the former promoter was studied in **Table 2**. In **Entry 1,** since $BF_3{\cdot}Et_2O$ activated the STol group in acceptor **6** at -30 °C, hardly any product **15** was obtained. Then with temperature cooled to -50 °C and -60 °C in **Entry 2** and **3**, the yield went up to 29% and 40%, respectively. However, a continued reduction of temperature to -70 °C did not increase the yield in **Entry 6,** indicating that -60 °C is proper for producing **15**. In addition to this, the quantity of catalyst was also studied in the Table. In **Entry 4,** less product was obtained by adding more catalyst at -60 °C. With less $BF_3{\cdot}Et_2O$ promoter **(Entry 5)**, the yield also appeared to decrease, because it needs a longer time to finish the reaction which is detrimental for a stable experimental condition.

Similarly, for constructing compound **17**, low temperature is beneficial to obtain product **17** in decent yield. Compared to **Entry 7-9**, **Entry10** provided a higher yield (23%) using the same amount of catalyst. However, deceasing the temperature in **Entry 13** did not result in a better output than in **Entry 10**. In addition, comparison of **Entry 10**, **11** and **12** demonstrated that reducing the catalyst quantity could enhance the reaction yield, whereas increasing the quantity may decrease the yield and the best result acquired is shown in **Entry 12**.

Subsequently, different conditions were also studied for synthesizing pentasaccharide **16**. From the results, in **Entry 14**, **15** and **18,** -60 °C is the optimal temperature to construct **16** with yield reaching 37%. In **Entry 17,** doubling the amount of promoter cut down the yield from 37% to 25% at the same temperature, while decreasing it to 0.2 equiv. did not affect the yield in **Entry 16**.

Table 2. Conditions studied to furnish compounds **15-17**

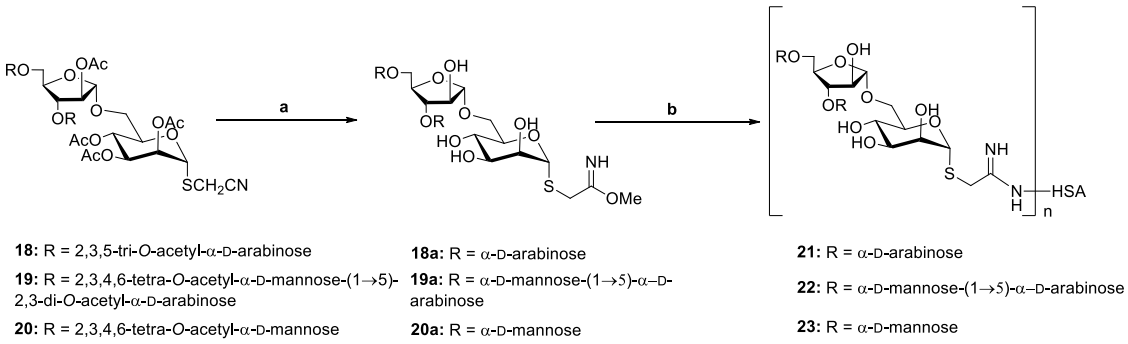
Entry	Donor	Acceptor	Temperature	Catalyst (equiv.)	Product	Yield
1	**8**	**6**	-30 °C	0.5	**15**	< 10%
2	**8**	**6**	-50 °C	0.5	**15**	29%
3	**8**	**6**	-60 °C	0.5	**15**	40%
4	**8**	**6**	-60 °C	1	**15**	33%
5	**8**	**6**	-60 °C	0.2	**15**	37%
6	**8**	**6**	-70 °C	0.5	**15**	35%
7	**10**	**6**	-30 °C	0.5	**17**	< 10%
8	**10**	**6**	-50 °C	0.5	**17**	< 10%
9	**10**	**6**	-60 °C	0.5	**17**	18%
10	**10**	**6**	-70 °C	0.5	**17**	23%
11	**10**	**6**	-70 °C	1	**17**	11%
12	**10**	**6**	-70 °C	0.2	**17**	32%
13	**10**	**6**	-90 °C	0.5	**17**	17%
14	**13**	**6**	-50 °C	0.5	**16**	35%
15	**13**	**6**	-60 °C	0.5	**16**	37%
16	**13**	**6**	-60 °C	0.2	**16**	37%
17	**13**	**6**	-60 °C	1	**16**	25%
18	**13**	**6**	-70 °C	0.5	**16**	34%

*In the table, all the conditions are donor being 3 equiv. and acceptor being 1 equiv.

Finally, promoted by NIS/AgOTf, glycoylations were performed between the three synthesized donors (**15**, **16** and **17**) and acceptor **14**, respectively. In **Scheme 3,** monosaccharide **14** is a known

compound synthesized by enzymatic hydrolysis[23], where there is a SCH_2CN group at the anomeric centre in **14** that may be damaged by using the same reagent. Since in SCH_2CN the cyano group is an electron withdrawing group, it can reduce the electronic cloud density around sulfur. Besides, furanose (donors **15**, **16** and **17**) is general more activated than pyranose (acceptor **14**) in glycosylation. We propose the catalyst would activate the STol groups in the donor first. In this step, the reactions were carried out at -10 °C, -30 °C and -50 °C, respectively, and -30 °C provided the best yield. Finally, two tetrasaccharides (**18** and **20**) and one hexasaccharide (**19**) were obtained for further protein conjugation via thiocyanomethyl group.

2.2 Preparation of the glycoconjugates



18: R = 2,3,5-tri-*O*-acetyl-α-D-arabinose
19: R = 2,3,4,6-tetra-*O*-acetyl-α-D-mannose-(1→5)-2,3-di-*O*-acetyl-α-D-arabinose
20: R = 2,3,4,6-tetra-*O*-acetyl-α-D-mannose

18a: R = α-D-arabinose
19a: R = α-D-mannose-(1→5)-α–D-arabinose
20a: R = α-D-mannose

21: R = α-D-arabinose
22: R = α-D-mannose-(1→5)-α–D-arabinose
23: R = α-D-mannose

Scheme 4. Reagents and conditions: (a) **21-23**, MeONa/MeOH, 35 °C, 48 h; (b) rHSA, sodium tetraborate buffer, glycosidic reagent/rHSA (molar ratio 200 : 1), pH 9.5, 25 °C, 24 h.

As shown in **Scheme 4**, compounds **18-20** were submitted to *one pot* deacetylation and activation to obtain the corresponding 2-deprotected iminomethoxyethyl (IME) thioglycosides **18a-20a**. The reactions were performed according to a previously reported procedure[17,23], working at 35°C in order to achieve a conversion around 50% of each intermediate into the corresponding IME derivative (**Table 3**), as resulting from ESI-MS analysis (see **Figure S1-S3** in supplementary information).

The coupling reactions between activated glycans **18a-20a** and the carrier protein, namely rHSA, were carried out according to a previously reported protocol for IME-activated saccharides that is specific for lysine residues[17]. Starting from literature conditions, different reaction parameters (temperature, saccharide/protein ratio, reaction temperature and time, presence of

organic solvent) were tested in order to reach satisfactory conjugation yields. For this purpose, reaction mixtures were analysed by direct injection in Hydrophilic Interaction Liquid Chromatography (HILIC-UV). In HILIC chromatography, the stationary phase (for our column composed of amide-derivatized silica) interacts with the sugar moieties of glycoproteins or glycoconjugates thanks to its high hydrophilicity, and the resulting elution order reflects the degree of glycosylation. Nowadays, HILIC analysis at intact protein level is considered a consolidated approach in glycoprofiling studies[29].

Collecting all the different observations, the final glycosylation protocol was developed. The HILIC-UV trace of *neo*-glycoprotein **21-23** obtained in the optimized conditions were reported in **Figure S4** supplementary data, where the absence of chromatographic peaks at the retention time of rHSA indicated that all three glycosylations were completed after 24 h (**Table 3**). Finally, MALDI-ToF analysis was performed for the characterization of the different *neo*glycoproteins. Based upon the mass difference between un-conjugated HSA and each of the 3 conjugated compounds, it was possible to estimate the average level of conjugation (average number of oligosaccharides attached) for each sample (**Table 3**).

Table 3. Results of the coupling reaction by **18-20**

Compound	IME-derivative (%) [(a)]	Product (%) [(b)]	n° sugar
18	**18a** (49%)	**21** (>98%)	14.5
19	**19a** (53%)	**22** (>98%)	12.4
20	**20a** (54%)	**23** (>98%)	14.3

2.3 Biological ex-vivo evaluation of the glycoconjugates

Ex-vivo antigenic evaluation has been performed with *neo*-glycoproteins **21-23**, in order to evaluate the affinity of putative epitopes for human antibodies. This approach is an elective method for the screening of new antigens because it is directly related to the recognition of human epitopes and allows an appropriate selection of candidates for successive *in vivo* testing. Thus, indirect ELISA (**Figure 4**) was applied using serum obtained from infected patient (30 individuals) to detect the potential of **21** (ManAra3-rHSA), **22** (Man3Ara3-rHSA) and **23** (Man3Ara-rHSA) as

antigens and 16 healthy members' serum as controls (details such as age and nationality are shown in Table S5). The results from this analysis demonstrated a statistically significant increase of the antibody reactivity in TB subjects compared with healthy control ones for *neo*glycoproteins **21** and **22** ($p \leq 0.0004$ and $p \leq 0.01$ respectively).

In the test, the obtained cut-off value of the rHSA-based ELISA was 0.380 (mean = 0.128, SD = 0.084), while the cut-off value of the **21**, **22** and **23**-based ELISA was 0.482 (mean = 0.168, SD = 0.105), 0.298 (mean = 0.106, SD = 0.064), and 0.307 (mean = 0.121, SD = 0.062), respectively. In these conditions on a total of 30 TB serum samples, 17 were determined as positive for product **21** (mean = 0.767, SD = 0.619), 13 for *neo*glycoprotein **22** (mean = 0.471, SD = 0.539) and only 2 for **23** (mean = 0.175, SD = 0.125).

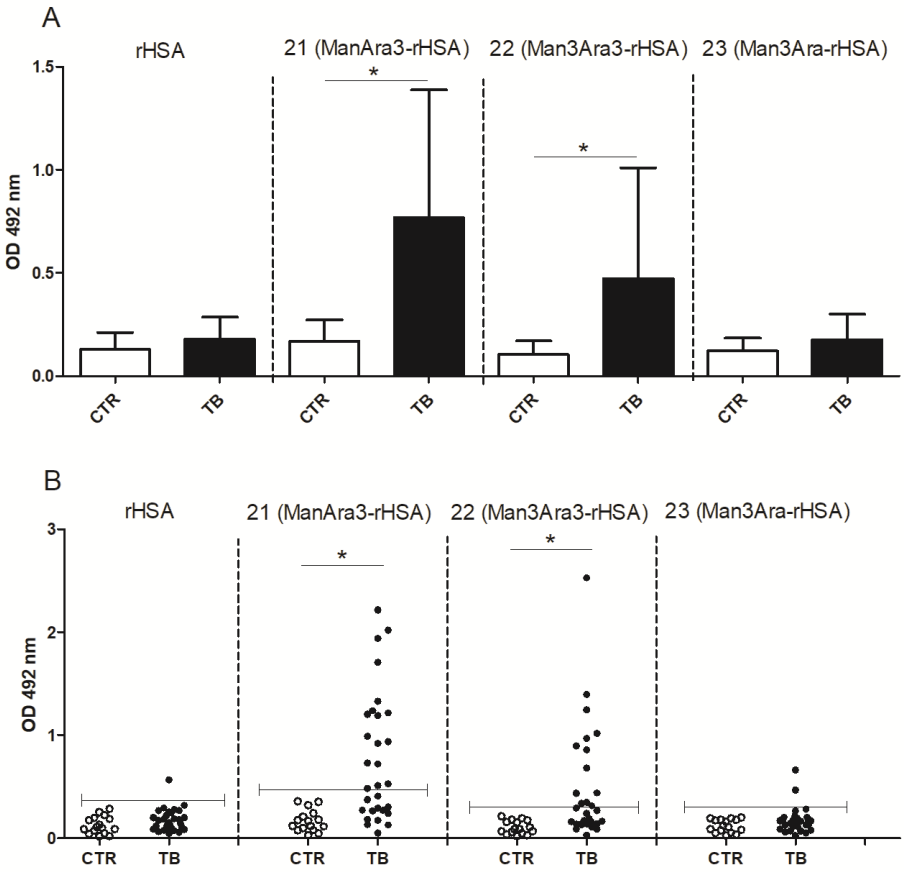


Figure 4. Antibodies (IgG) levels reactive against *neo*glycoproteins **21**, **22** and **23** in 30 TB patients and 16 healthy subjects (CTR).

In the figure, rHSA was used as protein control. Panel A: Histograms report OD mean values and standard deviation of TB and control samples. Panel B: Distribution of IgG level of samples. Horizontal lines represents the cut off values calculated for each antigens used in the study as the average of negative controls + (3 × SD of negative controls). The sera above horizontal line were considered positive and sera below the line were negative

according to the cut off value. OD = Optical Density; CTR: Controls; TB = Tuberculosis. $P \leq 0.05$ = *.

The analysis of antibody specificity to different *neo*glycoproteins of sera from M.TB patients demonstrated that the D-Ara motif branched with D-Ara α(1→5) and α(1→3), contained in compound **21**, is well recognized by antibodies from 17 out of 30 patients. The replacement of the two D-Ara residues with two D-Man α(1→5) units (compound **23**), completely abrogated the reactivity of antibodies. The activity is partially maintained when two additional units of D-Man-α(1→5) are introduced on the branched D-Ara motif to obtain compound **22**.

Interestingly, the analysis of the 13 patients positively responding to the *neo*glycoprotein **22** are almost all revealing positive for glycoconjugate **21** (in **Figure 5**) (except for one patient that is purely responding towards both sugars), while only about 70% of the 17 patients responding for antigen **21** are positive for **22**. These results may indicate that the epitope of **22** is correlated with the branched D-Ara motif contained in **21** and the introduction of two additional mannose molecules at the end of the chain reduces the recognition of this epitope.

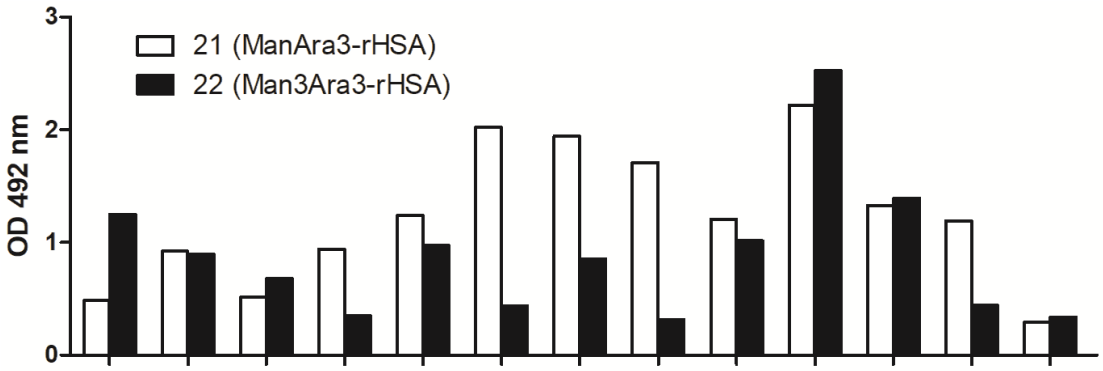


Figure 5. OD values obtained with the M.TB patients that respond positively against compound **22**

As one of the most convenient and rapid technologies in immunoassay, ELISA plays an essential role in TB *ex-vivo* biological evaluation because of its sensitivity and specificity for human antibodies. However, most of the research used mice as the experimental model[16, 30, 31], which may lead to different results than humans epitopes. In this work, we use the serum obtained from different infected patients to evaluate the prepared compounds and our study demonstrates

that the branched D-Ara motif included in different parts of the AM structure can have significance as a human epitope and thus relevant in developing novel TB vaccine candidates.

3. Conclusion

A novel enzymatic approach was developed to selectively remove acetyls from a peracetylated arabinose thioglycoside furnishing a key building block for synthesizing three branched AM analogues. The flow chemistry application through immobilized CALB (Nvozyme 435) in the column bioreactor not only shortened the reaction time, but also enhanced the output significantly on the gram scale. After required steps of glycosylations, three oligosaccharides derived from AM were furnished and conjugated with rHSA. In the ELISA test, the three glycoconjugates showed different affinities to human antibodies present in the serum of M.TB infected patients, with compound **21** obtaining the best results. The fact that the number of patients responding to **21** and the mean response observed by *ex-vivo* assay to **21** were much greater than the responses obtained by the two other analogues prepared with the same synthetic approach, demonstrates that the branched arabinose motif contained in *neo*-glycoprotein **21** is a putative human epitope of LAM. Thus, *neo*-glycoprotein based on branched D-Ara motif are prone to induce production of specific human antibodies against LAM and are worthy of further investigation.

In order to find out the best epitopes of AM and design more effective antituberculosis vaccine candidates, **21** can be regarded as the lead compound to prepare more related compounds including the use of suitable carrier proteins for *in-vivo* evaluation. It is noteworthy that this is the first work using the chemo-enzymatic approach and it represents a method to rapidly synthesize new branched analogues in order to better elucidate the antigenic structures of AM. In this context, the use of biological evaluation by *ex-vivo* tests is also helpful to identify the optimal human epitope(s) and design novel efficient AM-based TB glycovaccine candidates for further evaluation. Furthermore, studies such as *in vivo* evaluations on suitable animal models will also be considered in the future for selected compounds.

4. Experimental Section

4.1 General method

All chemicals were purchased as reagent grade and used without further purification. All reactions were carried out under Ar atmosphere and anhydrous conditions with freshly distilled solvents, unless otherwise noted. Reactions were monitored by thin layer chromatography (TLC) on a pre-coated plate of silica gel 60 F254 (Merck) and detection by charring with sulfuric acid. Solvents were evaporated under reduced pressure and below 40 °C (water bath). Column chromatography was performed on silica gel 60 (230-400 mesh, Merck). All the new compounds were fully characterized by ^{1}H and ^{13}C NMR, as well as HRMS. ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz with Bruker AVANCE DRX 400 spectrometer, respectively. The chemical shifts were referenced to the solvent peak, 7.26 ppm (^{1}H) and 77.16 ppm (^{13}C) for $CDCl_3$ at 25 °C, and coupling constants were given in Hz. Complete assignment of all NMR signals was performed using a combination of ^{1}H,^{1}H-COSY and ^{1}H,^{13}C-HSQC experiments. For characterization of the synthetic intermediates and oligosaccharides high-resolution mass spectra (HRMS) were recorded with a Bruker Micro-TOF spectrometer in electrospray ionization (ESI) mode, using Tuning-Mix as reference. The continuous flow reactions were performed using a R2+/R4 series flow reactor, commercially available from Vapourtec equipped with Omnifit glass columns. For the preparation of the different *neo*-glycoprotein, rHSA (recombinant human serum albumin) is derived from rice grains (gene-modified rice endosperm) and obtained by chromatographic purification (>99% by SDS-PAGE) was provided by Healtgen biotechnology Corp (Wuhan, China).

4.2 Synthesis of the AM analogues

4.2.1 4-Methylphenyl 2,3-di-*O*-acetyl-1-thio-α-D-arabinofuranoside **(4)**

4.2.1.1 Batch method: To a solution of *t*-BuOH (12 mL) with 4% water (0.84 mL) added, compound **2** (600 mg, 1.6 mmol) and CALB enzyme (1.5 g) were added. Then the reaction was stirred vigorously at 1000 rpm at 60 °C for 15 h. After filtrating the enzyme, the solution was concentrated under vacuum before purification by column chromatography (cyclohexane: ethyl acetate = 2:1), with white foam **4** obtained (360 mg, 65%).

4.2.1.2 Flow method: A solution (25 mg/mL) of substrate **2** was prepared in a mixture water/*tert*-butanol (96:4). The bioreactor (prepared packing 345 mg CALB into a glass column; volume: 1.54 mL) was washed with the flow stream (water/*tert*-butanol 96:4) at 0.5 mL/min for 5 min at RT and 5 min at 60 °C. The substrate solution (3.6 mL) was then recirculated at 60 °C through the bioreactor (flow 52 µL/min). The reaction was monitored by HPLC and after for 18 h the bioreactor was washed with water/*tert*-butanol (96:4) at 0.5 mL/min for 10 min at 60 °C. The solvent was evaporated under reduced pressure and the crude was purified by flash chromatography (cyclohexane: ethyl acetate = 1:1) to give pure **4** (53 mg, 67%). R_f = 0.6 (cyclohexane: ethyl acetate = 1:1). $[\alpha]_D^{25}$ +174.1 (c 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.47 (d, *J* = 2.5 Hz, 1H, H-1), 5.30 (t, *J* = 2.5 Hz, 1H, H-2), 5.12 (m, 1H, H-3), 4.35 (m, 1H, H-4), 3.93-3.79 (m, 2H, H-5, H-5'), 2.34 (s, 3H, Tol), 2.14 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.02-1.99 (m, 1H, OH). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.46, 169.67, 138.16, 132.83, 129.85, 129.58, 91.10 (C-1), 82.59, 81.71, 77.08, 61.57, 21.14, 20.81, 20.76. HR ESI-TOF MS (*m/z*): calcd for

$C_{16}H_{20}O_6SNa$ $[M+Na]^+$, 363.0873; found, 363.0880.

4.2.2 Ethyl 2,3-*O*-acetyl-1-thio-α-D-arabinofuranoside **(5)**

To a solution of *t*-BuOH (12 mL) with 4% water (0.84 mL) added, compound **2** (510 mg, 1.6 mmol) and CALB enzyme (1.5 g) were added. Then the reaction was stirred vigorously at 1000 rpm at 60 °C for 15 h. After filtrating the enzyme, the solution was concentrated under vacuum before purification by column chromatography (cyclohexane: ethyl acetate = 3:2), with white foam **5** obtained (191 mg, 43%). R_f = 0.5 (cyclohexane: ethyl acetate = 1:1). $[\alpha]_D^{25}$ +162.5 (c 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.33 (m, 1H, H-1), 5.14 (t, *J* = 2.0 Hz, 1H, H-2), 5.08 (m, 1H, H-3), 4.25 (m, 1H, H-4), 3.92-3.79 (m, 2H, H-5, H-5'), 2.74-2.61 (m, 2H, SCH_2), 2.11 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.10 (m, 1H, OH), 1.3 (t, *J* = 7.4 Hz, 1H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.51, 169.76, 87.57 (C-1), 82.28, 82.17, 77.29, 61.66, 25.17, 20.81, 20.79, 14.72. calcd for $C_{11}H_{18}O_6SNa$ $[M+Na]^+$, 301.0716; found, 301.0724.

4.2.3 4-Methylphenyl 2-*O*-acetyl-1-thio-α-D-arabinofuranoside **(6)**

4.2.3.1 Batch method: To a solution of *t*-BuOH (12 mL) with 8% water (0.84 mL) added, compound **2** (600 mg, 1.6 mmol) and CALB enzyme (1.5 g) were added. Then the reaction was stirred vigorously at 1000 rpm at 60 °C for 80 h. After filtrating the enzyme, the solution was concentrated under vacuum before purification by column chromatography (cyclohexane: ethyl acetate = 1:1), with white foam **6** obtained (304 mg, 65%).

4.2.3.2 Flow method: A solution of **2** (25 mg/mL) was prepared in a mixture water/*tert*-butanol (98:2). The bioreactor (prepared as above described) was washed with the flow stream (water/*tert*-butanol 98:2) at 0.5 mL/min for 5 min at RT and 5 min at 60 °C (P = 75 psi). The substrate solution (36 mL) was then recirculated through the bioreactor at 104 µL/min (60 °C, P =75 psi). The reaction was monitored by HPLC and after 48 h the bioreactor was washed with water/*tert*-butanol (98:2) at 0.5 mL/min for 10 min (60 °C). The solvent was evaporated under reduced pressure and the crude was purified by flash chromatography (cyclohexane: ethyl acetate = 1:1) to yield 550 mg of pure **4** (80% yield). R_f = 0.28 (cyclohexane: ethyl acetate = 1:1). $[\alpha]_D^{25}$ +184.9 (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.51 (d, *J* = 3.2 Hz, 1H, H-1), 4.93 (t, *J* = 3.3 Hz, 1H, H-2), 4.30 (m, 1H, H-4), 4.15 (m, 1H, H-3), 3.91 (m, 1H, H-5), 3.80 (m, 1H, H-5'), 3.49 (s, 1H, OH), 2.33 (s, 3H, Tol), 2.12 (s, 3H, OAc). ^{13}C NMR (100 MHz, $CDCl_3$) δ 172.00, 138.32, 132.93, 129.96, 129.67, 89.66 (C-1), 86.84, 82.74, 77.48, 77.16, 76.84, 76.25, 61.29, 21.26, 20.95. HR ESI-TOF MS (*m/z*): calcd for $C_{14}H_{18}O_5SNH_4$ $[M+NH_4]^+$, 316.1213; found, 316.1214.

4.2.4 Ethyl 2-*O*-acetyl-1-thio-α-D-arabinofuranoside **(7)**

To a solution of *t*-BuOH (12 mL) with 8% water (0.84 mL) added, compound **2** (600 mg, 1.6

mmol) and CALB enzyme (1.5 g) were added. Then the reaction was stirred vigorously at 1000 rpm at 60 °C for 80 h. After filtrating the enzyme, the solution was concentrated under vacuum before purification by column chromatography (cyclohexane: ethyl acetate = 1:1), with white foam **7** obtained (212 mg, 48%). R_f = 0.28 (cyclohexane: ethyl acetate = 1:1). $[\alpha]_D^{25}$ +164.8 (c 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.31 (d, *J* = 2.8 Hz, 1H, H-1), 4.73 (t, *J* = 3.0 Hz, 1H, H-2), 4.13 (m, 1H, H-4), 4.03 (m, 1H, H-3), 3.85 (m, 1H, H-5), 3.72 (m, 1H, H-5'), 3.29 (d, *J* = 3.9 Hz, 1H, OH), 2.70-2.54 (m, 2H, SCH_2), 2.06 (s, 3H, Ac), 1.25 (t, *J* = 7.4 Hz, 3H, CH_3). ^{13}C NMR (100 MHz, $CDCl_3$) δ 171.80, 87.18 (C-1), 86.06, 82.59, 76.52, 61.37, 25.13, 20.84, 14.68. $C_{11}H_{18}O_6SNa$ $[M+Na]^+$, 259.0611; found, 259.0621.

4.2.5 4-Methylphenyl (2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→5)-2,3-di-*O*-acetyl-1-thio-α-D-arabinofuranoside **(11)**

The dried compound **4** (300 mg, 0.88 mmol), **10** (650 mg, 1.32 mmol) and activated 4Å MS (1 g) were mixed together in anhydrous CH_2Cl_2 (10 mL) stirred for 1 h at RT under argon. Then $BF_3{\cdot}Et_2O$ (63 μL, 0.44 mmol) was added and the reaction was left for 30 min at -60 °C before quenching by triethylamine. After filtering by Celite and concentration, the resulting crude product was purified by column chromatography (cyclohexane : ethyl acetate = 3:2) getting **11** (325 mg, 55%) as colorless syrup. R_f = 0.25 (cyclohexane: ethyl acetate = 3:2). $[\alpha]_D^{25}$ +133.8 (c 1, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (d, *J* = 8.1 Hz, 2H, Ph), 7.13 (d, *J* = 8.1 Hz, 2H, Ph), 5.47 (s, 1H, H-1^{Ara}), 5.31-5.27 (m, 4H, H-2^{Ara}, H-2^{Man}, H-3^{Man}, H-4^{Man}), 5.14 (m, 1H, H-3^{Ara}), 4.90 (d, *J* = 1.0 Hz, 1H, H-1^{Man}), 4.39 (m, 1H, H-4^{Ara}), 4.02 (m, 1H, H-5^{Ara}), 3.82 (m, 1H, H-5'^{Ara}), 4.30 (m, 1H, H-6^{Man}), 4.10 (m, 1H, H-6'^{Man}), 4.07 (m, 1H, H-5^{Man}), 2.33 (s, 3H, Ac), 2.14 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.97 (s, 3H, Ac). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.62, 170.15, 170.04, 169.89, 169.67, 169.63, 138.06, 132.84, 129.81, 129.46, 98.03 (C-1^{Man}), 91.15(C-1^{Ara}), 81.75, 81.50, 77.50, 69.29, 69.06, 68.70, 66.38, 65.98, 62.28, 21.13, 20.85, 20.75, 20.73, 20.70, 20.65, 20.63. $C_{30}H_{38}O_{15}SNa$ $[M+Na]^+$, 693.1824; found, 693.1831.

4.2.6 4-Methylphenyl (2,3,5-tri-*O*-acetyl-α-D-arabinofuranosyl)-(1→3)-[2,3,5-tri-*O*-acetyl-α-D-arabinofuranosyl-(1→5)-]-2-*O*-acetyl-1-thio-α-D-arabinofuranoside **(15)**

The dried compound **6** (100 mg, 0.34 mmol), **8** (420 mg, 1.00 mmol) and activated 4Å MS (600 mg) were mixed together in anhydrous CH_2Cl_2 (5 mL) stirred for 1 h at RT under argon. Then $BF_3{\cdot}Et_2O$ (25 μL, 0.17 mmol) was added and the reaction was left for 30 min at -60 °C before quenching by triethylamine. After filtering by Celite and concentration, the resulting crude product was purified by column chromatography (cyclohexane: ethyl acetate = 1:1) getting **15** (110 mg, 40%) as colorless syrup. R_f = 0.3 (cyclohexane: ethyl acetate = 1:1). $[\alpha]_D^{25}$ +177.6 (c 0.6, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.40 (d, *J* = 8.1 Hz, Ph), 7.11 (d, *J* = 8.1 Hz, Ph), 5.47 (m, 1H, H-1$^{Ara\text{-}A}$), 5.27 (s, 1H, H-1$^{Ara\text{-}B}$), 5.21 (t, *J* = 1.9 Hz, 1H), 5.15 (m, 1H), 5.12 (m, H-1$^{Ara\text{-}B'}$), 5.12 (m, 1H), 5.01 (m, 1H), 4.96 (m, 1H), 4.44-4.35 (m, 3H), 4.27-4.18 (m, 5H), 3.92 (dd, J_1 = 4.3 Hz, J_2 =

11.6 Hz, 1H), 3.76 (dd, J_1 = 4.3 Hz, J_2 = 11.6 Hz, 1H), 2.99 (s, 3H, Tol), 2.12 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.09 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.08 (s, 3H, Ac). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.53, 170.47, 170.11, 169.94, 169.52, 169.41, 137.76, 132.39, 130.22, 129.74, 105.62 (C-$1^{Ara\text{-}B}$), 105.29 (C-$1^{Ara\text{-}B'}$), 91.02 (C-$1^{Ara\text{-}A}$), 82.41, 81.32, 81.12, 80.90, 80.80, 80.68, 77.21, 76.86, 65.20, 63.21, 63.02, 21.11, 20.86, 20.75, 20.71, 20.63. HR ESI-TOF MS (*m/z*): calcd for $C_{36}H_{46}O_{19}SNa$ $[M+Na]^+$, 837.2246; found 837.2216.

4.2.7 4-Methylphenyl (2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→5)-(2,3-di-*O*-acetyl-α-D-arabinofuranosyl)-(1→3)-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→5)-(2,3-di-*O*-acetyl-α-D-arabinofuranosyl)-(1→5)-]-2-*O*-acetyl-1-thio-α-D-arabinofuranoside **(16)**

To a solution of **11** (830 mg, 1.24 mmol) in 16 mL wet CH_2Cl_2 (20% H_2O) was added NIS (558 mg, 2.48 mmol), AgOTf (318 mg, 1.24 mmol) and TTBP (744 mg, 3 mmol). The solution was stirred for 1 h until no **11** left. The crude was purified by column chromatography (cyclohexane: ethyl acetate = 1:2) to give **12** (629 mg, 90%) as colorless syrup. All the **12** was immediately put into 6 mL dry CH_2Cl_2 under argon and then CCl_3CN (1.1 mL, 11.1 mmol) and DBU (166 μL, 1.11 mmol) were added to the solution at ice bath. The solution was stirred for 1 h at 0 °C and solution was removed under vacuo and purified by column chromatography (cyclohexane: ethyl acetate = 1:1) with 1% triethylamine added to afford **13** (705 mg, 90%) as colorless syrup. **13** should be used without delay for its poor stability. The dried compound **6** (100 mg, 0.34 mmol), **13** (705 mg, 1.00 mmol) and activated 4Å MS (1.5 g) were mixed together in anhydrous CH_2Cl_2 (10 mL) stirred for 1 h at RT under argon. Then $BF_3{\cdot}Et_2O$ (25 μL, 0.17 mmol) was added and the reaction was left for 30 min at -60 °C before quenching by triethylamine. After filtering by Celite and concentration, the resulting crude product was purified by column chromatography (cyclohexane: ethyl acetate = 1:2) getting **16** (172 mg, 37%) as colorless syrup. R_f = 0.4 (cyclohexane: ethyl acetate = 1:2). $[\alpha]_D^{25}$ +84.5 (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) 7.42 (d, J = 8.1 Hz, 1H, Ph), 7.12 (d, J = 8.1 Hz, 1H, Ph), 5.48 (s, 1H, H-$1^{Ara\text{-}A}$), 5.33 (m, 7H), 5.29 (s, 1H, H-$1^{Ara\text{-}B}$), 5.25 (t, J = 1.8 Hz, 1H), 5.19 (d, J = 1 Hz, 1H), 5.15-5.14 (m, 2H), 5.13 (s, 1H, H-$1^{Ara\text{-}B'}$), 5.05 (d, J = 4.9 Hz, 1H), 4.92 (m, 2H, H-1^{Man}, H-$1^{Man'}$), 4.46 (m, 1H), 4.34-4.31 (m, 2H), 4.23 (dd, J_1 = 1.4 Hz, J_2 = 5.6 Hz, 1H), 4.17-4.11 (m, 6H), 4.04-4.01 (m, 2H), 3.91- 3.88 (m, 1H), 3.81-3.75 (m, 3H), 2.34 (s, 3H, Tol), 2.17 (s, 6H, Ac), 2.17 (s, 3H, Ac), 2.14 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.12 (s, 6H, Ac), 2.10 (s, 3H, Ac), 2.06 (s, 3H, Ac), 2.06 (s, 3H, Ac), 1.99 (s, 3H, Ac), 1.98 (s, 3H, Ac). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.64, 170.37, 170.28, 170.12, 169.99, 169.91, 169.89, 169.70, 169.64, 169.61, 137.68, 132.37, 130.37, 129.72, 105.46 (C-$1^{Ara\text{-}B}$), 105.21 (C-$1^{Ara\text{-}B'}$), 98.14 (C-1^{Man}), 98.12 (C-$1^{Man'}$) 91.09 (C-$1^{Ara\text{-}A}$), 82.55, 82.40, 82.27, 81.53, 81.36, 81.22, 80.82, 77.45, 76.89, 69.26, 69.11, 69.07, 68.75, 68.68, 66.34, 66.23, 65.96, 65.36, 62.27, 21.10, 20.86, 20.74, 20.71, 20.64, 20.62. HR ESI-TOF MS (*m/z*): calcd for $C_{60}H_{78}O_{35}SNa$ $[M+Na]^+$, 1413.3937; found 1413.3970.

4.2.8 4-Methylphenyl (2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl-(1→5)-]-2-*O*-acetyl-1-thio-α-D-arabinofuranoside **(17)**

The dried compound **6** (100 mg, 0.34 mmol), **10** (490 mg, 1.00 mmol) and activated 4Å MS (1 g) were mixed together in anhydrous CH_2Cl_2 (7 mL) stirred for 1 h at RT under argon. Then $BF_3{\cdot}Et_2O$ (25 μL, 0.17 mmol) was added and the reaction was left for 50 min at -70 °C before quenching by triethylamine. After filtering by Celite and concentration, the resulting crude product was purified by column chromatography (cyclohexane : ethyl acetate = 2:3) getting **17** (172 mg, 32%) as colorless syrup. R_f = 0.4 (cyclohexane: ethyl acetate = 2:3). $[\alpha]_D^{25}$ +117.8 (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (s, 1H, H-1^{Ara}), 5.37-5.25 (m, 6H), 5.18 (s, 1H), 5.09 (d, *J* = 1.6 Hz, 1H, H-1^{Man}), 4.94 (d, *J* =1.0 Hz, 1H, H-1$^{Man'}$), 4.53-4.52 (m, 1H), 4.32-4.23 (m, 3H), 4.20-4.19 (m, 1H), 4.13-4.05 (m, 2H), 4.04-3.99 (m, 2H), 3.87 (t, *J* = 3.9 Hz, 1H), 2.32 (s, 3H, Tol), 2.17 (s, 3H, Ac), 2.13 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.08 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 2.00 (s, 3H, Ac), 1.96 (s, 3H, Ac). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.55, 170.40, 170.03, 169.86, 169.77, 169.74, 169.70, 169.62, 138.03, 132.91, 129.81, 129.63, 98.10 (C-1^{Man}), 97.48 (C-1$^{Man'}$), 91.06 (C-1^{Ara}), 82.34, 82.19, 81.53, 69.40, 69.32, 69.13, 68.93, 68.74, 68.65, 66.24, 65.98, 62.59, 62.32, 21.13, 20.84, 20.68, 20.65, 20.60. HR ESI-TOF MS (*m/z*): calcd for $C_{42}H_{54}O_{23}SNa$ $[M+Na]^+$, 981.2669; found 981.2682.

4.2.9 Cyanomethyl (2,3,5-tri-*O*-acetyl-α-D-arabinofuranosyl)-(1→3)-[2,3,5-tri-*O*-acetyl-α-D-arabinofuranosyl-(1→5)-]-(2-*O*-acetyl-α-D-arabinofuranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranoside **(18)**

The dried **15** (40 mg, 0.049 mmol), **14** (27 mg, 0.073 mmol) and activated 4Å MS (100 mg) were mixed together in anhydrous CH_2Cl_2 (2 mL) for 1 h at RT. The reaction was put at -30 °C for 5 min before NIS (13 mg, 0.059 mmol) and AgOTf (15 mg, 0.059 mmol) were added. Upon for stirring at -30 °C for 50 min, the reaction was diluted with CH_2Cl_2, after filtering by Celite, the organic phase was washed with saturated aq. $Na_2S_2O_3$, water and dried over $MgSO_4$, and then concentrated under reduced pressure. The residue was finally purified by silica gel column chromatography with cyclohexane: ethyl acetate (1:2) as the eluent to afford **18** (31 mg, 60%) as foamy solid. R_f = 0.3 (cyclohexane: ethyl acetate = 1:2). $[\alpha]_D^{25}$ +196.0 (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.47 (s, 1H, H-1^{Man}), 5.37 (m, 1H), 5.23 (m, 1H, H-1$^{Ara\text{-}B}$), 5.22-5.20 (m, 2H), 5.14 (m, 1H, H-1$^{Ara\text{-}B'}$), 5.14-5.10 (m, 2H), 5.05-5.03 (m, 1H), 4.98 (m, 1H, H-1$^{Ara\text{-}A}$), 4.98-4.95 (m, 2H), 4.46-4.42 (m, 2H), 4.29-4.18 (m, 7H), 3.91-3.85 (m, 2H), 3.74 (dd, J_1 = 2.3 Hz, J_2= 11.6 Hz, 1H), 3.57 (d, *J* = 17.3 Hz, SCH_2CH), 3.50 (dd, J_1 = 2.3 Hz, J_2 = 11.6 Hz, 1H), 3.36 (d, *J* = 17.3 Hz, SCH_2CH), 2.17 (s, 3H, Ac), 2.12 (s, 3H, Ac), 2.10 (s, 18H, Ac), 2.06 (s, 3H, Ac), 1.99 (s, 3H, Ac). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.55, 170.45, 170.23, 170.13, 170.11, 169.83, 169.71, 169.65, 169.39, 115.98, 105.73 (C-1$^{Ara\text{-}B}$), 105.52 (C-1$^{Ara\text{-}B'}$), 104.74 (C-1$^{Ara\text{-}A}$), 82.13, 81.45, 81.28, 80.81 (C-1^{Man}), 80.78, 80.58, 80.14, 79.60, 70.49, 69.47, 66.53, 65.56, 65.13, 63.23, 62.73, 20.85, 20.73, 20.69, 20.63, 20.54, 14.77. HR ESI-TOF MS (*m/z*): calcd for $C_{43}H_{57}NO_{27}SNa$ $[M+Na]^+$, 1074.2731; found 1074.2735.

4.2.10 Cyanomethyl (2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→5)-(2,3-di-*O*-acetyl-α-D-arabinofuranosyl)-(1→3)-[(2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→5)-(2,3-di-*O*-acetyl-α-D-arabinofuranosyl)-(1→3)-]-(2-*O*-acetyl-α-D-arabinofuranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranosid

e **(19)**

The dried **16** (45 mg, 0.032 mmol), **14** (18 mg, 0.049 mmol) and activated 4Å MS (100 mg) were mixed together in anhydrous CH_2Cl_2 (2 mL) for 1 h at RT. The reaction was put at -30 °C for 5 min before NIS (8.5 mg, 0.038 mmol) and AgOTf (10 mg, 0.038 mmol) were added. Upon for stirring at -30 °C for 50 min, the reaction was diluted with CH_2Cl_2, after filtering by Celite, the organic phase was washed with saturated aq. $Na_2S_2O_3$, water and dried over $MgSO_4$, and then concentrated under reduced pressure. The residue was finally purified by silica gel column chromatography with cyclohexane: ethyl acetate (1:3) as the eluent to afford **18** (26 mg, 49%) as foamy solid. R_f = 0.25 (cyclohexane: ethyl acetate = 1:3). $[\alpha]_D^{25}$ +70.4 (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.45 (s, 1H, H-1^{Man-A}), 5.35 (m, 1H), 5.28 (m, 6H), 5.21(m, 1H, H-1^{Ara-A}), 5,21-5.17 (m, 2H), 5.15-5.10 (m, 3H), 5.00-4.97 (m, 2H), 5.10 (m, 1H, H-1^{Ara-B}), 4.97 (m, 1H, H-1$^{Ara-B'}$), 4.89 (m, 2H, H-1^{Man-B}, H-1$^{Man-B'}$), 4.31-4.23 (m, 3H), 4.18 (m, 2H), 4.13-4.07 (m, 6H), 4.02-3.96 (m, 2H), 3.89-3.77 (m, 3H), 3.72-3.68 (m, 2H), 3.58 (d, *J* = 17 Hz, 1H), 3.50 (m, 1H), 3.36 (d, *J* = 17 Hz, 1H), 2.15 (s, 3H, Ac), 2.13 (s, 9H, Ac), 2.10 (s, 3H, Ac), 2.08 (s, 15H, Ac), 2.04 (s, 3H, Ac), 2.02 (s, 6H, Ac), 1.96 (s, 3H, Ac), 1.95 (s, 3H, Ac), 1.94 (s, 3H, Ac). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.60, 170.39, 170.33, 170.06, 169.90, 169.88, 169.85, 169.66, 169.62, 169.58, 116.00, 105.58 (C-1^{Ara-B}), 105.46 (C-1$^{Ara-B'}$), 104.69 (C-1^{Ara-A}), 98.12 (C-1^{Man-B}, C-1$^{Man-B'}$), 82.42, 82.04, 81.94, 81.75, 81.22, 81.07, 80.50 (C-1^{Man-A}), 79.75, 77.53, 70.53, 69.47, 69.22, 69.10, 69.06, 68.74, 68.67, 66.51, 66.44, 66.08, 65.92, 65.66, 65.21, 62.24, 20.84, 20.71, 20.69, 20.60, 20.53, 14.77. HR ESI-TOF MS (*m/z*): calcd for $C_{67}H_{89}NO_{43}SNH_4$ $[M+NH_4]^+$, 1645.4867; found 1645.4871.

4.2.11 Cyanomethyl (2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl)-(1→3)-[2,3,4,6-tetra-*O*-acetyl-α-D-mannopyranosyl -(1→5)-]-(2-*O*-acetyl-α-D-arabinofuranosyl)-(1→6)-2,3,4-tri-*O*-acetyl-1-thio-α-D-mannopyranosid e **(20)**

The dried **17** (40 mg, 0.041 mmol), **14** (22 mg, 0.061 mmol) and activated 4Å MS (100 mg) were mixed together in anhydrous CH_2Cl_2 (2 mL) for 1 h at RT. The reaction was put at -30 °C for 5 min before NIS (11 mg, 0.049 mmol) and AgOTf (12.5 mg, 0.049 mmol) were added. Upon for stirring at -30 °C for 50 min, the reaction was diluted with CH_2Cl_2, after filtering by Celite, the organic phase was washed with saturated aq. $Na_2S_2O_3$, water and dried over $MgSO_4$, and then concentrated under reduced pressure. The residue was finally purified by silica gel column chromatography with cyclohexane: ethyl acetate (1:2) as the eluent to afford **18** (27 mg, 55%) as foamy solid. R_f = 0.3 (cyclohexane: ethyl acetate = 1:2). $[\alpha]_D^{25}$ +129.7 (c 0.5, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$) δ 5.46 (m, H-1^{Man-A}), 5.39 (dd, J_1 = 1.3 Hz, J_2 = 3.2 Hz, 1H), 5.33-5.27 (m, 6H), 5.24-5.17 (m, 3H), 5.10 (m, H-1^{Man-B}), 5.00 (m, 1H), 4.99-4.98 (m, 2H, H-1$^{Man-B'}$, H-1^{Ara}), 4.35-4.21 (m, 5H), 4.14-4.06 (m, 2H), 4.03-3.82 (m, 5H), 3.60-3.54 (m, 2H), 3.49 (d, *J* = 17 Hz, 1H), 2.18 (s, 3H, Ac), 2.16 (s, 3H, Ac), 2.15 (s, 3H, Ac), 2.11 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.10 (s, 3H, Ac), 2.07 (s, 3H, Ac), 2.05 (s, 3H, Ac), 2.04 (s, 3H, Ac), 1.99 (m, 6H, Ac), 1.96 (s, 3H, Ac). ^{13}C NMR (100 MHz, $CDCl_3$) δ 170.56, 170.40, 170.23, 170.03, 169.87, 169.85, 169.80, 169.71, 169.68, 169.66, 169.58, 116.17, 105.52 (C-1^{Ara-A}), 98.44 (C-1^{Man-B}), 97.32 (C-1$^{Man-B'}$), 81.83, 81.70, 81.56, 80.73 (C-1^{Man-A}), 70.47, 69.40, 69.37, 69.15, 69.02, 68.93, 68.80, 66.54, 66.17,

66.03, 65.79, 65.66, 62.49, 62.41, 20.85, 20.81, 20.75, 20.70, 20.66, 20.63, 20.60, 20.58, 20.53, 14.85. HR ESI-TOF MS (*m/z*): calcd for $C_{67}H_{89}NO_{43}SNH_4$ $[M+NH_4]^+$, 1213.3599; found 1213.3605.

4.3 Preparation of neo-glycoproteins

Compounds **18**–**20** (0.005-0.01 mmol) were dissolved in 3 mL of dry methanol and 1 wt% of sodium methoxide (0.18 mmol) was added. The reaction was reacted for 48 h under nitrogen at 35 °C, after which time the reaction mixture was concentrated *in vacuo* and the solid formed was characterized by ESI-MS analysis (See **Figures S1-S3** in the additional information). The MS analysis were carried out on a LTQ-MS (Thermo Electron, San Jose, CA) with an electrospray ionization (ESI) source controlled by X-calibur software 1.4 (Thermo Finnigan, San Jose, CA). The samples were dissolved in methanol and introduced into the mass spectrometer with a syringe pump at a flow rate of 10 µL/min. Full scan MS experiments were carried out under the following instrumental conditions: positive ion mode, mass range 300–1500 m/z, source voltage 4.6 kV, capillary voltage 36 V, sheath gas 6, auxiliary gas 1, capillary temperature 250 °C, tube lens voltage 80 V.

rHSA derived from gene-modified rice endosperm, was selected as carrier protein in order to have a pure (>99% by SDS-PAGE) homogeneous and non-antigenic protein. The reaction solutions were analysed by Hydrophilic Interaction Liquid Chromatography (HILIC) using an UV detector. Thus, an aliquot of 10 µL of each reaction mixture was brought to pH 6 by adding 1M HCl (2.3 µL), diluted with water to reach a final concentration of 0.5 mg/mL and directly injected in the chromatographic system. Chromatographic separations were performed on an Agilent HPLC series 1100 system, equipped with mobile-phase online degasser, quaternary pump, autosampler, column thermostated compartment, and diode array detector. For data acquisition and analysis, the ChemStation software version Rev. B.04.01 was used in a Microsoft Windows XP environment.

The chromatographic conditions were optimized starting from an already reported method[29]. The selected HILIC column was an AdvanceBio Glycan Mapping column (150 × 2.1 mm, 2.7 µm) from Agilent Technologies (Palo Alto, CA, USA). The mobile phase was composed of ACN and water both containing 0.1% TFA. The elution gradient was from 28% to 51% B in 35 min. The injection volume was fixed at 5 µL, the column temperature at 60 °C and the flow rate was set at 0.25 mL/min. According to the results obtained in the optimization study, the final glycosylation method entails a rHSA concentration of 2.0 mg/mL, a glycoside/protein molar ratio of 200/1 and the use of sodium tetraborate buffer (100 mM, pH 9.5). Thus, rHSA was dissolved in the buffer and the resulting solution was mixed with IME-glycoside. The reaction mixture was vortexed for 1 min and incubated for 24 h at 25 °C under continuous stirring. At the end of the reaction, the samples were purified by ultrafiltration in order to remove reagents and salts (seven 10 min steps of centrifugation at 13000 g and 4 °C) using centrifuge 5804-R (Eppendorf s.r.l., Milan, Italy) and Millipore's Amicon® Ultra filters with a nominal molecular weight limit of 10 kDa and load capacity of 500 µL. HSA conjugates were finally collected in water and lyophilized. MALDI-ToF MS analysis was performed for the characterization of the different *neo*-glycoproteins (see **Figure S5).**

The experiments were performed on an Autoflex III instrument (Bruker Daltonics, Inc., Bremen, Germany), equipped with a SmartBeam Nd:YAG laser emitting at 355 nm. Each mass spectrum represents an accumulation of 3000 laser shots obtained in the linear mode, with a repetition rate of 100 Hz, using DHB as the matrix. Calibration was performed using ProMix 3 (LaserBio Labs, Valbonne, FR).

Based upon the mass difference between the peak summit for the un-conjugated rHSA and each of the 3 conjugated saccharides, using the molecular weight of the different IME-oligosaccharide, it was estimated the average level of conjugation (average number of oligosaccharides attached): **21** (ManAra3-rHSA) 14.24; **22** (Man3Ara3-rHSA) 12.21; **23** (Man3Ara) 14.22.

4.4 Biological ex-vivo Immunological evaluation

4.4.1 **Studied population**: The population considered in the study included 30 subjects with newly diagnosed and untreated active pulmonary TB, as well as 16 healthy individuals without any history of TB exposure (hereafter indicated as TB unexposed controls). The diagnosis of active TB was confirmed by *M. tuberculosis* culture isolation in all cases. A detailed analysis of the main demographic features of each study group are shown in the Supplementary information (**Table S5**).

4.4.2 **ELISA assay**: Antibodies directed against *neo*-glycoproteins were determined as previously described by ELISA assay with minor modifications[32]. ELISA assay was performed in 96-well microtiter plates (Nunc Maxisorp, Invitrogen, Italy) coated with rHSA, **21**, **22** and **23** (1 microg/well) diluted in 100 µL of carbonate buffer (pH = 9.6) and incubated 1 h at RT and overnight at 4 °C. The wells were then washed (2 times with 200 µL phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBS-T). The plate was incubated (RT, 1 h) with 100 mL of serum (1:100). Plates were incubated 1 h at RT with anti-human IgG Secondary Antibody HRP conjugated (Invitrogen, Italy). All samples were measured in duplicate and for each serum, difference in mean ODs of antigen- and buffer-coated wells was calculated.

4.4.3 **Statistical analysis and cut off determination**: Data was reported as the mean values ± standard deviation (SD). Comparisons between two groups were performed by Student's t-test. *p* values<0.05 were considered to be statistically significant. GraphPad Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA) was used for all statistical analyses and graphs. By utilizing the formula (Average of negative controls + (3 X Standard deviation of negative controls)), the cut of value was set for each protein/glycoprotein.

4.4.4 **Ethics statement**: The Ethics Committee of the University of Rome “Tor Vergata” (Rome, Italy) approved the study protocol (protocol number 173/19).

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