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Development of a plate-based scintillation proximity assay for the mycobacterial AftB enzyme involved in cell wall arabinan biosynthesis

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ABSTRACT

A number of mycobacterial arabinosyltransferases, such as the Emb proteins, AftA, AftB, AftC, and AftD have been characterized and implicated to be involved in the cell wall arabinan assembly. These arabinosyltransferases are essential for the viability of the organism and are logically valid targets for developing new anti-tuberculosis agents. For instance, Ethambutol, a first line anti-tuberculosis drug, targets the Emb proteins involved in the formation of the arabinan of cell wall arabinogalactan. Among these arabinosyltransferases, the terminal β-(1→2) arabinosyltransferase activity has been associated with AftB. The predicted topology of AftB in *Mycobacterium tuberculosis* has 10 N terminal transmembrane domains and a C terminal hydrophilic domain similar to the Emb proteins. It has a conserved GT-C motif and is difficult to express. In a cell free assay, synthetic disaccharide, α-D-Ara*f*-(1→5)-α-D-Ara*f*-octyl, has been used as a substrate to explore the function of AftB. In our work, the disaccharide was synthesized in its pentenylated and biotinylated form, and the enzymatic product formed was identified as the β-(1→2) arabinofuranose adduct. When synthetic tri- and tetra-saccharides were used as substrates, a mixture of products containing both β-(1→2) and α-(1→5) linkages were formed. Therefore, the biotinylated disaccharide was selected to develop a scintillation proximity assay.

1. Introduction

Tuberculosis (TB) is still a major public health problem.[1] Particularly with the emergence of multi-drug-resistant (MDR-TB) tuberculosis and, more recently, extensively drug-resistant (XDR-TB) tuberculosis clinical isolates.[2] MDR-TB is defined as resistance to at least two of the most powerful first-line anti-TB drugs, isoniazid (INH) and rifampin. XDR-TB is defined as MDR-TB plus resistance to any fluoroquinolone, and at least to one of the second-line anti-TB drugs, capreomycin, kanamycin or amikacin. The recent reports of XDR-TB in HIV co-infected patients have renewed global awareness of drug-resistant TB[3] and prompted the need for research on developing new anti-TB drugs and identify new drug targets.

Two first-line drugs, INH and ethambutol (EMB), target the biosynthesis of the mycobacterial cell-wall, which is essential for the viability of the *Mycobacterium tuberculosis* (*M. tuberculosis*).[4] The mycobacterial cell envelope comprises of four macromolecules: lipoarabinomannan (LAM), mycolic acids, arabinogalactan (AG), and peptidoglycan.[5–8] The galactan domain of AG is linked to peptidoglycan through a diglycosylphosphoryl linker of L-Rhap-(1→3)-α-D-GlcNAc, and the arabinan domain is attached to mycolic acids, forming the mycolyl-arabinogalactan-peptidoglycan (mAGP) complex. The arabinan contains α-(1→5), α-(1→3), and β-(1→2) arabinofuranosyl (Ara*f*) linkages that forms different structural motifs of arabinan.[6,9] Enzymes associated with the synthesis of these molecules have all been found to be essential.[10–13]

The Ara*f* residues of the arabinan originate from the pentose phosphate shunt pathway and the immediate donor is decaprenylphosphoryl-D-arabinofuranose (DPA).[14,15] The biosynthetic pathway for the DPA formation has been recently elucidated. Phosphoribosepyrophosphate (pRpp) is converted to 5-phosphodecaprenylribose (DPPR), which is dephosphorylated to form decaprenylphosphoryl-D-ribose (DPR). Finally, DPR is epimerized to form DPA.[16] For the assembly of the arabinan from DPA, in the ten years of study that focused on arabinosyltransferases (AraT), it has been established that the Emb proteins (EmbA, EmbB, and EmbC) were required for the arabinan synthesis and worked not only on the formation of the non-reducing end of the arabinan but also in the assembly of the internal arabinan.[17–19] Recently, four additional AraTs have been reported. As illustrated in Figure 1, one of the enzymes, AftA,

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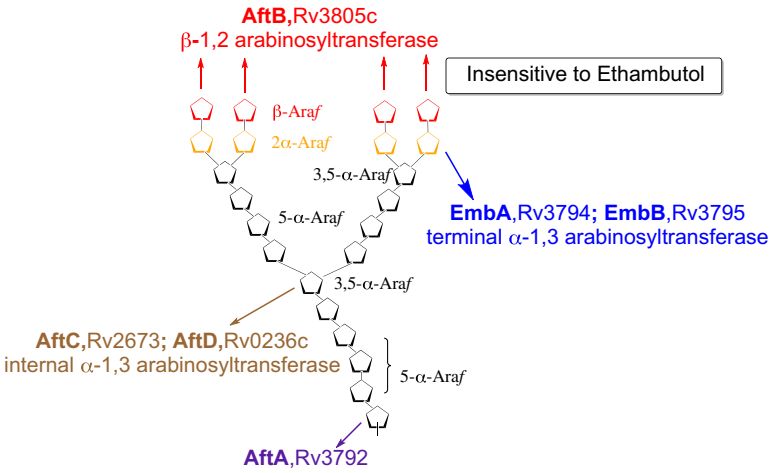


Figure 1. Biosynthesis of arabinan in AG in *M. tuberculosis*. There are three Ara_{22} mers (only one is shown) on the galactan backbone. All Ara*f* residues are donated by DPA. Key AraTs (six in total) identified till date are shown. These are only for the AG synthesis. It is interesting to note that both EmbA and EmbB have similar functions and AftC and AftD also, have similar functions. AftA is unique in that it only recognizes galactan and donates the first Ara*f* residues.

is responsible for recognizing the galactan and donating the initial key α-Ara*f* residues.[10] AftB on the other hand, is involved in the synthesis of the terminal β-Ara*f* cap. The disaccharide with the octyl aglycon (α-D-Ara*f*-(1→5)-α-D-Ara*f*-octyl) has been used as acceptor in the functional characterization of AftB to confirm that AftB was a terminal β-(1→2) AraT, and this activity was found to be insensitive to EMB.[11] Recently, two α-(1→3) branching AraT enzymes, AftC (Rv2673) and AftD (Rv0236c), have been identified by constructing a deletion mutant of MSMEG2785 (homolog of Rv2673) in *Mycobacterium smegmatis* (*M. smegmatis*)[12] and overexpression of Rv0236c in *M. smegmatis*,[13] respectively. Whereas EmbA and EmbB were suggested to be α-(1→3) AraT for synthesizing terminal Araβ1→2Araα1→5(Araβ1→2Araα1→3)Araα1→5Ara-(α1→ (Ara_6) motif,[17] Rv2673 has been shown to be a α-(1→3) AraT for synthesizing internal arabinan in AG.

One of the primary criteria for selecting a protein for screening for inhibitors is the essentiality of the encoding gene for growth. AftB fits this criterion, as it is essential in *M. tuberculosis*. Moreover, the function of AftB is unique in that, it is a retaining enzyme and its function is to terminate growing arabinan chain in AG (Fig. 1). AG contributes to the structural integrity of the bacterial cell. Ongoing drug development efforts have recently identified benzothiazinone, as an inhibitor of DprE1 (Rv 3790, an enzyme identified in the epimerization of DPR to DPA).[20] This enzyme is also not inhibited by EMB. Thus the arabinan is validated as an attractive target for developing new class of inhibitors not only on the premises of success of EMB, but also for the fact that many of these newly identified enzymes are essential.[10–13] Finding inhibitors for separate enzymes in one essential biosynthetic pathway might be beneficial to avert drug resistance.

In this work, we developed a plate-based assay to screen inhibitors for AftB. We chose *M. smegmatis* as the source of AftB since studies have shown that AftB is a conserved protein although Rv3805c is essential in *M. tuberculosis*. Biochemical phenotype of the deletion mutant of an orthologue NCgl2780 in *Corynebacterium glutamicum* could be fully complemented with AftB from *M. tuberculosis*.[11] First, oligosaccharides with a pentenyl aglycon (Ara_2-Ara_4, **1**–**3** in Fig. 2) were examined in a cell-free assay using p[^{14}C]Rpp as an indirect arabinosyl donor. Later, biotinylated disaccharide (**4** in Fig. 2) was synthesized and utilized in a

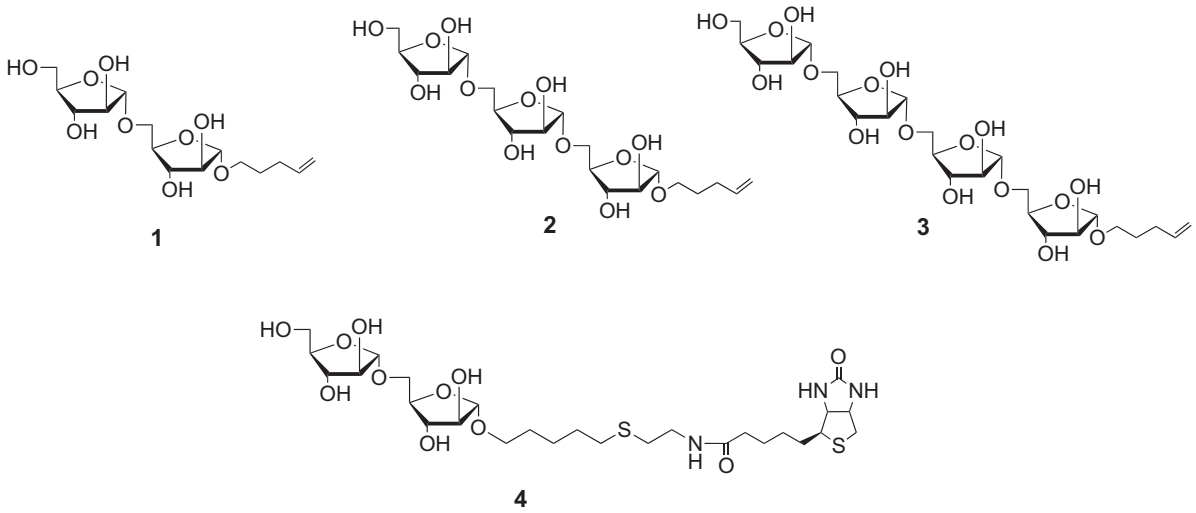


Figure 2. Acceptors **1–4** used in this study.

scintillation proximity assay (SPA).[21,22] The plate-based SPA assay was constructed and optimum conditions were developed.

2. Results and discussion

2.1. Chemical synthesis of acceptors 1, 2, and 3

The synthesis of arabinan acceptors **1**, **2**, and **3** was accomplished using a linear strategy. Glycosylation of known disaccharide **5**[23,24] with known trichloroacetimidate **6**[23] using standard TMSOTf-catalyzed activation (Scheme 1) proceeded smoothly with complete α-selectivity and the corresponding trisaccharide **7** was obtained in 95% yield. Desilylation afforded **8** (96%), which was subsequently coupled to glycosyl trichloroacetimidate **6** under TMSOTf activation to give the desired tetrasaccharide **9** in 84% yield. Removal of the TIPS protecting group gave **10**.

Finally, global deprotection of the three linear arabinosides was carried out using a three-step protocol (Scheme 2). Birch reduction of **5**, **8**, and **10** followed by peracetylation of the intermediates furnished the peracetylated pentenyl arabinosides that could be easily purified by silica gel column chromatography. Deacetylation under basic conditions finally gave the target arabinosyl acceptors **1**, **2**, and **3** in good overall yields.

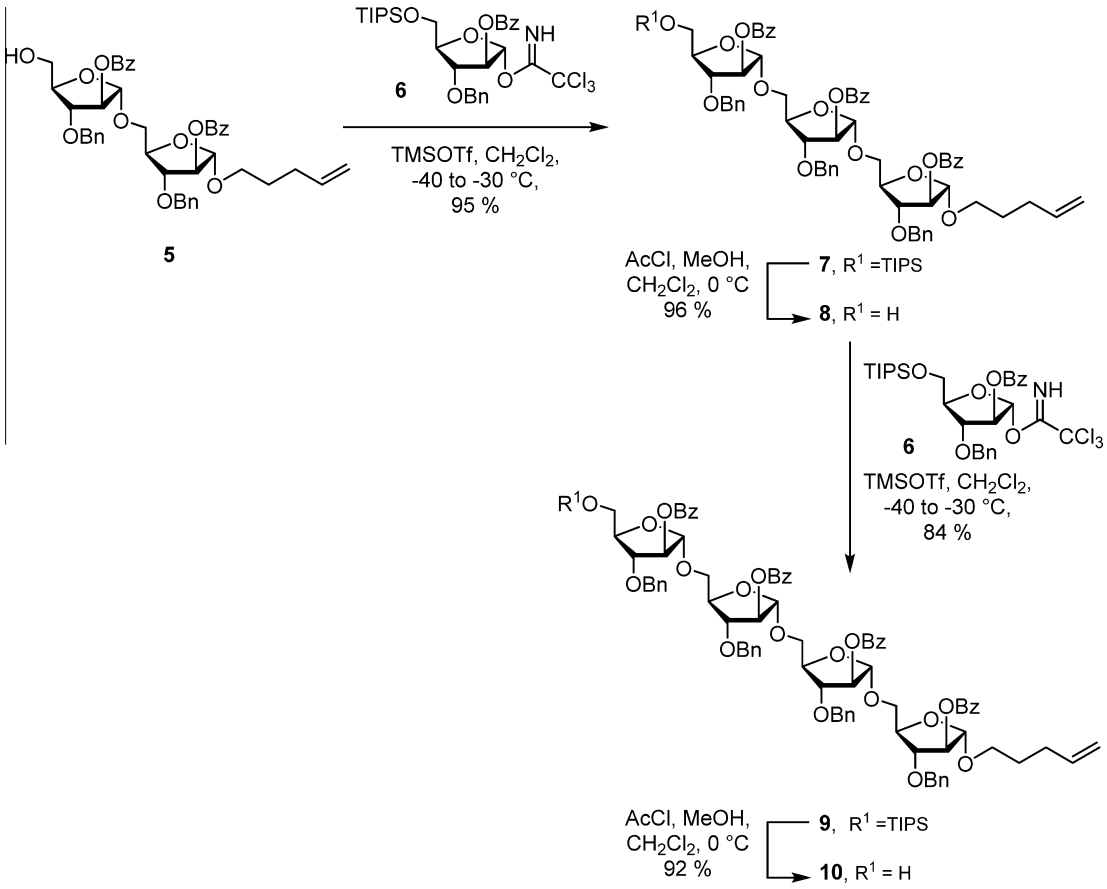
2.2. Enzymatic assays using pentenyl aglycon acceptors

Compounds **1**, **2**, and **3** (Fig. 2) with pentenyl aglycon were tested for their ability to function as acceptors in a cell-free assay using p[^{14}C]Rpp (that was converted to DPA in situ) as an indirect arabinosyl donor.[16] After a typical reaction,[25] products were extracted with ethanol and unincorporated p[^{14}C]Rpp removed by passing through an anion exchange column (SAX). The eluate from the SAX column was dried and the residue was partitioned between 1-butanol and water. The 1-butanol layer was dried and the residue was applied to TLC. TLC analysis revealed formation of radioactive products (lanes 1′, 2′, and 3′ in Fig. 3A), that migrated slower compared to the corresponding acceptor (lanes 1, 2, and 3 in Fig. 3A).

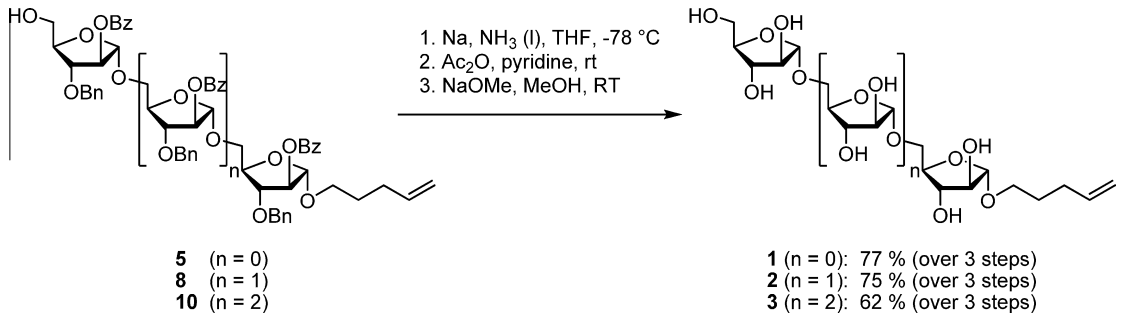
To confirm that [^{14}C]-arabinose had been added to the acceptor, an aliquot of the material extracted with 1-butanol (2000 dpm) was hydrolyzed with 2 M TFA and the hydrolysate was subjected to TLC. Autoradiography showed that the radioactivity was exclusively associated with arabinose (Fig. 3B) indicating that one extra Ara*f* was transferred from DPA to the acceptors used in this study.

When the disaccharide acceptor **1** was used (lane 1 of Fig. 3), the band corresponding to the biosynthetic product β-D-Ara*f*-(1→?)-α-D-Ara*f*-(1→5)-α-D-Ara*f*-pentenyl (lane 1′ of Fig. 3A) migrated slower than the trisaccharide acceptor **2**, α-D-Ara*f*-(1→5)-α-D-Ara*f*-(1→5)-α-D-Ara*f*-pentenyl (lane 2 of Fig. 3A) indicating that the enzymatic product was different and did not incorporate additional α-(1→5) Ara*f* onto the acceptor. Linkage analysis on the enzymatic product revealed the presence of *t*-Ara*f*, *2*-Ara*f* and a *5*-Ara*f* (Fig. 1S).

Therefore, combining the previous data that showed both α-(1→5) and β-(1→2) containing trisaccharide products were formed when α-D-Ara*f*-(1→5)-α-D-Ara*f*-octyl was used as an acceptor,[11] the disaccharide acceptor with pentenyl aglycon showed more specific recognition of AftB arabinosyltransferase. Only the product containing β-(1→2)-Ara*f* formed in the cell-free assay.



Scheme 1.



Scheme 2.

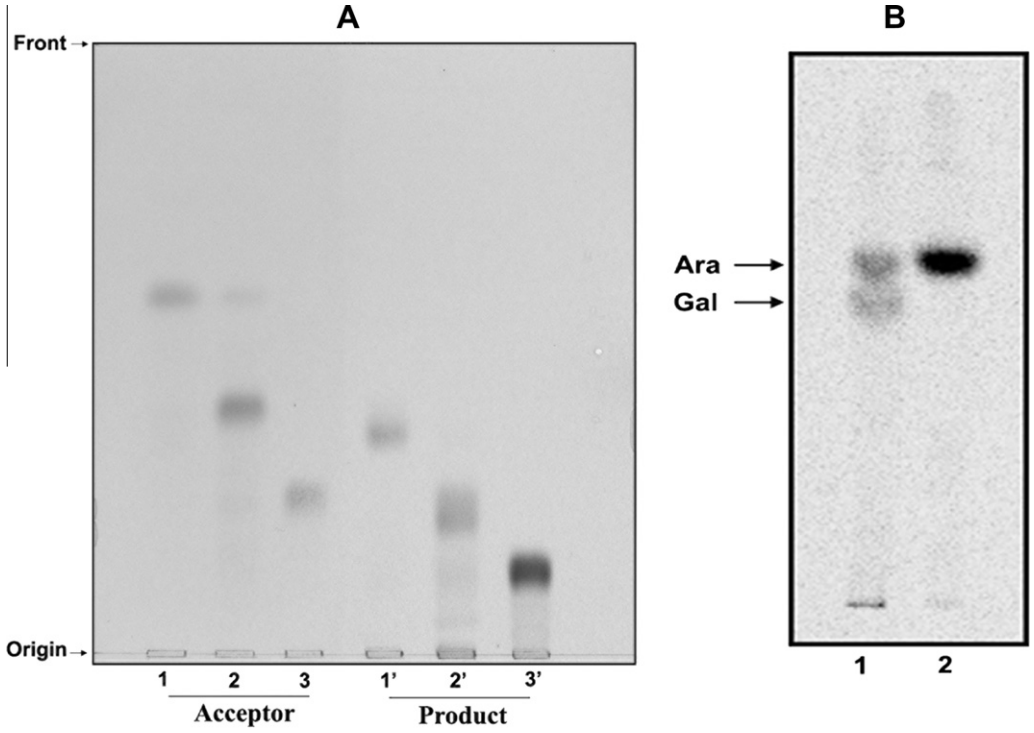


Figure 3. (A) TLC analysis of biosynthetic products from enzymatic reaction with acceptors **1**, **2**, and **3**. Acceptors were visualized after spraying with α-naphthol (lanes 1, 2, and 3), and the radioactive products were revealed by autoradiography (lanes 1′, 2′, and 3′). An aliquot of the product labeled with p[^{14}C]Rpp from the eluate after the SAX column followed by 1-butanol extraction was applied to TLC plate, developed in $CHCl_3$/MeOH/NH_4OH/H_2O (65:25:0.4:3.6) and exposed for four days. The migration of the enzymatically synthesized products was slower than the corresponding acceptors. (B) Radioactive AG (lane1) and the enzymatic product from acceptor **1** (lane 2) were hydrolyzed with 2 M TFA, and applied to TLC. Chromatogram was developed in pyridine/ethyl acetate/acetic acid/water (5:5:1:3) and subjected to autoradiography.

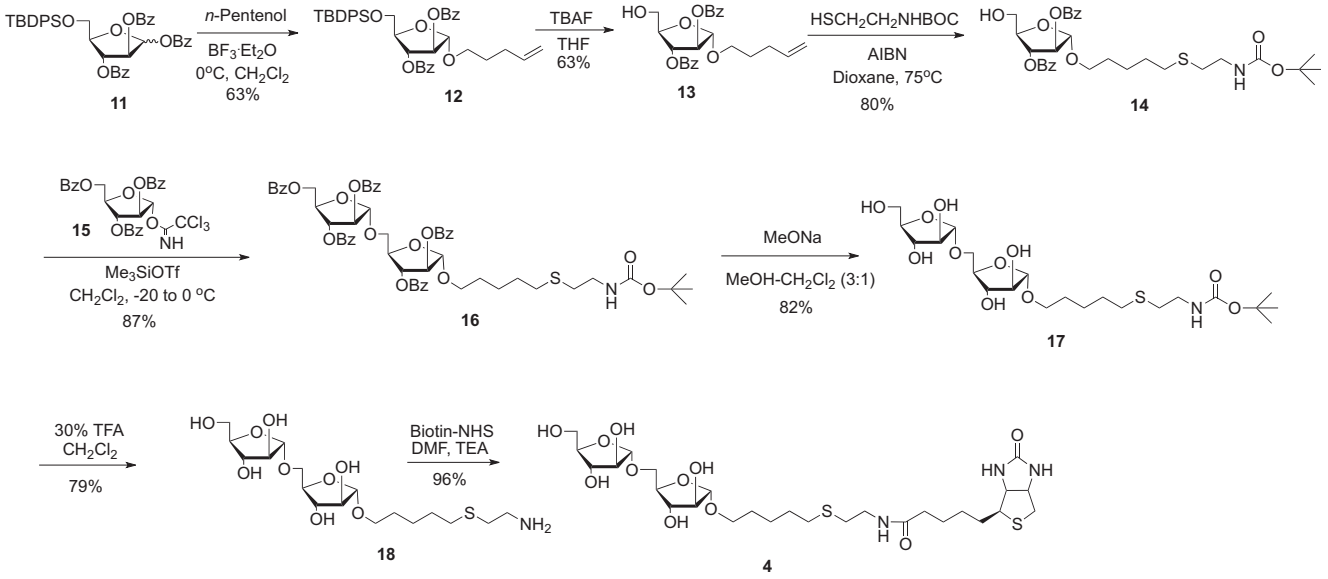
For trisaccharide acceptor **2** (lane 2 of Fig. 3A), the major β-(1→2) and minor α-(1→5) product (tetrasaccharides) was formed per TLC (lane 2′ of Fig. 3A) analysis that showed that the acceptor is recognized by both AftB and an α-1,5 AraT in comparison to acceptor **1**. Tetrasaccharide acceptor **3** yielded a pentasaccharide (lane 3′ of Fig. 3). We reasoned that this substrate should not be recognized by AftB since a similar four Ara*f* linear stretch is not present in AG. The enzymatic product was isolated, digested with *Cellulomonas endo*-arabinanase[25] and the product formed was examined by Dionex High-Performance Anion Exchange Chromatography (HPAEC). The product formed after the enzyme digestion co-chromatographed with a synthetic tetrasaccharide-α-Ara*f*-[(1→3,5)]$_2$-α-Ara*f*-(1→5)-α-Ara*f*, suggesting AftC α-(1→3) branching AraT of acceptor **3** (Fig. 2S).

2.3. Chemical synthesis of biotinylated acceptor (4)

During the chemical synthesis of the biotinylated acceptor (Scheme 3), an *n*-pentenyl group was introduced using building block **11**[25,26] as a functional spacer for further synthesis.[27] The *n*-pentenyl glycoside **12** was deprotected at 5-OH to obtain **13**. Radical elongation with 2-[(*tert*-butoxycarbonyl)amino]-1-ethanethiol in the presence of AIBN at 75 °C formed sulfide spacer glycoside **14** in high yield (81%). Monosaccharide building block **14** was coupled with perbenzoylated α-D-arabinofuranosyl trichloroacetimidate (**15**)[28] to afford disaccharide **16** (87%).[29] After deprotection of **16** by MeONa/MeOH and 30% TFA to remove benzoyl and BOC groups, respectively, compounds **17** and **18** were obtained. The spacer glycoside **18** could be linked with a commercial *N*-hydroxysuccinimide ester reagent to form the biotinylated acceptor **4**.[30]

2.4. Enzymatic assays using biotinylated acceptor (4)

The biotinylated acceptor **4**, *S*-D-Biotinoyl-5-(2-amino-1-thioethyl)-pentyl 5-*O*-(α-D-arabinofuranosyl)-α-D-arabinofuranoside, was used in the arabinosyltransferase assay with a similar procedure as described for acceptors **1–3**. The major enzymatic product



Scheme 3. Chemical synthesis of the biotinylated acceptor (**4**).

was β-(1→2) Ara*f* (Band I in Fig. 4A) and minor with α-(1→?) Ara*f* (Band II in Fig. 4A), both of the products were shown to contain [^{14}C]Ara*f* by TFA hydrolysis (Fig. 4B). In a separate experiment, on a streptavidin coated plate, when biotinylated acceptor **4**, crude mycobacterial cell lysate and pRpp, followed by monoclonal antibody CS35 was added sequentially, positive color reaction was obtained after adding the developing reagent. CS35 is known to recognize β-Ara*f*-(1→2)-α-Ara*f*.[31–33] Positive reaction of the enzymatic product to CS35 indicated that proper trisaccharide was formed with the acceptor **4.** However, since ELISA typically involves several washing steps not favoured in assay, we moved on to developing the assay for screening purpose.

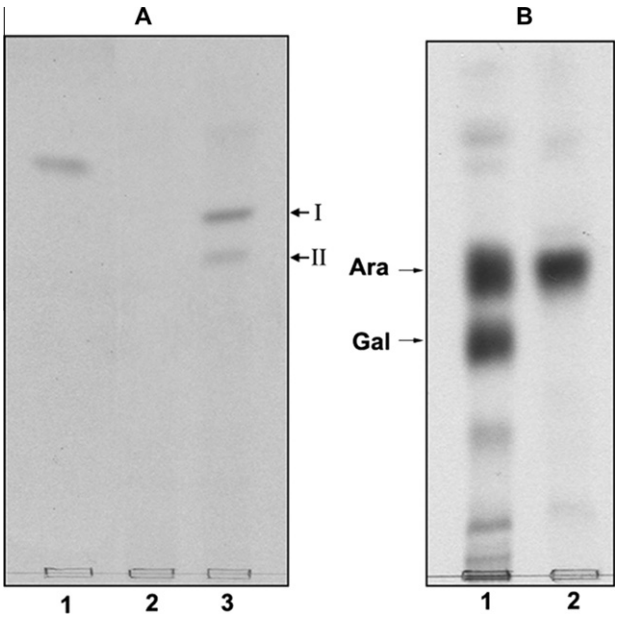


Figure 4. Enzymatic assays using the biotinylated acceptor **4**. (A) TLC analysis of the biosynthetic products from biotinylated acceptor **4**. Acceptor **4** was visualized by α-naphthol (lane 1); the radioactive reactions without (lane 2) or with (lane 3) the acceptor were detected by autoradiography. (B) Radioactive AG (lane1) and the products (lane 2) were hydrolyzed with 2 M TFA, and applied to TLC.

2.5. Developing SPA assay for AftB

A schematic representation of a scintillation proximity assay (SPA) for AftB enzyme is illustrated in Figure 5A. In this assay, AftB catalyzes the addition of a ^{3}H-labeled Ara*f* to the biotinylated acceptor **4** to generate biotinylated trisaccharide product with β-(1→2) Ara*f* residue. Once the streptavidin-coated SPA beads are added to the reaction mixture, this ^{3}H-labeled biotinylated product is captured by the SPA beads as a result of the specific biotin/streptavidin binding.[34] Thus, the ^{3}H is put to proximity of the scintillant impregnated within the bead. Such proximity results in a β-emission that can be readily detected in MicroBeta scintillation count plate reader. The unreacted p[^{3}H] Rpp and its degradation products (DPPR, DPR, DPA, etc.) remain in solution and are sufficiently distant from the scintillant so that no significant signal is observed. Therefore, the SPA technology enables assays to be carried out without the intermediate purification steps and can be used directly for microtitreplate assay.

A typical SPA assay consisted of incubating biotinylated acceptor **4**, radio-labeled p[^{3}H]Rpp, and membrane/P60 enzyme source for a specific amount of time. P60 is the cell wall enriched particulate fraction which improved the efficiency of pRpp transformation to DPA. The resulting reaction was terminated with addition of ethanol and SPA beads were incubated overnight with reaction mixture. Typically, the assay reaction with acceptors resulted in about 150 cpm more counts than the control reaction (reaction without addition of biotinylated acceptor (**4**)). In this assay, both the radio-labeled biotinylated product and unlabeled acceptor (**4**) can be bound to the beads. A maximal SPA signal should be obtained when the biotin-binding capacity of SPA beads exceeds the amount of acceptor used in a typical assay. This prediction was investigated by determining the optimal relationship between the SPA signal and the SPA beads quantity (Fig. 5B). A maximal SPA signal was obtained when the beads quantity was 1.0 mg/assay. Another variable in the SPA assay was the amount of the enzyme used. As shown in Figure 5C, the maximal SPA signal was achieved at enzyme concentrations of 0.4 mg/assay (membrane/P60, 5:3). In contrast, SPA assays conducted with enzyme quantity more than 0.4 mg resulted in significant incomplete binding of the radio-labeled product since the surface of SPA beads was covered by the P60 particles that blocked biotin/streptavidin binding. Therefore,

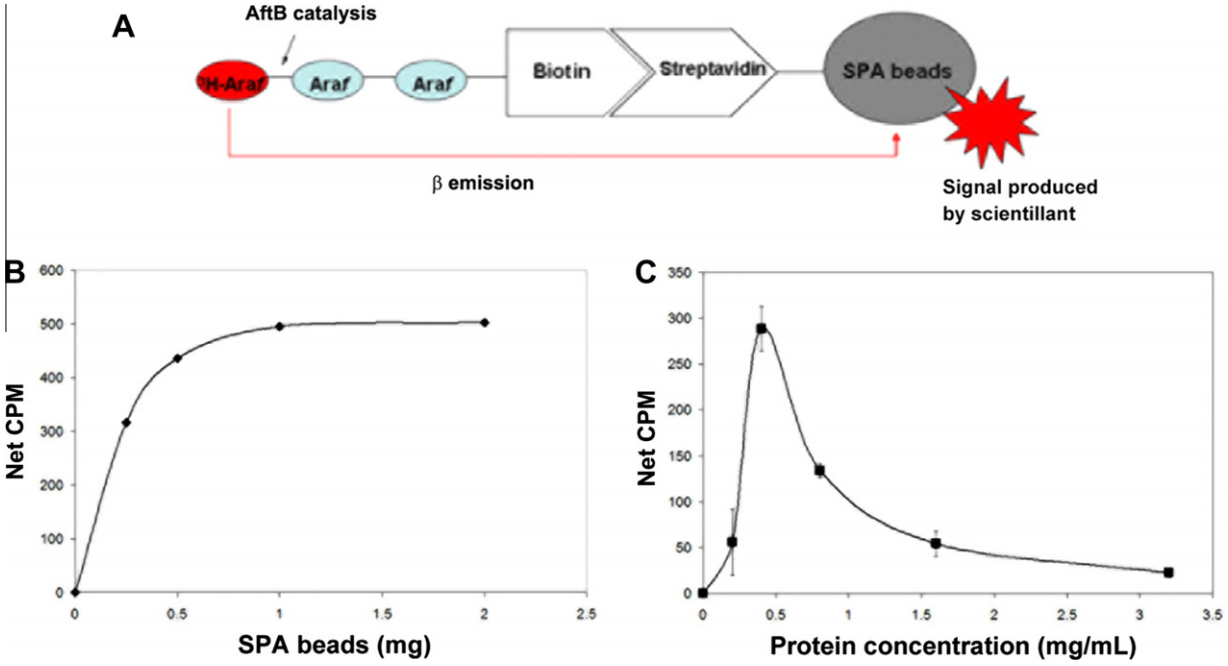


Figure 5. The SPA assay using p[^{3}H]Rpp. (A) Schematic representation of the principle of scintillation proximity assay (SPA) targeted at AftB. (B) Dependence of scintillation signals on SPA beads concentration. The concentration of acceptor **4** was 300 μM in the assay. The data was expressed as net CPM after subtracting the background (reaction without adding SPA beads). (C) Effect of protein concentrations on net SPA signals after subtracting the background (reaction without membranes). The enzyme used in the assay was a mixture of membrane and P60 at the ratio of 5:3.

the radioactive product formed was not proportional to the amount of enzyme used in the assay.

In order to improve the homogeneity in the system and increase efficiency of the assay, DP[^{3}H]A was used in the reaction instead of p[^{3}H]Rpp to avoid the incomplete binding of enzymatic product to SPA beads. Igepal CA-630 (Sigma) was used as a detergent in this assay to enhance DP[^{3}H]A solubility. Compared to the assay using p[^{3}H]Rpp, DP[^{3}H]A assay had a more homogenous appearance and P60 was eliminated from the assay resulting in higher efficiency of biotin/streptavidin binding. A variable quantity of membranes as the enzyme source was used in the assay, and as can be seen in Figure 6A, the addition of increasing amounts of enzyme up to 0.5 mg of membranes resulted in a proportional increase in product formation. The product formation achieved a saturation starting at the point of using 0.5 mg of membranes and the reaction rate arrived at the maximum when using 1.0 mg of membranes in the reaction. An optimal assay should include approximately 0.5 mg of membrane proteins. The kinetic parameter of AftB was investigated using different concentrations of the substrate (acceptor **4**). As shown in Figure 6B, the apparent K_m for acceptor **4** was found to be 40.9 μM when 0.5 mg of membranes were used in assay.

On the basis of the SPA assay developed to measure the activity of KasA and KasB enzymes in mycolic acid biosynthesis in *M. tuberculosis*[22] we believe that the assay described in this work could be transformed into a fast, sensitive, and plate-based assay for screening of inhibitors against AraT activities. Here, we have developed a SPA-assay to evaluate an important and essential AraT (AftB) that is functional in synthesizing β-(1→2) Ara*f*, a terminal Ara*f* required to tether mycolic acid in cell wall. The method has potential for developing into assay for screening of inhibitors.

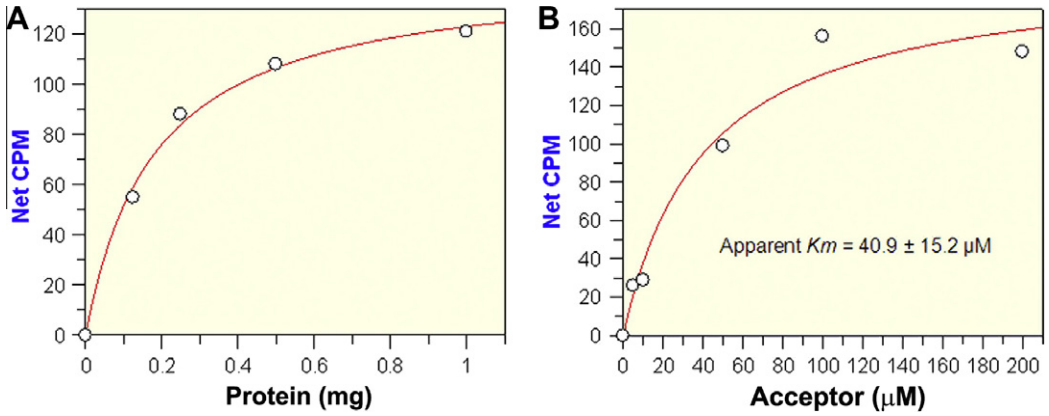


Figure 6. The SPA assay using DP[^{3}H]A. (A) Dependence of signal generation on enzyme amounts (0, 0.125, 0.25, 0.5, 1.0 mg). (B) Effect of acceptor **4** concentration (0, 5, 10, 50, 100, 200 μM) on the formation of enzymatic product captured by SPA beads. The data obtained was subjected to analysis using GraFit 5.0, and expressed as net CPM after subtracting the background (reaction without enzyme or acceptor **4**).

3. Experimental procedures

3.1. General methods

Radioactive p[^{14}C]Rpp (300 mCi/mmol) and p[^{3}H]Rpp (60 mCi/mmol) were generated from uniformly labeled D-[^{14}C]glucose and D-[^{3}H]glucose, respectively, (American Radiolabeled Chemicals Inc.) as described.[15] Streptavidin-coated polyvinyl toluene SPA scintillation beads were purchased from GE-Healthcare. Protein concentrations were determined using the BCA assay (Pierce). All chemicals were purchased from Sigma/Aldrich and were used without further purification. Reagent grade dichloromethane (CH_2Cl_2), tetrahydrofuran (THF), and toluene were passed through an activated neutral alumina column prior to use. Reagent grade methanol (MeOH) was dried over activated molecular sieves prior to use. Pyridine and triethylamine (Et_3N) were distilled over CaH_2 prior to use. Thin layer chromatography (TLC) was performed on silica gel G60 aluminium-backed plates (Merck). Column chromatography was performed using silica gel (70–230 mesh). Unless otherwise stated, all reactions were performed at room temperature under nitrogen or argon. Glycosylation reactions were performed in oven-dried glassware under an inert atmosphere. Drying after organic extractions was done over anhydrous Na_2SO_4. ^{1}H NMR spectra were recorded at 300 or 400 MHz and ^{13}C NMR spectra were recorded at 75 or 100 MHz.

3.2. Chemical synthesis of acceptors 1, 2, and 3

3.2.1. Pent-4-enyl 5-*O*-(5-*O*-(2-*O*-benzoyl-3-*O*-benzyl-5-*O*-triisopropysilyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranoside (7)

Disaccharide **5**[23,24] (0.330 g, 0.447 mmol) and imidate **6**[23] (0.390 g, 0.605 mmol) were combined, azeotroped with toluene (5 × 20 mL) and dried in vacuo overnight. The mixture was dissolved in anhydrous CH_2Cl_2 (12 mL), cooled to −40 °C and TMSOTf (0.012 mL, 0.063 mmol) was added. After 30 min at −40 to −30 °C, the reaction was quenched by addition of Et_3N, the solution was stirred for 10 min at room temperature, and the solvents were removed in vacuo. Purification by flash silica gel column chromatography (pure toluene to toluene/EtOAc, 50:1) yielded trisaccharide **7** (0.520 g, 95%) as a colorless oil; R_f = 0.15 (hexanes/EtOAc, 7:1); $[\alpha]_D$ +78.9 (*c* 0.90, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.08–8.00 (m, 6H), 7.59–7.51 (m, 3H), 7.44–7.39 (m, 6H), 7.37–7.17 (m, 15H), 5.89–5.76 (m, 1H), 5.47 (d, *J* = 1.4 Hz, 1H), 5.43–5.42 (m, 2H), 5.31 (s, 1H), 5.27 (s, 1H), 5.16 (s, 1H), 5.07–4.94 (m, 2H), 4.81 (d, *J* = 12.1 Hz, 1H), 4.65 (d, *J* = 12.1 Hz, 1H), 4.64 (d, *J* = 12.1 Hz, 1H), 4.62 (d, *J* = 12.1 Hz, 1H), 4.51 (d, *J* = 12.1 Hz, 1H), 4.48 (d, *J* = 12.1 Hz, 1H), 4.39–4.34 (m, 1H), 4.26–4.14 (m, 3H), 4.09 (s, 2H), 3.96 (dd, *J* = 4.1, 11.2 Hz, 1H), 3.90 (dd, *J* = 3.9, 11.2 Hz, 1H), 3.82–3.68 (m, 5H), 3.53–3.44 (m, 1H), 2.20–2.13 (m, 2H), 1.78–1.69 (m, 2H), 1.08–0.94 (m, 21H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.4, 165.1, 165.1, 138.1, 137.8, 137.7, 133.2, 133.1, 129.7, 129.6, 129.4, 128.4, 128.2, 128.2, 128.1, 127.7, 127.6, 127.5, 127.4, 127.4, 114.7, 106.0, 105.9, 83.9, 83.2, 83.2, 82.7, 82.0, 81.9, 81.8, 81.7, 81.4, 72.2, 72.0, 71.8, 66.6, 65.5, 65.2, 62.5, 30.1, 28.5, 17.8, 11.7. IR ($CHCl_3$): ν_{max} 2944, 1718, 1451, 1292, 1267, 1113, 1067, 1021 cm^{-1}. MALDI-HRMS: *m/z* (M+Na)$^+$ calcd for $C_{71}H_{84}NaO_{16}Si$: 1243.5426, obsd 1243.5439.

3.2.2. Pent-4-enyl 5-*O*-(5-*O*-(2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranoside (8)

A solution of **7** (0.520 g, 0.426 mmol) in anhydrous MeOH/CH_2Cl_2 (4:1, 10 mL) was cooled to 0 °C and acetyl chloride (0.30 mL, 4.2 mmol) was added. After 4 h at 0 °C, the reaction was quenched by addition of saturated aqueous $NaHCO_3$-solution (15 mL). The aqueous layer was extracted with CH_2Cl_2 (4 × 30 mL). The combined organic extracts were washed with brine (50 mL), dried with Na_2SO_4, filtered and evaporated. Purification by flash silica gel column chromatography (hexanes/EtOAc, 3:1) yielded **8** (0.434 g, 96%) as a colorless oil; R_f = 0.15 (hexanes/EtOAc, 3:1); $[\alpha]_D$ +103.1 (*c* 1.2, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.07–7.98 (m, 6H), 7.61–7.52 (m, 3H), 7.46–7.17 (m, 21H), 5.89–5.76 (m, 1H), 5.45 (d, *J* = 1.3 Hz, 1H), 5.40 (d, *J* = 1.6 Hz, 1H), 5.36 (d, *J* = 1.3 Hz, 1H), 5.30 (s, 1H), 5.25 (s, 1H), 5.14 (s, 1H), 5.07–4.93 (m, 2H), 4.80 (d, *J* = 12.1 Hz, 1H), 4.67 (d, *J* = 12.1 Hz, 1H), 4.63 (d, *J* = 11.7 Hz, 1H), 4.59 (d, *J* = 11.7 Hz, 1H), 4.51 (d, *J* = 12.1 Hz, 1H), 4.43 (d, *J* = 12.1 Hz, 1H), 4.37–4.33 (m, 1H), 4.24–4.20 (m, 1H), 4.19–4.16 (m, 1H), 4.12–4.10 (m, 1H), 4.04–4.00 (m, 1H), 3.97–3.92 (m, 2H), 3.85 (dd, *J* = 4.0, 11.3 Hz, 1H), 3.80–3.68 (m, 4H), 3.57–3.45 (m, 2H), 2.19–2.12 (m, 2H), 1.77–1.68 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.5, 165.3, 165.2, 138.2, 137.8, 137.6, 133.4, 133.3, 133.3, 129.8, 129.7, 129.5, 129.5, 129.4, 128.5, 128.3, 128.3, 127.8, 127.7, 127.7, 127.6, 127.5, 114.8, 106.2, 106.1, 106.0, 83.3, 83.2, 82.7, 82.1, 82.0, 81.7, 81.4, 72.3, 72.1, 66.8, 65.6, 65.5, 61.9, 30.2, 28.6. IR ($CHCl_3$): ν_{max} 2922, 1720, 1451, 1311, 1296, 1265, 1109, 1068, 1026 cm^{-1}. MALDI-HRMS: *m/z* (M+Na)$^+$ calcd for $C_{62}H_{64}NaO_{16}$: 1087.4092, obsd 1087.4104.

3.2.3. Pent-4-enyl 5-*O*-(5-*O*-(5-*O*-(2-*O*-benzoyl-3-*O*-benzyl-5-*O*-triisopropysilyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranoside (9)

Trisaccharide **8** (0.490 g, 0.460 mmol) and imidate **6**[23] (0.386 g, 0.598 mmol) were combined, azeotroped with toluene (5 × 10 mL) and dried in vacuo overnight. The mixture was dissolved in anhydrous CH_2Cl_2 (10 mL), cooled to −40 °C and TMSOTf (0.012 mL, 0.63 mmol) was added. After 45 min at −40 to −30 °C, the reaction was quenched by addition of Et_3N, the solution was stirred for 20 min at room temperature and the solvents were removed in vacuo. Purification by flash silica gel column chromatography (hexanes/EtOAc, 8:1 to 7:1) yielded tetrasaccharide **9** (0.432 g, 94%) as a colorless oil; R_f = 0.10 (hexanes/EtOAc, 7:1); $[\alpha]_D$ +88.9 (*c* 0.83, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.04–7.97 (m, 8H), 7.60–7.50 (m, 4H), 7.44–7-14 (m, 28H), 5.87–5.74 (m, 1H), 5.42 (d, *J* = 1.3 Hz, 1H), 5.38–5.36 (m, 3H), 5.26 (s, 1H), 5.23 (s, 1H), 5.20 (s, 1H), 5.12 (s, 1H), 5.05–4.91 (m, 2H), 4.77 (d, *J* = 12.1 Hz, 1H), 4.62 (d, *J* = 12.1 Hz, 1H), 4.60 (d, *J* = 12.1 Hz, 1H), 4.57 (d, *J* = 12.2 Hz, 1H), 4.55 (d, *J* = 12.1 Hz, 1H), 4.48 (d, *J* = 12.2 Hz, 1H), 4.44 (d, *J* = 12.1 Hz, 1H), 4.42 (d, *J* = 12.1 Hz, 1H), 4.34–4.29 (m, 1H), 4.20–4.00 (m, 7H), 3.91 (dd, *J* = 4.1, 11.3 Hz, 1H), 3.87–3.60 (m, 8H), 3.47 (td, *J* = 6.6, 9.6 Hz, 1H), 2.17–2.10 (m, 2H), 1.76–1.66 (m, 2H), 1.04–0.93 (m, 21H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.2, 165.0, 164.9, 164.9, 137.9, 137.6, 137.6, 137.5, 133.1, 133.0, 129.6, 129.5, 129.3,129.3, 128.2, 128.1, 128.1, 128.0, 128.0, 127.6, 127.5, 127.5, 127.4, 127.4, 127.3, 127.3, 127.2, 114.6, 105.9, 105.8, 83.9, 83.1, 83.1, 83.0, 82.6, 82.0, 81.9, 81.8, 81.7, 81.6, 81.4, 72.2, 72.0, 71.9, 71.8, 66.6, 65.5, 65.3, 65.1, 62.5, 30.2, 28.6, 17.9, 11.8. IR ($CHCl_3$): ν_{max} 3005, 2944, 2862, 1718, 1446, 1292, 1267, 1113, 1072 cm^{-1}. MALDI-HRMS: *m/z* (M+Na)$^+$ calcd for $C_{90}H_{102}NaO_{21}Si$: 1569.6581, obsd 1569.6546.

3.2.4. Pent-4-enyl 5-*O*-(5-*O*-(5-*O*-(2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranosyl)-2-*O*-benzoyl-3-*O*-benzyl-α-D-arabinofuranoside (10)

A solution of **9** (0.310 g, 0.200 mmol) in anhydrous MeOH/CH_2Cl_2 (4:1, 5 mL) was cooled to 0 °C and acetyl chloride (0.14 mL, 1.9 mmol) was added. The mixture was stirred for 6 h, while slowly warming to room temperature. The reaction was

quenched by addition of saturated aqueous $NaHCO_3$-solution (20 mL). The aqueous layer was extracted with CH_2Cl_2 (4 × 40 mL). The combined organic extracts were dried with Na_2SO_4, filtered and evaporated. Purification by flash silica gel column chromatography (hexanes/EtOAc, 3:1–2:1) yielded **10** (0.255 g, 92%) as a colorless solid; mp 42–44 °C; R_f = 0.30 (hexanes/EtOAc, 3:2); $[\alpha]_D$ +115.8 (*c* 0.52, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 8.09–8.00 (m, 8H), 7.62–7.54 (m, 4H), 7.48–7.40 (m, 8H), 7.38–7.20 (m, 20H), 5.91–5.78 (m, 1H), 5.47 (d, *J* = 1.3 Hz, 1H), 5.44–5.43 (m, 2H), 5.38 (d, *J* = 1.3 Hz, 1H), 5.31 (s, 1H), 5.29 (s, 1H), 5.25 (s, 1H), 5.16 (s, 1H), 5.09–4.95 (m, 2H), 4.81 (d, *J* = 12.2 Hz, 1H), 4.70–4.43 (m, 7H), 4.39–4.35 (m, 1H), 4.28–4.11 (m, 5H), 4.07–4.03 (m, 1H), 3.99–3.68 (m, 9H), 3.60–3.46 (m, 2H), 2.22–2.14 (m, 2H), 1.82–1.70 (m, 3H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 165.5, 165.2, 165.2, 165.1, 138.2, 137.8, 137.8, 137.5, 133.4, 133.2, 129.7, 129.6, 129.5, 129.5, 129.4, 128.4, 128.3, 128.2, 127.8, 127.7, 127.6, 127.6, 127.5, 114.7, 106.2, 106.1, 106.0, 83.3, 83.2, 82.8, 82.2, 82.0, 81.8, 81.5, 72.3, 72.1, 72.1, 72.0, 66.7, 65.7, 65.6, 61.9, 30.2, 28.6. IR ($CHCl_3$): ν_{max} 2926, 1722, 1602, 1452, 1316, 1298, 1268, 1114, 1071, 1028 cm^{-1}. MALDI-HRMS: *m/z* (M+Na)$^+$ calcd for $C_{81}H_{82}NaO_{21}$: 1413.5246, obsd 1413.5264.

3.2.5. Pent-4-enyl 5-*O*-(α-D-arabinofuranosyl)-α-D-arabinofuranoside (1)

Disaccharide **5**[23,24] (0.060 g, 0.081 mmol) was dissolved in anhydrous THF (5 mL) under argon, cooled to −78 °C and ammonia (about 20 mL) was condensed into the flask. Small pieces of sodium (washed with *n*-pentane) were then added to the solution until the color of the reaction mixture turned dark blue. After 40 min at −78 °C, MeOH was added until the blue color disappeared. Small pieces of sodium were again added to the mixture until the blue color of the solution persisted. After 50 min at −78 °C, the reaction was quenched by addition of MeOH (3 mL), the cooling bath was removed and the ammonia was evaporated using a stream of nitrogen. The solution was diluted with MeOH (5 mL), neutralized with Amberlite IR-120 and filtered. The resin was washed with MeOH and the solution was concentrated in vacuo. The residue was dissolved in pyridine (3 mL) and acetic anhydride (3 mL) and the solution was stirred at room temperature for 18 h. The solvents were evaporated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc, 3:1–2:1) to give the peracetylated disaccharide (0.039 g, 86% over two steps) as a colorless oil; R_f = 0.10 (hexanes/EtOAc, 2:1); $[\alpha]_D$ +94.2 (*c* 0.33, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.86–5.72 (m, 1H), 5.15–5.12 (m, 3H), 5.03–4.91 (m, 5H), 4.43 (dd, *J* = 3.0, 11.5 Hz, 1H), 4.29–4.13 (m, 3H), 3.90 (dd, *J* = 4.1, 11.5 Hz, 1H), 3.73–3.61 (m, 2H), 3.43 (td, *J* = 6.3, 9.6 Hz, 1H), 2.14–2.05 (m, 17H), 1.71–1.62 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 170.2, 170.1, 169.8, 169.4, 138.0, 114.9, 105.5, 105.5, 81.9, 80.9, 80.8, 66.8, 65.5, 63.3, 30.1, 28.6, 20.7, 20.6. IR ($CHCl_3$): ν_{max} 3026, 2923, 1739, 1364, 1113, 1062, 1048, 974 cm^{-1}. MALDI-HRMS: *m/z* (M+Na)$^+$ calcd for $C_{25}H_{36}NaO_{14}$: 583.2003, obsd 583.1987.

The peracetylated disaccharide (0.036 g, 0.064 mmol) was dissolved in anhydrous MeOH (1.3 mL) and 0.5 M NaOMe in MeOH (0.13 mL, 0.065 mmol) was added. The mixture was stirred at room temperature for 24 h and then neutralized with Amberlite IR-120. The mixture was filtered, the resin washed with MeOH and the solvents were evaporated. Purification by Sephadex G-10 column chromatography (H_2O/EtOH, 1:1) gave **1** (0.020 g, 89%) as a colorless solid; R_f = 0.60 (MeOH/EtOAc, 1:4); $[\alpha]_D$ +103.2 (*c* 1.0, MeOH). ^{1}H NMR (300 MHz, CD_3OD): δ 5.90–5.77 (m, 1H), 5.06–4.93 (m, 2H), 4.95 (d, *J* = 1.4 Hz, 1H), 4.84 (d, *J* = 1.5 Hz, 1H), 4.03–3.61 (m, 16H), 3.43 (td, *J* = 6.5, 9.5 Hz, 1H), 2.18–2.10 (m, 2H), 1.72–1.64 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD): δ 139.4, 115.2, 109.6, 109.5, 85.9, 83.6, 83.5, 83.1, 79.1, 78.8, 68.2, 68.1, 63.1, 31.4, 30.0. ESI-HRMS: *m/z* (M+Na)$^+$ calcd for $C_{15}H_{26}NaO_9$: 373.1475, obsd 373.1467.

3.2.6. Pent-4-enyl 5-*O*-(5-*O*-(α-D-arabinofuranosyl)-α-D-arabinofuranosyl)-α-D-arabinofuranoside (2)

Trisaccharide **8** (0.055 g, 0.052 mmol) was dissolved in anhydrous THF (4.5 mL) under argon, cooled to −78 °C and ammonia (about 20 mL) was condensed into the flask. Small pieces of sodium (washed with *n*-pentane) were then added to the solution until the color of the reaction mixture turned dark blue. After 35 min at −78 °C, MeOH was added until the blue color disappeared. Small pieces of sodium were again added to the mixture until the blue color of the solution persisted. After 30 min at −78 °C, the reaction was quenched by addition of MeOH (3 mL), the cooling bath was removed and the ammonia was evaporated using a stream of nitrogen. The solution was diluted with MeOH (5 mL), neutralized with Amberlite IR-120 and filtered. The resin was washed with MeOH and the solution was concentrated in vacuo. The residue was dissolved in pyridine (2 mL) and acetic anhydride (1.5 mL) and the solution was stirred at room temperature for 20 h. The solvents were evaporated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc, 1:1) to give the peracetylated trisaccharide (0.037 g, 92% over two steps) as a colorless oil; R_f = 0.15 (hexanes/EtOAc, 1:1); $[\alpha]_D$ +79.6 (*c* 0.67, $CHCl_3$). ^{1}H NMR (300 MHz, $CDCl_3$): δ 5.86–5.72 (m, 1H), 5.14–5.11 (m, 6H), 5.03–4.91 (m, 5H), 4.44 (dd, *J* = 2.7, 11.3 Hz, 1H), 4.28–4.13 (m, 4H), 3.95–3.87 (m, 2H), 3.74–3.63 (m, 3H), 3.43 (td, *J* = 6.2, 9.7 Hz, 1H), 2.15–2.06 (m, 23H), 1.72–1.63 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.5, 170.2, 170.1, 170.1, 169.8, 169.6, 169.3, 138.0, 114.9, 105.5, 105.4, 81.9, 81.5, 81.5, 80.9, 80.9, 66.8, 65.5, 65.3, 63.2, 30.1, 28.6, 20.7, 20.6, 20.6. IR ($CHCl_3$): ν_{max} 3011, 2928, 1742, 1372, 1113, 1065, 973 cm^{-1}. MALDI-HRMS: *m/z* (M+Na)$^+$ calcd for $C_{34}H_{48}NaO_{20}$: 799.2637, obsd 799.2617.

The peracetylated trisaccharide (0.033 g, 0.042 mmol) was dissolved in anhydrous MeOH (1 mL) and 0.5 M NaOMe in MeOH (0.084 mL, 0.042 mmol) was added. The mixture was stirred at room temperature for 13 h and then neutralized with Amberlite IR-120. The mixture was filtered, the resin washed with MeOH and the solvents were evaporated. Purification by Sephadex G-10 column chromatography (H_2O/EtOH, 1:1) gave **2** (0.0166 g, 81%) as a colorless solid; R_f = 0.20 (CH_2Cl_2/MeOH, 4:1); $[\alpha]_D$ +94.2 (*c* 0.83, MeOH). ^{1}H NMR (300 MHz, CD_3OD): δ 5.90–5.76 (m, 1H), 5.06–4.92 (m, 2H), 4.94–4.93 (m, 2H), 4.84 (s, 1H), 4.10–3.55 (m, 23H), 3.42 (td, *J* = 6.5, 9.7 Hz, 1H), 2.17–2.09 (m, 2H), 1.72–1.63 (m, 2H). ^{13}C NMR (75 MHz, CD_3OD): δ 139.4, 115.3, 109.7, 109.6, 109.5, 85.9, 84.1, 83.7, 83.5, 83.2, 83.1, 79.2, 79.1, 78.8, 68.2, 68.1, 63.1, 31.4, 30.0. ESI-HRMS: *m/z* (M+Na)$^+$ calcd for $C_{20}H_{34}NaO_{13}$: 505.1897, obsd 505.1898.

3.2.7. Pent-4-enyl 5-*O*-(5-*O*-(5-*O*-(α-D-arabinofuranosyl)-α-D-arabinofuranosyl)-α-D-arabinofuranosyl)-α-D-arabinofuranoside (3)

Tetrasaccharide **10** (0.050 g, 0.036 mmol) was dissolved in anhydrous THF (4 mL) under argon, cooled to −78 °C and ammonia (about 20 mL) was condensed into the flask. Small pieces of sodium (washed with *n*-pentane) were then added to the solution until the color of the reaction mixture turned dark blue. After 40 min at −78 °C, MeOH was added until the blue color disappeared. Small pieces of sodium were again added to the mixture until the blue color of the solution persisted. After 40 min at −78 °C, the reaction was quenched by addition of MeOH (3 mL), the cooling bath was removed and the ammonia was evaporated using a stream of nitrogen. The solution was diluted with MeOH (5 mL), neutralized with Amberlite IR-120 and filtered. The resin was washed with MeOH and the solution was concentrated in vacuo. The residue was dissolved in pyridine (3 mL) and acetic anhydride (2 mL) and

the solution was stirred at room temperature for 16 h. The solvents were evaporated and the residue was purified by flash silica gel column chromatography (hexanes/EtOAc, 1:1–1:2) to give the peracetylated tetrasaccharide (0.028 g, 78% over two steps) as a colorless oil; R_f = 0.30 (hexanes/EtOAc, 1:2); $[\alpha]_D$ +111.7 (*c* 0.23, $CHCl_3$). ^1H NMR (300 MHz, $CDCl_3$): δ 5.86–5.73 (m, 1H), 5.14–4.91 (m, 14H), 4.44 (dd, *J* = 2.7, 11.4 Hz, 1H), 4.28–4.14 (m, 5H), 3.95–3.87 (m, 3H), 3.75–3.62 (m, 4H), 3.43 (td, *J* = 6.2, 9.7 Hz, 1H), 2.17–2.00 (m, 29H), 1.72–1.63 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 170.6, 170.3, 170.2, 170.2, 170.2, 169.9, 169.7, 169.4, 138.0, 114.9, 105.4, 105.4, 105.3, 105.2, 81.9, 81.8, 81.6, 81.5, 81.4, 80.9, 80.9, 80.8, 66.7, 65.2, 65.1, 63.2, 30.1, 28.5, 20.8, 20.7, 20.6, 20.6. IR ($CHCl_3$): ν_{max} 3026, 2933, 1739, 1369, 1108, 1062, 1041, 969 cm^{-1}. MALDI-HRMS: *m/z* $(M+Na)^+$ calcd for $C_{43}H_{60}NaO_{26}$: 1015.3271, obsd 1015.3244.

The peracetylated tetrasaccharide (0.025 g, 0.025 mmol) was dissolved in anhydrous MeOH (0.5 mL) and 0.5 M NaOMe in MeOH (0.050 mL, 0.025 mmol) was added. The mixture was stirred at room temperature for 48 h and then neutralized with Amberlite IR-120. The mixture was filtered, the resin washed with MeOH and the solvents were evaporated. Purification by Sephadex G-10 column chromatography (H_2O/EtOH, 1:1) gave **3** (0.0125 g, 80%) as a colorless solid; R_f = 0.15 (MeOH/EtOAc, 1:5); $[\alpha]_D$ +114.3 (*c* 0.63, MeOH). ^1H NMR (300 MHz, $CDCl_3$): δ 5.90–5.77 (m, 1H), 5.06–4.80 (m, 6H), 4.11–3.61 (m, 21H), 3.43 (td, *J* = 6.5, 9.8 Hz, 1H), 2.17–2.10 (m, 2H), 1.72–1.63 (m, 2H). ^{13}C NMR (75 MHz, $CDCl_3$): δ 139.4, 115.3, 109.7, 109.5, 85.9, 84.1, 83.7, 83.5, 83.2, 83.1, 79.1, 78.8, 68.2, 63.1, 31.4, 30.0. ESI-HRMS: *m/z* $(M+Na)^+$ calcd for $C_{25}H_{42}NaO_{17}$: 637.2320, obsd 637.2314.

3.3. Chemical synthesis of biotinylated acceptors (4)

3.3.1. Pent-4-enyl 2,3-di-*O*-benzyl-5-*O*-*t*-butyldiphenylsilyl-α-D-arabinofuranoside (12)

To a solution of compound **11**[25] (1.98 g, 2.83 mmol) and *n*-pentenol (576 μL, 5.66 mmol) in dry CH_2Cl_2 (20 mL) at 0 °C was added $BF_3{\cdot}Et_2O$ (716 μL, 1.43 mmol) dropwise. The reaction mixture was stirred for 2 h at 0 °C, and then at room temperature for 1 h, before Et_3N (1.2 mL) was added. The mixture was diluted with CH_2Cl_2, washed with a saturated solution of $NaHCO_3$, dried over Na_2SO_4, concentrated, and purified by chromatography on silica gel (hexane/EtOAc, 10:1→8:1) to give compound **12** (1.20 g, 63.8%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.12 (d, 2H, *J* = 7.2 Hz), 8.04 (d, 2H, *J* = 6.9 Hz), 7.78–7.30 (m, 16H), 5.97–5.84 (m, 1H), 5.67 (dd, 1H, *J* = 1.2, 5.1 Hz), 5.51 (d, 1H, *J* = 1.5 Hz), 5.13–5.01 (m, 2H), 4.46–4.41 (m, 1H), 4.09–4.06 (m, 2H), 3.89–3.81 (m, 1H), 3.64–3.57 (m, 1H), 2.29–2.24 (m, 2H), 1.87–1.80 (m, 2H), 1.17–1.09 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 165.8, 165.7, 138.4, 135.9, 134.6, 133.5, 133.4, 130.5, 130.1, 129.9, 129.7, 129.5, 128.6, 128.1, 127.9, 115.1, 105.9, 83.1, 82.5, 77.7, 66.9, 63.9, 30.5, 29.0, 27.0, 26.2, 19.5.

3.3.2. Pent-4-enyl 2,3-di-*O*-benzyl-α-D-arabinofuranoside (13)

To a solution of compound **12** (1.10 g, 1.66 mmol) in dry THF (10 mL) at room temperature was added dropwise the solution of TBAF (866 mg, 3.3 mmol) in THF (5 mL). After stirring at room temperature for 1 h, the reaction mixture was diluted with CH_2Cl_2, washed with a saturated solution of $(NH_4)_2SO_4$ and brine. The organic phase was dried over Na_2SO_4, and concentrated. The residue was purified by chromatography on silica gel (hexane/EtOAc, 4:1→2:1) to give compound **13** (442 mg, 62.7%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.10–8.05 (m, 4H), 7.63–7.58 (m, 2H), 7.49–7.45 (m, 4H), 5.86–5.79 (m, 1H), 5.54 (d, 1H, *J* = 1.6 Hz), 5.45 (d, 1H, *J* = 4.4 Hz), 5.24 (s, 1H), 5.04–4.96 (m, 2H), 4.35–4.31 (m, 1H), 4.05–3.96 (m, 2H), 3.83–3.77 (m, 1H), 3.58–3.52 (m, 1H), 2.18 (m, 2H), 1.80–1.73 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.4, 165.6, 138.3, 133.8, 130.1, 130.0, 129.9, 129.4, 129.3, 128.8, 128.7, 115.2, 105.7, 83.8, 82.0, 78.0, 67.0, 62.6, 30.5, 28.9.

3.3.3. 5-[2-[(*t*-Butoxylcarbonyl)amino]-1-thioethyl]-pentyl 2,3-di-*O*-benzyl-α-D-arabinofuranoside (14)

A solution of compound **13** (140 mg, 0.33 mmol), AIBN (30 mg, 0.15 mmol), 2-[(*t*-butoxycarbonyl)amino]-1-ethanethiol (900 μL, 5.0 mmol) in dry dioxane (5 mL) was thoroughly degassed by N_2 before being reacted at 75 °C (preheated oilbath). TLC detected the processing of the reaction. After the consumption of the starting material (8 h), the mixture was concentrated and loaded to silica gel column (hexane/EtOAc, 2:1) to give compound **14** (160 mg, 80.8%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.08–8.00 (m, 4H), 7.60–7.55 (m, 2H), 7.46–7.41 (m, 4H), 5.49 (m, 1H), 5.39 (m, 1H), 5.20 (m, 1H), 4.29 (dd, 1H, *J* = 4.0, 8.0 Hz), 3.99–3.93 (m, 2H), 3.74 (m, 1H), 3.53–3.49 (m, 1H), 3.25 (t, 2H, *J* = 6.4 Hz), 2.58 (m, 2H), 2.44 (m, 2H), 1.64–1.46 (m, 6H), 1.42 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.4, 165.6, 133.8, 130.1, 130.0, 129.4, 129.3, 128.8, 128.7, 105.7, 83.8, 82.0, 78.1, 67.4, 62.6, 40.1, 32.4, 31.9, 29.6, 29.3, 28.6, 28.5, 25.6. ESI-MS *m/z* 504.13 $[M-Boc+2H]^+$, 603.93 $[M+H]^+$, 620.00 $[M+NH_4]^+$, 626.27 $[M+Na]^+$, 642.27 $[M+K]^+$.

3.3.4. 5-[2-[(*t*-Butoxylcarbonyl)amino]-1-thioethyl]-pentyl 5-*O*-(2,3,5-tri-*O*-benzyl-α-D-arabinofuranosyl)-2,3-di-*O*-benzyl-α-D-arabinofuranoside (16)

To a solution of compound **14** (120 mg, 0.20 mmol) and compound **15**[28] (145 mg, 0.24 mmol) in dry CH_2Cl_2 (5 mL) was added Me_3SiOTf (5.4 μL, 0.030 mmol) under N_2 at −20 °C. The reaction mixture was stirred for 1 h allowing the temperature to gradually rise to 0 °C, then neutralized with Et_3N and concentrated. The residue was purified by silica gel column (hexane/EtOAc, 4:1→2:1) to give compound **16** (180 mg, 86.5%). ^1H NMR (400 MHz, $CDCl_3$): δ 8.07–7.90 (m, 10H), 7.55–7.20 (m, 15H), 5.60 (m, 2H), 5.55–5.26 (m, 2H), 5.48 (s, 1H), 5.43 (s, 1H), 5.18 (s, 1H), 4.82–4.77 (m, 1H), 4.72 (dd, 1H, *J* = 4.4, 8.4 Hz), 4.66–4.61 (m, 1H), 4.41 (m, 1H), 4.21 (m, 1H), 3.94 (m, 1H), 3.75–3.69 (m, 1H), 3.49–3.44 (m, 1H), 3.25 (m, 2H), 2.56 (m, 2H), 2.44 (m, 2H), 1.60–1.53 (m, 4H), 1.57 (m, 2H), 1.41 (m, 9H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 166.5, 166.0, 165.9, 165.7, 165.5, 156.0, 133.9, 133.7, 133.5, 133.2, 130.2, 130.1, 130.0, 129.9, 129.5, 129.2, 128.8, 128.7, 128.6, 128.5, 106.1, 105.8, 82.7, 82.1, 82.0, 81.7, 81.5, 78.2, 78.1, 67.3, 66.4, 63.9. ESI-MS *m/z* 1047.93 $[M+H]^+$, 1065.07 $[M+NH_4]^+$, 1070.33 $[M+Na]^+$.

3.3.5. 5-[2-[(*t*-Butoxylcarbonyl)amino]-1-thioethyl]-pentyl 5-*O*-(α-D-arabinofuranosyl)-α-D-arabinofuranoside (17)

To a solution of compound **16** (130 mg, 0.12 mmol) in dry CH_2Cl_2 (3 mL) and dry MeOH (9 mL) was added MeONa (1 M in MeOH, 360 μL). After 2 h of stirring at room temperature, the reaction mixture was neutralized with Amberlite IR-120 (H^+) resin, filtered, and the residue was purified by silica gel column ($CHCl_3$/MeOH, 8:1→6:1) to give compound **17** (53 mg, 81.5%). NMR (400 MHz, $CDCl_3$): δ 5.12 (br, 1H), 5.06 (s, 1H), 4.96 (s, 1H), 4.12–3.96 (m, 7H), 3.83–3.70 (m, 4H), 3.43 (m, 1H), 3.30 (m, 2H), 2.64 (m, 2H), 2.54 (m, 2H), 2.22 (br, 2H), 1.60 (m, 4H), 1.45 (m, 9H), 1.26 (m, 2H). ^{13}C NMR (100 MHz, $CDCl_3$): δ 156.2, 108.2, 108.0, 85.8, 83.8, 81.1, 80.8, 79.8, 78.1, 77.4, 67.8, 66.6, 62.1, 40.0, 32.4, 31.8, 29.9, 29.5, 29.2, 28.6, 25.5. ESI-MS *m/z* 528.00 $[M+H]^+$, 550.27 $[M+Na]^+$.

3.3.6. 5-(2-Amino-1-thioethyl)-pentyl 5-*O*-(α-D-arabinofuranosyl)-α-D-arabinofuranoside (18)

Compound **17** (50 mg, 0.017 mmol) was dissolved in CH_2Cl_2 (2 mL) and then treated with TFA (1 mL) at 0 °C. After stirring for 0.5 h, the mixture was concentrated and purified by silica gel column ($CHCl_3$/MeOH, 1:1) to give compound **18** (32 mg, 79.0%). ^1H

NMR (400 MHz, D_2O): δ 5.05 (d, 1H, J = 0.8 Hz), 4.99 (d, 1H, J = 2.0 Hz), 4.15–4.12 (m, 1H), 4.10 (m, 1H), 4.08–4.02 (m, 2H), 3.99–3.96 (m, 1H), 3.94–3.91 (m, 1H), 3.87–3.77 (m, 3H), 3.75–3.66 (m, 2H), 3.58–3.52 (m, 1H), 3.19 (t, 2H, J = 6.4 Hz), 2.82 (t, 2H, J = 6.4 Hz), 2.58 (t, 2H, J = 7.2 Hz), 1.64–1.57 (m, 4H), 1.47–1.41 (m, 2H). ^{13}C NMR (100 MHz, D_2O): δ 107.5, 107.3, 84.0, 81.9, 81.0, 80.9, 76.6, 76.5, 68.4, 66.8, 61.2, 38.4, 30.6, 28.3, 28.2, 28.1, 24.5. ESI-MS m/z 428.13 $[M+H]^+$, 450.27 $[M+Na]^+$.

3.3.7. *S*-D-Biotinoyl-5-(2-amino-1-thioethyl)-pentyl 5-*O*-(α-D-arabinofuranosyl)-α-D-arabinofuranoside (4)

Compound **18** (8 mg, 0.047 mmol) was dissolved in DMF (0.8 mL), and the pH was adjusted to 9.0 using Et_3N. NHS-biotin (16 mg, 0.047 mmol) was added and the reaction was stirred at room temperature for 12 h. The solvent was removed by Savant and the residue was purified by silica gel column ($CHCl_3$/MeOH, 1:1) to give biotinylated acceptor **4** (12 mg, 96.0%). ^1H NMR (400 MHz, D_2O): δ 5.05 (s, 1H), 4.99 (s, 1H), 4.58 (m, 1H), 4.41 (m, 1H), 4.13–4.03 (m, 3H), 3.97 (m, 1H), 3.91 (m, 1H), 3.87–3.82 (m, 1H), 3.78 (m, 1H), 3.75–3.66 (m, 2H), 3.54 (m, 1H), 3.39 (m, 2H), 3.32 (m, 1H), 2.99 (m, 1H), 2.96 (d, 1H, J = 4.8 Hz), 2.92 (s, 1H), 2.76 (m, 4H), 2.69 (t, 2H, J = 6.4 Hz), 2.58 (t, 2H, J = 7.2 Hz), 2.25 (t, 2H, J = 6.8 Hz), 1.71–1.55 (m, 8H), 1.44–1.39 (m, 4H). ESI-MS m/z 654.13 $[M+H]^+$, 676.47 $[M+Na]^+$.

3.4. Preparation of mycobacterial membranes and cell wall enriched fraction (P60)

Wild-type *M. smegmatis* mc^2155 cells were grown in 7H9 containing oleic acid-albumin-dextrose-catalase (OADC) to mid-log phase (O.D. ~0.7). Cells were harvested by centrifugation, washed and re-suspended in ice cold buffer A containing 50 mM MOPS (pH 7.9), 5 mM 2-mercaptoethanol and 10 mM $MgCl_2$. Cells were disrupted by three passages through a French Press at 1500 psi. The suspension was centrifuged at 27,000*g* for 60 min at 4 °C. The cell wall pellet was removed and the supernatant re-centrifuged at 100,000*g* for 2 h at 4 °C. The supernatant was discarded and the pellet of enzymatically active membranes was gently re-suspended in 500 μl of buffer A; the protein concentrations of the membrane fractions were typically between 15 and 20 mg/ml. The 27,000*g* pellet was suspended in 10 ml of buffer A and Percoll was added to achieve a 60% suspension. The suspension was mixed and centrifuged at 27,000*g* for 60 min at 4 °C. The resulting flocculent, white layer was collected and washed three times with buffer A at 12,000*g* to yield P60. This fraction was re-suspended in 1 ml of buffer A to yield a protein concentration of 5–6 mg/ml.

3.5. Arabinosyltransferase assays

Typical reaction mixtures contained buffer A, 62.5 μM ATP, 3.8 μM p[^{14}C]Rpp (500,000 dpm), acceptors **1**, **2**, **3**, or **4** (300 μM), membranes (0.5 mg) and P60 (0.3 mg) in a total volume of 200 μL. The reaction mixtures were incubated at 37 °C for 1 h and then terminated by adding 200 μL of ethanol. The resulting mixture was centrifuged at 14,000 rpm and the supernatants were loaded onto prepacked strong anion exchange (SAX) columns (Burdick and Jackson). The columns were eluted with 2 ml of 50% ethanol. The eluate was evaporated to dryness and partitioned between the two phases (1:1) of water saturated 1-butanol and water. The 1-butanol fractions were measured for radioactive incorporation by liquid scintillation counting.

3.6. Analytical procedures

For TLC analysis of the enzymatic products, radiolabeled product, an aliquot of the 1-butanol extract, was dried under air and the residue was reconstituted in Milli-Q water (10 μL) for analysis by silica gel TLC. The plates were developed in $CHCl_3$/MeOH/NH_4OH/H_2O (65:25:0.4:3.6) or $CHCl_3$/MeOH/1 M NH_4OAc/NH_4OH/H_2O (180:140:9:9:23), followed by autoradiography at −70 °C using Biomax MR film (Kodak) and acceptors were visualized using α-naphthol spray reagent. For analysis of the sugar composition of the enzymatic products, the water layer after 1-butanol extraction was purified by Biogel P-2 column, eluted with Milli-Q water, and approximately 2000 dpm of the purified product was hydrolyzed in 200 μl of 2 M trifluoroacetic acid (TFA) at 120 °C for 2 h. TFA was removed under a stream of air and the hydrolysate was analyzed on silica gel TLC plate developed in pyridine/ethyl acetate/acetic acid/water (5:5:1:3) followed by autoradiography as described above. Radioactive spots were identified by co-chromatography with standard radioactive arabinose and galactose from radioactive [^{14}C] labeled AG.

3.7. Preparation of DP[^{3}H]A

The reaction containing 1 mg of wild-type *M. smegmatis* membranes, 62.5 μM ATP, 10 mM NADH, 500,000 dpm of p[^{3}H]Rpp, and buffer A in final volume of 160 μL was incubated at 37 °C for 1 h, then stopped by the addition of 3 mL of $CHCl_3$/MeOH (2:1), and the mixture was centrifuged. The supernatant was removed from the pellet, and 340 μL of buffer A was added to form two phases. After thorough mixing and a brief centrifugation, the aqueous phase was discarded. The organic phase was backwashed with $CHCl_3$/MeOH/H_2O (3:47:48). The sample was then dried under air at room temperature. The radiolabeled material was quantified and stored in $CHCl_3$/MeOH/H_2O/NH_4OH (65:25:3.6:0.5) at −20 °C.

3.8. SPA beads stock solution

SPA beads were suspended in PBS with 20% glycerol to a concentration of 10 mg/mL. This stock solution was stored at 4 °C as long as one month. A typical assay used 100 μL of this solution which contains 1 mg of SPA beads.

3.9. AftB enzyme assay using SPA technology

The typical reaction mixture contained the following components in a final volume of 50 μL: buffer A, 62.5 μM ATP, 20 μM p[^{3}H]Rpp (200,000 dpm), biotinylated acceptor **4** (100 μM), and indicated amount of membranes and P60. In the cases when using DP[^{3}H]A (10,000 dpm), Igepal CA-630 (sigma) was used in the assay at the final concentration of 0.1%. Reactions were incubated at 37 °C for 1 h, terminated by adding indicated amount of SPA beads suspension. The mixture was then incubated overnight at room temperature with gentle shaking. All incubation and shaking were performed in 96-well plate (Wallac 1450-401) with sealing tape (Wallac 1450-461). The [^{3}H]Ara*f* incorporation was measured by a Wallac MicroBeta scintillation count plate reader (Model 1450) using a parameter in SPA mode. Because the efficiency of capture by the SPA beads was not known, results were generally expressed as CPM.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmc.2010.07.040.

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