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# Reconstitution of Functional Mycobacterial Arabinosyltransferase AftC Proteoliposome and Assessment of Decaprenylphosphorylarabinose Analogues as Arabinofuranosyl Donors

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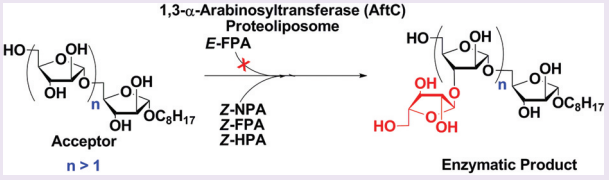
Supporting Information

**ABSTRACT:** Arabinosyltransferases are a family of membrane-bound glycosyltransferases involved in the biosynthesis of the arabinan segment of two key glycoconjugates, arabinogalactan and lipoarabinomannan, in the mycobacterial cell wall. All arabinosyltransferases identified have been found to be essential for the growth of *Mycobcterium tuberculosis* and are potential targets for developing new antituberculosis drugs. Technical bottlenecks in designing enzyme assays for screening for inhibitors of these enzymes are (1) the enzymes are membrane proteins and refractory to isolation; and (2) the sole arabinose donor, decaprenylphosphoryl-D-arabinofuranose is sparingly produced and difficult to isolate, and commercial substrates are not available. In this study, we have synthesized several analogues of decaprenylphosphoryl-D-arabinofuranose by varying the chain length and investigated their arabinofuranose (Ara*f*) donating capacity. In parallel, an essential arabinosyltransferase (AftC), an enzyme that introduces α-(1→3) branch points in the internal arabinan domain in both arabinogalactan and lipoarabinomannan synthesis, has been expressed, solubilized, and purified for the first time. More importantly, it has been shown that the AftC is active only when reconstituted in a proteoliposome using mycobacterial phospholipids and has a preference for diacylated phosphatidylinositoldimannoside ($Ac_2PIM_2$), a major cell wall associated glycolipid. α-(1→3) branched arabinans were generated when AftC—liposome complex was used in assays with the (*Z*,*Z*)-farnesylphosphoryl D-arabinose and linear α-D-Ara*f*-(1→5)$_{3-5}$ oligosaccharide acceptors and not with the acceptor that had a α-(1→3) branch point preintroduced.




According to the 14th (2010) annual tuberculosis report by the World Health Organization (WHO), there were an estimated 9.4 million new cases of tuberculosis (TB) worldwide.[1,2] Increase in the incidence of new TB cases is seen mostly within developing countries of Africa, Asia, and Latin America. The emergence of MDR-TB (Multidrug Resistant TB) and XDR-TB (Extensive Drug Resistant TB) strains are worrisome as these are virtually untreatable with the existing panel of anti-TB drugs. It is estimated, in 2009, that 3.3% of all new TB cases are MDR-TB positive and XDR-TB positive cases are distributed in 58 countries. It is important to find new affordable and effective antituberculosis drugs to reduce the physical and economic burden of TB patients. The mycobacterial cell wall is regarded as a validated target for developing new anti-TB drugs, as first-line anti-TB drugs including ethambutol and isoniazid inhibit synthesis of arabinan and mycolic acids, key constituents of the cell wall of *Mycobacterium tuberculosis*. The cell wall provides a permeability barrier, shielding the bacterium from degradative forces as well as modulating host immune responses to its own needs and presenting special problems for disease chemotherapy. The cell envelope possesses two major structural components: the plasma membrane and the wall proper. The plasma membrane is characterized by the presence of the phosphatidylinositol mannosides lipomannan and lipoarabinomannan (LAM) but, in other respects, appears typical of bacterial membranes. The cell wall however, is highly distinctive and characterized by a "core" consisting of covalently linked mycolic acids, the unusual heteropolysaccharide-arabinogalactan, and peptidoglycan (mAGP complex). mAGP is composed of three arabinan chains attached to the homogalactan core. The homoarabinan chains are composed of linear α-D-Ara*f* residues with branching produced by 3,5-linked α-D-Ara*f* residues substituted at both positions by α-D-Ara*f* residues. On the other hand, the α-(1→5)-linked arabinan chains of LAM terminate in either one of the two well-defined motifs, namely, a branched $Ara_6$ or a linear $Ara_4$, the relative proportion of which reflects the amount of α-(1→3)-linkage branching of the α-(1→5)-linked arabinan backbone, prior to termination by β-(1→2)-arabinosylation. It

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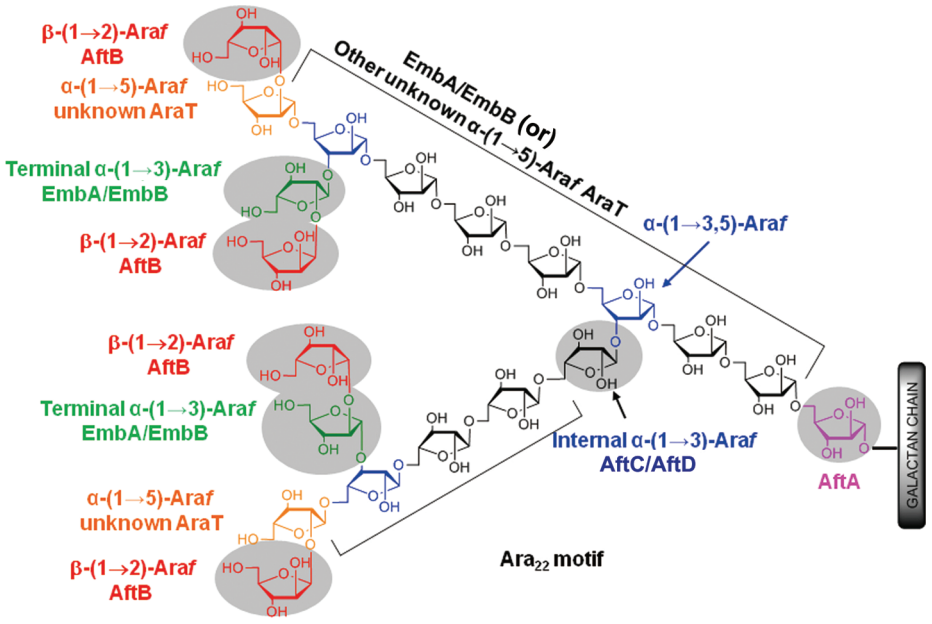


**Figure 1.** Structure and putative biosynthesis of arabinan motif in AG in *Mycobacterium* spp. There are three $Ara_{22}$ (one is drawn in the figure) motifs on the galactan backbone in AG. All Ara*f* residues are donated by DPA. The AraTs (six in total) identified are shown. AftA (Rv3792) is a priming AraT and donates the first Ara*f* on the galactan.[13] AftB (Rv3805c) is a capping AraT and terminates the arabinan chain presumably after branching has been introduced by EmbA/EmbB.[12,14] Both AftC (Rv2673) and AftD (Rv0236c) have been shown to exhibit internal branching (α-(1→3)-AraT) activity.[15,20] All AraTs are essential in *M. tuberculosis* and have not been isolated for detailed functional studies. EmbA, EmbB, and AftD could be bifunctional enzymes. AftC has been shown to be nonessential in *M. smegmatis*.

has been shown that all Ara*f* residues in mycobacterial D-arabinan originate from the pentose phosphate pathway/hexose monophosphate shunt, and the immediate precursor is decaprenylphospho-D-arabinofuranose (DPA). The biosynthetic pathway for the DPA formation has been recently elucidated.[3,4] Recently, an enzyme, decaprenylphosphoryl-β-D-ribose-2′-epimerase (DprE), involved in the synthesis of decaprenylphosphoryl-D-arabinofuranose (DPA), the arabinose donor for arabinan synthesis, has been identified as the target for benzothiazinone and dinitrobenzamide derivatives, new classes of antituberculosis candidates.[5,6] These compounds are now in a phase 2 clinical trials.[7]

Although the structural scaffold and the biosynthetic steps in mycobacterial arabinan formation have been almost completely elucidated,[8−12] screening for inhibitors against arabinosyltransferase (AraT) activity has not been pursued because of difficulties associated with obtaining soluble, active proteins for biochemical assays. This is largely due to the fact that all known mycobacterial AraTs are membrane proteins with multiple membrane spanning domains. As shown in Figure 1, AraTs dedicated to the arabinogalactan (AG) synthesis pathway include AftA (Rv3792), apparently responsible for the transfer of the first Ara*f* residue to the galactan domain of AG,[13] the terminal β-(1→2)-capping AftB (Rv3805c),[14] AftC (Rv2673) involved in the synthesis of the internal α-(1→3)-branching of the arabinan domain of AG,[15] and the EmbA and EmbB proteins involved in the formation of the [β-D-Ara*f*-(1→2)-α-D-Ara*f*]$_2$-3,5-α-D-Ara*f*-(1→5)-α-D-Ara*f*- ($Ara_6$) termini of arabinan.[12] On the contrary, interestingly, only three enzymes, EmbC, AftC, and AftD (Rv0236c), have been implicated in lipoarabinomannan (LAM) arabinan synthesis.[16,17,20] The donor substrate (DPA) used in the AraT enzymatic assays is produced by *Mycobacterium* sp. at very low level and is not commercially available. Preparation of DPA from mycobacteria is laborious and time-consuming and is often obtained in poor yield[18] and therefore not suitable for development of high throughput assays. Moreover, due to the long chain length in the lipid, DPA has poor solubility in aqueous buffers. As an alternate, phosphoribose pyrophosphate (pRpp) has been utilized in many assays.[19−21] This highly soluble precursor can be transformed to DPA *in situ* in an enzymatic reaction.[3] However, because of the five steps involved in the conversion of pRpp to DPA, overall product formation is often inefficient (approximately 4% overall yield).[21]

In this study, we were able to express (after inducing with acetamide) *AftC* in *Mycobacerium smegmatis* (*M. smegmatis*), purify the recombinant protein, and reconstitute it in a proteoliposome retaining the AraT activity in enzymatic assays. In addition, we synthesized several DPA analogues carrying shorter lipid chains (C10, C15, and C35). These compounds (Figure 2A), (ω,*Z*)-nerylphosphoryl D-arabinose (*Z*-NPA), (ω,*Z*,*Z*)-farnesylphosphoryl D-arabinose (*Z*-FPA), (ω,*E*,*E*)-farnesylphosphoryl D-arabinose (*E*-FPA), and (ω,*E*,*E*,*Z*,*Z*,*Z*,*Z*)-heptaprenylphosphoryl D-arabinose (*Z*-HPA), were assessed as artificial donors for AraT assays with specific synthetic linear arabinofuranosyl acceptors.

## RESULTS AND DISCUSSION

**Chemical Syntheses of DPA Analogues (Scheme 1).** The chemical syntheses of all DPA analogues used a common intermediate, 2,3,5-(tri-*tert*-butyldimethylsilyl)-D-arabinosyl phosphate, that was obtained by coupling dibenzyl phosphate with 2,3,5-(tri-*tert*-butyldimethylsilyl)-D-arabinosyl bromide.[22,23] The β-anomer of the arabinoside was produced in 75% yield in the α/β mixture (as estimated by $^1$H NMR). In the reaction step of coupling arabinosyl bromide with dibenzyl phosphate, a rigorous drying of bromide and dibenzyl phosphate intermediates in advance was necessary to improve the proportion of β-anomer in the products

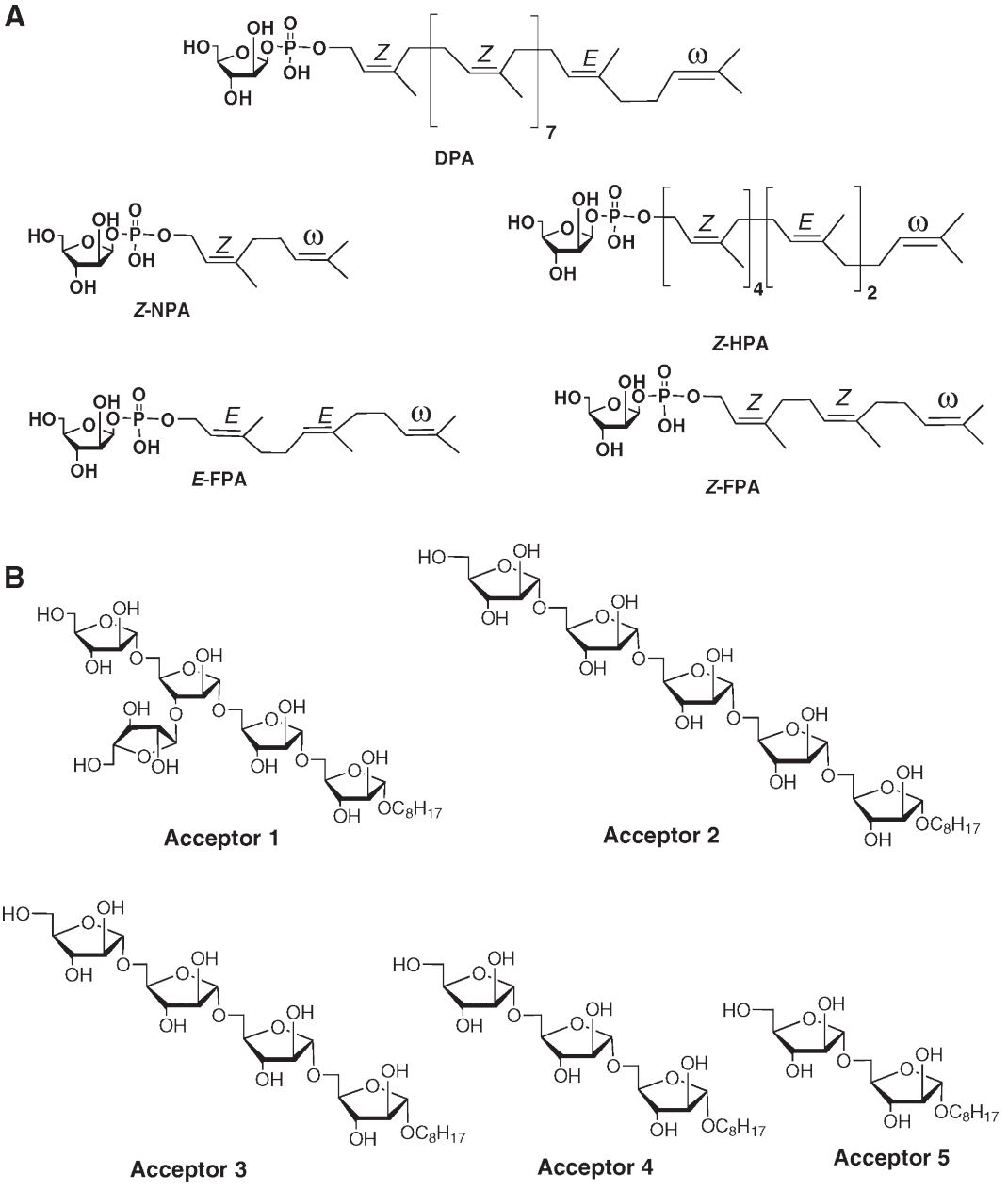


**Figure 2.** (A) Structure of DPA and DPA analogues used in this study. "*Z*", "*E*", and "$\omega$" represent the configuration of the double bonds in the lipids in DPA and DPA analogues. Abbreviations: ($\omega$,*Z*)-nerylphosphoryl D-arabinose (*Z*-NPA), ($\omega$,*Z*,*Z*)-farnesylphosphoryl D-arabinose (*Z*-FPA), ($\omega$,*E*,*E*)-farnesylphosphoryl D-arabinose (*E*-FPA), ($\omega$,*E*,*E*,*Z*,*Z*,*Z*)-heptaprenylphosphoryl D-arabinose (*Z*-HPA). (B) The structure of the acceptors **1**−**5** used in the assays. Acceptor **1** is a branched pentasaccharide that was used for probing a α-(1→5) AraT activity, and acceptors **2**−**5** are linear oligosaccharides from dimer to pentamer that were used for probing the α-(1→3) AraT (AftC) or $\beta$-(1→2) AraT (AftB).

of arabinosyl phosphate. Although the $\beta$-anomer could not be completely separated from the α-anomer by column chromatography, the early eluting fractions were enriched in the $\beta$-anomer (~88%). Alcohols containing the lipid chains (C10, C15, C35) were activated by forming trichloroacetimidate intermediates. Although the trichloroacetimidates were sensitive to moisture and temperature, they could be stored at −20 °C in a desiccator for several days. Finally, the arabinosyl phosphate was coupled with trichloroacetimidates in toluene. Deprotection of the coupled products with ammonium fluoride in a 15% methanolic ammonium hydroxide followed by chromatography on silica gel gave pure DPA analogues. (Details in Supporting Information Scheme S1).

Chemical synthesis of DPA described initially used phosphoramidite-phosphite triester methodology, which yielded a $\beta$/α ratio of 0.25.[24] However, only the $\beta$-anomer of DPA has proven to be a suitable donor for Araf. In our present work for the synthesis of DPA analogues, an optimized approach was applied to increase the proportion of the $\beta$-anomer in an anomeric mixture ($\beta$/α ratio of 3) and with this anomeric mixture also increased the yield of the product three-fold compared to using the ones synthesized earlier.

**Cell-Free Assay.** We first wanted to determine whether the DPA analogues would be effective in a cell-free assay using membrane preparation (100,000 × *g* pellet) from *M. smegmatis*


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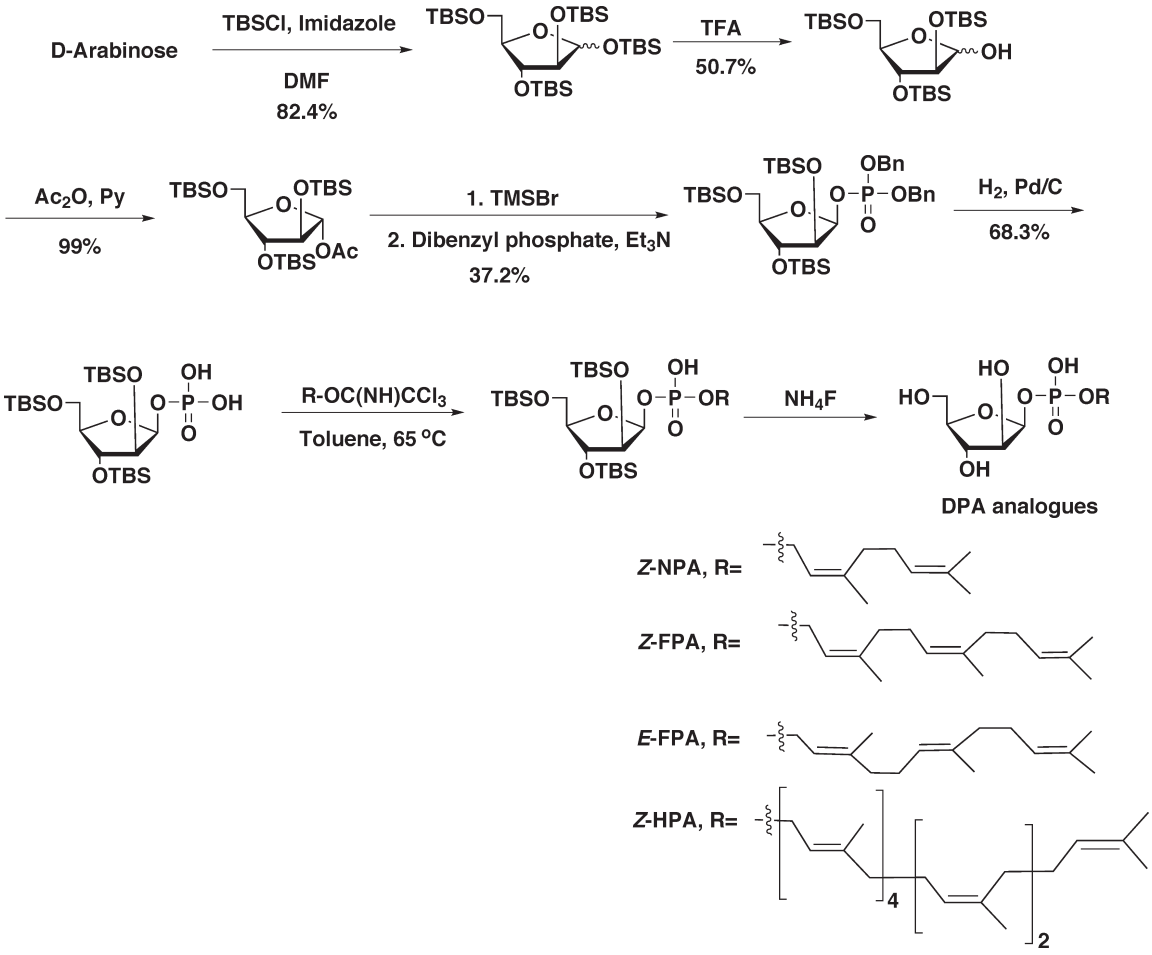
**Scheme 1. Synthesis of DPA Analogues**




as the enzyme source. One specific pentasaccharide (acceptor **1**, structure shown in Figure 2B), octyl (α-D-Araf)$_2$-(1→3,5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf, was used as an acceptor (200 $\mu$M concentration) to assay for α-(1→5) AraT activity.[21] This acceptor was selected because of its efficiency and specificity in a cell-free assay in which a mixture of all AraT activities are present. In order to examine the contribution of the length (C10, C15, or C35) and configuration of the double bonds (*Z* or *E*) in the lipid chains to the donor efficiency in the assay, we assessed the ability of *Z*-NPA, *Z*-FPA, *E*-FPA, and *Z*-HPA in serving as donor substrates. The enzymatic reaction mixture without further processing was per-*O*-methylated and directly analyzed by MALDI-TOF-MS. As shown in Figure 3, molecular ions corresponding to unreacted acceptor ($m/z$ 967) and an enzymatic product ($m/z$ 1127) as sodium adducts were observed. Additionally, several phosphatidylinositol mannoside (PIMs) and other endogenous components present in the membranes could also be detected (methylated $PIM_2$ at $m/z$ 877, methylated $PIM_3$ at $m/z$ 1081, methylated $PIM_4$ at $m/z$ 1285, methylated $PIM_5$ at $m/z$ 1489, methylated $PIM_6$ at $m/z$ 1693). By quantification of the relative intensity of the acceptor ($m/z$ 967) and the product ($m/z$ 1127) reflected in the mass spectrum using a standard curve (see Methods), the conversion rate (activity) of DPA analogues could be estimated. The *Z*-FPA exhibited the best Araf-donating activity (Figure 3B). However, the *E*-FPA that possessed the same length of lipid as *Z*-FPA had no Araf-donating activity (Figure 3C). The *Z*-NPA and *Z*-HPA exhibited moderate conversions (Figure 3A and D).

**AraT Competition Assay.** To confirm the AraT activities of DPA analogues, we evaluated the ability of unlabeled polyprenyl-P-Araf (*Z*-NPA, *Z*-FPA, *E*-FPA, and *E*-HPA) to inhibit the incorporation of labeled Araf from DP[$^{14}$C]A generated *in situ* from the p[$^{14}$C]Rpp into the product. The results showed that the addition of *Z*-FPA led to 54% inhibition of the DP[$^{14}$C]A (Figure 4B). In contrast, *E*-FPA only marginally affected the DP[$^{14}$C]A incorporation (11% inhibition), indicating a limited competition of the lipids in the *E*-configuration. In addition, *Z*-NPA and *Z*-HPA showed 10% and 35% inhibitory ability, respectively. As is evident from the TLC (Figure 4A), formation of DPA and DPR (decaprenylphosphoryl-D-ribofuranose) also decreased dramatically using *Z*-FPA as competitor. It indicates that one of the biosynthetic steps in the transformation of p[$^{14}$C]Rpp to DP[$^{14}$C]A could also be inhibited by *Z*-FPA.

Thus, we have been successful in identifying the differential inhibitory properties of the DPA analogues in the AraT assay using DP[$^{14}$C]A as donor. The enzyme has a preference for *Z*-FPA compared to all other DPA analogues synthesized in this work (Figure 4B).

**Expression of *AftC* and Development of AraT Assays Using AftC-Proteoliposome and DPA Analogues.** A recombinant His$_6$-tagged AftC was efficiently produced in *M. smegmatis* mc$^2$155/pJAM/Rv2673 upon induction of the expression of

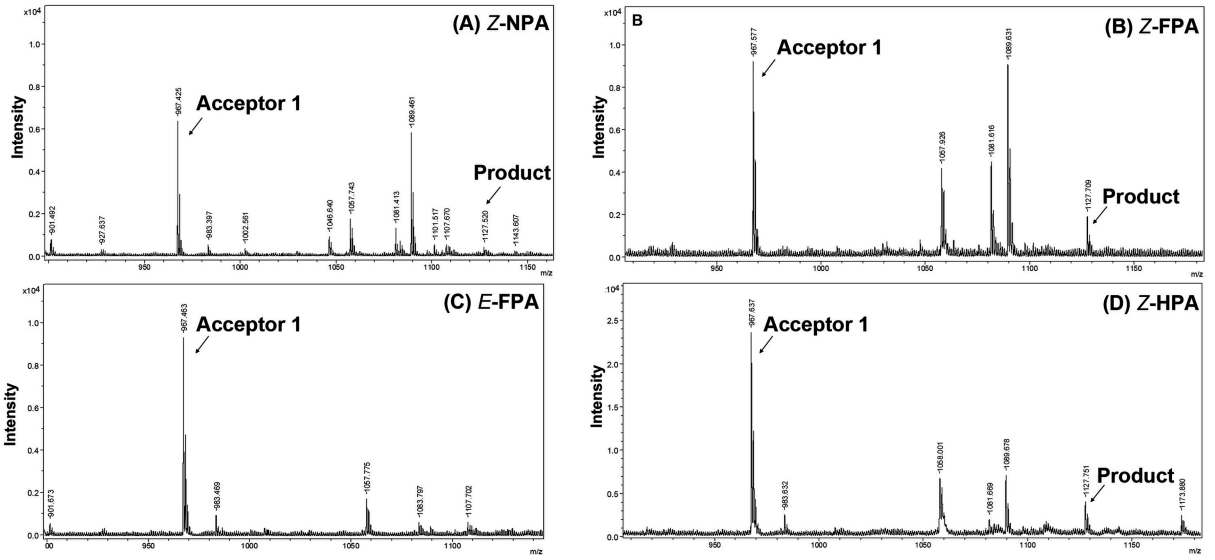



**Figure 3.** MALDI-TOF-MS spectra of enzymatic product by using *Z*-NPA (A), *Z*-FPA (B), *E*-FPA (C), and *Z*-HPA (D) in the cell-free assay using membranes from *M. smegmatis*; *m*/*z* 967 represents the methylated acceptor **1** and *m*/*z* 1127 represents the methylated product. Several endogenous components, such as PIMs, can be observed in the mass spectra as the product is not purified from the reaction mixture.

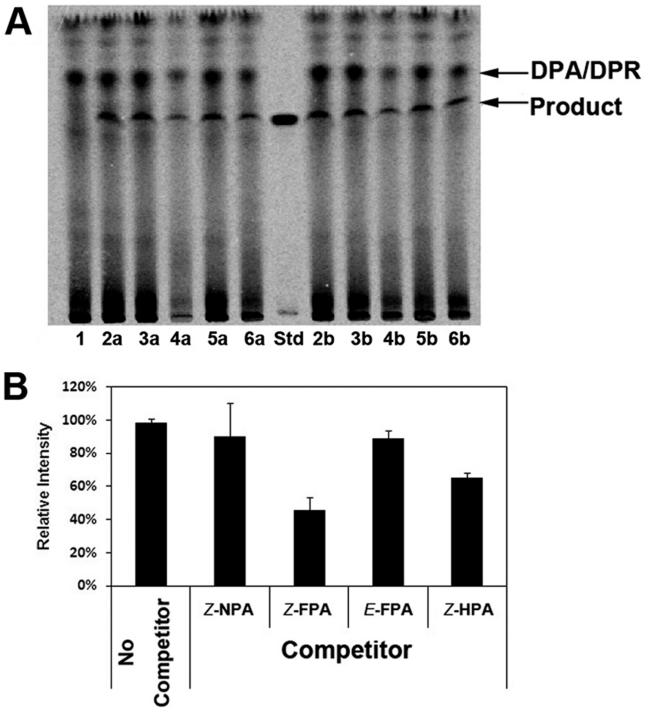



**Figure 4.** Competitive assay. (A) TLC profile of the reactions (using pR[$^{14}$C]-pp) in competition against the DPA analogues including *Z*-NPA (lanes 3a and 3b), *Z*-FPA (lane 4a and 4b), *E*-FPA (lane 5a and 5b), and *Z*-HPA (lane 6a and 6b). Lanes 2a and 2b represent the reaction without any competitor (negative control). Lane 1 exhibits the reaction without using acceptor **1** (positive control). The std lane represents the enzymatic product purified by column chromatography; "a" and "b" represent the duplicate of one competitive assay. The bands that migrated above the enzymatic product contained the [$^{14}$C]-DPA and [$^{14}$C]-DPR that were produced from p[$^{14}$C]Rpp. (B) Comparison of the scintillation intensity of the competitive assays using the DPA analogues as the competitors. The relative intensity was evaluated according to the assay without using the competitors, which was normalized to 100%.

the *aftC* gene with acetamide.[25] The $His_6$-tagged recombinant protein could be detected by Western blot (migrating at ~38 kDa) in the transformants. Cells from AftC overexpressor were disrupted by sonication and solubilized in 1% Igepal CA-630, a detergent proven to retain AraT activity.[14] The resulting supernatant after centrifugation was nickel-affinity column purified by eluting with imidazole. Fractions (50−200 mM imidazole) containing $His_6$-tagged AftC were monitored by SDS-PAGE and Coomassie stain (Figure S1 in Supporting Information), and ~200 $\mu$g of partially purified protein was obtained from 1 L of cell culture. The identity of the AftC was confirmed using in-gel trypsin digestion and analysis of the peptides by mass spectrometry, matching the masses with MASCOT searching (score 122, Figure S2 in Supporting Information).

Enzymatic assays were next designed to compare the ability of *M. smegmatis* mc$^2$155/pJAM and mc$^2$155/pJAM/Rv2673 cell-free extracts to transfer Ara*f* from the DPA analogues onto acceptor **2**. No significant increase in product formation was observed (1.3-fold increase over the control). However, when only the affinity purified recombinant AftC protein was used in the assay, we were unable to detect any product. On the other hand, when recombinant AftC was combined with *M. smegmatis* membranes from the overexpressing strain, product formation increased 2.3-fold (results not shown).

We reasoned that the failure of the recombinant protein to catalyze product formation was perhaps due to a lack of normal environment of membrane or misfolding of the protein. It has been reported that GT-C membrane proteins require lipids for folding to retain structure and glycosyltransferase activity.[26] On the basis of this hypothesis, we developed a reconstitution system forming a liposome using lipid extracts. At first we used commercially available dipalmitoyl phosphatidylcholine (DPPC) to reconstitute, but the resulting liposome failed to generate active enzyme. Then the purified AftC was reconstituted with native lipid ($CHCl_3$/$CH_3OH$/$H_2O$ (10:10:3)) extracts from *M. tuberculosis* H37Rv cells to form AftC proteoliposome. Since both *Z*-FPA (C15) and *Z*-HPA (C35) worked well in the AraT assay,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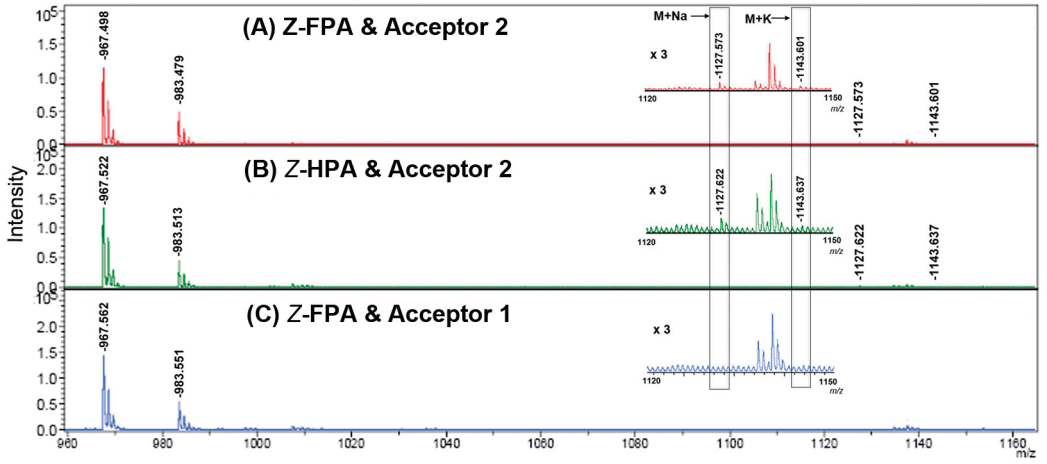


**Figure 5.** MALDI-TOF-MS in the positive ionization mode of the enzymatic products using the AftC proteoliposome and DPA analogues. The substrates including the acceptors and donors (DPA analogues) used in the assay were (A) acceptor **2** and *Z*-FPA; (B) acceptor **2** and *Z*-HPA; (C) acceptor **1** and *Z*-FPA. The *m*/*z* 1127 (+Na) and *m*/*z* 1143 (+K) represent the enzymatic product, and *m*/*z* 967 (+Na) and *m*/*z* 983 (+K) represent the unconsumed acceptor.

we used these compounds as sugar donors in separate reactions from which AftC would transfer Araf(s) to the acceptors.

In our earlier work, we had chemically synthesized arabinofuranosyl acceptors that represent various structural domains in the arabinan of AG and LAM. In the cell-free assay where all AraT activities are present, using p[$^{14}$C]Rpp as a donor, we were able to demonstrate that a disaccharide acceptor $Ara_2$, octyl α-D-Araf-(1→5)-α-D-Araf (acceptor **5**), yielded the specific product octyl β-(1→2)-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf, and octyl (α-D-Araf)$_2$-(1→3,5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf (acceptor **1**) gave a hexa-arabinoside[21] with a single Araf residue added on to the acceptor. Thus the acceptor specificity was evident even when a mixture of AraTs was present in an assay. We then asked which arabinan structure would AftC proteoliposome prefer for its activity.

Five different arabinofuranosyl acceptors (1 mM concentration) were tested in the enzymatic reactions using the AftC proteoliposome and DPA analogues. One of the acceptors was a branched pentasaccharide (acceptor **1**), and the others were linear oligosaccharides (acceptors **2**−**5**, structures shown in Figure 2B) including $Ara_5$, octyl α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf (acceptor **2**); $Ara_4$, octyl α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf (acceptor **3**); $Ara_3$, octyl α-D-Araf-(1→5)-α-D-Araf-(1→5)-α-D-Araf (acceptor **4**); and $Ara_2$, octyl α-D-Araf-(1→5)-α-D-Araf (acceptor **5**).

Direct mass spectral analyses of the reaction mixtures revealed that the products formed in reactions with acceptors **2**−**4** only and showed evidence of transfer of a single Araf onto each of the acceptors tested. No products were detected for either acceptor **1** or **5**. We concluded that AftC can only transfer Araf when a minimum of three Araf residues are present in a linear structure as in acceptors **2**−**4** in order to introduce an α-D-Araf-(1→3) branch point[15,17] (Figure S3 in Supporting Information). In fact, in AG or LAM internal α-(1→3) branching is evident only after three linear α-(1→5)-Araf residues have been assembled.[27,28] Consequentially, the $Ara_2$ (acceptor **5**) could not form a trisaccharide with AftC as it is recognized as a substrate by AftB to form a β-(1→2)-Araf linkage[14,29] (Figure 1). Furthermore, linear $Ara_5$ acceptor **2** could generate an enzymatic product with a mass at 1127 $[M + Na]^+$ and 1143 $[M + K]^+$ as shown in Figure 5A and B when using *Z*-FPA and *Z*-HPA as a donor, respectively; acceptor **1** with a α-(1→3)-Araf branch already introduced could not yield any product with AftC proteoliposome (panel C, Figure 5). This acceptor **1** has been proven to be specific for a α-(1→5) arabinosyltransferase that has yet to be identified.[21]

AftC has recently been reported to display a α-(1→3) branching AraT activity on a synthetic linear α-(1→5) linked $Ara_5$ acceptor *in vitro*.[15] One characteristic feature of AftC is that, unlike other AraTs described, the gene is nonessential in *M. smegmatis* and knockout mutants could be generated, an attribute reminiscent of the Emb proteins.[12,16] Phenotypic analysis of the mutants in *Corynebacterium glutamicum* and *M. smegmatis* showed that this enzyme is responsible for α-(1→3) branching of the inner core of arabinan domain of AG (Figure 1) and LAM. Although AftC belongs to the glycosyltransferase superfamily C (GT-C) and is a membrane-associated protein with 10−12 membrane spanning domains,[10] we have been successful in overexpressing and purifying a soluble form of AftC and have shown that it retains activity in an *in vitro* assay after successful reconstitution in proteoliposome using mycobacterial lipids. In addition, we were able to show that use of DPA can be substituted using some of the short chain synthetic DPA analogues, preferably the analogue containing a moderate-length lipid (C15). In mass spectrometric analysis and quantification of the enzymatic product formed (Supporting Information Figure 5), *Z*-FPA exhibited the highest Araf-donating activity, and 19.4% conversion was observed in a typical 2 h assay (Figure 3B). However, the *E*-FPA that possessed the same length of lipid as *Z*-FPA had no ions at *m*/*z* 1127 and hence was considered to have zero percent conversion (Figure 3C). *Z*-NPA (7.9% conversion) and *Z*-HPA (17.4% conversion) exhibited low to moderate conversions (Figure 3A and D). Furthermore, we have also shown that the DPA analogues containing lipids with isoprenes in the *Z*-configuration are competitive substrates for DPA in the AraT assays. DPA has a unique stereoconfiguration and contains all of the isoprene residues at the α-end in the *Z* configuration and only one *trans* (*E*)-isoprene residue at its ω-end (Figure 2).[30] This is in contrast with most bacteria that use C55-undecaprenol phosphate, consisting of 11 isoprene units in

the $\omega$-di-*E*/octa-*Z* configuration. Our data suggests that for optimal Ara*f* donating capability, isoprene units in the $\omega$-di-*Z* configuration as in *Z*-FPA are sufficient and it is not necessary to introduce *E*-configuration in the $\omega$-end as in the *Z*-HPA. We believe that lipids in the *Z*-configuration are favored because structurally *Z*-FPA is to some extent similar to DPA, which also contains *Z*-isoprene units. Moreover, there is precedence in the literature showing that membrane-associated enzymes involved in undecaprenyl-dependent pathways often accept shorter (10—15 carbon) lipid substituents *in vitro*.[31,32] The enhanced solubility of *Z*-FPA (C15) could be a second favorable factor for its optimal Ara*f*-donating activity. In our hands *Z*-NPA ($\omega$-mono-*Z* configuration) could donate an Ara*f* residue to the acceptor although not efficiently. This was in contrast to the earlier report[33] that showed no activity. One reason for this discrepancy is perhaps thepredominant presence of α-anomer in the earlier preparation.

All AraTs described are membrane proteins of the GT-C family. One characteristic feature of these proteins is that these are membrane bound, which beat all odds for isolation and purification. Even if these are isolated with difficulty, after expression and purification, GT-C membrane proteins are mostly insoluble and aggregate easily.[26] Therefore, developing an efficient assay to pursue functional studies on AftC or to screen for inhibitors has been problematic. We have been able to express and purify AftC and have shown that it has α-(1→3) AraT activity when reconstituted in a proteoliposome. Repeated attempts to express AftC and obtain it in large amounts in *E. coli* pLySS, BL21, or C43 strains[34] have failed, suggesting toxicity of the protein in a nonmycobacterial host. Experiments with other host strains of *E. coli* for expression of *aftc* are currently ongoing.

*In vitro* assay using the purified recombinant AftC and DPA failed to give any product. However, with the successful reconstitution of AftC-proteoliposome and replacement of DPA with analogues *Z*-FPA or *Z*-HPA, we were able to improve the *in vitro* assay with product formation in reasonable amounts. For restoring AraT activity for AftC, reconstructing a lipid environment seemed to be a crucial factor. The inactivity of the proteoliposome using DPPC might because (1) DPPC does not mimic the mycobacterial membrane well since it is positively charged and bacterial membranes are negatively charged; (2) DPPC is not present in mycobacterium and therefore it is not recognized by AftC; (3) a combination of phospholipids from *M. tuberculosis* are necessary for creating a "real" mycobacterial membrane environment mimicking the cell membrane that will foster proper folding of AraT. Mycobacterial lipid extracts are dominated with the presence of the phospholipids such as the family of phosphatidylinositol mannosides (PIMs). We reasoned that these lipids are required to mimic the membrane environment. When we fractionated the lipid extract into individual bands using preparative TLC, a lipid (Band IV) (Supporting Information Figure S4A and Chart S4B) showed better activity than other lipids for fabricating AftC proteoliposome (Supporting Information, Figure S4C). Mass spectral analysis revealed Band IV contained predominantly diacylPIM$_2$ ($Ac_2PIM_2$) with considerable heterogeneity in the fatty acyl composition ($Ac_2PIM_2$-16:0/16:0/16:0/19:0; 16:0/16:0/18:0/19:0; 16:0/16:0/18:0/18:0) (Supporting Information, Figure S6), indicating that these phospholipids are important aspects in retaining AftC AraT activity. Since these experiments were carried out in the absence of membranes, this lipid-dependent activity could be a function of intrinsic interactions between the donor lipid and the enzyme. We further speculate that $Ac_2PIM_2$ supports an active conformation of the enzyme. More work needs to be done to understand this result.

D-Ara*f* is unique to mycobacteria and is a constituent of the two most important macromolecules, AG and LAM, that play significant physiological and biological roles. The enzymes involved in the polymerization of AG and LAM have been suggested to present future opportunities for developing new therapeutics.[30] The facts that Ethambutol, a first line antituberculosis drug, inhibits arabinan synthesis[35] and new compounds have been identified that are capable of inhibiting the formation of DPA, also inhibiting *M. tuberculosis* grown intracellularly, support this notion.[5] Our present study describes isolation of one important AraT whose functional studies can now be pursued as a result of its availability in a recombinant form. AftC is essential in *M. tuberculosis*,[36] although a knockout mutant can be obtained in *M. smegmatis*. Thus, it is important to develop assays for the screening of small molecule inhibitors. The assay developed bypasses use of radioactive pRpp, isolation of DPA, and use of membrane preparations in which all AraTs are present.

Our ongoing efforts encompass (1) expression of *aftC* to produce larger quantities in *E.* coli to pursue functional aspects of AftC, (2) replacement of the aglycon on the acceptors with an "octylamine" tag for development of novel assays based on carbohydrate microarrays for small molecule screening, (3) demonstration that AftC is a valid anti-TB target, and (4) refinement of several assay parameters to increase the conversion rate of the enzymatic reaction, including optimization of detergents for protein dissolution and lipids for proteoliposome formation.

## METHODS

**Syntheses of the DPA Analogues.** The syntheses of DPA analogues were performed according to established methods.[37,38] The detailed synthetic procedure and spectroscopic data are presented in the Supporting Information.

**Cell-Free Assays and Structural Analyses of the Enzymatic Products.** Typical assay reaction mixtures contained buffer A that has 50 mM MOPS (pH 7.9), 5 mM 2-mercaptoethanol and 10 mM $MgCl_2$, ATP (62.5 $\mu$M), DPA analogues (1 mM), acceptor **1** (200 $\mu$M), and membranes (1.0 mg) in a total volume of 100 $\mu$L. The cell membranes were prepared from *M. smegmatis* according to the established procedure.[21] The reaction mixtures were incubated at 37 °C for 2 h and terminated by adding 666 $\mu$L of 1:1 $CHCl_3$/MeOH to make a final solution of $CHCl_3$/MeOH/$H_2O$, 10:10:3. The reaction mixture was centrifuged at 14,000 rpm for 10 min. The supernatant was then evaporated to dryness. The dried reaction mixtures without further processing were per-*O*-methylated, and resulting residues were dissolved in 50 $\mu$L of $CHCl_3$. One microliter of sample was mixed with 1 $\mu$L of 2,5-dihydroxy benzoic acid (DHB, 10 mg mL$^{-1}$ in 50% acetonitrile, 0.1% trifluoroacetic acid). The mixture was spotted on the MALDI target and allowed to air-dry. The sample was analyzed by an Ultraflex-TOF/TOF mass spectrometer (Bruker Daltonics, Billerica, MA) in positive ion, reflector mode using a 25 kV accelerating voltage. External calibration is done using a peptide calibration mixture (4 to 6 peptides) on a spot adjacent to the sample. The data was processed in the FlexAnalysis software (version 2.4, Bruker Daltonics).

**Competitive Assay.** The competition assays were performed according to the typical radioactive assay using DPA analogues as competitors. In brief, reaction mixtures contained buffer A (as above, pH 7.9) with 62.5 $\mu$M ATP, 0.8 $\mu$M p[$^{14}$C]Rpp (100,000 dpm), acceptor **1** (300 $\mu$M), 80 $\mu$M unlabeled competitors (*Z*-NPA, *Z*-FPA,

*E*-FPA or *E*-HPA), membranes (0.5 mg) and cell wall extracts (P60, 0.3 mg)[19] in a total volume of 160 μL. The reaction mixtures were incubated at 37 °C for 1 h and then terminated by adding 160 μL of ethanol. The resulting mixture was centrifuged at 14,000 × *g*, and the supernatants were passed through prepacked strong anion exchange (SAX) columns. The columns were eluted with 2 mL of water. The eluent was evaporated to dryness and partitioned between the two phases (1:1) of water saturated 1-butanol and water. Using liquid scintillation counting, the 1-butanol fractions were measured for radioactivity incorporation. For TLC analysis of the enzymatic products formed in the competition assay, an aliquot of the 1-butanol fraction was dried under air, and the residue was reconstituted in Milli-Q water (10 μL) for analysis by silica gel TLC. The TLC plate was chromatographed in $CHCl_3$/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).

**Overexpression of AftC (Rv2673) in *M. smegmatis*.** The entire coding sequence of *Rv2673* was PCR amplified from *M. tuberculosis* H37Rv genomic DNA using the primers Rv2673pJAMS forward-(5′-cggagatctgtgtacggtgcgctggtgacgg-3′) and Rv2673pJAMS reverse(5′-ccctctagaccgctggccctcccgctcgg-3′), excised with *Bgl*II and XbaI, and cloned into the compatible *Bam*HI and XbaI restriction sites of the expression vector pJAM2.[25] The resulting plasmid, pJAMRv2673, allows the inducible expression of *Rv2673* under control of the acetamidase promoter. The recombinant protein produced with this system had a hexa-histidine at its carboxyl terminus allowing its purification using metal affinity columns and detection by immunoblotting with the monoclonal Penta-His antibody from QIAGEN. Synthesis of Rv2673 in $mc^2$155/pJAMRv2673 cells grown at 37 °C in MM63 broth was induced during log phase with 0.2% acetamide for 12 h. The *M. smegmatis* control strain carried the empty plasmid, pJAM2. Cells were harvested by centrifugation (3,000 rpm) and frozen at −80 °C until further use.

**Generation of $His_6$-AftC.** Frozen cells were thawed on ice and suspended in 50 mM Tris-HCl buffer (pH 7.9) containing 150 mM NaCl (pH 8.0), and protease inhibitor (EDTA-free, Roche), DNAase, and Igepal CA-630 [1.0% (v/v)] were added to the cell suspension. Cells were disrupted by probe sonication on ice (10 cycles at 60 s on and 90 s off). Cell debris was removed by centrifugation (10,000 × *g*, 15 min, 4 °C). Soluble cell lysate was applied to affinity column containing Ni-NAT agarose (0.4 mL, QIAGEN). The column was washed with 20 mL of Tris-HCl buffer with 0.1% Igepal CA-630. Then, a gradient elution buffer (Tris-HCl) containing 5 to 300 mM of imidazole (pH 8.0) and 0.1% Igepal CA-630 was applied to elute the column over 15 column volumes. Fractions containing $His_6$-AftC (150 mM-200 mM of imidazole) were identified by SDS-PAGE and Western blot. Protein identity was confirmed using trypsin digestion and Mascot search engine (Proteomics and Metabolomics Facility located at Colorado State University). The $His_6$-Rv2673 was desalted on PD-10 column (GE Healthcare) and stored in Tris-HCl with 10% (v/v) glycerol at −80 °C until further use.

**AftC Liposome Reconstitution.** $CHCl_3$/$CH_3OH$-extracted lipids (5 mg) from *M. tuberculosis* $H_{37}Rv$ obtained from the Tuberculosis Research Material Contract (NIH) at Colorado State University were suspended in $CHCl_3$ and then dried under a gentle stream of nitrogen. One milliliter of the suspension buffer (1% Igepal CA-630 in 50 mM buffer A) was added to the film. The solution was left at RT for 2 h and was then sonicated. Purified recombinant AftC (200 μg) was added to the lipid−detergent mixture. The mixture was allowed to stand for 30 min on ice. Activated and washed BioBeads (300 mg) were added, and the mixture was stirred overnight at 4 °C to allow gentle removal of the detergent and incorporation of the AftC into the lipid bilayer. The BioBeads were removed, 250 μL of the fresh batch was added, and the suspension was stirred for 1 h at 4 °C. The mixture was centrifuged for 15 min (10,000 × *g*, 4 °C) and separated into a pellet and a milky supernatant containing AftC proteoliposome. The protein concentration of proteoliposome was determined by BCA method.

**Arabinosyltransferase Assays Using AftC Proteoliposome and DPA Analogues and Analysis by MALDI-TOF-MS.** A typical reaction mixture of 100 μL total volume contained 2 μM of Rv2673 proteoliposome, 1 mM acceptor **1**−**5**, and 2 mM Ara*f* donor (*Z*-FPA or *Z*-HPA) in buffer A. Reactions were incubated at RT for 2 h at 37 °C and then terminated by adding 666 μL of 1:1 $CHCl_3$/MeOH to make a final $CHCl_3$/MeOH/$H_2O$ of 10:10:3. The reaction mixture was centrifuged at 14,000 rpm for 10 min. The supernatant was evaporated to dryness in a SpeedVac. The dried samples were directly per-*O*-methylated and then subjected to MALDI-TOF-MS in the positive ionization mode according to the methods described previously.[21] Since the penta-arabinosyl acceptor and the hexa-arabinosyl enzymatic product are expected to have very similar properties during MALDI-TOF-MS and are present together in the sample and their ions collected in the same laser acquisition, we hypothesized that the area of the M + $Na^+$ ions peaks would be proportional to the abundance of the actual oligosaccharides present. This hypothesis was tested using known ratios of synthetic octyl hexa-arabinoside and synthetic octyl penta-arabinoside (molar ratios of 1, 0.5, 0.25, and 0.125 were tested). Indeed a linear relationship between the ratio of the two oligosaccharides and the ratio of their respective M + $Na^+$ ion peaks was found. A standard curve was generated as presented in Supporting Information Figure S5. This standard curve was then used to calculate the ratio of product to substrate from the ratio of the M + $Na^+$ ion peaks in actual samples.

## ■ ASSOCIATED CONTENT

**Supporting Information.** This material is available free of charge *via* the Internet at http://pubs.acs.org.

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