Synthesis of the Mycobacterial Arabinose Donor β-D-Arabinofuranosyl-1-monophosphoryldecaprenol, Development of a Basic Arabinosyl-Transferase Assay, and Identification of Ethambutol as an Arabinosyl Transferase Inhibitor

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Abstract: Mycobacterial diseases, such as tuberculosis and leprosy, are serious human pathogens, in which the cell wall arabinans of arabinogalactan and lipoarabinomannan are essential components of the bacterial cell wall. The chemical synthesis of the key mycobacterial arabinose donor [1-^{14}C]-β-D-arabinofuranosyl-1-monophosphoryldecaprenol **2** is described by an application of the phosphoramidite-phosphite triester methodology to form the β-arabinofuranosyl, allylic phosphodiester. The synthesis uses a novel *tert*-butyl dimethylsilyl arabinofuranosyl protection strategy, which allows for a regioselective C-1 acid hydrolysis and final full deprotection with ammonium fluoride under mild conditions. A basic arabinosyl transfer assay was developed to study the incorporation of **2** into the cell wall arabinans of mycobacteria. The incorporation was proportional with respect to both the concentration of membrane protein and the acceptor. The epimeric substrate [1-^{14}C]-α-D-arabinofuranosyl-1-monophosphoryldecaprenol was inactive and noninhibitorial in this assay. The antimycobacterial drug ethambutol was found to be active suggesting that its mode of action is as an inhibitor of arabinosyl transfer.

Introduction

Mycobacterial diseases such as tuberculosis and leprosy have plagued man since the earliest civilizations with *Mycobacterium tuberculosis* infection still the leading killer by a single infectious agent in the world today.[1a-c] As a consequence of the HIV epidemic and the lack of potency of current multidrug regimens, mutational drug resistance has become more evident, and the need to develop a novel series of antituberculosis agents has become a high priority.[2ab] Many of the current antimycobacterial agents, such as ethambutol (EMB) have resulted from the random screening of compound libraries and not from rational drug design.[3] Early studies conducted with EMB demonstrated that the *S,S* absolute stereochemistry (Figure 1) was essential for activity[4ab] and its bacteriostatic properties were exclusive to mycobacteria and closely related actinomycetes.[5] More recently, studies on the biochemical affects of EMB have shown a selective decrease in the arabinose content of the mycobacterial cell wall,[6a-c] leading to increased permeability, bacteriostasis, and noted synergistic effects of EMB with other antimycobacterial drugs.[7a-c] However, the precise mode of action of EMB is unknown. It is envisaged that through an understanding of the basic biochemistry and molecular biology of arabinan metabolism the primary mode of EMB action will emerge.

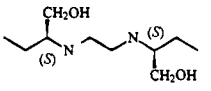

Figure 1. Ethambutol.

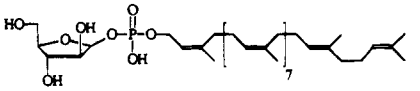

Figure 2. β-D-Arabinofuranosyl-1-monophosphoryldecaprenol, **1**.

The D-arabinan segments of the mycobacterial cell wall also provide attractive targets for new drug development due to the xenobiotic status in the human host. Recently, a key mycobacterial arabinose donor β-D-arabinofuranosyl-1-monophosphoryldecaprenol, **1** (Figure 2), was identified within the lipid extracts of *Mycobacterium smegmatis* and implicated in the biogenesis of the two major cell wall polysaccharides arabinogalactan (AG) and lipoarabinomannan (LAM).[8] In addition **1** has proved to be the only intermediate, thus far isolated, involved in arabinan biosynthesis. Unfortunately the low stability and poor biological availability through metabolic

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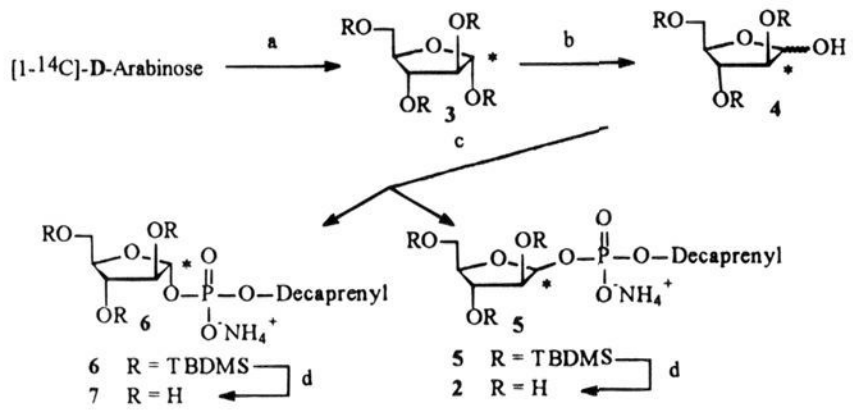
Scheme 1[a]



[a] (a) TBDMSCl, imidazole, DMF (65%); (b) (i) TFA, CH_2Cl_2 (ii) NH_4OH, MeOH, −20 °C (77%); (c) (i) decaprenol, $(CNCH_2CH_2O)$-$(^iPr_2N)PCl$, EtN^iPr_2, CH_2Cl_2 (ii) **4**, tetrazole (iii) H_2O_2, THF (overall **5**, 11% and **6**, 48%); (d) NH_4F, MeOH (**2**, 95% and **7**, 93%).

whole-cell labeling of [1-^{14}C]-β-D-arabinofuranosyl-1-monophosphoryldecaprenol, **2**, has hindered studies of arabinan metabolism. As a consequence a synthetic strategy to synthesize **2** has been developed, in order to explore the biosynthesis of AG and LAM and the mode of action of EMB.

Results and Discussion

Synthesis of 2. The classical synthesis of polyprenyl phosphosugars by Jeanloz and Warren involves a two stage process, firstly the preparation of an appropriately protected sugar phosphate in the correct salt form and, secondly, the coupling of the protected sugar phosphate with the polyprenol using the coupling reagent triisopropylbenzenesulfonyl chloride.[9] More recently, this approach has been improved by the coupling of sugar phosphates with activated polyprenyl trichloroacetimidates.[10] Utilization of this strategy would require, firstly, an efficient synthesis of β-D-arabinofuranosyl-1-phosphate which had not been described, the major problem being that the α-anomer is strongly favored, and, secondly, it is a multistep process which would be difficult to convert to a microscale synthesis of the desired radiolabeled product. These factors lead us to explore a new synthetic strategy to overcome both the highly unsaturated nature of the polyprenol moiety and the high lability of the arabinofuranosyl, allylic phosphodiester.

Recent advances in the synthesis of oligonucleotide derivatives have resulted in new methods for synthesizing phosphodiesters, the most notable being the phosphoramidite-phosphotriester approach,[11] which has been utilized to synthesize a wide variety of phosphodiesters apart from the classical 3′-5′ oligonucleotide linkage.[12ab] The technique has been also used to synthesize a number of phosphomonoesters including sugar 1-phosphates and allylic phosphates.[13a−c,14] We planned to utilize this technique to synthesize **2**, thus, simplifying the overall synthesis by combining the formation of the sugar phosphate and the prenol phosphodiester coupling to one step.

The key to the synthesis (Scheme 1) was the development of a novel silyl based arabinofuranosyl protection strategy. Treatment of the starting material [1-^{14}C]-D-arabinose with *tert*-butyldimethylsilyl chloride (TBDMSCl) in the presence of imidazole and DMF afforded **3** the tetra-protected arabinofuranoside as colorless crystals. The product was subjected to a selective C-1 acid hydrolysis using TFA. A selective quench of ammonium hydroxide in methanol at −20 °C minimized C-5 hydrolysis. Column chromatography afforded the triprotected arabinoside **4** as an α/β mixture (3:1).

The phosphoramidite coupling was then performed under standard conditions using 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite and coupling firstly decaprenol[15] then **4**. Notably, deprotection of the cyanoethyl group by the conventional use of ammonium hydroxide in methanol resulted in much degradation, which was circumvented by the use of potassium hydroxide in methanol as a more basic hydroxide source. The anomeric products were separable by flash column silica gel chromatography. Facile deprotection of the *tert*-butyldimethylsilyl protecting groups with ammonium fluoride yielded **2** and its α anomer **7** respectively, quantitatively.

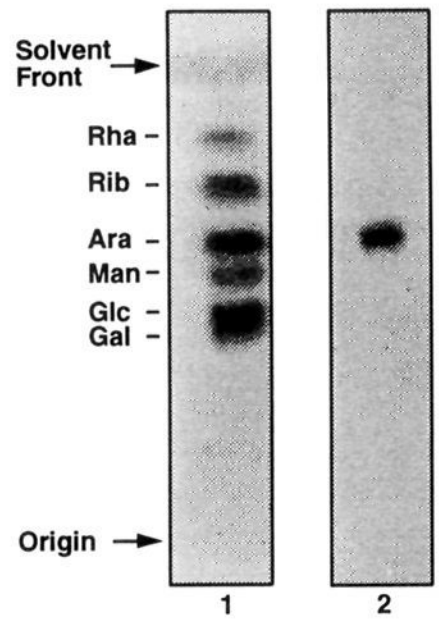


Figure 3. Polymeric product analysis: lane 1, sugar standards (phthalic acid stain) and lane 2, Product hydrolysate (autoradiogram).

Biological Studies of 2. Using **2** a basic arabinosyl transfer assay was then developed using the enzymatically active membrane fraction of *M. smegmatis*. The incorporation into wall material was assayed using descending paper chromatography in a similar manner to that described by Ito *et al.* for the incorporation of galactosyl phosphorylpolyprenols into the lipoteichoic acids of *Bacillus coagulans*.[16] The mycolylarabinogalactan rich (P60) particulate cell wall fraction obtained as a byproduct of the membrane preparation was utilized as the acceptor for these experiments.[17] Optimum conditions for the glycosyl transfer were at pH 8 and in the presence of the divalent cation magnesium. Incorporation was considerably boosted by coincubation with a variety of sugar nucleotides which may be attributable to the *in situ* generation of various acceptors. The radiolabel in the polymeric fraction was found to reside exclusively in arabinose after re-extraction from the paper chromatogram followed by sugar analysis (Figure 3). The incorporation was found to be linear with respect to membrane protein and acceptor P60 concentration (Figure 4). A related time course experiment showed a smooth incorporation of **2** with respect to time (Figure 5) and the fact that the α anomer

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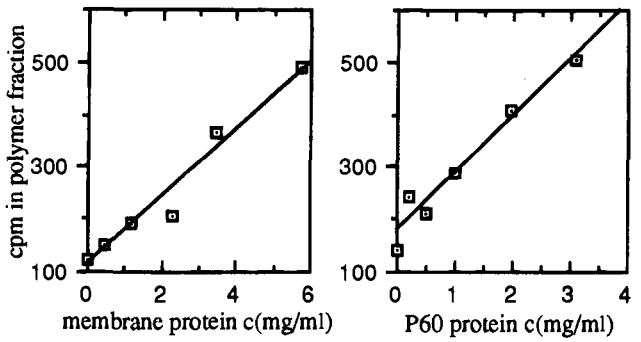

Figure 4. The effect of membrane and P60 concentration.

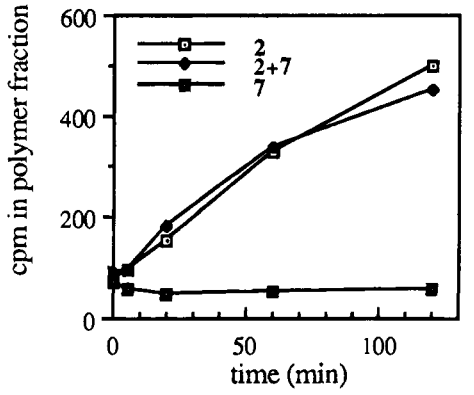

Figure 5. Incorporation of β-**2**, α-**7**, and a equimolar α + β mixture (**2** + **7**) into polymer relative to time.

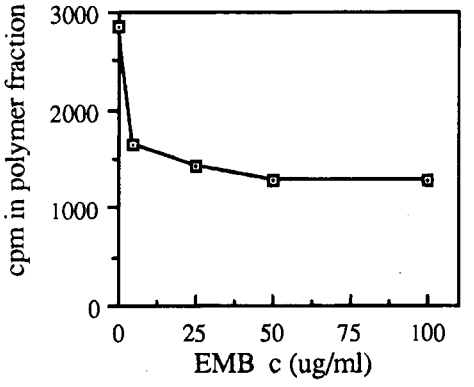

Figure 6. Ethambutol inhibition curve.

7 was inactive. Coincubation of **2** with **7** resulted in no inhibition of the incorporation of **2**. This feature is analogous with other glycosyl transferase systems where the enzyme shows high anomeric specificity and where the epimeric substrate is neither active or inhibitorial.[18a-c] EMB clearly inhibited the arabinosyl transfer (Figure 6), to a residual activity of around 40% at 50 μg/mL. The residual activity was unaffected by coincubation with a variety of enzymic cofactors, which may be attributable to **2** being a substrate to more than one arabinosyl transferase within the AG and LAM biosynthetic pathways. The arabinan components of AG and LAM are distinguished by several different arabinosyl linkages,[19] which are indicative of the involvement of a number of arabinosyl transferases within their biosynthetic pathways. Thus, these result strongly suggest that the primary site of EMB action is as an inhibitor of one or more arabinosyl transferases. EMB has a simple molecular structure and is probably an arabinan mimetic acting on a specific biosynthetic step within the AG/LAM pathway. It is also noteworthy that while much research has gone into azasugars and their analogues as an aid to study glycosyl processing and in the development of new drugs, EMB with its simple molecular structure possessing such potent biological properties has been in clinical usage for over 30 years without a full understanding of its mode of action.

Experimental Section

Radiolabeled Synthesis. General. All chemicals were purchased to the highest purity available from the Aldrich Chemical Company and used without any further purification, including anhydrous solvents acetonitrile, dichloromethane, toluene, and tetrahydrofuran and excluding decaprenol (Ficaprenol C50) which was purchased from the Sigma Chemical Company and [1-^{14}C]-D-arabinose which was purchased from American Radiochemicals. ^{1}H-NMR, ^{13}C-NMR, and ^{31}P-NMR were measured at 500, 75, and 121 MHz. All non-phosphorylated samples were measured in $CDCl_3$. Phosphorylated samples were measured in a mixture of $CDCl_3$:CD_4OD:ND_4OD (600:300:30). ^{31}P-NMR was set to an external reference of neat phosphoric acid at $\delta_P 0$. Both HRMS FAB ($^+$ve) and ($^-$ve) were performed using a *m*-nitrobenzyl alcohol matrix. Column chromatography was performed using silica gel 60 (230–400 mesh) purchased from the Aldrich Chemical Company.[20] All reactions were performed in oven dried glassware and under an argon blanket.

[1-^{14}C]-D-1,2,3,5-(Tetra-*tert*-butyldimethylsilyl) arabinose (3). [1-^{14}C]-D-Arabinose (1 mCi, 3.8 mg) in ethanol/water 9:1 was concentrated *in vacuo* and further dried by coevaporation with dry acetonitrile (2 × 3 mL) before being placed in a high vacuum dessicator for 2 h. The [1-^{14}C]-D-arabinose was diluted by the addition of 28.1 mg of cold D-arabinose. Thus, D-arabinose (27 mg, 0.2 mmol) was dissolved in DMF (1 mL) to which was added sequentially imidazole (68 mg, 1 mmol) and *tert*-butyldimethylsilylchloride (144 mg, 0.96 mmol). The mixture was then stirred at room temperature for 10 min before being heated at 70 °C for 2 h and left at 4 °C overnight. The resultant colorless solid was filtered and recrystallized from hot chloroform (500 μl) and 5% ammonium hydroxide in methanol (2 mL). The product was then filtered off and dried *in vacuo* to yield **3** (81 mg, 65%).

[1-^{14}C]-D-2,3,5-(Tri-*tert*-butyldimethylsilyl)arabinose (4). To a solution of **3** (80 mg, 0.13 mmol) in chloroform (3 mL) was added TFA (0.75 mL), and the mixture was stirred for 1 min before being poured into a stirred solution of ammonium hydroxide (1.5 mL) in methanol (10 mL) at −20 °C. The mixture was then allowed to warm to room temperature before partitioning between chloroform (20 mL) and water (20 mL). The organic phase was taken and concentrated *in vacuo* and purified by silica gel column chromatography using firstly, petroleum ether/toluene (3:1) to afford residual **3** (12.7 mg, 16%) and, secondly, petroleum ether/ethyl acetate 15:1 to afford **4** (50.3 mg, 77%)-which cochromatographed with an authentic sample of **9**[21] on silica gel tlc R_f 0.55 (petroleum ether/ethyl acetate15:1).

Ammonium [1-^{14}C]-β-D-2,3,5-(Tri-*tert*-butyldimethylsilyl)arabino-1-monophosphoryldecaprenol (5) and Ammonium [1-^{14}C]-α-D-2,3,5-(Tri-*tert*-butyldimethylsilyl)arabino-1-monophosphoryldecaprenol (6). Decaprenol (78.3, 112 μmol) was placed in a round bottomed flask and dried by coevaporation with anhydrous toluene (2 × 3 mL). Dichloromethane (1.5 mL) and *N,N*-diisopropylethylamine (23 μl, 134 μmol) were added. The flask was then cooled to 0 °C before the dropwise addition of 2-cyanoethyl *N,N*-diisopropylchlorophosphoramidite (28 μl, 123 μmol). The mixture was then allowed to warm to room temperature and stirred for 30min before being cooled to 0 °C. **4** (60.6 mg, 68 μmol) in dichloromethane (1 mL) and tetrazole (11.3 mg, 162 μmol) were added and the mixture was allowed to warm to room temperature and left to stir for 4 h. The solvent was then removed under reduced pressure and the thick syrup was then titurated with petroleum ether (3 x 2 mL). The petrol washings were collected and

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filtered through a glass wool plug, concentrated under reduced pressure to yield a colorless oil. The oil was redisolved in tetrahydrofuran (3 mL) and hydrogen peroxide (30% v/w, 20 μl, 308 μmol) was added. The mixture was stirred for 30 s before the addition of potassium hydroxide 5% in methanol (6 mL). The mixture was then stirred for 30 min before partitioning between water (10 mL) and chloroform (10 mL). The organic phase was taken and washed with saturated brine and concentrated *in vacuo*. Silica gel column chromatography using a gradient of chloroform/methanol/ammonium hydroxide 465:25:4 to 265:25:4 afforded two major products firstly **5** (15.6 mg, 11%) R_f 0.55 (165:25:4 chloroform/methanol/ammonium hydroxide) and secondly **6** (69.0 mg, 48%) R_f 0.50 (165:25:4 chloroform/methanol/ammonium hydroxide).

[1-^{14}C]-β-D-arabinofuranosyl-1-monophosphoryldecaprenol (2). **5** (15.6, 12.2 μmol) direct from the column was concentrated into a 16 × 100ml screw topped tube containing a stir bar and 5% ammonium hydroxide in methanol (8 mL) and ammonium fluoride (100 mg) were added. The tube was then capped and placed in a stirring heating block at 55 °C for 3 days, chloroform (6 mL) was added and the resulting precipitated ammonium fluoride was filtered off. The eluent was then concentrated *in vacuo* and purified by silica gel column chromatography using a chloroform:methanol:ammonium hydroxide gradient 165:25:4 to 65:125:4 to yield **2** (62 × 10^{6}cpm, 10.8 mg, 95%) R_f 0.60 (65:25:4 chloroform/methanol/ammonium hydroxide) (see autoradiogram in supporting information (Figure 7)).

[1-^{14}C]-α-D-arabinofuranosyl-1-monophosphoryldecaprenol (7). **7** was prepared using the procedure described above for the preparation of **2**. Yielding **7** (268 × 10^6 cpm, 42.6 mg, 93%) R_f 0.55 (65:25:4 chloroform/methanol/ammonium hydroxide).

Arabinosyl Transferase Assays. Materials. *M. smegmatis* mc^2-155 was obtained from W. R. Jacobs, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY. Ethambutol, UDP-*N*-acetylglucosamine, UDP-galactose, GDP-mannose, ATP, MOPS, 2-mercaptoethanol, and magnesium chloride were purchased from the Sigma Chemical company, U.S.A. TDP-rhamnose was prepared as described by Okazaki.[22] Percoll was purchased from Pharmacia, Sweden. Nonidet P40 was purchased from Boehringer Mannheim, Germany. Protein concentrations were determined using the BCA assay kit purchased from Pierce, USA. Descending paper chromatography utilized Whatmann 3MM paper. Cellulose TLC was performed on Baker-flex cellulose plastic plates from the J. T. Baker Chemical Company. Autoradiograms were produced by exposure of the resulting TLC plates to Kodak X-Oman AR film at −70 °C.

Preparation of Membranes and P60. *M. smegmatis* mc^{2}155 cells (10 g) were harvested at mid log (o.d. 0.4), washed, resuspended in ice cold buffer A which contained 50 mM MOPS buffer pH 8, 5 mM 2-mercaptoethanol, and 10 mM magnesium chloride (30 mL) and subjected to probe sonication at 4 °C for a total time of 10 min in ten 60 s pulses with 90 s cooling intervals between pulses. The whole sonicate was then centrifuged at 27 000g at 4 °C for 12 min. The supernatant was taken and recentrifuged at 100 000g for 1 h to yield a clear membraneous pellet. The pellet was resuspended with buffer A (400 μl). The protein concentration of the membrane fraction was determined to be typically 15−20 mg/ mL.

The 27 000 g pellet was resuspended with buffer A to a final volume of (20 mL). The suspension was split into two centrifuge tubes, and Percoll was added to each tube to achieve a 60% suspension. The tubes were then centrifuged at 27 000 g for 1 h. The particulate cell wall fraction which migrated as a diffuse band, approximately two-thirds of the volume height from the bottom of the tube, was then collected and washed three times with buffer A, before being finally resuspended in buffer A (5 mL) to give the cell wall P60 fraction with a typical protein concentration of 8−10 mg/mL.

Basic Arabinosyl Transfer Assay. The assay for arabinose incorporation from [1-C^{14}]-β-**D**-arabinofuranosyl-1-monophosphoryl-decaprenol **2** into polymer, consisted of **2** (20 000 cpm, 9 μM), 50 mM MOPS buffer pH 8, 10 mM magnesium chloride, 5 mM 2-mercaptoethanol, O.1% Nonidet P-40, 0.1 mM TDP-rhamnose, 0.1 mM UDP-*N*-acetylglucosamine, O.1 mM UDP-galactose, O.1 mM GDP-mannose, 0.4−0.5 mg of membranes (estimated protein concentration) and 0.2−0.3 mg of the P60 particulate cell wall fraction (estimated protein concentration) in a final volume of 80 μl. After incubation for 1 h at 37 °C the reaction mixture was subjected to descending paper chromatography using the eluent isobutyric acid/0.5 M ammonium hydroxide (5:3 by volume). The radioactivity retained at the origin of the chromatogram was counted to determine incorporation into polymer.

The Effects of Membrane and P60 Acceptor Concentration. This experiment used the same basic assay described above but varying concentrations of membranes (0−5.8 mg/mL) with a constant cell wall P60 concentration (3.1 mg/mL) or alternatively P60 (0−3.1 mg/mL) with a constant membrane protein concentration (5.8 mg/mL) in a final volume of 80 μl.

Product Analysis. The basic assay was scaled up 8-fold to a final volume of 640 μl which contained **2** (120 000 cpm). The reaction mixture was incubated at 37 °C for 1 h and then subjected to descending paper chromatography using the protocol described earlier. The origin of the paper chromatogram was taken and extracted by washing with water (3 × 2 mL). The washings were then filtered, concentrated, and finally hydrolyzed by treatment with 2 M TFA (200 μl) for 1 h at 120 °C. Sugar compositional analysis on the product was performed using cellulose tlc and using a three fold development in the eluent formic acid/water/*tert*-butyl alcohol/methyl ethyl ketone (15:15:40:30). The plate was then exposed to autoradiography before being stained with phthalic acid−butanol−aniline reagent to locate the ribose, arabinose, glucose, mannose, and rhamnose standards.

Time Course Assay. Three sets of five tubes were prepared. The first set contained **2** (20 000 cpm each), the second set contained **2** (20 000 cpm) and **7** (20 000 cpm), and the third set of tubes contained **7** (20 000 cpm). The basic assay mixture was then added to each tube which were then incubated at 37 °C, and at time intervals of 0, 5 min, 20 min, 1 h, and 2 h the reactions were stopped by the addition of ethanol (1 mL). The contents of the tubes were then loaded onto paper, and incorporation was assayed as described above.

The Effect of Ethambutol on the Basic Assay. Five tubes were prepared according to the basic assay protocol but were scaled up 4-fold. Thus, **2** (48 000 cpm) was incubated in a total reaction mixture of 320 μl containing EMB at the concentrations of 0, 5, 25, 50, and 100 μg/ mL. After incubation for 1 h the incorporation into polymer was determined by the standard methodology.

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Supporting Information Available: Contained is the TLC autoradiogram of **2** (Figure 7) and the synthesis of model compounds β-D-arabinofuranosyl-1-monophosphoryl farnesol and α-D-arabinofuranosyl-1-monophosphorylfarnesol complete with ^{1}H and ^{13}C NMR spectra (18 pages). This material is contained in many libraries on microfiche, immediately follows this article in the microfilm version of this journal, and can be ordered from the ACS, and can be down loaded from the Internet; see any current masthead for ordering information and Internet access instructions.

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