Synthesis and Rapid Purification of ^{32}P-Labeled Photoactive Analogs of Farnesyl Pyrophosphate

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SUMMARY

To study the binding sites of protein prenyltransferases, we have synthesized photoaffinity labeling analogs that contain photoactive benzophenone units. Here we describe the syntheses and a method for the rapid purification of two ^{32}P-labeled analogs; phosphorylation was accomplished by reacting the corresponding alcohols with CCl_3CN and [^{32}P]-H_3PO_4 in CH_3CN and the purification was performed using C_{18} reversed-phase cartridges. Analog [^{32}P]-**3a** was prepared from 2.4 mg of starting alcohol **4a** and was obtained in 3.3% yield, 50% radiochemical purity and 480 Ci/mol specific activity. Further purification by preparative thin layer chromatography yielded material with a radiochemical purity of 90%. A second analog, [^{32}P]-**3b**, was prepared from 1.2 mg of alcohol **4b** and was obtained in 1.0% yield, 54% radiochemical purity and 700 Ci/mol specific activity. Further purification by preparative thin layer chromatography yielded material with a radiochemical purity of 91%.

Key words: Farnesyl Pyrophophate Analog, Geranylgeranyl Pyrophosphate Analog, Photoaffinity Labeling, Benzophenone, Diphosphate.

INTRODUCTION

Protein prenyltransferases catalyze the attachment of farnesyl or geranylgeranyl isoprenoids to specific cysteine residues in proteins.[1] Recently, these proteins have attracted considerable attention due to the observation that Ras protein, a protein involved in many forms of cancer, is farnesylated. Since farnesylation is essential for Ras protein function, inhibition of this modification may block the oncogenic effects of mutant Ras proteins. For this purpose, a number of inhibitors of protein farnesyl transferase have been prepared.[2,3] To understand how protein farnesyl transferase and the related enzyme, protein geranylgeranyl transferase, recognize and differentiate between their respective substrates we are studying the prenyl group binding site of these proteins by photoaffinity labeling.[4,5] To date, we have prepared eleven different analogs of farnesyl pyrophosphate (**1**) and geranylgeranyl pyrophosphate (**2**).[6,7] To identify the site of crosslinking for each of these molecules, it is necessary to have a simple procedure for the preparation of radioactive forms of these analogs. For this purpose,

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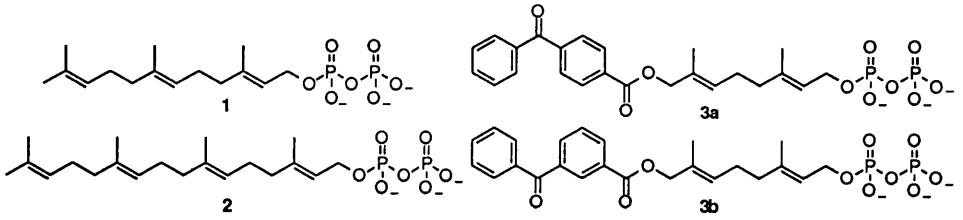
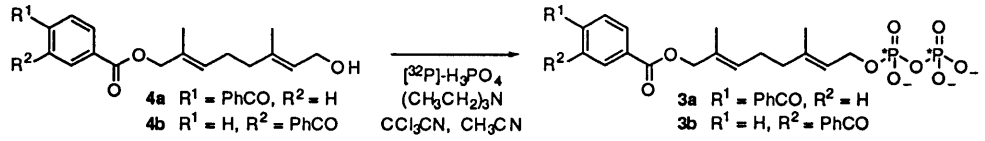
many investigators in this area have prepared ^{3}H-labeled compounds.[8-10] ^{32}P-labeled analogs are attractive alternatives because they allow the label to be introduced in the last step of the synthesis and they can be prepared in very high specific activity. Recently, Bukhtiyarov, Allen, and Omer reported an elegant approach for the preparation of ^{32}P labeled analogs of diazotrifluoropropionate-containing diphosphates.[11] Here, we describe the syntheses and a simple method for rapid purification of the ^{32}P-labeled analogs **3a** and **3b** shown below.



RESULTS AND DISCUSSION

In our initial synthesis, **3a** and **3b** were prepared by converting **4a** and **4b** to their corresponding allylic chlorides followed by displacement with $[n\text{-butyl})_4N]_3HP_2O_7$.[12] To prepare the ^{32}P-labeled analogs a different procedure was employed; this modification was made to avoid having to prepare $[n\text{-butyl})_4N]_3HP_2O_7$ on a small scale suitable for radioactive synthesis. Unlabeled **3a** was first prepared using this alternative procedure by reacting **4a** (3.6 mg) with anhydrous H_3PO_4 and CCl_3CN in CH_3CN using conditions described by Cramer shown below.[13,14]



The crude reaction product was then applied to a small reversed-phase C_{18} cartridge and the resin was eluted with a CH_3CN step gradient. The absorbance at 256 nm (indicative of the benzophenone chromophore) for each fraction is shown in Figure 1. Thin layer chromatography analysis of the column fractions indicated that **3a** was present in the 40% and 50% CH_3CN fractions and that the 70% and 80% CH_3CN fractions contained mainly starting material (**4a**) and impurities. The crude reaction product and the 40% and 50% CH_3CN fractions from the reversed-phase column were also analyzed by HPLC. Figure 2A shows an HPLC chromatogram from a sample of crude product. Compound **3a**

is present as a small peak that elutes at 21 min. Figure 2B shows a chromatogram of a sample from the 40% CH_3CN fraction. Integration of the peak areas from this fraction indicated a purity of 54% for **3a**; the main impurity present was the monophosphate (that eluted at 26 min) resulting from incomplete phosphorylation.

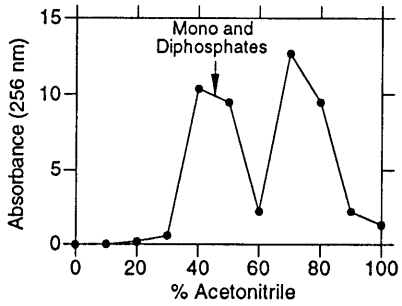


Figure 1 Purification of **3a** by Sep-Pak Chromatography.

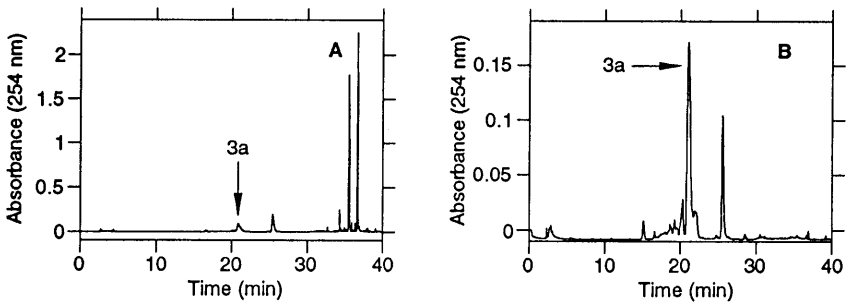


Figure 2 HPLC analysis of (A) crude **3a** and (B) **3a** after Sep-Pak chromatography.

No attempt was made to optimize these conditions since it was know from previous investigations that longer reaction times or higher phosphate/alcohol ratios result in the formation of polyphosphate or dimeric products. The identity of **3a** as the desired product was confirmed by coinjection with **3a** prepared via the chloride and the yield of **3a** was determined by UV spectroscopy using the extinction coefficient previously determined for **3a**. Thus, using this direct pyrophosphorylation procedure, **3a** was prepared in 21% yield and 54% chemical purity. Given the simplicity of this procedure and the facile purification that yielded material of reasonable purity, this was deemed a useful method for the preparation of ^{32}P labeled benzophenone-containing farnesyl pyrophosphate analogs.

[^{32}P]-**3a** was then prepared from 2.4 mg of **4a** and 5 mCi of [^{32}P]-H_3PO_4 using the procedure outlined above for **3a**. Fractionation of the crude material using a Sep-Pak column was then performed as described for **3a** except that the gradient consisted of 5% CH_3CN steps between 30% and 50% CH_3CN. Samples from each fraction were separated by thin layer chromatography and analyzed using a phosphorimager. Based on this analysis, material eluted with 35% CH_3CN was determined to be the most pure. A thin layer chromatography analysis of this sample quantitated by phosphorimaging is shown in Figure 3A. Thus, [^{32}P]-**3a** was obtained in 3.3% yield, 50% radiochemical purity and 480 Ci/mol specific activity. [^{32}P]-**3a** was further purified by preparative thin layer chromatography. A thin layer chromatography analysis of the resulting material quantitated by phosphorimaging is shown in Figure 3B. Area integration of the data revealed a radiochemical purity of 90%.

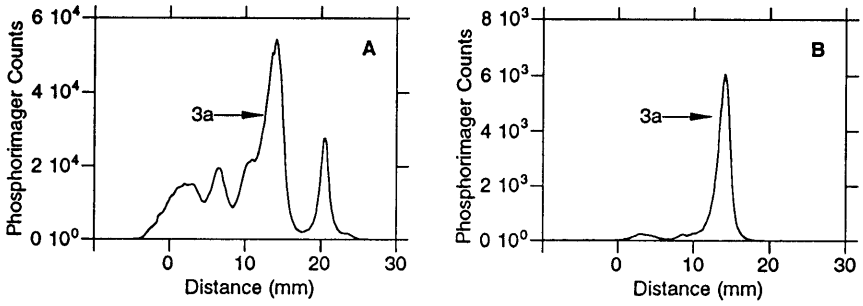


Figure 3 Phosphorimaging analysis of [^{32}P]-**3a** samples fractionated by thin layer chromatography. (A) [^{32}P]-**3a** purified by Sep-Pak chromatography and (B) purified by preparative thin layer chromatography.

Compound [^{32}P]-**3b** was prepared on a smaller scale using 1.2 mg of **4b** and 5 mCi of [^{32}P]-H_3PO_4 employing the procedure outlined above for **3a**. Using this smaller scale reaction, [^{32}P]-**3b** was obtained in 1.0% yield, 56% radiochemical purity and 700 Ci/mol specific activity. A thin layer chromatography analysis of the product quantitated by phosphorimaging is shown in Figure 4A and a similar analysis of [^{32}P]-**3b** purified by preparative thin layer is shown in Figure 4B. Area integration of the latter revealed a radiochemical purity of 91%.

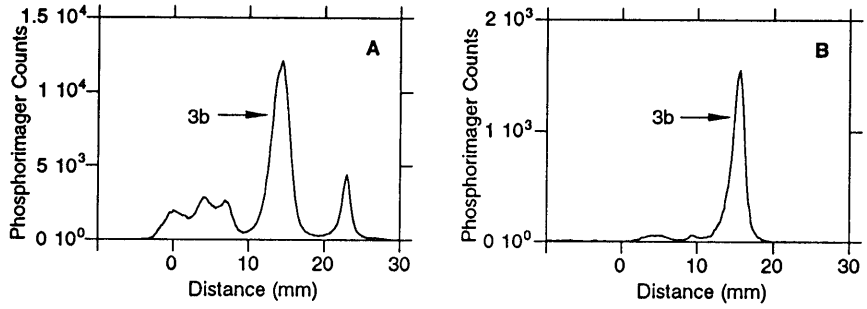


Figure 4 Phosphorimaging analysis of [^{32}P]-**3b** samples fractionated by thin layer chromatography. (A) [^{32}P]-**3b** purified by Sep-Pak chromatography and (B) purified by preparative thin layer chromatography.

This paper describes a convenient procedure for the synthesis and purification of radiolabeled analogs of farnesyl pyrophosphate with moderate specific activities (480 - 700 Ci/mol). Material with a specific activity as high as 5,700 Ci/mol has been prepared using this procedure although the reduced concentration of carrier H_3PO_4 present in high specific activity reactions (still performed on a 5 mCi scale) results in a lower yield of diphosphate product (0.2%). It should be noted that this limited amount of product can still be effectively purified and recovered using the reversed-phase purification described herein. Thus, this facile procedure should be useful for the preparation of a wide variety of radiolabeled farnesyl pyrophosphate analogs.

EXPERIMENTAL

Materials. All reactions were conducted under dry nitrogen and stirred magnetically. Thin layer chromatography was performed on precoated (0.25 mm) silica gel 60F-254 plates purchased from E. Merck and visualized by UV irradiation, autoradiography, or phosphorimaging. CH_3CN was distilled from CaH_2. Sep-Pak Plus columns (C_{18} Environmental) were obtained from Waters. [^{32}P]-H_3PO_4 (specific activity 8500-9120 Ci/mmol) was obtainined from DuPont NEN. Compounds **4a** and **4b** were prepared as previously described.[7]

Instrumentation. UV spectra were obtained using a Hewlett Packard 8452A spectrophotometer. HPLC separations were carried out using a Beckman model 127/166 instrument

equiped with a diode array UV detector and a Phenomenex Luna C_{18} column (5 μm, 4.6 x 250 mm). Liquid scintillation counting was carried out using a Beckman LS3801 instrument and phosphorimaging analysis was performed with a Moleular Dynamics 445 SI Phosphorimager.

(*E,E*)-8-*O*-(4-Benzoylbenzoyl)-3,7-dimethyl-2,6-octadiene-1-diphosphate (3a). Anhydrous H_3PO_4 (1.6 mg, 20 μmol) was prepared by lyophilization of 110 μL of a 1% (v/v) of H_3PO_4. The lyophilization was performed by placing a 10 mL round bottom flask containing the frozen solution of H_3PO_4 in a 500 mL lyophilization jar containing P_2O_5 on silica gel (Fluka) under a layer of glass wool. To the anhydrous H_3PO_4 was added a solution of triethylamine (4.1 mg, 40 μmol) in CH_3CN (50 μL). Alcohol **4a** (3.6 mg, 10 μmol) in 200 μL of CH_3CN containing 20% CCl_3CN (v/v) was then added and the reaction was allowed to stir under N_2 for 2 h after which time the volatile components were removed under a stream of N_2. The crude reaction mixture was then dissolved in 1 mL of 25 mM NH_4HCO_3 and applied to a Sep-Pak C_{18} cartridge equilibrated in the same solution. The cartridge was washed with 4 mL of 25 mM NH_4HCO_3 and eluted with a step gradient of increasing [CH_3CN] (2 mL per step, 10% CH_3CN per step) from 0% CH_3CN to 100% CH_3CN for a total of 10 2 mL fractions. The absorbance at 256 nm was determined for each sample to locate the benzophenone-containing fractions. Samples (100 μL) from each fraction were also concentrated ten fold and analyzed by thin layer chromatography in 2-propanol/NH_4OH/H_2O (6:3:1, v/v/v). The 40% and 50% CH_3CN-containing fractions were also analyed by HPLC. HPLC analysis was carried out by injecting 100 μM samples, eluting with a flow rate of 0.8 mL/min using a gradient of solvent A (25 mM NH_4HCO_3) and solvent B (CH_3CN) and detecting at 256 nm. Elution was performed by isocratic elution with 20% solvent B for 10 min followed by a 3 min linear gradient from 80% to 65% solvent A, further isocratic elution with 35% solvent B for 14 min, an additional 8 min linear gradient from 65% to 0% solvent A and isocratic elution with 100% B for 10 min. The 40% CH_3CN-containing fraction was evaporated under a stream of N_2 to yield **3a** (1.14 mg) in 21% yield. This material was estimated to be 54% pure by HPLC analysis. Compound **3a** prepared via this procedure and analyzed by HPLC co-eluted with **3a** synthesized by previous methods.

[α,β(n)^{32}P]-(*E,E*)-8-*O*-(4-Benzoylbenzoyl)-3,7-dimethyl-2,6-octadiene-1-diphosphate ([^{32}P]-3a). Carrier free [^{32}P]-H_3PO_4 (5 mCi) was diluted with unlabeled H_3PO_4 (1.2 mg, 13 μmol) to a specific activity of 400 Ci/mol and lyophilized over P_2O_5 as described for the unlabeled material above. To the anhydrous H_3PO_4 was added a solution of triethylamine (2.5 mg, 25 μmol) in CH_3CN (35 μL). Alcohol **4a** (2.4 mg, 6.3 μmol) in 200 μL of CH_3CN and CCl_3CN (50 μL) were then added and the reaction was allowed to stir under N_2 for 2 h after which time the volatile components were removed under a stream of N_2. The crude reaction mixture was then dissolved in 1

mL of 25 mM NH_4HCO_3 and applied to a Sep-Pak C_{18} cartridge equilibrated in the same solution. The cartridge was washed with 4 mL of NH_4HCO_3 and eluted with a step gradient of increasing CH_3CN concentration. The following fractions expressed as a percentage of CH_3CN were collected: 10% (2 mL), 20% (2mL), 30%, (2 mL), 35% (2 x 1 mL), 40% (2 x 1 mL), 45% (2 x 1 mL), 50% (2 x 1 mL), 60% (2 mL) 70% (2mL), 80% (2 mL), 90% (2 mL), 100% (2 mL). A sample from each fraction (3 μL) was separated by thin layer chromatography (R_f for **3a** = 0.47) in 2-propanol/NH_4OH/H_2O (6:3:1, v/v/v) and analyzed by phosphorimager to quantify each radiolabeled component present. The first 1 mL fraction collected upon elution with 35% CH_3CN/ NH_4HCO_3 was found to be the most pure and was evaporated under a stream of N_2. The yield of [^{32}P]-**3a** was determined by UV spectroscopy using the extinction coefficient reported for **3a** (ε_{262} = 18,600 $M^{-1}cm^{-1}$).[7] Compound [^{32}P]-**3a** was obtained in 3.3% yield with a specific activity of 482 Ci/mol and a radiochemical purity of 50%. The [^{32}P]-**3a** obtained above was further purified by preparative thin layer chromatography in 2-propanol/NH_4OH/H_2O (6:3:1, v/v/v) to yield material with a radiochemical purity of 90%.

[α,β(n)^{32}P]-(*E,E*)-8-*O*-(3-Benzoylbenzoyl)-3,7-dimethyl-2,6-octadiene-1-diphosphate ([^{32}P]-3b). Carrier free [^{32}P]-H_3PO_4 (5mCi) was diluted with unlabeled H_3PO_4 (0.6 mg, 6.5 μmol) to a specific activity of 800 Ci/mol and lyophilized over P_2O_5 as described for the unlabeled material above. To the anhydrous H_3PO_4 was added a solution of triethylamine (1.3 mg, 13 μmol) in CH_3CN (35 μL). Alcohol **4b** (1.2 mg, 3.1 μmol) in 200 μL of CH_3CN and CCl_3CN (50 μL) were then added and the reaction was allowed to stir under N_2 for 2 h after which time the volatile components were removed under a stream of N_2. The crude product was purified as described for **3a**. Again, the first 1 mL fraction collected upon elution with 35% CH_3CN/ NH_4HCO_3 was found to be the most pure (R_f for **3b** = 0.51) by thin layer chromatography in 2-propanol/NH_4OH/H_2O and was evaporated under a stream of N_2. The yield of [^{32}P]-**3b** was determined by UV spectroscopy using the extinction coefficient reported for **3b** (ε_{222} = 39,100 $M^{-1}cm^{-1}$, ε_{258} = 24,100 $M^{-1}cm^{-1}$).[7] Compound [^{32}P]-**3a** was obtained in 1.0% yield with a specific activity of 700 Ci/mol and a radiochemical purity of 56%. The [^{32}P]-**3b** obtained above was further purified by preparative thin layer chromatography in 2-propanol/NH_4OH/H_2O (6:3:1, v/v/v) to yield material with a radiochemical purity of 90%.

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