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Design and Synthesis of a Transferable Farnesyl Pyrophosphate Analogue to Ras by Protein Farnesyltransferase

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The posttranslational addition of a farnesyl moiety to the Ras oncoprotein is essential for its membrane localization and is required for both its biological activity and ability to induce malignant transformation. We describe the design and synthesis of a farnesyl pyrophosphate (FPP) analogue, 8-anilinogeranyl pyrophosphate **3** (AGPP), in which the ω-terminal isoprene unit of the farnesyl group has been replaced with an aniline functionality. The key steps in the synthesis are the reductive amination of the α,β-unsaturated aldehyde **5** to form the lipid analogue **6**, and the subsequent conversion of the allylic alcohol **7** to the chloride **8** via Ph_3PCl_2 followed by displacement with $[(n\text{-Bu})_4N]_3HP_2O_7$ to give AGPP (**3**). AGPP is a substrate for protein farnesyltransferase (FTase) and is transferred to Ras by FTase with the same kinetics as the natural substrate, FPP. AGPP is highly selective, showing little inhibitory activity against either geranylgeranyl-protein transferase type I (GGTase I) ($K_i = 0.06\ \mu$M, $IC_{50} = 20\ \mu$M) or squalene synthase ($IC_{50} = 1000\ \mu$M). AGPP is the first efficiently transferable analogue of FPP to be modified at the ω-terminus that provides a platform from which additional analogues can be made to probe the biological function of protein farnesylation. AGPP is the first example of a class of compounds that are alternate substrates for protein isoprenylation that are not inhibitors of squalene synthase.

Introduction

Mutated forms of cellular Ras genes are among the most common genetic abnormalities in human cancer, occurring in 30% of all neoplasms.[1] Ras proteins are synthesized as cytosolic precursors which localize to the inner leaflet of the plasma membrane only after undergoing a series of well-defined posttranslational modifications.[2] The first and obligatory step in this processing is the transfer of the 15-carbon isoprene farnesyl from farnesylpyrophosphate (FPP, **1**) to a cysteine located four residues from the Ras C-terminus by protein farnesyltransferase (FTase).[2a,3] While farnesylation is essential for Ras to become membrane associated and induce cellular transformation,[2b−d] it is unknown whether the prenyl group functions as a hydrophobic membrane anchor,[4] or by targeted protein−protein recognition.[5] FTase inhibitors (FTIs) have been the subject of intense research because they prevent Ras membrane localization and function.[6] However, inhibition of protein prenylation by FTIs precludes the study of events downstream of farnesylation which may be important to membrane localization and/or protein−protein recognition. These downstream events are potentially interesting alternate targets for interfering with Ras function.

Synthetic modifications of enzymatic substrates are useful in studying the interactions between small molecules and proteins because they allows critical structures involved in ligand binding and catalysis to be identified. Analogues of FPP that are alternative transferable substrates for FTase have been utilized to study farnesylation and subsequent downstream processing events.[4f] Simple FPP analogues stripped of most isoprenoid features such as methyl groups and unsaturation are transferred to Ras by FTase.[4f] Although the structure of these lipids attached to the Ras farnesylation site can affect further processing, the precise structure of these analogues appears to have little effect on the ability of Ras to activate effector pathways once the modified Ras has become membrane-localized.[4f] Other FPP analogues that have been modified at the farnesyl C3 position of FPP with vinyl or cyclopropyl substituents as well as the *Z,E* and *E,Z* isomers of FPP are also alternative substrates for FTase.[7] While 3-vinyl FPP and its farnesol

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analogue act as alternative substrates for FTase in H-Ras-F cells and transformed NIH3T3 fibroblasts,[8] respectively, it is unknown whether the above FPP isomers support full function of Ras in vivo.

Studies aimed at understanding the biological role of protein isoprenylation are limited by the lack of FPP analogues containing heteroatoms and more widely varied structures that are transferable to Ras by FTase. There are a number of challenges to the design of structurally diverse, transferable, isoprenoid analogues. The ability of FTase to transfer lipid analogues to target proteins is exquisitely sensitive to the structure of the ω-terminal group of the prenyl chain.[4f,9] Incorporation of photoreactive functionality and heteroatoms on the ω-terminus of FPP analogues that would allow further study of downstream processing events, only generated FTIs.[9] Recently, Waldmann and co-workers have successfully synthesized ω-arylated photoprobes that act as alternative substrates for the prenyltransferase geranylgeranyltransferase II.[10] Inspection of the four crystal structures of FTase with and without bound substrates[11] indicates that there is sufficient room for modest modification at the ω-terminus of FPP. Although the synthesis of ω-arylated farnesol analogues has been achieved via a carbon−carbon linkage,[12] we reasoned that a transferable analogue would result from introduction of a heteroatom linkage between a geranyl pyrophosphate moiety and an aniline ring.

We report the design, synthesis and functional characterization of 8-anilinogeranyl pyrophosphate (**3**) (AGPP), which is recognized and transferred to Ras by FTase with in vitro kinetic properties that are indistinguishable from that of FPP.

Results and Discussion

Design and Synthesis. We conceptually composed 8-anilinogeranyl pyrophosphate (AGPP) **3** by replacing the terminal isoprene of FPP **1** with an aniline functionality (Figure 1). We chose aniline because it is a convenient platform for the incorporation of a large number of highly functionalized synthons into the 8-anilinogeranyl skeleton. The synthesis of AGPP was accomplished in five steps from commercially available geranyl acetate **4** as illustrated in Scheme 1. Previous attempts to generate the α,β-unsaturated aldehyde **5** using only SeO_2,[13] or SeO_2 and *tert*-butyl hydroperoxide[14] resulted in poor yields due to numerous side reactions including

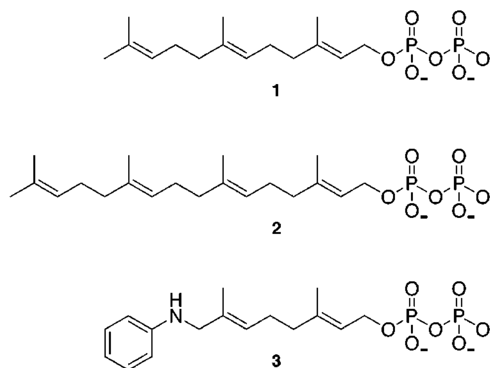

Figure 1. Structure of farnesyl pyrophosphate **1**, geranylgeranyl pyrophosphate **2**, and transferable analogue 8-anilinogeranyl pyrophosphate **3** (AGPP).

over-oxidation to the α,β-unsaturated carboxylic acid, or difficulty in product isolation. We found that a two-step oxidation of **4** with SeO_2 and *tert*-butyl hydroperoxide, followed by MnO_2, resulted in the selective conversion of the ω-methyl group to the all trans aldehyde **5** in 73% yield.

The key step in the synthesis of AGPP **3** was formation of acetate **6** by reductive amination of aniline with aldehyde **5** using $NaBH(OAc)_3$.[15] We required a very mild reductive amination in this scheme, since we envision incorporating anilines with sensitive functionality in the future. We selected $NaBH(OAc)_3$ because it is a very mild reducing agent and gives consistently higher yields with fewer side products than conventional reductive amination reagents such as $NaCNBH_3$/MeOH, BH_3−pyridine, and catalytic hydrogenation.[15d] Only one example of $NaBH(OAc)_3$ reductive amination employing an α,β-unsaturated aldehyde, cinnamaldehyde, has been described.[15d,16] A slight excess of $NaBH(OAc)_3$ in 1,2-dichloroethane at 25 °C resulted in the allylic aniline **6** in 85% yield. Integration of the ^{1}H NMR spectrum of amine **6** indicated that a mixture of *cis/trans* isomers about the 6,7 double bond was formed in a 7:93 ratio. The acetate **6** was hydrolyzed to alcohol **7** with K_2CO_3/MeOH/H_2O in 95% yield. The geometry of the *cis/trans* isomers was confirmed by NOE experiments performed on alcohol **7** by irradiating the resonances of the C8 methylene protons at 3.65 ppm (the desired *E (trans)* isomer) and 3.71 ppm (*Z (cis)* isomer) (see Supporting Information). Attempts to separate the isomers by column chromatography, silica-HPLC, or reverse-phase HPLC were unsuccessful.

The presence of the amine in our farnesol analogue precluded the implementation of standard methods to activate the lipid alcohol **7** for pyrophosphorylation (Ph_3P, CCl_4;[17] $SOCl_2$;[18] NCS, Me_2S[19]). Attempts at employing

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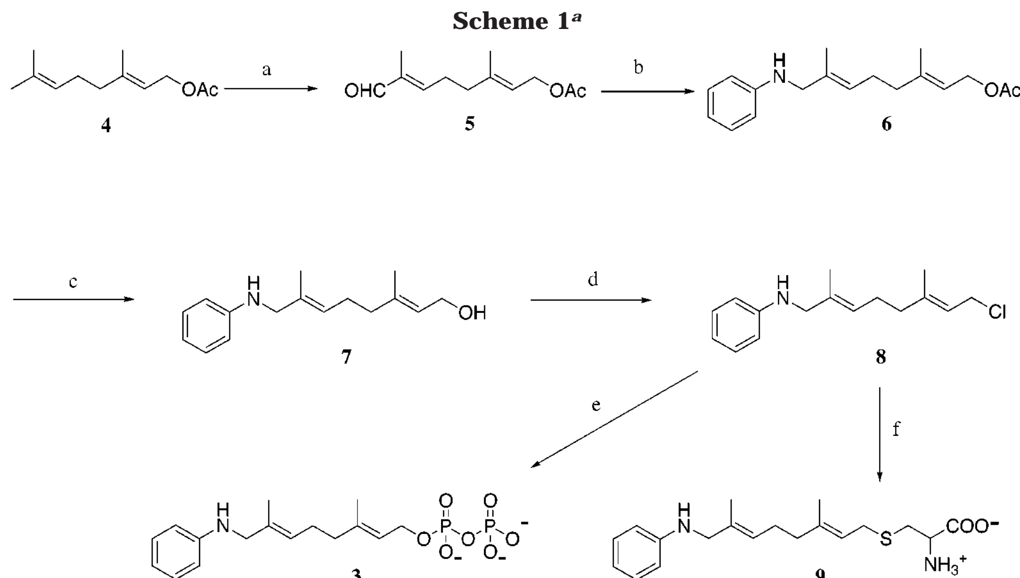
Scheme 1[a]



[a] Reaction conditions: (a) *tert*-butyl hydroperoxide, SeO_2, salicylic acid, CH_2Cl_2; MnO_2, CH_2Cl_2; (b) aniline, $NaBH(OAc)_3$, HOAc, $ClCH_2CH_2Cl$; (c) K_2CO_3, H_2O, MeOH; (d) $(Ph)_3PCl_2$, Hünig's base, CH_3CN; (e) $[(n\text{-Bu})_4N]_3HP_2O_7$, CH_3CN; (f) L-cysteine, NH_3, MeOH.

these procedures resulted in low or no yield of allylic chloride **8** due to amine oxidation, polymerization, or difficulty in product isolation. Sandri and Viala found mild conditions for converting homoallylic alcohols into their corresponding bromides using Ph_3PBr_2.[20] Utilizing Ph_3PCl_2 in MeCN, we were able to convert allylic alcohol **7** into the chloride **8** in 75% yield. The chloride **8** is unstable and decomposes upon short-term storage at −20 °C, apparently into polymeric materials. Consequently, alcohol **7** was converted to pyrophosphate **3** without isolating the intermediate allylic chloride **8**. Other previously reported farnesyl chloride analogues were not isolated prior to displacement reactions due to their instability.[7b] Displacement of the allylic chloride **8** with tris(tetra-*n*-butylammonium) hydrogen pyrophosphate[21] followed by ion exchange chromatography and reverse-phase HPLC gave AGPP (**3**) in 61% overall yield from alcohol **7**. AGPP decomposes upon prolonged exposure to room light.

FTase transfers the lipid portion of FPP to a cysteine residue on Ras forming a thioether linkage. To verify that AGPP was appropriately transferred to Ras by FTase, we required an authentic sample of the product of FTase catalyzed transfer of the anilinogeranyl chain to cysteine. We modified the procedure of Brown et al.[22] to synthesize cysteine adduct **9** from alcohol **7** via chloride **8** in 65% yield.

Biological Evaluation of AGPP: Inhibition Studies. AGPP inhibited FTase-catalyzed farnesylation of Ras in a concentration dependent manner when measured in the presence of increasing concentrations of AGPP and at a fixed concentration of [^{3}H]FPP (Figure 2, filled circles). The K_i (which is equivalent to the K_m for an alternative substrate competitive inhibitor) for AGPP was calculated using the K_m value for FPP (0.04 μM)[23] along with velocity measurements from Figure 2. These data were fit to eq 1 to obtain a $K_i = K_m = 0.03$ μM (see Experimental Procedures) that is essentially identical to that of FPP. The relative affinity of an inhibitor toward an enzyme can be expressed as the IC_{50} value, since this value is directly related to the K_i of the inhibitor. The IC_{50} value for AGPP (0.5 μM) calculated from eq 2 was comparable to the IC_{50} concentration reported by Moores et al.[24] for FPP (0.34 μM).

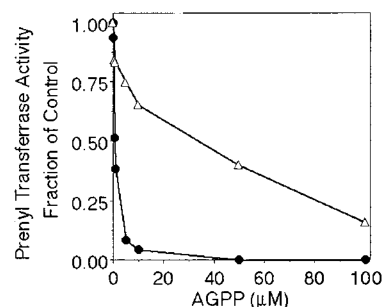


Figure 2. Differential inhibition of farnesyltransferase and CAAX geranylgeranyltransferase by AGPP. The assay mixtures contained (in a final volume of 25 μL) various components as described in Experimental Procedures. After incubation for 15 min at 37 °C, the amount of [^{3}H]prenyl group transferred to the appropriate protein substrate [Ras-CVLS for FTase (filled circles) and Ras-CVLL for CAAX GGTase (open triangles)] was measured by precipitation with ethanol−HCl. The assays contained 10 ng of recombinant rat FTase, 5 μM Ras, and 0.6 μM [^{3}H]FPP (33000 dpm/pmol) or 100 ng of recombinant rat CAAX GGTase, 5 μM Ras-CVLL, and 1 μM [^{3}H]GGPP (33000 dpm/pmol) and the indicated concentration of unlabeled AGPP. The 100% of control values were 1.1 and 5.5 pmol of [^{3}H]farnesyl or [^{3}H]geranylgeranyl transferred per tube. Each value is the average of duplicate incubations and is representative of two separate experiments.

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Geranylgeranyltransferase-I (GGTase I) catalyzes the transfer of a geranylgeranyl group from GGPP to proteins having a C-terminal CAAX sequence where X is leucine.[2a] Both CAAX-based tetrapeptides and other FPP analogues have been shown to be selective inhibitors of FTase relative to GGTase I.[25] To evaluate the selectivity of AGPP for these enzymes, we examined its inhibitory activity against recombinant GGTase I, as measured by incorporation of radioactivity from [^{3}H]geranylgeranylpyrophosphate into Ras-CVLL. As expected for an FPP analogue,[26] AGPP inhibits GGTase I activity much less efficiently than it inhibits FTase (Figure 2, open triangles). A $K_i = 0.06\ \mu$M for GGTase I was estimated for AGPP when the literature K_m value for GGPP (0.003 μM)[27] was used with eq 1. The IC_{50} value calculated for GGTase I inhibition by AGPP (20 μM) compared favorably to the reported IC_{50} value for FPP (16 μM).[24] Therefore, AGPP is similar to FPP in that it is a 40-fold better inhibitor of FTase than of GGTase I.

[^{3}H]AGPP Is a Substrate for Farnesyltransferase In Vitro. The observed inhibition kinetics could be a manifestation of the ability of AGPP to act as an alternative substrate for FTase. Even though the data implied that the structure of AGPP was recognized by FTase, previously reported ω-terminal modified FPP analogues are not transferred very efficiently by FTase.[9] To evaluate the potential of AGPP to serve as a FTase substrate, [^{3}H]-AGPP was prepared by a similar route as described above, incorporating tritium with a specific activity of 17 Ci/mmol (synthesis to be reported elsewhere). [^{3}H]-AGPP was an alternative substrate for FTase when incubated with Ras-CVLS as a cosubstrate. Figures 3A and 3C show that FTase incorporated radioactivity from [^{3}H]AGPP into Ras in a time and concentration dependent fashion at 37 °C. The incorporated radioactivity was detected as a band of the expected molecular weight of farnesylated Ras on SDS-polyacrylamide gels (Figure 3B). The saturation curve from Figure 3C was almost identical to that obtained with [^{3}H]-FPP,[3a] suggesting that FTase has a similar affinity for AGPP and FPP. Either nonradiolabeled AGPP or FPP, but not alcohol **7** or farnesol, were found to inhibit the time and concentration dependent transfer of [^{3}H]AGPP to Ras (data not shown). Figure 3D shows that [^{3}H]AGPP and [^{3}H]FPP are transferred with comparable affinities by FTase. These sets of data suggest that the *cis/trans* isomer mixture of AGPP does not interfere with the FTase catalyzed transfer of lipid to Ras. We applied initial velocity measurements from Figure 3D and a $K_m = 0.63\ \mu$M[23b] for Ras-CVLS to eq 3 to calculate the kinetic parameters k_{cat} and k_{cat}/K_m for AGPP and FPP. These values are tabulated in Table 1. The kinetic values we obtained for FPP were identical to those reported in the literature.[23b]

Verification of appropriate transfer of the anilinogeranyl group to cysteine was accomplished by hydrolyzing the [^{3}H]AGPP-modified Ras and comparing the digestion products to the authentic AG-cysteine standard **9**. The modified Ras was digested with Pronase E and the butanol-soluble products were analyzed by reverse phase chromatography. The chromatographic mobility of the major radioactive product of this digestion was consistent with authentic AG-cysteine **9**.

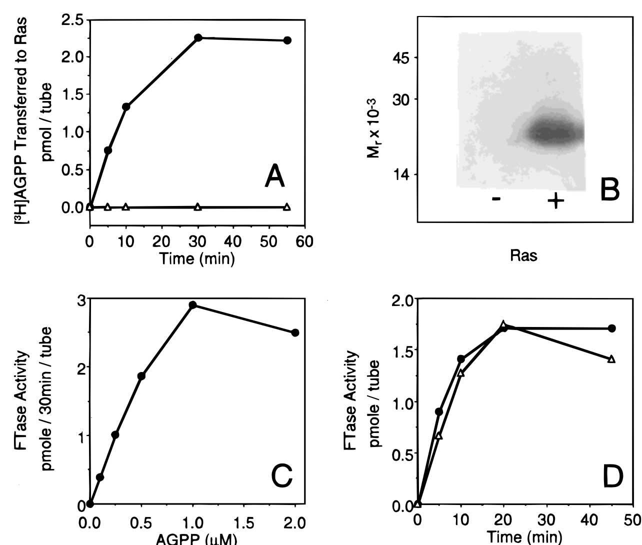

Figure 3. Transfer of anilinogeranyl from [^{3}H]AGPP to Ras by farnesyltransferase. (A) Each reaction contained 1 μM [^{3}H]-AGPP (37400 dpm/pmol) and 10 ng of recombinant FTase in the absence (open triangles) or presence (filled circles) of 5 μM Ras. Duplicate samples were incubated for the indicated times at 37 °C, and ethanol−HCl precipitated radioactivity was quantified. (B) The autoradiograph shows the migration of [^{3}H]-AG modified Ras on a 12.5% SDS-polyacrylamide gel of an aliquot from the reaction carried out for 1 h in the presence (+) or absence (−) of Ras. The gel was treated with Amplify solution (Amersham), dried, and exposed to Kodak X-OMAT AR film for 2 days at −70 °C. The positions of molecular weight markers placed in an adjacent lane is indicated. (C) The AGPP substrate saturation curve for FTase was determined by varying the amount of [^{3}H]AGPP in the standard reaction described in A. Assays were carried out in duplicate for 30 min at 37 °C, and the ethanol−HCl precipitable radioactivity measured. The data are representative of three independent experiments. (D) FTase (10 ng) was incubated in a final volume of 25 μL in the presence of 1.0 μM [^{3}H]AGPP (open triangles) or [^{3}H]FPP (filled circles) and 5 μM Ras. After samples were incubated for the indicated times at 37 °C, the amount of [^{3}H]-prenyl transferred to Ras was determined. Each value is the average of duplicate incubations and is representative of five separate experiments.

Table 1. Steady-State Kinetic Parameters

substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})
FPP	0.04	0.09	2×10^6
AGPP	0.03	0.07	2×10^6

AGPP Does Not Inhibit Squalene Synthase Activity. Squalene synthase is involved in cholesterol biosynthesis and has an important role as a secondary regulatory protein in that pathway. Squalene synthase catalyzes the reductive head-to-head coupling of two FPP molecules to give squalene.[28] FPP analogues have been found through design and screening of chemical libraries that are potent inhibitors of FTase but are not inhibitors of squalene synthase.[29] However, other FTIs, particularly those competitive for FPP binding, are inhibitors of

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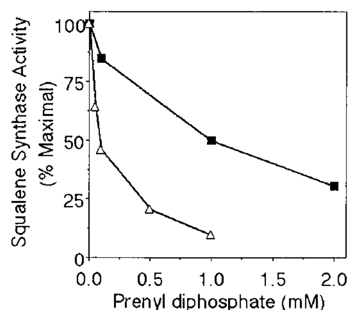

Figure 4. Inhibition of squalene synthase by AGPP. The initial rates of squalene synthase activity were determined from bovine brain microsomal preparations in the presence of the indicated concentration of unlabeled AGPP (filled squares) or unlabeled FPP (open triangles). Each value is the average of duplicate incubations and is representative of three separate experiments.

squalene synthase.[28] An important objective in the development of our alternative FPP substrates for FTase was the generation of molecules that do not interfere with other FPP utilizing enzymes. AGPP was found to be a far less potent in vitro inhibitor of squalene synthase activity than FPP, with an $IC_{50} = 1000\ \mu M$ (Figure 4). We tested for the possibility that AGPP might act as a substrate to form a homo-coupled product and the possibility that it might form hetero-coupled products with FPP. No organic soluble products resulting from squalene synthase-catalyzed coupling reactions of [^{3}H]AGPP were detected. Apparently, the structural differences between AGPP and FPP do not allow AGPP to interfere with squalene synthase activity. AGPP is the first example of a class of compounds that are alternate substrates for protein isoprenylation that are not inhibitors of squalene synthase.

Conclusion. AGPP is the prototype of a class of alternative substrates for FTase that possess a modified ω-terminus. The reductive amination of aniline with an aliphatic α,β-unsaturated aldehyde and $NaBH(OAc)_3$ provides a robust and versatile method to access a new class of amino-functionalized isoprenyl analogues. An alternative route to the synthesis of allylic chlorides from their corresponding alcohols using dichlorotriphenylphosphorane has also been demonstrated.

Experimental Procedures

General Chemical Procedures. Melting points are uncorrected. All reactions were conducted under dry argon, and all reactions and chromatography procedures were conducted under diminished light. Analytical TLC was performed on precoated (0.25 mm) silica gel 60F-254 (Merck) plates and developed with 30% ethyl acetate in hexane, except where noted otherwise. Preparative TLC was performed on precoated silica gel plates (E. Merck #13793-7). Visualization was achieved either by UV irradiation, anisaldehyde–sulfuric acid spray followed by heating, or subjecting the plates to a 5% ethanolic phosphomolybdic acid solution followed by heating. Flash chromatography was performed on Merck silica gel 60 (230–400 mesh ASTM). NMR spectra were obtained in $CDCl_3$ (unless otherwise noted) at 200 MHz or at 500 MHz. Chemical shifts for the following deuterated solvents are reported in ppm downfield using the indicated reference peaks: $CDCl_3$ ($CDCl_3$ internal peak: 7.27 ppm for ^{1}H, 77.4 ppm for ^{13}C), C_6D_6 (C_6D_6 internal peak: 7.37 ppm for ^{1}H), DMSO-d_6 (DMSO-d_6 internal peak: 39.5 ppm for ^{13}C). Mass spectra were obtained from the University of Kentucky Mass Spectra Facility. Combustion analyses were performed by Atlantic Microlabs, Inc., Norcross, GA.

(*E,E*)-3,7-Dimethyl-1-acetoxy-2,6-octadien-8-al (5). Geranyl acetate was oxidized as described previously[14] with some modification. A 40 mL (0.36 mol) amount of 90% *tert*-butyl hydroperoxide was poured into a stirred suspension of 1.11 g (0.010 mol) of SeO_2, 1.4 g (0.010 mol) of salicylic acid, and 75 mL of CH_2Cl_2 in a 250 mL round-bottom flask. The resulting solution was allowed to stir to homogeneity at room temperature before being placed at 0 °C. After 10 min, 21.5 mL (0.10 mol) of geranyl acetate **4** was introduced. The mixture was stirred for 5 h at 0 °C, and then stirred for 24 h at room temperature. The solution was diluted with 100 mL of toluene, and solvents were removed under reduced pressure. The residue was then dissolved in toluene, washed with 5% $NaHCO_3$ to remove H_2SeO_3, saturated $CuSO_4$, saturated aqueous $Na_2S_20_3$ (2×), water, and brine, dried over $MgSO_4$, and filtered. The solvent was removed under reduced pressure to afford a crude oil, which was dissolved in 100 mL of dry CH_2Cl_2 and placed at 0 °C. A 40.95 g (0.47 mol) amount of activated MnO_2 was added, and the suspension was stirred for 5 h at 0 °C, and then stirred overnight at room temperature. The mixture was filtered over a short bed of Celite and washed thoroughly with dry CH_2Cl_2. Solvent was evaporated under reduced pressure to yield a crude, pale yellow oil which was purified by flash chromatography on silica gel (hexane (1000 mL), 5% EtOAc in hexane) affording 6.04 g of unreacted **4** and 10.68 g (73% based on consumed **4**) of **5** as a colorless oil; TLC: (R_f 0.38 aldehyde **5**, R_f 0.19 alcohol); ^{1}H NMR (C_6D_6, 500 MHz) δ 9.47 (s, 1H), 5.98 (t, 1H, $J = 6.9$ Hz), 5.48 (t, 1H, $J = 6.9$ Hz), 4.75 (d, 2H, $J = 6.8$ Hz), 2.11 (q, 2H, $J = 7.3$ Hz), 1.92 (t, 2H, $J = 7.3$ Hz), 1.91 (s, 3H), 1.79 (s, 6H), 1.59 (s, 3H); ^{13}C NMR (50.3 MHz) δ 195.47, 171.39, 153.70, 140.73, 140.08, 120.01, 61.48, 38.13, 27.33, 21.37, 16.77, 9.62.

8-Aniline-3,7-dimethyl-1-acetoxy-2,6-octadiene (6). The reductive amination procedure of Abdel-Magid et al.[15d] was modified as follows. Into a 250 mL three-neck flask was introduced 150 mL of 1,2-dichloroethane, followed by 7.88 g (37.48 mmol) of **5**, 3.76 mL (41.22 mmol) of fresh aniline, and 2.58 mL (44.89 mmol) of glacial acetic acid. After stirring for 0.5 min at room temperature, 11.12 g (52.47 mmol) of NaBH-$(OAc)_3$ was added to the above solution. The solution was stirred for 5 h at room temperature, and then quenched by pouring into a separatory funnel containing 200 mL of 5% $NaHCO_3$. The product was extracted with ether (3 × 75 mL), dried ($MgSO_4$), and concentrated to give a pale yellow, viscous oil. Purification by flash chromatography (10% EtOAc in hexane) yielded 9.15 g (85%) of a light-straw colored to colorless oil; TLC: (R_f 0.46 **6**); ^{1}H NMR (200 MHz) δ 7.18 (m, 2H), 6.69 (m, 1H), 6.62 (m, 2H), 5.38 (m, 2H), 4.60 (d, 2H, $J = 7.2$ Hz), 3.82 (s, 1H), 3.65 (s, 2H), 2.33–2.02 (m, 4H), 2.08 (s, 3H), 1.72 (s, 3H), 1.69 (s, 3H); ^{13}C NMR (50.3 MHz) δ 171.42, 148.84, 142.12, 133.13, 129.43, 125.71, 118.93, 117.44, 113.13, 61.68, 52.03, 39.50, 26.21, 21.37, 16.74, 15.00. Anal. Calcd for $C_{18}H_{25}NO_2$: C, 75.22; H, 8.77; N, 4.87. Found: C, 75.38; H, 8.63; N, 4.93.

8-Aniline-3,7-dimethyl-2,6-octadien-1-ol (7). To a 500 mL three-neck flask were added acetate **6** (8.00 g, 27.8 mmol) and 250 mL of methanol. Potassium carbonate (11.53 g, 83.4 mmol) dissolved in 20 mL of water was added, and the reaction mixture was stirred at room temperature overnight. The mixture was then concentrated to ca. 100 mL on a rotary evaporator. Water was added (100 mL), and the mixture was extracted with ether (4 × 100 mL). The combined extracts were washed with brine (100 mL), dried ($MgSO_4$), and concentrated to yield 6.48 g (95%) of **7** as a viscous, light-straw colored to colorless oil; TLC: (R_f 0.20 **7**); ^{1}H NMR (200 MHz) δ 7.18 (m, 2H), 6.71 (m, 1H), 6.61 (m, 2H), 5.40 (m, 2H), 4.13 (d, 2H, $J = 6.9$ Hz), 3.65 (s, 2H), 2.30–1.98 (m, 4H), 1.69 (s, 6H); ^{13}C NMR (50.3 MHz) δ 148.84, 139.45, 132.94, 129.45, 125.83, 124.09, 117.46, 113.18, 59.67, 52.01, 39.51, 26.24, 16.55, 15.04. Puri-

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fication by flash chromatography (30% EtOAc in hexane) yielded the analytical sample of **7**. Anal. Calcd for $C_{16}H_{23}NO$: C, 78.32; H, 9.45; N, 5.71. Found: C, 78.13; H, 9.46; N, 5.88.

8-Aniline-1-chloro-3,7-dimethyl-2,6-octadiene (8). The allylic chloride was synthesized as described for homoallylic alcohols by Sandri and Viala[20] with some modification. Into a 100-mL round-bottom flask were added alcohol **7** (1.24 g, 5.05 mmol), *N,N*-diisopropylethylamine (1.41 mL, 8.09 mmol), and 30 mL of dry acetonitrile. The solution was stirred at 0 °C for 10 min. Solid dichlorotriphenylphosphorane (2.45 g, 7.58 mmol) was then added evenly to the reaction mixture over a 7 min period. After the final addition of Ph_3PCl_2, the reaction was allowed to stir at 0 °C for an additional 40 min. The mixture was then loaded directly onto a silica gel column and purified by flash chromatography (5% EtOAc in hexane) to yield 1.00 g (75%) of pure chloride **8** as a colorless oil (which rapidly turns brown upon exposure to light); TLC: (R_f 0.62 **8**); ^{1}H NMR (200 MHz) δ 7.19 (m, 2H), 6.96 (m, 3 H), 5.31 (m, 2H), 4.02 (d, 2H, $J = 7.6$ Hz), 3.68 (s, 2H), 2.18−1.86 (m, 4H), 1.69 (s, 3H), 1.63 (s, 3H); ^{13}C NMR (50.3 MHz) δ 145.47, 141.27, 133.67, 131.60, 129.38, 122.19, 120.86, 117.68, 55.25, 41.25, 38.89, 26.05, 16.20, 15.35.

8-Aniline-3,7-dimethyl-2,6-octadiene Pyrophosphate (3). To a 50-mL glass centrifuge tube was added 7.45 g (7.6 mmol) of tris(tetrabutylammonium) hydrogen pyrophosphate[21] and 12 mL of dry acetonitrile. After vortexing, the milky white solution was then centrifuged at 2000 rpm for 10 min. The clear supernatant was decanted into a flame-dried, 100-mL, round-bottomed flask charged with 1.00 g (3.8 mmol) of chloride **8**. The solution was allowed to stir at room temperature for 3 h. Solvent was removed, and the pale white residue was dissolved in 3 mL of ion exchange buffer (ion exchange buffer was generated by dissolving ammonium bicarbonate (2.0 g, 25.3 mmol) in 1.0 L of 2% (v/v) isopropyl alcohol/water). The resulting milky white solution was loaded onto a preequilibrated 2 × 30-cm column of Dowex AG 50W-X8 (100−200 mesh) cation-exchange resin (NH_4^+ form). The flask was washed with buffer (2 × 5 mL), and both washes were loaded onto the column before elution with 190 mL (two column volumes) of ion exchange buffer. The cloudy white eluant was collected in a 600-mL freeze-drying flask, frozen, and lyophilized to yield 3.17 g of an off-white solid. A portion of the crude AGPP was dissolved in 25 mM NH_4HCO_3 to a final concentration of ca. 5 mM AGPP. The colorless solution was loaded onto an analytical Vydac C_4 (214TP54) column and eluted under the following gradient at a flow rate of 1 mL/min: 0−11 min 100% A, 11−12 min 80% A, 12−24 min 80% A, 24−27 min 5% A, 27−31 min 5% A, 31−36 min 100% A, 36−45 min 100% A. Solvents: A = water; B = 0.01% (v/v) TFA in 2-propanol. The desired peak ($t_R \approx 18.8$ min) was immediately collected in a vial containing 25 mM NH_4HCO_3, frozen, and lyophilized yielding **3** as a white solid (1.05 g overall, 61%); ^{1}H NMR (D_2O, 200 MHz) δ 7.26 (m, 2H), 6.86 (m, 3H), 5.42 (m, 2H), 4.47 (t, 2H, $J = 6.7$ Hz), 3.69 (s, 2H), 2.25−2.01 (m, 4H), 1.69 (s, 3H), 1.64 (s, 3H); ^{13}C NMR (D_2O, 50.3 MHz) δ 145.70, 135.39, 132.46, 129.78, 122.92, 122.76, 122.19, 118.34, 65.65 (d, $J = 5.3$ Hz), 54.35, 41.40, 28.18, 18.41, 16.63; ^{31}P NMR (D_2O, 202.4 MHz) δ −6.87 (1P, d, $J = 22$ Hz), −10.84 (1P, dt, $J = 22$ Hz). LRMS (FAB$^+$, H$^+$ glycerol matrix): (M$^+$ + H$^+$) 406.2. LRMS (FAB$^-$, H$^+$ glycerol matrix): (M$^+$ − H$^+$) 404.1.

8-Aniline-3,7-dimethyl-2,6-octadiene-*S*-L-cysteine (9). The cysteine adduct was synthesized as described by Brown et al.[22] with some modification. The alcohol **7** (0.50 g, 2.04 mmol) was first converted into chloride **8** as described above. Crude chloride **8** was then added to a 25-mL pear shaped flask, followed by 0.32 g (2.67 mmol) of L-cysteine and 10 mL of a 2 N NH_3 solution in MeOH. The solution began to form a precipitate after 15 min, and stirring was continued for 1 d. The reaction mixture was then filtered and the solid washed with 10 mL of MeOH:H_2O (9:1 v/v). Aliquots of the filtrate were loaded onto a preparative Vydac C_{18} (218TP1010) column and eluted under the following gradient at a flow rate of 5 mL/min: 0−5 min 90% A, 5−20 min 5% A, 20−23 min 5% A, 23−27 min 90% A, 27−31 min 90% A. Solvents: A = 25 mM NH_4HCO_3; B = CH_3CN. The desired peak ($t_R \approx 15.7$ min) was collected, concentrated, and lyophilized yielding **9** (0.46 g overall, 65% from alcohol **7**) as a white solid, mp 151−153 °C (dec); ^{1}H NMR (DMSO-d_6, 500 MHz) δ 7.61 (bs, 1H), 7.02 (m, 2H), 6.53 (m, 2H), 6.47 (m, 1H), 5.82 (bs, 1H), 5.33 (t, 1H, $J = 5.9$ Hz), 5.17 (t, 1H, $J = 7.8$ Hz), 3.52 (s, 2H), 3.32 (bs, 3H), 3.28 (dd, 1H, $J = 3.4$ Hz), 3.15 (ddd, 1H, $J = 8.3$ Hz), 2.99 (dd, 1H, $J = 3.4$ Hz), 2.64 (dd, 1H, $J = 9.3$ Hz), 2.10 (q, 2H, $J = 7.3$ Hz), 1.99 (t, 2H, $J = 7.3$ Hz), 1.63 (s, 3H), 1.59 (s, 3H); ^{13}C NMR (DMSO-d_6, 125.7 MHz) δ 168.31, 149.01, 138.12, 132.66, 128.61, 123.94, 120.37, 115.21, 112.00, 53.51, 50.25, 38.91, 32.48, 28.53, 25.64, 15.84, 14.34. Anal. Calcd for $C_{19}H_{28}N_2O_2S \cdot 0.5H_2O$: C, 63.83; H, 8.18; N, 7.84; S, 8.97. Found: C, 64.02; H, 7.92; N, 7.74; S, 8.89. LRMS (MALDI): (MH$^+$) 349.20.

General Biological Materials and Methods. [1-^{3}H]-Farnesyl pyrophosphate ([^{3}H]FPP, 15 Ci/mmol) and [1-^{3}H]-geranylgeranyl pyrophosphate ([^{3}H]GGPP, 15 Ci/mmol) were obtained from American Radiochemical Co. Recombinant Ras-CVLS and its derivative Ras-CVLL were produced in bacteria as described previously.[30] Farnesyl cysteine (F-Cys) was synthesized as described by Kamiya et al.[31] and purified by preparative TLC. All other solvents and chemicals were reagent grade and purchased from standard commercial sources.

Production of Recombinant CAAX GGTase-1 and FTase in Sf9 Cells. Recombinant FTase was prepared by coinfection of fall army worm ovarian (Sf9) cells with recombinant baculoviruses encoding the α- and β-subunits of rat FTase and purified as previously described.[30] The FTase α-subunit had been modified to contain a 6xHistidine affinity tag at its N-terminus to allow rapid purification using Ni^{2+}-Sepharose affinity chromatography as described.[32] A cDNA clone that encodes the GGTase-1 β-subunit was kindly provided by Dr. Guy James (University of Texas, Southwestern Medical Center, Dallas). Recombinant baculoviruses encoding the GGTase-1 β-subunit were generated by cotransfection of Sf9 cells with pVL-GGTB and linear BacPAK6 viral DNA (Clontech) and plaque purified as described.[32]

Assay for Farnesyltransferase Activity. Farnesyltransferase activity was assayed by measuring the amount of [^{3}H]-farnesyl or [^{3}H]anilinogeranyl transferred from [^{3}H]FPP or [^{3}H]AGPP to recombinant Ras, as described previously.[33] Unless otherwise stated, each reaction mixture contained the following components in a final volume of 25 μL: 50 mM Tris (pH 7.4), 20 mM KCl, 0.2% octyl β-glucopyranoside, 1 mM dithiothreitol, 10−20 ng recombinant FTase, 5 μM Ras, 3 mM $MgCl_2$, 50 μM $ZnCl_2$, and 0.6 μM [^{3}H]FPP (33000 dpm/pmol; American Radiochemical Co.) or 0.1−5 μM [^{3}H]AGPP (37400 dpm/pmol). Following incubation for the indicated times at 37 °C, the amount of [^{3}H]prenyl transferred was measured by ethanol−HCl precipitation and filtration on glass fiber filters with modification as previously described.[30] A blank value was determined in parallel incubation mixtures containing no enzyme. This blank value was subtracted from each reaction before calculating pmol [^{3}H]prenyl transferred.

Assay for CAAX Geranylgeranyltransferase-1 Activity. CAAX geranylgeranyltransferase activity was assayed by measuring the amount of [^{3}H]geranylgeranyl transferred from [^{3}H]GGPP to recombinant Ras CVLL, as described previously.[26] Unless otherwise stated, each reaction contained the following components in a final volume of 25 μL: 50 mM Tris-HCl (pH 7.4), 5 mM dithiothreitol, 20 μM Zwittergent (3−14), 1 μM [^{3}H]GGPP (33000 dpm/pmol; American Radiochemical Co.), 5 μM Ras CVLL, 5 μM $ZnCl_2$, 5 mM $MgCl_2$, 100 ng recombinant CAAX GGTase-1, and the indicated amount of unlabeled AGPP. Following incubation at 37 °C for 15 min, the amount of [^{3}H]geranylgeranyl transferred was measured

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by ethanol–HCl precipitation followed by filtration on glass fiber filters. A blank value was determined in parallel incubation mixtures containing no enzyme and was subtracted from each reaction.

Digestion of [^{3}H]AGPP-Modified Ras. An aliquot from a standard FTase reaction carried out at 37 °C for 30 min in the presence of [^{3}H]AGPP and Ras was subjected to Pronase E digestion as previously described.[34] The labeled products, extracted with *n*-butanol, were analyzed on C_{18} reverse-phase TLC plates developed in CH_3CN:H_2O:HOAc (75:25:1 v/v/v). Radioactive zones were located with a Bioscan Imaging System 200-IBM.

Assay for Squalene Synthase Activity. Initial rates of squalene synthase activity present in bovine brain microsome fractions were assayed as previously described[30] with minor modifications. Typical reaction mixtures contained crude bovine brain microsomes (0.2 mg membrane protein), 55 mM HEPES (pH 7.4), 5.5 mM $MgCl_2$, 11 mM KF, 1 mM NADPH, and 0.05 mM [^{3}H]FPP (105 cpm/pmol) in a total volume of 100 μL. Unlabeled AGPP and FPP were added at the indicated concentrations. Assay mixtures were incubated for 30 min at 37 °C, and the reactions terminated by the addition of 50 μL of 40% (w/v) KOH and then 100 μL of 95% ethanol. The resulting mixtures were incubated at 60 °C for 2 h and then cooled to room temperature. Authentic carrier squalene (20 μg) was added to each reaction tube, which were then extracted with petroleum ether (3×). The lipid extracts were pooled, washed with water (3×), and evaporated under a stream of N_2. The lipid residue was redissolved in 100 μL of $CHCl_3$/CH_3-OH (2:1 v/v) and an aliquot (30 μL) was taken to determine the amount of labeled lipid formed by scintillation spectrometry. The remaining sample was analyzed on Silica gel G 60 TLC plates (Merck) by developing with hexane.[35] In all analyses the squalene standard was located by anisaldehyde spray reagent,[36] and radioactive zones were located with a Bioscan Imaging System 200-IBM.

To evaluate the potential of AGPP as an alternative substrate for squalene synthase, the above assay was repeated as described with some minor modification. Where indicated, 0.05 mM [^{3}H]FPP was substituted with 0.05 mM [^{3}H]AGPP (304 cpm/pmol), and no unlabeled isoprenyl analogues were added. After incubation, the reactions were extracted, concentrated, and analyzed by TLC for possible products resulting from any [^{3}H]AGPP coupling reactions.

Kinetic Analysis. Equation 1 describes the expression for the relative velocity or fractional activity in the presence and absence of a competitive inhibitor[37]

$$y = 1 - [\mathrm{I}/(\mathrm{I} + K_\mathrm{i}(1 + S/K_\mathrm{m}))] \quad (1)$$

where y is the fractional inhibition of enzyme activity, I is the concentration of the inhibitor AGPP, S is the concentration of the substrate FPP or GGPP, and K_m and K_i are the respective Michaelis constants for the substrate and inhibitor. Equation 2 describes the expression for the concentration of inhibitor that yields 50% inhibition of the relative enzyme activity (the IC_{50} value)[37]

$$[\mathrm{I}]_{0.5} = (1 + S/K_\mathrm{m})K_\mathrm{i} \quad (2)$$

where $[I]_{0.5}$ is the IC_{50} value for AGPP. Equation 3 describes the dependence of the steady-state velocity on substrate concentration for a two-substrate random-equilibrium mechanism for FTase with FPP or AGPP and Ras-CVLS to form the prenylated product Ras-C(isoprenoid)-VLS[23a]

$$k_\mathrm{cat} = \frac{\nu(SR + \alpha R + \beta S + \alpha\beta)}{E_\mathrm{T}SR} \quad (3)$$

where ν is the velocity of product formation, S is the concentration of the isoprenyl substrate FPP or AGPP, R is the concentration of the protein acceptor Ras-CVLS, α is the K_m of FPP or AGPP, β is the K_m for Ras-CVLS, and E_T is the total enzyme concentration.

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Supporting Information Available: ^{1}H, ^{13}C, ^{31}P NMR, and MS spectra for compound **3**; ^{1}H and ^{13}C NMR spectra for compound **6**; ^{1}H, ^{13}C NMR, and NOE spectra for compound **7**; ^{1}H and ^{13}C NMR spectra for compound **8**; ^{1}H, ^{13}C NMR, and MS spectra for compound **9**. This material is available free of charge via the Internet at http://pubs.acs.org.

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