Stereochemical Analysis of the Reaction Catalyzed by Human Protein Geranylgeranyl Transferase†

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ABSTRACT: Protein geranylgeranyltransferase type I (PGGTase-I) catalyzes the nucleophilic substitution reaction between the C_{20} geranylgeranyl diphosphate (GGPP) and a protein-derived thiol to form a thioether linkage. Here, we describe the stereochemical outcome, at the isoprenoid C1, of the reaction catalyzed by human PGGTase-I. To accomplish this, the pentapeptide *N*-dansyl-GCVLL was first enzymatically prenylated by human PGGTase-I with either (*S*)-[1-^{2}H]farnesyl diphosphate or (*S*)-[1-^{2}H]GGPP. The prenylated products were then degraded to dipeptides using carboxypeptidase Y. After HPLC purification, the prenylated dipeptide products were analyzed by ^{1}H NMR spectroscopy. The final spectra were compared with the spectra from the same product obtained via chemical synthesis to deduce the stereochemistry of the PGGTase-I-catalyzed reaction. This comparison showed that the reaction proceeds with inversion of configuration with no detectable (<6%) racemization. These results are more consistent with an associative-type mechanism, but they cannot be used to rule out a dissociative mechanism involving a rigid, solvent-sequestered, tight ion pair.

Protein geranylgeranyltransferase type I (PGGTase-I)[1] catalyzes the nucleophilic substitution reaction between the C_{20} geranylgeranyl diphosphate (GGPP) and a protein-derived thiol to form a thioether linkage as shown in Figure 1. PGGTase-I catalyzes the alkylation of proteins that have a C-terminal sequence of CaaX, where C is the residue that is modified, "a" are both aliphatic residues, and X is Phe or Leu (*1*, *2*). Protein substrates for PGGTase-I include the γ-subunit of heterodimeric G-proteins and Ras-related proteins such as Rac and Rap (*3*, *4*). Short peptides with the minimal four-residue sequence can be modified by PGGTase-I (*1*, *2*). PGGTase-I differs from the type II PGGTases, which require C-terminal sequences of CXC, XXCC, or CCXX (*5*−*7*). Little is known about the chemical mechanism of the enzyme-catalyzed reaction by PGGTase-I, but much has been discovered about the chemical mechanism of the related enzyme, protein farnesyltransferase (PFTase).

PFTase catalyzes the modification of CaaX-containing proteins (where X is Cys, Ser, Gln, Met, or Ala) with a C_{15} farnesyl group derived from farnesyl diphosphate (FPP) (*1*, *8*, *9*). Ras is an important example of a protein that is farnesylated (*10*). Mutant forms of Ras proteins are present in 30% of all human cancers, and farnesylation is required for their oncogenic activity (*11*, *12*). Furthermore, a number of human cancers involve mutations in other proteins upstream of Ras and may be blocked by inhibiting farnesylation. Because of this, PFTase has been the target for the development of inhibitors that arrest the growth of tumor cells (*13*−*16*). Much has been learned about PFTase through many studies on the chemical mechanism of the enzyme-catalyzed reaction. Different studies have indicated both associative and dissociative character for the mechanism of this reaction (*17*−*21*). We are interested in defining the chemical mechanism for the PGGTase-I-catalyzed reaction to discern the similarities and differences between the PFTase and PGGTase-I. Such information may be particularly useful for the design of inhibitors that are specific for PFTase but leave PGGTase-I activity unimpeded.

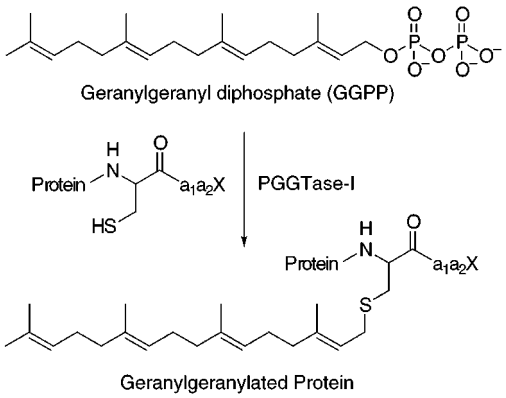

FIGURE 1: Reaction catalyzed by protein geranylgeranyltransferase type I.

Many studies suggest that PFTase and PGGTase-I are similar. They are both heterodimeric, with a common α subunit but distinct β subunits (*22*). PGGTase-II differs from

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[1] Abbreviations: PFTase, protein farnesyltransferase; FPP, farnesyl diphosphate; PGGTase-I, protein geranylgeranyltransferase type I; GGPP, geranylgeranyl diphosphate; IPTG, isopropyl-β-thiogalactoside; DTT, dithiothreitol; TLC, thin-layer chromatography; PMSF, phenylmethanesulfonyl fluoride; EDTA, ethylenediaminetetraacetic acid; FPLC, fast protein liquid chromatography; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography; FAB-MS, fast atom bombardment mass spectrometry; EI-MS, electrospray ionization mass spectrometry; BSA, bovine serum albumin.

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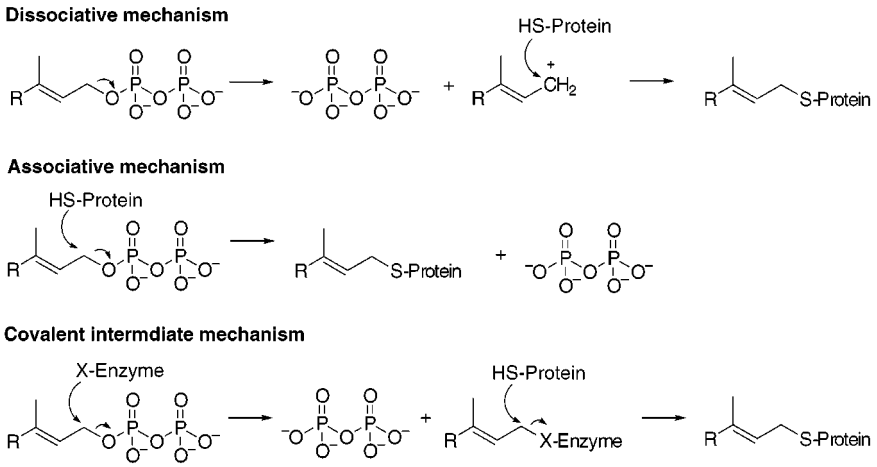

FIGURE 2: Possible mechanisms for the reaction catalyzed by protein geranylgeranyltransferase type I.

both PFTase and PGGTase-I in that it requires an additional protein, denoted as the escort protein, for activity (*23*, *24*). Photoaffinity labeling experiments have shown that the β subunits of both PFTase and PGGTase-I bind the prenyl diphosphate substrate (*25*−*28*). The enzymes from the same sources display similar kinetic mechanisms. PGGTase-I and PFTase from mammalian sources proceed via a random sequential mechanism, but the preferred kinetic pathway is ordered with the prenyl diphosphate substrate binding first (*29*−*32*). Enzymes from the yeast source manifest no random element to their sequential pathway, with the prenyl diphosphate substrate binding before the protein substrate (*33*−*35*). Both enzymes require zinc for activity (*36*, *37*). For the PFTase, the zinc metal ion has been shown to play a catalytic role, where the substrate-derived sulfur nucleophile binds to the zinc as a thiolate (*38*, *39*). However, the two enzymes also manifest interesting differences in their metal ion requirements; while PFTase requires millimolar concentrations of Mg^{2+} for full activity, PGGTase-I does not (*36*, *40*, *41*). In addition, PFTase from rat has been crystallized with FPP bound, suggesting that the appropriate prenyl diphosphate is recognized by the length of the active site (*42*−*44*). It has been proposed that PGGTase-I has a hydrophobic binding pocket that is deeper than the binding pocket of PFTase to allow for the additional isoprene unit.

To begin to compare the chemical mechanisms of the PFTase- and PGGTase-I-catalyzed reactions, we have determined the stereochemical course of the PGGTase-I reaction to distinguish between the possible associative, dissociative, or covalent intermediate reaction pathways illustrated in Figure 2 (*45*, *46*). By using enantiomerically pure C-1 deuterated substrates with known configuration at C-1 of the prenyl group, ^{1}H NMR spectroscopy can be used to determine the stereochemical course of the enzyme-catalyzed reaction. In the prenylated product, the C-1 protons of the isoprenyl group are in close proximity to the chiral α-carbon of the Cys residue and are thus diastereotopic.[2] Two studies have been reported addressing the stereochemical course of the PFTase-catalyzed reaction. In 1996, Gibbs and coworkers showed that the human PFTase catalyzes farnesylation predominantly with inversion of configuration (*19*). More recently, Edelstein et al. also showed inversion of configuration at the C-1 prenyl position with PFTase from yeast (*20*). Using a method that allowed for the direct interpretation of the ^{1}H NMR spectrum of a enzymatically derived dipeptide, the authors detected no racemization at the reacting carbon. This study also demonstrated that peptides with more than four residues resulted in severe spectral overlap that did not allow the C-1 protons to be distinguishable (*20*).

The study presented here describes the stereochemical outcome of the reaction catalyzed by human PGGTase-I using the methodology outlined in Figure 3. The pentapeptide *N*-dansyl-GCVLL (**3**) was first enzymatically prenylated by human PGGTase-I with either (*S*)-[1-^{2}H]FPP (**1**) or (*S*)-[1-^{2}H]GGPP (**2**). The prenylated products (**4** and **5**) were then degraded to dipeptides using carboxypeptidase Y (CPY). After HPLC purification, the prenylated dipeptide products (**6** and **7**) were analyzed by ^{1}H NMR spectroscopy. The final spectra were compared with the spectra from the farnesylated product obtained via chemical synthesis to deduce the stereochemistry of the PGGTase-I-catalyzed reaction.

EXPERIMENTAL PROCEDURES

Materials. Reagents, unless otherwise noted, were obtained from Aldrich. Carboxypeptidase Y and alkaline phosphatase were purchased from Sigma. Trifluoroacetic acid (TFA) was obtained from Fisher. *N*-Dansyl-GCVLL was obtained from Bio-Synthesis (Lewisville, TX). Preparative HPLC employed a Rainin Dynamax Microsorb C_{18} column (2.14 cm × 25 cm with a 5 cm guard column), and analytical HPLC employed a Rainin Dynamax Microsorb C_{18} column (25 × 0.45 cm with a 5 cm guard column). Analytical TLC was performed on precoated (0.25 mm) silica gel 60F-254 plates purchased from E. Merck. Flash chromatography silica gel (60−120 mesh) was obtained from E. M. Science. Dowex AG50W-X8 resin was obtained from Bio-Rad. Sep-Pak C_{18} cartridges were obtained from Waters.

*[1,1-$^2H_2$]Farnesol ([1,1-$^2H_2$]**10**).* Methyl farnesoate (**12**, 4.2 g, 18 mmol) was added to THF (50 mL) and cooled to −78 °C. To this was added dropwise 36 mL of a 1.0 M solution of $LiAl^2H_4$ in THF (1.5 g, 36 mmol) over 10 min. The reaction was allowed to proceed at −78 °C for 1 h and then warmed to room temperature for an additional 2 h. The reaction was quenched with a saturated solution of aqueous

[2] The two stereogenic centers specified in this abbreviation are the Cys Cα (α) and the farnesyl C-1 (1).

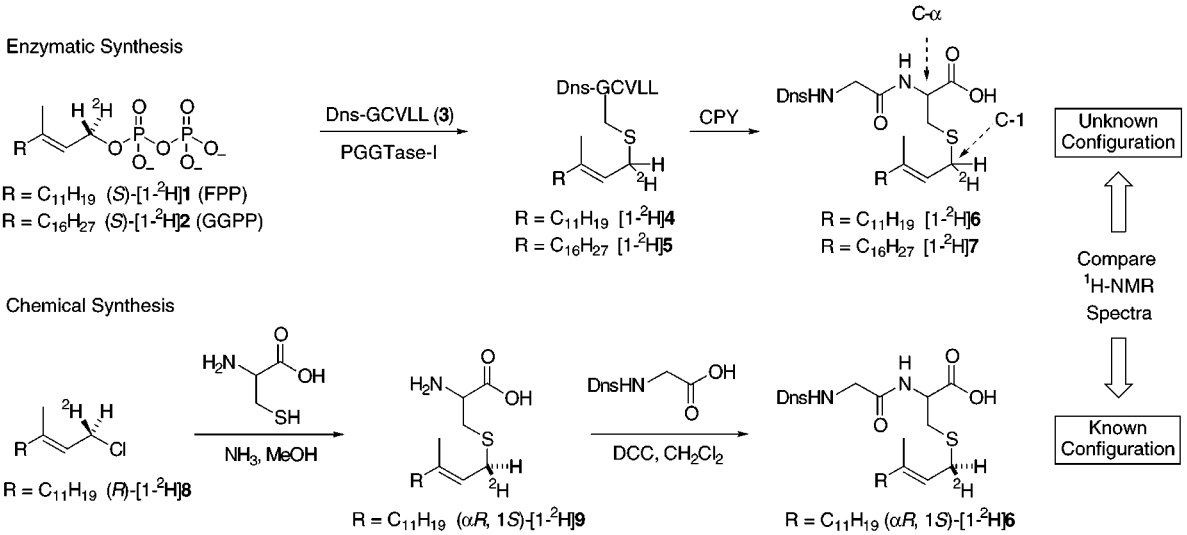

FIGURE 3: Strategy for determining the stereochemical course of the reaction catalyzed by protein geranylgeranyltransferase type I.

citric acid (70 mL) and extracted with EtOAc (3 × 200 mL). The organic layers were combined and washed with H_2O (200 mL) and brine (200 mL) and dried with $MgSO_4$. Evaporation under reduced pressure yielded crude product that was purified by flash chromatography using EtOAc/hexanes (1:10, v/v) to yield 3.0 g (77% yield) of [1,1-$^2H_2$]-**10** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.59 (s, 6H), 1.67 (s, 6H), 1.98−2.09 (m, 8H), 5.08−5.10 (m, 2H), 5.39 (s, 1H); ^{13}C (75.4 MHz, $CDCl_3$) δ 16.55, 16.82, 18.24, 26.86 (primary C), 26.25, 27.27, 40.11, 40.25, 59.57 (m) (secondary C), 123.81, 124.35, 124.87 (tertiary C), 131.87, 135.88, 140.26 (quarternary C).

*[1-^{2}H]Farnesal ([1-^{2}H]**11**) (47).* In a dry 100 mL round-bottom flask were combined 1.05 g (12.1 mmol) of MnO_2 and 6 mL of dry CH_2Cl_2. The flask was sealed and placed under N_2 (g) before the addition of 227 μL (235 mg, 1.05 mmol) [1-$^2H_2$]farnesol ([1-$^2H_2$]**10**). After 1.5 h, 1.26 g (14.5 mmol) of MnO_2 was added, followed by 1.18 g (13.4 mmol) after an additional 3.0 h. The reaction was stirred overnight for a total of 22 h followed by filtration through Celite to remove the MnO_2, and the solvent was removed by rotary evaporation. Crude material (109 mg) was purified by flash chromatography on silica gel (1.5 × 30 cm) with 10:1 (v/v) toluene/EtOAc to yield 63.9 mg (27.5%) of pure product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.15 (s, 3H), 1.59 (s, 6H), 1.74 (s, 3H), 2.00 (m, 4H), 2.23 (m, 4H), 5.08 (m, 2H), 5.87 (s, 1H); ^{13}C-DEPT NMR (75 MHz, $CDCl_3$) δ 16.0, 17.6, 17.7, 25.7 (primary C), 26.6, 27.0, 29.6, 40.6 (secondary C), 122.4, 124.1, 127.4 (tertiary C), 131.5, 136.5, 163.9 (quaternary C).

*(S)-[1-^{2}H]Farnesol ((S)-[1-^{2}H]**10**).* Following a standard procedure (*48*), 58.3 mg (0.264 mmol) of [1-^{2}H]farnesal ([1-^{2}H]**11**) was mixed with 650 μL of (*R*)-alpine borane dissolved in THF (0.5 M, 0.317 mmol) under a blanket of N_2 (g) in a 10 mL oven-dried round-bottom flask. The reaction was stirred at room temperature for 4 h before quenching it with 7.4 μL (0.132 mmol) of acetaldehyde. The THF was removed by rotary evaporation, and the reaction flask was placed under high vacuum to remove the pinene, a side product of the reaction. Dry Et_2O, 300 μL, was added to the sealed reaction flask under N_2 (g), and ethanolamine (20.6 μL, 0.264 mmol) was added via syringe. The precipitate that formed was removed by filtration and washed with Et_2O. The combined Et_2O layers were dried over $MgSO_4$, and the solvent was removed by rotary evaporation. The crude material was purified by flash chromatography on silica gel (1.5 × 2.0 cm) with toluene/EtOAc (10:1, v/v). The product, 40.9 mg (69.8% yield), was obtained as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.62 (s, 6H), 1.70 (s, 6H), 2.07 (m, 8H), 4.15 (d, J = 6.3, 1H), 5.12 (m, 2H), 5.44 (d, J = 6.9, 1H); ^{13}C-DEPT NMR (75 MHz, $CDCl_3$) δ 16.2, 16.4, 17.8, 25.9 (primary C), 26.5, 26.9, 39.7, 39.9, 59.2 (t, J = 21.5) (secondary C), 123.4, 123.9, 124.5 (tertiary C), 131.5, 135.5, 140.0 (quaternary C).

*(S)-[1-^{2}H]Farnesyl-(R)-α-methoxy-α-(trifluoromethyl)-phenyl Acetate ((1S, αR)-[1-^{2}H]**20**).* Using an established procedure (*49*), (*S*)-[1-^{2}H]farnesol (1.0 mg, 4.5 μmol) was dissolved in dry pyridine (100 μL) in a sealed flask flushed with N_2 (g). (*S*)-α-Methoxy-α-(trifluoromethyl)phenylacetyl chloride [(*S*)-MTPA-Cl] (16 mg, 12 μL, 64 μmol) was added to the flask, and the reaction was stirred under N_2 (g) for 3 h. One drop of H_2O was added to quench the reaction, and the crude product was dissolved in CH_2Cl_2. The solution was then washed sequentially with 0.1 M HCl, 1 M $NaHCO_3$, and H_2O, and the organic layer was dried over $MgSO_4$. Following concentration, 3.6 mg of crude material was purified by chromatography on silica gel (0.5 × 5 cm) with toluene/EtOAc (5:2, v/v) to yield 1.7 mg (89%) of product. ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.59 (s, 6H), 1.68 (s, 3H), 1.72 (s, 3H), 1.95−2.00 (m, 2H), 2.03−2.13 (m, 6H), 3.56 (s, 3H), 4.79 (d, J = 7.0, 0.999H), 4.83 (d, J = 7.0, 0.001H), 5.07−5.12 (m, 2H), 5.39 (d, J = 7.0, 1H), 7.38−7.42 (m, 3H), 7.51−7.53 (m, 2H). Decoupling by irradiation at 5.4 ppm gives two singlets at 4.79 (0.998 H) and 4.83 (0.002 H) ppm, indicating >99% *de*.

*(R)-[1-^{2}H]Farnesyl Chloride ((R)-[1-^{2}H]**8**).* Following a published procedure (*50*), 13.2 mg (988 μmol) of *N*-chlorosuccinimide was added to a flamed-dried 10 mL flask. The flask was sealed under N_2 (g) before the addition of 200 μL of dry CH_2Cl_2 followed by cooling to −30 °C [acetonitrile/CO_2 (s) bath]. Dimethyl sulfide, 11 μL (150 μmol), was added, and the reaction flask was briefly warmed to 0 °C before cooling it to −40 °C. (*S*)-[1-^{2}H]Farnesol {(*S*)-[1-^{2}H]**10**, 20.0 mg, 89.1 μmol}, dissolved in 300 μL of

dry CH_2Cl_2, was added to the flask. The reaction flask was allowed to warm to 0 °C over 1 h, react at this temperature for an additional 1 h, and then warmed to room temperature for 0.5 h. The completed reaction mixture was extracted with cold pentane. The organic phase was washed with cold saturated NaCl, and the layers were separated. The aqueous layer was washed with 3 portions (3.0 mL each) of cold pentane. Finally, all organic layers were combined and washed with three portions (3 mL each) of cold, saturated NaCl, dried over $MgSO_4$, and concentrated under reduced pressure to yield 14.0 mg (64.5%) of product. The crude material from this reaction was used without further purification. ^{1}H NMR (200 MHz, $CDCl_3$) δ 1.60 (s, 6H), 1.68 (s, 3H), 1.73 (s, 3H), 2.06 (m, 8H), 4.09 (d, $J = 8$, 1H), 5.45 (d, $J = 8$, 1H), 5.09 (m, 2H).

*(S)-[1-^{2}H]Farnesyl Diphosphate ((S)-[1-^{2}H]**1**).* Following a modification of the published procedure (*50*), (*R*)-[1-^{2}H]-farnesyl chloride ((*R*)-[1-^{2}H]**8**, 14.0 mg, 57.4 μmol) and 300 μL of dry CH_3CN were added to a 10 mL flask. Tris(tetra-*n*-butylammonium) hydrogen diphosphate was then added (227 mg, 248 μmol), and the reaction was stirred for 6 h under N_2 (g) at room temperature. The crude product was applied to a cation exchange column (Dowex AG50-X8 resin, 1 × 20 cm) equilibrated in 49:1 25 mM NH_4HCO_3/2-propanol (the resin was initially converted to the ammonium salt with 3:1 H_2O/NH_4OH) to exchange the tetra-*n*-butylammonium groups for ammonium groups. Fractions containing product were lyophilized to dryness. Finally, the crude product was purified using a C_{18} Sep-Pak cartridge. The column was initially washed with 10 mL of CH_3CN (solvent B) and 30 mL of 25 mM NH_4HCO_3 (solvent A) before loading about 15 mg of crude material in 10 mL of solvent A. The column was eluted with 20 mL of solvent A and 4.0 mL of each of the following, collecting 2.0 mL fractions: 20, 40, 50, and 100% B. Fractions were analyzed by TLC using 6:3:1 (v/v/v) 2-propanol/NH_4OH/H_2O as the solvent system; the remainder of the crude material was purified by repeating this procedure. Fractions containing product were pooled, concentrated by rotary evaporation, and lyophilized. Pure product, 19.2 mg (77.7% yield), was obtained as a white solid. ^{1}H NMR (300 MHz, D_2O pH 8.0 with ND_4OD) δ 1.45 (s, 6H), 1.51 (s, 3H), 1.55 (s, 3H), 1.93 (m, 8H), 4.29 (broad t, 1H), 5.01 (m, 2H), 5.28 (d, $J = 6$, 1H); ^{31}P NMR (D_2O, pH 8.0 with ND_4OD, with one drop of 1% EDTA in D_2O, 121.4 MHz) δ −8.67 (d, $J = 21.4$, 1P), −11.05 (d, $J = 21.4$, 1P).

Enzymatic Hydrolysis of (S)-[1-^{2}H]Farnesyl Diphosphate. In a glass vial (1 × 3 cm) were mixed 2.0 mg of (*S*)-[1-^{2}H]-FPP ((*S*)-[1-^{2}H]**1**), 50 μL of 10× dephosphorylating buffer (100 mM Tris·HCl, pH 8.3, 10 mM $MgCl_2$, 1.0 mM $ZnCl_2$), 5.0 μL (25 DEA units) of alkaline phosphatase, and H_2O to a final volume of 500 μL. This was allowed to react at 37 °C for 4 h before the product was extracted into CH_2Cl_2. The crude alcohol was esterified using (*S*)-MTPA-Cl as described above for (*S*)-[1-^{2}H]**10**. From the decoupled ^{1}H NMR spectrum (irradiated at 5.4 ppm) obtained of the Mosher ester, (1*S*, α*R*)-[1-^{2}H]**20**, the diastereomeric purity of this material was found to be 78% *de* (peak integration had a ratio of 1.0:8.1 minor to major diastereomer).

*[1-$^2H_2$]Geranylgeraniol ([1-$^2H_2$]**17**).* In a 10 mL round-bottom flask was prepared 476 μmol of Al^2H_3 in situ by mixing 15.0 mg (357 μmol) of $LiAl^2H_4$ in 1.0 mL of dry Et_2O with an excess of $AlCl_3$ (26.0 mg, 0.196 mmol) at −30 °C in a sealed flask under N_2 (g) (*51*, *52*). After observing the formation of the side product, LiCl (s), and allowing the reaction flask to warm to room temperature, 203.5 mg (609 μmol) of ethyl geranylgeranoate (**13**) was added dissolved in 1.0 mL of dry Et_2O. After 6.5 h, the reaction was not complete as judged by TLC (95:5, v/v, hexanes/EtOAc). An additional 241 μmol of Al^2H_3 in 1.0 mL of Et_2O (prepared as described above) was added to the reaction mixture via syringe. The reaction was allowed to proceed an additional 6.5 h and then quenched with a few drops of H_2O. The reaction was washed with 2.0 mL of dilute H_2SO_4 and 2.0 mL of H_2O, dried over $MgSO_4$, filtered, and concentrated in vacuo. Pure product, 104 mg (61.6% yield), was obtained after flash chromatography on silica gel (1.5 × 30 cm) with 5:2 (v/v) toluene/EtOAc. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.60 (s, 9H), 1.67 (s, 6H), 2.06 (m, 12H), 5.10 (m, 3H), 5.41 (s, 1H); ^{13}C-DEPT NMR (75 MHz, $CDCl_3$) δ 16.1, 16.4, 17.8, 25.8 (primary C), 26.4, 26.7, 26.8, 39.6, 39.7, 39.8, 58.8 (quintet, $J = 21$) (secondary C), 123.2, 123.9, 124.2, 124.4 (tertiary C), 131.4, 135.0, 135.5, 140.0 (quaternary C). EI-MS calcd $C_{20}H_{32}{}^2H_2O$ $[M]^+$ 292.3, found 292.3.

*[1-^{2}H]Geranylgeranial ([1-^{2}H]**18**).* In a 50 mL round-bottom flask was dissolved 103.6 mg (375 μmol) of [1-$^2H_2$]-geranylgeraniol ([1-$^2H_2$]**17**) in 10 mL of dry hexanes. MnO_2 (0.5 g, 15 equiv) and Na_2CO_3 (0.5 g, 15 equiv) were added 3 times at 45 min intervals to the reaction flask, while the contents were stirred vigorously with a magnetic stir bar (*53*). After 6 h, the reaction was filtered through Celite, dried over $MgSO_4$, refiltered, and concentrated in vacuo. Pure product, 50.2 mg (46.3% yield), was obtained by flash chromatography on silica gel (1.5 cm × 30 cm) eluting with 5:2 (v/v) toluene/EtOAc. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.58 (s, 3H), 1.59 (s, 3H), 1.66 (s, 3H), 1.93−2.06 (m, 8H), 2.15 (s, 3H), 2.21 (m, 4H), 5.07 (m, 3H), 5.87 (s, 1H); ^{13}C-DEPT NMR (75 MHz, $CDCl_3$) δ 16.0, 16.1, 17.6, 17.7, 25.7 (primary C), 25.7, 26.6, 26.8, 39.6, 39.7, 40.7 (secondary C), 122.5, 124.0, 124.4, 127.4 (tertiary C), 131.1, 135.1, 136.6, 163.9 (quaternary C).

*(S)-[1-^{2}H]Geranylgeraniol ((S)-[1-^{2}H]**17**).* [1-^{2}H]Geranylgeranial ([1-^{2}H]**18**) (50.2 mg, 174 μmol) was reduced with 417 μL of (*R*)-alpine borane (0.5 M solution in THF, 209 μmol) as described above for compound (*S*)-[1-^{2}H]**10**. Crude product was subjected to flash chromatography on silica gel (1.5 × 30 cm) eluted with 5:2 (v/v) toluene/EtOAc, giving 39.0 mg (81.5% yield) of pure product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.62 (s, 6H), 1.68 (s, 6H), 1.96−2.10 (m, 12H), 4.12 (d, $J = 6$, 1H), 5.10 (m, 3H), 5.41 (d, $J = 7$, 1H); ^{13}C-DEPT NMR (75 MHz, $CDCl_3$) δ 16.1, 16.4, 17.8, 25.8 (primary C), 26.4, 26.7, 26.8, 39.6, 39.7, 39.8, 59.1 (t, $J =$ 22) (secondary C), 123.3, 123.8, 124.2, 124.4 (tertiary C), 131.4, 135.0, 135.5, 140.0 (quaternary C).

*(S)-[1-^{2}H]Geranylgeranyl-(S)-α-methoxy-α-(trifluoromethyl)phenyl Acetate ((1S, αS)-[1-^{2}H]**21**).* Esterification of (*S*)-[1-^{2}H]**17** with (*R*)-MTPA-Cl was performed as described above for compound (*S*)-[1-^{2}H]**10**, and ^{1}H NMR analysis indicated a diastereomeric purity of >99% *de* for the product, (1*S*, α*S*)-[1-^{2}H]**21**.

*(R)-[1-^{2}H]Geranylgeranyl Chloride ((R)-[1-^{2}H]**19**).* As described above for compound (*R*)-[1-^{2}H]**11**, 37.0 mg (135 μmol) of (*S*)-[1-^{2}H]**17** was reacted with 1.1 equiv of *N*-chlorosuccinimide (20.6 mg, 155 μmol) and dimethyl

sulfide (10.9 μL, 149 μmol) to yield 26.1 mg (62.6%) of product. ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.59 (s, 9H), 1.67 (s, 3H), 1.72 (s, 3H), 1.96−2.15 (m, 12H), 4.08 (d, J = 7.8, 1H), 5.06−5.10 (m, 3H), 5.43 (d, J = 7.8, 1H); ^{13}C-DEPT NMR (75 MHz, $CDCl_3$) δ 16.1, 16.2, 17.7, 25.8 (primary C), 26.2, 26.6, 26.8, 39.5, 39.7, 39.8, 41.0 (t) (secondary C), 120.2, 123.5, 124.2, 124.4 (tertiary C), 131.3, 135.0, 135.6, 142.9 (quaternary C).

*(S)-[1-^{2}H]Geranylgeranyl Diphosphate ((S)-[1-^{2}H]**2**).* Compound (*R*)-[1-^{2}H]**18** (25.2 mg, 81.3 μmol) was added to a sealed flask containing 151 mg (167 μmol) of tris(tetra-*n*-butylammonium) hydrogen diphosphate and 0.5 mL of dry CH_3CN. This was stirred for 2.0 h before an additional 72.5 mg (80.4 μmol) of tris(tetra-*n*-butylammonium) hydrogen diphosphate was added to the reaction. After a total of 5.0 h, the reaction was concentrated to remove solvent, and the product was purified as described above for compound (*S*)-[1-^{2}H]**1**. The crude product (89.5 mg) after Sep-Pak purification yielded 31.2 mg (76.4% yield) of pure product, (*S*)-[1-^{2}H]**2**, as a white solid. ^{1}H NMR (300 MHz, D_2O at pH 8.0 with ND_4OD) δ 1.38 (s, 6H), 1.41 (s, 3H), 1.46 (s, 3H), 1.52 (s, 3H), 1.72−1.99 (m, 12H), 4.24 (t, J = 6.3, 1H), 4.88−4.02 (m, 3H), 5.24 (d, J = 6.6, 1H); ^{31}P NMR (121 MHz, D_2O at pH 8.0 with ND_4OD and one drop of 0.1% EDTA in D_2O) δ −9.36 (d, J = 20.7, 1P) and −5.65 (d, J = 20.7, 1P). HR-FAB-MS calcd $C_{20}H_{35}{}^2HO_7P_2$ $[M-H]^-$ 450.1922, found 450.1904.

Enzymatic Hydrolysis of (S)-[1-^{2}H]GGPP. Compound (*S*)-[1-^{2}H]**2** was treated with alkaline phosphatase and esterified with (*R*)-MTPA-Cl to determine the enantiomeric purity of the material, as described above for (*S*)-[1-^{2}H]FPP. ^{1}H NMR analysis indicated a diastereomeric purity of 62% *de* (peak integration had a ratio of 4.3:1.0 major to minor diastereomer).

Purification of Human PGGTase-I. Following modifications of published procedures (*54*, *55*), wet cell paste [5 g, *E. coli* BL21(DE3)/pRD578] was suspended in 20 mL of sonicating buffer [50 mM Tris·HCl, pH 7.5, 1.0 mM EDTA, 1.0 mM ethylene glycol-*O*,*O*′-bis(2-aminoethyl)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), 1.0 mM dithiothreitol (DTT), 1.0 mM phenylmethylsulfonyl fluoride, 2.0 μg/mL leupeptin, 2.0 μg/mL antipain, and 10 μg/mL aprotinin]. The cells were pulse-sonicated on ice for 4 × 30 s. The cellular debris was removed by centrifugation at 30000*g* for 30 min. The clarified crude extract was applied to a HiLoad 26/10 Sepharose Q Fast Flow column (Pharmacia) for FPLC at a flow rate of 1.0 mL/min. The column was preequilibrated in low-salt buffer (50 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 50 μM $ZnCl_2$, and 10 mM 2-mercaptoethanol). After applying the protein sample, the column was washed with 100 mL of low-salt buffer flowing at 1.0 mL/min. The protein was then eluted with a 0−500 mM salt gradient over 500 min. The high-salt buffer was prepared by augmenting the low-salt buffer with 1 M NaCl. Fractions were pooled based on PGGTase-I enzyme activity (*26*, *56*). The partially purified sample was then dialyzed against low-salt buffer (2 × 1 L) and centrifuged at 30000*g* for 30 min to remove precipitated proteins. The dialyzed sample was applied to an anti-α-tubulin immunoaffinity column that was preequilibrated in buffer (20 mM Tris·HCl, 1 mM $MgCl_2$, 10 μM $ZnCl_2$, 5.0 mM 2-mercaptoethanol, and 50 mM NaCl) by gravity flow at a rate of about 3.0 mL/h. The column was washed overnight with buffer to remove unbound proteins until the UV absorbance (at 280 nm) of the eluant returned to the baseline. The protein was then eluted with the above buffer supplemented with 5.0 mM Asp-Phe. Active fractions were pooled, concentrated with an Amicon Centriprep 10, and stored in small aliquots with 20% glycerol (v/v) at −80 °C after flash-freezing with N_2 (l). A purification (from 5 g of wet cell paste) yielded 3.13 mg of PGGTase-I with a specific activity of 0.329 μmol·min^{-1}·mg^{-1}.

*Chemical Synthesis of (Cys-αR, Farnesyl-1S)-N-dansyl-Gly-Cys(S-[1-^{2}H]farnesyl)-OH ((αR, 1S)-[1-^{2}H]**6**).* Dry methanol (6 mL) was added to cysteine (25 mg, 0.21 mmol), and the solution was cooled to 0 °C. Anhydrous ammonia gas was bubbled into the reaction while (*R*)-[1-^{2}H]**8** (240 mg, 1.0 mmol), dissolved in methanol, was added. Ammonia bubbling was continued for an additional 50 min while stirring at 0 °C. The reaction was sealed and stirred for 3 h at 0 °C, followed by 1 h at room temperature. N_2 (g) was then bubbled through the reaction in order to remove the NH_3, and solvent was removed at reduced pressure. The product was partially purified by dissolving the crude residue in butanol and washing with H_2O. The butanol layer was dried with $MgSO_4$ and concentrated. The residue was then dissolved in MeOH and washed with hexane, followed by concentration in vacuo to yield 52 mg of partially purified farnesyl cysteine. Next, *N*-dansyl-Gly (59 mg, 0.19 mmol) was dissolved in 20 mL of CH_2Cl_2 with DCC (1,3-dicyclohexylcarbodiimide) (40 mg, 0.19 mmol), followed by addition of farnesyl cysteine (30 mg, 0.10 mmol) prepared as described above. The reaction was stirred at room temperature for 24 h under N_2. It was then filtered and concentrated. The product was purified by preparative reversed-phase HPLC (solvent A: 95% H_2O, 5% CH_3CN, and 0.2% TFA; solvent B: CH_3CN and 0.2% TFA) using a 30 min linear gradient from 50% solvent B to 100% solvent B (retention time = 35 min). ^{1}H NMR (500 MHz, $CDCl_3$) δ 1.285 (s, 6H), 1.596 (s, 3H), 1.678 (s, 3H), 1.937−2.098 (m, 8H), 2.478−2.510 (dd, 1H), 2.625−2.653 (dd), 2.998−3.071 (m, 2H), 3.431 (s, 6H), 3.604−3.656 (m, 1H), 3.771−3.857 (m, 1H), 4.089 (m, 1H), 5.045−5.107 (m, 3H), 5.766 (broad s, 1H), 5.945 (broad s, 1H), 7.706 (d, J = 7.5, 1H), 7.769−7.816 (m, 2H), 8.414 (d, J = 7.0, 1H), 8.608 (d, J = 8.5, 1H), 8.895 (d, J = 8.5, 1H). FAB-MS calcd for $C_{32}H_{46}N_3O_5S_2$ $[M+H]^+$ 616.3, found 616.4; calcd for $C_{32}H_{44}N_3O_5S_2$ $[M-H]^-$ 614.3, found 614.3; calcd for $C_{32}H_{45}N_3O_5S_2Na$ $[M+Na]^+$ 638.3, found 638.4; calcd for $C_{32}H_{45}N_3O_5S_2K$ $[M+K]^+$ 654.2, found 654.3.

*Enzymatic Synthesis of (Cys-αR)-N-Dansyl-Gly-Cys(R-[1-^{2}H]farnesyl)-OH ((αR, 1R)-[1-^{2}H]**6**).* The night before performing the enzymatic synthesis, the following components were mixed: 2.0 mg of *N*-dansyl-GCVLL, 10 mL of 500 mM Tris·HCl, pH 7.5, 500 μL of 1.0 M $MgCl_2$, 50 μL of 100 mM $ZnCl_2$, 200 μL of 500 mM DTT, 4.0 mg of dodecyl-β-D-maltoside, and 50 mL of H_2O. This solution was stored at 4 °C overnight to ensure the reduction of any disulfide-linked peptide dimer. Just prior to initiating the reaction, 35 mL of H_2O and 6.9 mg of (*S*)-[1-^{2}H]FPP dissolved in 2.0 mL of 25 mM NH_4HCO_3 were added, and the resulting mixture was allowed to warm to 30 °C. The final concentrations in the reaction were as follows: 50 mM Tris·HCl, pH 7.5, 5.0 mM $MgCl_2$, 50 μM $ZnCl_2$, 1.0 mM DTT, 0.04% dodecyl-β-D-maltoside, 140 μM FPP, and 27

μM *N*-dansyl-GCVLL. The reaction was initiated with 30 μL of stock PGGTase-I (11.2 μM) and allowed to react at 30 °C. Enzyme aliquots were added periodically: 30 μL at 0.5, 1.0, 2.0, 4.0, and 9.0 h and 90 μL at 24 and 30 h. The reaction was monitored by analytical scale reversed-phase HPLC using solvents A and B described above. The program was as follows at 1.0 mL/min: 15% B for 10 min, a gradient to 60% B in 5 min, a 30 min gradient to 90% B followed by 15 min at 90% B, and then the column was returned and reequilibrated in 15% B. Starting material (*N*-dansyl-GCVLL) eluted at approximately 20 min, and farnesylated *N*-dansyl-GCVLL eluted at 35 min. The completed reaction was centrifuged (16000*g* for 15 min) to separate the precipitated product. The supernatant was brought to 20 mM EDTA by the addition of 4.2 mL of 500 mM EDTA, pH 8.0, and the collected precipitate was suspended in 50 mM Tris·HCl, pH 7.5, and 20 mM EDTA (100 mL, final volume). Carboxypeptidase Y (2.0 mg) was dissolved in 40 μL of 20 mM Tris·HCl, pH 7.0, and 10 mM EDTA, and 20 μL was added to each reaction (supernatant and dissolved pellet). After the reaction was allowed to sit for 4 h and judged complete by analytical HPLC analysis (retention time of farnesylated dipeptide was 30 min), it was partially purified and concentrated on a Sep-Pak cartridge. The sample was loaded onto a prepared column (initially washed with 10 mL of solvent B and equilibrated with 20 mL of solvent A). The column was then washed with 10 mL of solvent A, 2 mL of 25% B, and 2 mL of 50% B, and the product was eluted with 100% B. Product elution was monitored by observing the dansyl fluorescence with a hand-held UV lamp. Fractions containing product were partially concentrated under a stream of N_2 (g) and purified by preparative scale reversed-phase HPLC. The program, at 10 mL/min, was as follows: 50% B for 10 min, 50−70% B in 30 min, and 70% B for 10 min. The retention time of the product, (αR, 1*R*)-[1-^{2}H]**6**, was 44 min using this elution profile. Enzymatic synthesis using undeuterated FPP produced the corresponding compound (αR)-**6**. LR-FAB-MS calcd for $C_{32}H_{46}N_3O_5S_2$ $[M+H]^+$ 616.3, found 616.4; calcd for $C_{32}H_{44}N_3O_5S_2$ $[M-H]^-$ 614.3, found 614.4.

(Cys-α*R)-N-Dansyl-Gly-Cys-(R-[1-*^{2}*H]geranylgeranyl)-OH (Enzymatic Synthesis) (*(αR*, 1R)-[1-*^{2}*H]7).* The preparation of (αR, 1*R*)-[1-^{2}H]**7** was conducted on a 200 mL scale and performed as described above, except for the addition of approximately 3.0 mg of *N*-dansyl-GCVLL and the use of (*S*)-[1-^{2}H]GGPP noted below. Just prior to initiating the reaction, the volume was adjusted to 200 mL with H_2O, and the reaction flask was allowed to warm to 30 °C. A 8.0 mM solution of (*S*)-[1-^{2}H]GGPP was prepared by dissolving approximately 4.0 mg in 1.0 mL of 25 mM NH_4HCO_3. The reaction was initiated with 50 μL of PGGTase-I (11.2 μM stock) and 200 μL of (*S*)-[1-^{2}H]GGPP. At 45 min intervals, additional enzyme and (*S*)-[1-^{2}H]GGPP were added. The reaction was monitored by analytical scale reversed-phase HPLC, as described above for the farnesylated peptide. The starting material (*N*-dansyl-GCVLL) eluted at 25 min, and geranylgeranylated *N*-dansyl-GCVLL eluted at 48 min. After the reaction was judged complete by HPLC analysis (4 h), the cloudy solution (due to the isolubility of the product) was brought to a concentration of 20 mM EDTA (8.3 mL of 500 mM EDTA, pH 8.0). Carboxypeptidase Y (3.0 mg of total protein dissolved in 30 μL of 50 mM Tris·HCl, pH 7.0, and 20 mM EDTA) was added in 10 μL aliquots over 1.0 h. The reaction was judged complete by HPLC analysis (retention time of geranylgeranylated dipeptide was 45 min) and partially purified and concentrated on Sep-Pak cartridges, as described above. Fractions containing product were partially concentrated under a stream of N_2 (g) and purified by preparative scale reversed-phase HPLC at 10 mL/min. The column was equilibrated in 75% B before loading the sample. The column was then washed for 10 min with 75% B before a gradient to 95% B in 30 min. The product, (αR, 1*R*)-[1-^{2}H]**7**, eluted at 50 min in this system. Fluorescent fractions were pooled, subjected to rotary evaporation, and lyophilized to dryness. LR-FAB-MS calcd $C_{37}H_{53}{}^2HN_3O_5S_2$ $[M+H]^+$ 685.3, found 685.4.

RESULTS

Synthesis of Chiral C-1 Deuterated Substrates. The preparation of chiral, monodeuterated substrates ((*S*)-[1-$^2H_2$]-**1** and (*S*)-[1-$^2H_2$]**2**) necessary for this study first required the synthesis of dideuterated alcohol precursors. Dideuterated farnesol ([1-$^2H_2$]**10**) was prepared using the route shown in Figure 4.

This procedure involved, first, the oxidation of farnesol to farnesal (**11**) using MnO_2, followed by further oxidation to methyl farnesoate (**12**) in the presence of MnO_2, NaCN, and CH_3OH. The methyl ester **12** was reduced with $LiAl^2H_4$ to yield [1-$^2H_2$]**10.** It should be noted that [1-$^2H_2$]**10** produced using this procedure is contaminated with over-reduced impurities that result from 1,4 addition of $LiAl^2H_4$ to the α,β-unsaturated ester (**12**). However, these impurities are easily removed by chromatography after the subsequent reoxidation step. Since an inexpensive source for geranylgeraniol was not available, a different route for the preparation of [1-$^2H_2$]**17** from farnesol (**10**), also shown in Figure 4, was developed. This route exploited recent chemistry developed by Gibbs and co-workers for the homologation of C_{15} isoprenoids to their C_{20} counterparts (*57*). First, farnesyl bromide (**13**), prepared from farnesol (**10**), was reacted with the dianion of ethyl acetoacetate to yield **14**. This β-keto ester (**14**) was deprotonated and the resulting enolate trapped as the enol triflate **15**. Further reaction involving methylation under Suzuki-type conditions yielded ethyl geranylgeranoate (**16**). To avoid the over-reduction problems described above in the synthesis of [1-$^2H_2$]**10**, the reduction of **16** to [1-$^2H_2$]-**17** was performed under milder conditions employing Al^2H_3 as the reductant (*58*). In both cases, the pure dideuterated alcohols ([1-$^2H_2$]**10** and [1-$^2H_2$]**17**) contained complete (>99%) deuteration at C-1 as judged from EI-MS analysis. For the preparation of the enantiomerically enriched alcohols, the dideuterated alcohols, [1-$^2H_2$]**10** and [1-$^2H_2$]**17**, were oxidized with MnO_2 to yield the corresponding deuterated aldehydes, [1-^{2}H]**11** and [1-^{2}H]**18**, followed by reduction employing (*R*)-alpine borane as summarized in Figure 5. The enantiomerically enriched alcohols (*S*)-[1-^{2}H]**10** and (*S*)-[1-^{2}H]**17** were converted to their respective Mosher esters (**20** and **21**) as shown in Figure 5 and analyzed by ^{1}H NMR to determine their enantiomeric purity and absolute configuration (*49*). In both cases, the (*S*) alcohols were produced with good enantiomeric purities, making them suitable for stereochemical analysis.

To synthesize the diphosphate compounds, the (*S*)-[1-^{2}H]-alcohols (**10** and **17**) were first activated as the (*R*)-[1-^{2}H]-

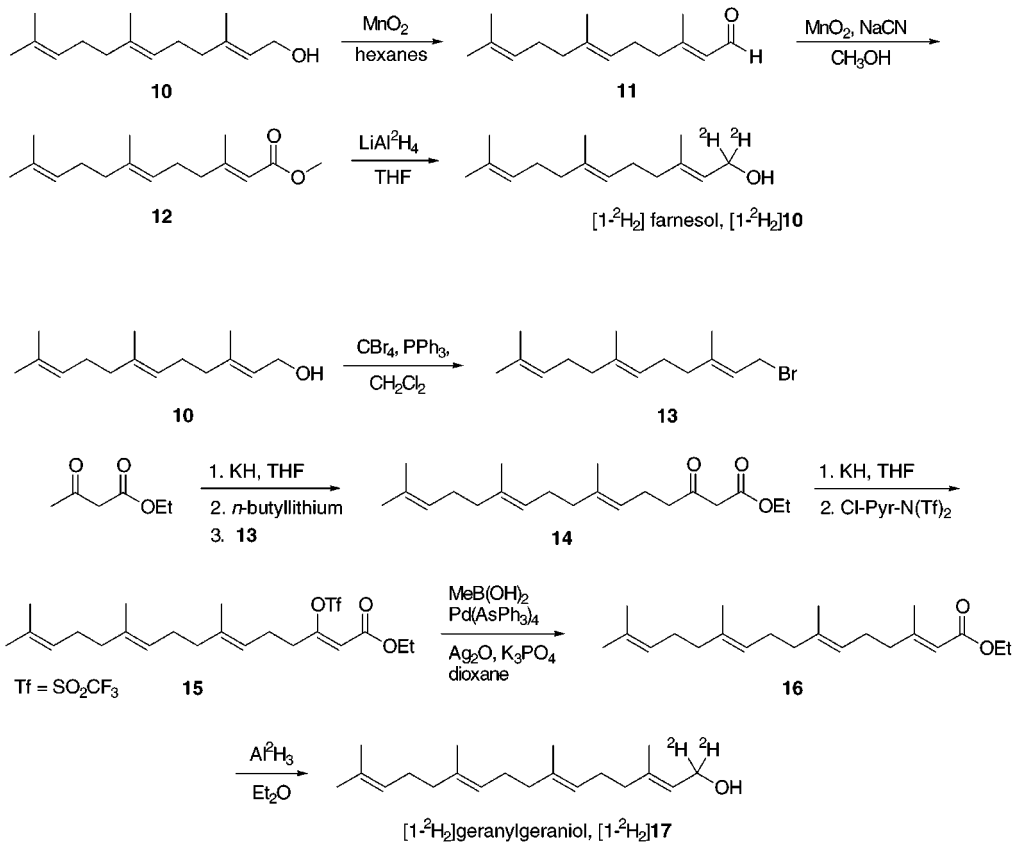

FIGURE 4: Syntheses of dideuteriofarnesol ([1-^{2}H$_2$]**10**) and dideuteriogeranylgeraniol ([1-^{2}H$_2$]**17**).

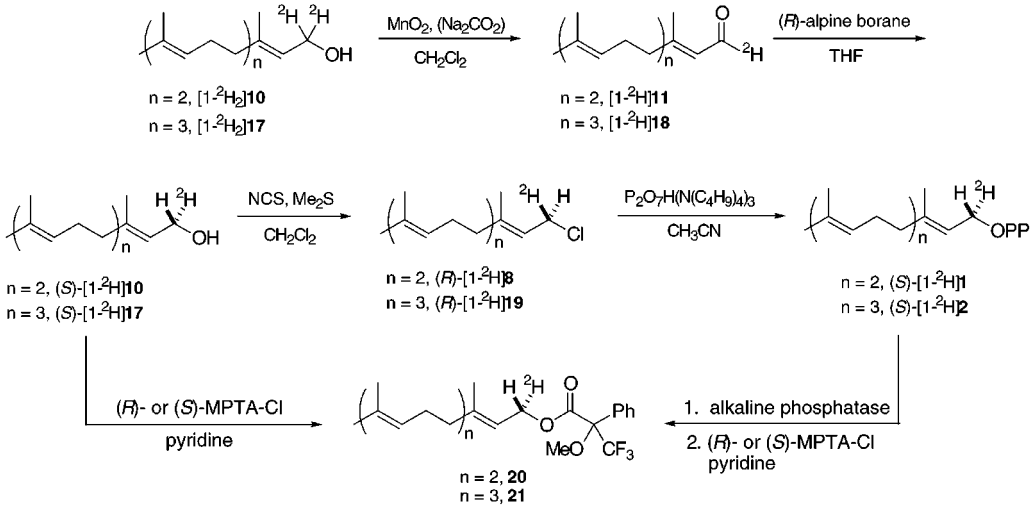

FIGURE 5: Syntheses and analyses of enantiomerically enriched forms of monodeuterated farnesol {(*S*)-[1-^{2}H]**10**}, geranylgeraniol {(*S*)-[1-^{2}H]**17**}, and the corresponding diphosphates {(*S*)-[1-^{2}H]**1** and (*S*)-[1-^{2}H]**2**}.

prenyl chlorides as shown in Figure 5 by a direct displacement reaction using *N*-chlorosuccinimide and dimethyl sulfide, resulting in the inversion of configuration at the C-1 positions yielding (*R*)-[1-^{2}H]**8** and (*R*)-[1-^{2}H]**19**. The chlorides were then displaced with [(*n*-Bu)$_4$N]$_3$HP$_2$O$_7$ which again inverted the configuation at C-1 and yielded the singly deuterated prenyl diphosphates ([1-^{2}H]**1** and [1-^{2}H]**2**) with (*S*) configurations. The enantiomeric purities of these substrates were examined by subjecting them to alkaline phosphatase hydrolysis and Mosher esterification as shown in Figure 5. Alkaline phosphatase cleaves phosphate esters between the O−P bond, retaining the original oxygen at the C-1 position and leaving the configuration at C-1 unaltered. Mosher esterification of the resulting alcohols then allowed for the assessment of the original enantiomeric purity of the prenyl diphosphates. Based on this analysis, (*S*)-[1-^{2}H]**1** had an enantiomeric purity of 78% *ee* measured by ^{1}H NMR spectral integration, while the (*S*)-[1-^{2}H]**2** had a 62% *ee* determined by the same method. Thus, the enantiomeric purity of these compounds is significantly lower than the original singly deuterated alcohols due to racemization that occurred either in the conversion to the prenyl chlorides and/or in the subsequent transformation to the diphosphates; similar results were obtained by Gibbs and co-workers in the preparation of both (*R*)- and (*S*)-[1-^{2}H]**1** for stereochemical analysis of PFTase (*19*). As was noted by Davisson et

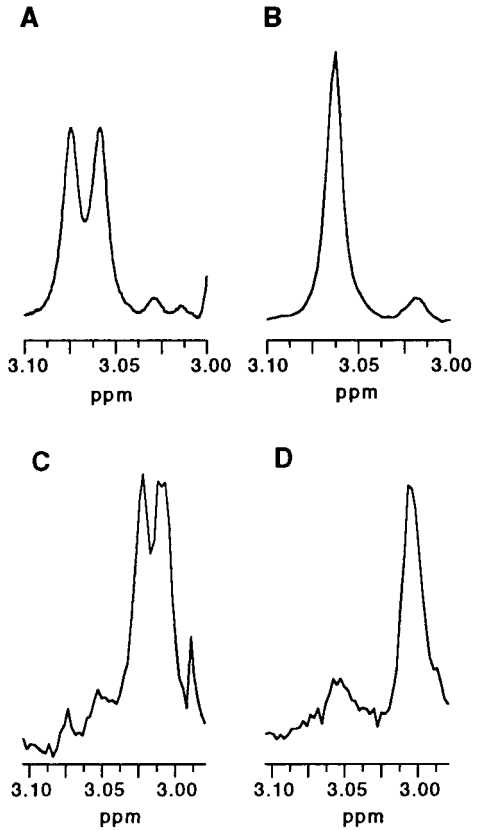

FIGURE 6: Expansion of the ^{1}H NMR spectra of [1-^{2}H]**6** showing the region containing the C-1 farnesyl proton resonances. (A) Spectrum of (α*R*, 1*S*)-[1-^{2}H]**6** obtained by chemical synthesis. (B) Spectrum of (α*R*, 1*S*)-[1-^{2}H]**6** obtained by chemical synthesis after decoupling at 5.1 ppm. (C) Spectrum of [1-^{2}H]**6** obtained via enzymatic synthesis with PGGTase-I. (D) Spectrum of [1-^{2}H]**6** obtained via enzymatic synthesis with PGGTase-I after decoupling at 5.1 ppm.

al., the developers of the phosphorylation procedure, the most likely explanation for this loss of stereochemical integrity is that it occurs in the prepration of the reactive allylic chlorides (*50*, *59*). Consistent with this view is our observation that attempts to prepare the diphosphates via the more reactive bromides by treating the alcohols with CBr_4 and Ph_3P resulted in complete racemization at C-1 (data not shown).

Assignment of Chemical Shifts for the Prenyl C-1 Protons in N-Dansyl-Gly-Cys([1-^{2}H]-farnesyl)-OH. To assign the chemical shifts of the C-1 farnesyl protons in *N*-dansyl-Gly-Cys([1-^{2}H]-farnesyl)-OH ([1-^{2}H]**6**), it was necessary to prepare at least one diastereomer of this compound via chemical synthesis as outlined in Figure 3. Enantiomerically enriched farnesyl chloride, (*R*)-[1-^{2}H]**8**, was used to alkylate cysteine in methanolic ammonia to produce (α*R*, 1*S*)-[1-^{2}H]-**9** which was subsequently coupled with *N*-dansyl-Gly using DCC to produce (α*R*, 1*S*)-[1-^{2}H]**6**. It should be noted that alkylation of cysteine under these conditions leads to inversion of configuration at the prenyl C-1, giving the C-1 position a (*S*) configuration. As shown in Figure 6A, ^{1}H NMR spectral analysis of this sample of (α*R*, 1*S*)-[1-^{2}H]**6** reveals one large doublet at 3.07 ppm and a smaller doublet at 3.02 ppm. Upon decoupling by irradiation at 5.10 ppm, which corresponds to the C-2 farnesyl proton, the two doublets simplify to a large and a small singlet shown in Figure 6B. This allowed us to assign the doublet at 3.07 ppm to the (α*R*, 1*S*)-[1-^{2}H]**6** diastereomer and the smaller doublet at 3.01 ppm as the (α*R*, 1*R*)-[1-^{2}H]**6** diastereomer. This was further confirmed by synthesizing (α*R*, 1*R*)-[1-^{2}H]**6** from (*S*)-[1-^{2}H]**8**. NMR analysis of this sample showed a large doublet at 3.01 ppm and a smaller doublet at 3.07 ppm consistent with the above assignments (data not shown). Unexpectedly, the chemical shifts of the C-1 protons and others in [1-^{2}H]-**6**, especially those from protons on the dansyl moiety, vary depending on the concentration of the sample and the amount of residual H_2O present. However, in all spectra obtained, the C-1 proton from the (α*R*, 1*S*)-[1-^{2}H]**6** diastereomer appeared downfield from the (α*R*, 1*R*)-[1-^{2}H]**6** diastereomer, making determination of the sterochemical course of the enzyme-catalyzed reaction possible using this NMR strategy.

^{1}H NMR Analysis of the Enzymatic Products. To analyze the stereochemistry of the enzymatic reaction products, human (h) PGGTase-I was used to prenylate the peptide *N*-dansyl-Gly-Cys-Val-Leu-Leu starting with either (*S*)-[1-^{2}H]**1** or (*S*)-[1-^{2}H]**2**. The resulting products were then digested with carboxypeptidase Y to remove the three C-terminal aliphatic residues, producing [1-^{2}H]**6** or [1-^{2}H]**7** whose configuration at the prenyl C-1 was unknown. Initial efforts focused on the farnesylation of *N*-dansyl-Gly-Cys-Val-Leu-Leu catalyzed by hPGGTase-I. The product of this reaction, following treatment with carboxypeptidase, yielded *N*-dansyl-Gly-Cys([1-^{2}H]-farnesyl)-OH ([1-^{2}H]**6**) which had also been prepared via chemical synthesis for the stereochemical analysis of the PFTase-catalyzed reaction, thereby enabling the absolute configuration of the enzymatically derived material to be determined. The enzymatic product was produced by incubating the peptide substrate (**3**) and (*S*)-[1-^{2}H]**1** in the presence of hPGGTase-I; several aliquots of the enzyme were added over a 30 h period to maximize the amount of conversion. It should be noted that the prenylated peptide precipitated during the reaction, generating a heterogeneous mixture containing the desired product both in the precipitate and in solution as determined by HPLC analysis; digestion of this reaction mixture with carboxypeptidase Y resulted in resolubilization of the product. HPLC analysis of this mixture after a 4 h incubation indicated complete hydrolysis of [1-^{2}H]**4** to [1-^{2}H]**6** had occurred. The reaction product was desalted using a small reversed-phase column and then purified via preparative scale HPLC. Mass spectrometric (FAB) and ^{1}H NMR analysis of this enzymatically derived product gave results identical to those obtained with the chemically synthesized material. Examination of the ^{1}H NMR spectrum of [1-^{2}H]**6** in the region near 3.0 ppm, shown in Figure 6C, reveals a large doublet at 3.01 ppm and a smaller doublet at 3.07 ppm. Decoupling of the spectrum by irradiation at 5.10 ppm resulted in a simplification of these two doublets to two singlets as was observed with the material obtained from chemical synthesis (see Figure 6D). Based on the assignments obtained from the sample of [1-^{2}H]**6** prepared by chemical synthesis, the presence of the large doublet at 3.01 ppm indicates that the major diastereomer contained in the enzymatically prepared sample is (α*R*, 1*R*)-[1-^{2}H]**6** while the minor component is (α*R*, 1*S*)-[1-^{2}H]**6**. Integration of the above decoupled spectrum (Figure 6D) indicates a ratio of 1.0:2.7 for the ratio of the minor:major diastereomer (46% *de*); however, accurate quantitation of this ratio is difficult since the smaller of the two peaks (the singlet at 3.06 ppm) is not substantially above the noise to give reliable data. Attempts to obtain better

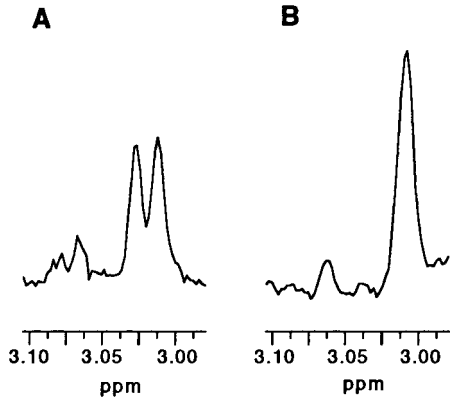

FIGURE 7: Expansion of the ^{1}H NMR spectra of [1-^{2}H]**7** showing the region containing the C-1 geranylgeranyl proton resonances. (A) Spectrum of [1-^{2}H]**7** obtained via enzymatic synthesis with PGGTase-I. (B) Spectrum of [1-^{2}H]**7** obtained via enzymatic synthesis with PGGTase-I after decoupling at 5.1 ppm.

quantification of the diastereomeric composition of [1-^{2}H]**6** via multicomponent analysis of the decoupled spectrum yielded values of 54−67% *de*, depending on the width of the spectral window employed in the calculation. Given the range of proton integration values obtained from various ^{1}H NMR spectra, it was important to estimate the experimental error in determining the integration values. This was done by comparing the experimentally obtained integrals for 10 clearly resolved different protons in the spectrum of [1-^{2}H]-**6** with the expected values; 2 experiments yielded errors of 6.1% and 5.5% for the average deviation in integration. Thus, the NMR method reported here cannot be used to quantify levels of racemization of less that 6%.

To obtain stereochemical information for the physiologically relevent isoprenoid substrate, GGPP, for hPGGTase-I, the corresponding geranylgeranylated peptide, [1-^{2}H]**7**, was prepared from (*S*)-[1-^{2}H]**2**. This was accomplished by a procedure similar to that used for the preparation of the farnesylated peptide described above except the GGPP was added in aliquots over the course of the reaction (GGPP is less soluble than FPP in buffered aqueous solution). Because GGPP is a better substrate than FPP for hGGPTase I, a significantly better yield of the final geranylgeranylated dipeptide was obtained, resulting in higher quality ^{1}H NMR spectra. Examination of the spectrum of [1-^{2}H]**7** in the region near 3.0 ppm, shown in Figure 7A, reveals a large doublet at 3.01 ppm and a smaller doublet at 3.07 ppm similar to the results described above for the farnesylated dipeptide. Decoupling of the spectrum by irradiation at 5.10 ppm simplified the spectrum of these two doublets to two singlets (see Figure 7B), consistent with the assignment of these signals to the C-1 isoprenoid protons. Integration of the above singlet signals from the decoupled spectrum (Figure 7B) indicates a ratio of 1.0:3.8 for the ratio of the minor:major diastereomer (58% *de*).

DISCUSSION

The study presented here describes the stereochemical analysis of the reaction catalyzed by human PGGTase-I using the methodology outlined in Figure 3. The diphosphate substrates (*S*)-[1-^{2}H]**1** (FPP) and (*S*)-[1-^{2}H]**2** (GGPP) were prepared using the routes outlined in Figures 4 and 5. Analysis of the enantiomeric purity of these compounds by Mosher esterification yielded values of 78% *ee* for (*S*)-[1-^{2}H]**1** and 62% *ee* for (*S*)-[1-^{2}H]**2**; these compounds were then used to enzymatically prenylate the peptide *N*-dansyl-GCVLL (**3**). The prenylated products, **4** and **5**, were degraded to dipeptides using carboxypeptidase Y and purified by HPLC to yield **6** and **7**. The farnesylated dipeptides were also prepared via chemical synthesis using enantiomerically enriched (*R*)-[1-^{2}H]**8** to clearly define the absolute configuration at the isoprenoid C-1 position. Assignment of the ^{1}H NMR signals from the diastereotopic C-1 protons in the chemically synthesized material permitted us to determine the absolute configuration of the enzymatically produced material. Since the enzymatic synthesis began with (*S*)-[1-^{2}H]**1** and resulted in the production of (α*R*, 1*R*)-[1-^{2}H]**6**, we concluded that the PGGTase-I-catalyzed reaction proceeds with inversion of configuration at the C-1 isoprenoid center. While these results allowed us to conclude that the reaction proceeds *predominantly* with inversion of configuration, it was not possible to determine whether some racemization had occurred. This ambiguity arose because FPP is a poor substrate for hPGGTase-I; it should be noted that the spectra shown in Figure 6C,D represent the best spectra from six attempts to enzymatically produce [1-^{2}H]**6** using PGGTase-I. Formation of only limited amounts of farnesylated product gave rise to ^{1}H NMR spectra manifesting low signal-to-noise ratios. Comparison of the *de* of [1-^{2}H]**6** prepared using PGGTase-I (46% *de*) with the *ee* of (*S*)-[1-^{2}H]**1** used in the enzymatic incubation (78% *ee*) suggests that significant racemization occurred in this reaction. However, further study of the spectra of the PGGTase-derived material via multicomponent analysis suggests that the *de* of this material may be substantially higher (as high as 67% *de*). Given these ambiguities and the error inherent in the proton integrals (ca. 6%), we believe there is insufficient evidence to conclude that significant racemization occurs when using FPP as a substrate for PGGTase-I. Clearer results were obtained using (*S*)-[1-^{2}H]**2** (GGPP) as a substrate. In this case, comparison of the diastereomeric purity of the product obtained via enzymatic synthesis, [1-^{2}H]**7** (58% *de*), with the enantiomeric purity of the diphosphate substrate, (*S*)-[1-^{2}H]**2** (62% *ee*), suggests that less than 4% racemization occurred in the enzyme-catalyzed reaction. This level of racemization is less than the error level of 6% estimated above for our NMR method. Thus, we conclude that the PGGTase-I-catalyzed reaction proceeds with no detectable (<6%) racemization.

The results presented above show that the alkylation reaction catalyzed by PGGTase-I proceeds with inversion of configuration. This is similar to results obtained with PFTase reported from our lab and others, as discussed above (*19, 20*). In the simplest interpretation, inversion of configuration suggests an associative mechanism (see Figure 2) where the nucleophile (the protein thiolate) directly displaces the diphosphate leaving group. These data are clearly inconsistent with the covalent intermediate mechanism shown in Figure 2 that was previously ruled out on the basis of kinetic measurements (*25, 35*). However, the results described here cannot be used to eliminate the possibility of a dissociative mechanism. If the alkylation reaction proceeds by a mechanism with some dissociative character where the farnesyl cation and the diphosphate leaving group form a tight ion pair within the active site of the enzyme, stereochemical analysis might also result in inversion of config-

uration. For example, such a result was observed for FPP synthase, where the mechanism of the enzyme-catalyzed reaction is thought to involve a dissociative pathway with the formation of a tight anion−cation pair (*60*−*62*).

Studies on yeast PFTase using fluorinated analogues and transition-state analogues suggest that there is some dissociative character to the enzyme-catalyzed reaction (*17*, *18*). However, there is also evidence to support an associative mechanism for the PFTase-catalyzed reaction. Work conducted on determining the α-secondary kinetic isotope effect, which allows associative and dissociative mechanisms to be distinguished, indicated the former for the PFTase-catalyzed reaction (*21*). In addition, studies on understanding the role of the Zn^{2+} metal ion in the active site of PFTase provide evidence that the peptide thiol is coordinated to the Zn^{2+}, indicating the presence of a strong nucleophile (*38*, *39*). Furthermore, the rate of thioether bond formation varies depending on the precise nature of thiolate−metal coordination consistent with an associative transition state (*63*). Crystallographic studies of PFTase have also yielded ambiguous results with regard to the enzyme mechanism. Examination of the structure of the PFTase ternary complex including the enzyme, a peptide substrate, and a FPP analogue reveals that the nucleophilic thiolate and the C-1 position of the isoprenoid are separated by 7.5 Å, making deductions about the mechanism difficult (*43*). Additional complexity is revealed in a series of crystal structures solved in the presence and absence of various metal ions which show significant variation in the conformation of the bound peptide substrate (*44*). In view of the uncertainties regarding the precise details of the PFTase mechanism, it is not possible to present an unambiguous picture of how PGGTase-I catalyzes its reaction. Nevertheless, the results presented here on the stereochemical course of the hPGGTase-I-promoted reaction indicate that this enzyme catalyzes alkylation via a mechanism in which C−O bond cleavage and C−S bond formation are coupled; this could occur in a single step or in a stepwise process in which the trajectory of nucleophilic attack is tightly controlled. Given that similar results were obtained with PFTase, it appears that these enzymes catalyze thiol alkylation via mechanisms that are closely related. Such functional similarities may make attempts of inhibitor design that are based on putative mechanistic differences difficult.

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SUPPORTING INFORMATION AVAILABLE

Experimental procedures for the synthesis of compounds **10**, **11**, **12**, **13**, **14**, **15**, and **16**, details of the error analysis of NMR integration, and an NMR spectrum of *N*-dansyl-Gly-Cys([1-^{2}H]farnesyl-OH ([1-^{2}H]**6**) are included (9 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

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