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On the Stereochemical Course of Human Protein-Farnesyl Transferase

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Abstract: The enzyme protein-farnesyl transferase (PFTase) catalyzes the farnesylation of the Ras protein and certain other proteins, using farnesyl diphosphate (FPP) as the prenyl source. Because of the important role of mutant Ras proteins in cancer, inhibitors of PFTase are of great interest as potential novel anticancer agents. The design of such agents would be aided by a greater knowledge of the mechanism of PFTase. We have determined the stereochemical course of PFTase using the two stereospecifically prepared isomers of [1-^{2}H]-FPP as substrates in conjunction with ^{1}H-NMR analysis of the farnesylated peptide products. This confirms that PFTase carries out the transfer of the farnesyl group with inversion of configuration. Combined with the results of studies on fluorinated analogs of FPP as PFTase substrates (Dolence, J. M.; Poulter, C. D. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92*, 5008), this indicates that the mechanistic course of PFTase is similar to that of the prototypical prenyltransferase enzyme, FPP synthase.

Mutant Ras proteins, which are the products of *ras* oncogenes, are involved in ~30% of all human cancers. It has been shown that, to be active, Ras proteins must be modified with a farnesyl moiety at a cysteine four residues from the carboxyl terminus in a specific sequence called a CAAX box. This modification is carried out by an enzyme, protein-farnesyl transferase (PFTase), which uses farnesyl diphosphate (FPP) as the source of the farnesyl moiety.[1−3] PFTase has been the subject of intense research interest because inhibitors of this enzyme block the action of mutant Ras proteins and halt the growth of *ras* transformed cells and, thus, might act as novel anticancer agents.[4−6] The design of more potent PFTase inhibitors would be facilitated by a greater understanding of the mechanism of this enzyme. Herein, we present stereochemical experiments that demonstrate PFTase carries out its reaction with inversion of configuration at the C-1 farnesyl center.

The yeast,[7] rat,[8] and human[9] forms of PFTase have been cloned and sequenced, but no three-dimensional structural information on any form of PFTase has been obtained.[10] However, site-directed mutagenesis experiments,[9,11] photoaffinity labeling studies,[12,13] and NMR studies of bound inhibi-

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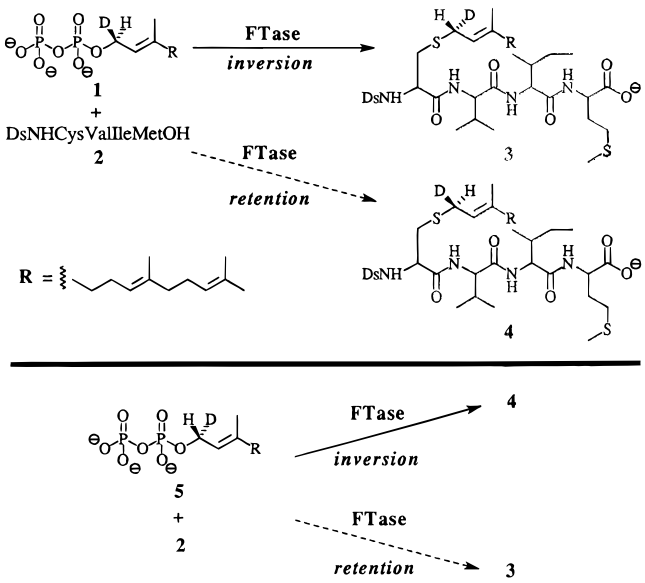
Scheme 1



tors[14,15] have provided limited information on the substrate binding sites. Initial steady-state kinetic experiments on PFTase indicated a random sequential mechanism.[16] Later, more detailed studies, including isotope trapping experiments, revealed a functionally ordered process, where FPP bound first, followed by the peptide substrate, chemistry, and then product release.[17] Recently, pre-steady-state kinetic experiments have verified the ordered nature of the mechanism. They have also demonstrated that FPP binding is followed by a conformational change and that product release is rate limiting.[18] Only one very recent report has directly addressed the chemical nature of the farnesylation reaction. Dolence and Poulter have found that the prenyl group of the 3-(trifluoromethyl) derivative of FPP is transferred by PFTase to the peptide substrate 1000-fold more slowly than the farnesyl group of FPP itself.[19] This indicates that the farnesylation reaction occurs by a mechanism with a significant degree of ionic character.

A second key chemical question involves whether PFTase transfers the farnesyl group with inversion or retention of configuration at the C-1 position of the farnesyl structure. These two alternatives can be distinguished using stereospecifically deuterium-labeled FPP (Scheme 1). Starting from (1*S*)-[1-^{2}H]-FPP (**1**) and the synthetic tetrapeptide substrate **2** (Ds = dansyl), inversion of configuration would produce farnesylated peptide **3**, while retention of configuration would yield **4**. Alternatively, with (1*R*)-[1-^{2}H]-FPP (**5**) and peptide **2**, inversion would produce **4** while retention would yield **3**. The stereochemical course of substitution reactions of allylic pyrophosphates with carbon nucleophiles has been extensively examined;[20−22] however, no stereochemical studies have been previously reported on their reaction with heteroatom nucleophiles. While inversion is the more likely mechanistic pathway, the enzyme has no apparent sequence homology with any other prenyltransferases,[9] and thus it is speculative to make any inferences for PFTase on the basis of previously observed results.

Experimental Section[23]

BocNH-Cys(SSEt)-Val-Ile-Met-OMe (8). The protected tripeptide BocNH-Val-Ile-Met-OMe (**7**) was prepared as shown in Scheme 2, using standard solution phase coupling reactions[24] as illustrated below for the synthesis of tetrapeptide **8**. Tripeptide **7** (408 mg, 0.86 mmol) was dissolved in CH_2Cl_2 (6.0 mL, distilled from CaH_2) and treated with trifluoroacetic acid (2.0 mL; protein sequencing grade, Sigma). After 1 h at room temperature, the solvent was removed, the residue was taken up in ∼10 mL of CH_2Cl_2, concentrated (three times), and finally dried under high vacuum for 1 h. The amine salt was then dissolved in CH_2Cl_2 (10.0 mL) and treated with triethylamine (0.27 mL, 1.89 mmol), 1-hydroxybenztriazole (HOBt; 139 mg, 1.03 mmol), BocNH-Cys(SSEt)-OH·dicyclohexylamine salt (438 mg, 0.95 mmol), and 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC; 198 mg, 1.03 mmol). After 48 h, the reaction mixture was taken up in additional CH_2Cl_2 (50 mL), washed with 5% HCl (4 × 20 mL) and water (20 mL), and dried ($MgSO_4$). Purification by flash chromatography (2.5% MeOH/$CHCl_3$; 2 × 15 cm silica gel, ∼15 mL fractions) afforded 465 mg (87%) of the desired protected tetrapeptide **8**.

DansylNH-Cys(SSEt)-Val-Ile-Met-OMe (9). Tetrapeptide **8** (453 mg, 0.71 mmol) was dissolved in CH_2Cl_2 (9.0 mL, distilled from CaH_2) and treated with trifluoroacetic acid (3.0 mL; protein sequencing grade, Sigma). After 1 h at room temperature, the solvent was removed, the residue was taken up in ∼15 mL of CH_2Cl_2, concentrated (three times), and finally concentrated under high vacuum for 1 h. The amine salt was then dissolved in CH_2Cl_2 (7.5 mL) and treated with triethylamine (0.20 mL, 1.42 mmol). The resulting clear solution was flushed with argon, cooled to 0 °C, and treated with dansyl chloride (172 mg, 0.71 mmol). After 1 h at room temperature in the dark, the reaction mixture was taken up in additional CH_2Cl_2 (75 mL), washed with aqueous $NaHCO_3$ (25 mL) and water (20 mL), and dried ($MgSO_4$). Purification by flash chromatography (2.5% MeOH/$CHCl_3$; 4 × 15 cm silica gel, ∼25 mL fractions) afforded 420 mg (77%) of the dansylated tetrapeptide **9**: ^{1}H NMR (300 MHz, $CDCl_3$/CD_3OD (∼25:75)) δ 0.8−1.0 (12H, m, 4 Ile and Val CH_3), 1.07 (3H, t, $-SSCH_2CH_3$), 2.06 (3H, s, $-SCH_3$), 2.34 (2H, m, $-SSCH_2CH_3$), 2.53 (2H, dd, Cys β CH_2), 2.89 (6H, s, dansyl $-N(CH_3)_2$), 4.05 (1H, t, Cys α CH), 4.14 and 4.22 (2H, two d, Ile and Val α CH), 4.61 (1H, dd, Met α CH), 7.27 (1H, d, naphthyl H), 7.58 (2H, app q, naphthyl H), 8.32 (2H, dd, naphthyl H), 8.58 (1H, d, naphthyl H); ^{13}C NMR (75.4 MHz, $CDCl_3$/CD_3OD (∼25:75)) δ 9.5, 12.7, 13.6, 14.0, 16.7, 17.9, 24.0, 29.1, 29.9, 30.7, 35.7, 39.1, 44.0, 50.5, 51.0, 55.0, 57.0, 58.2, 114.5, 118.6, 122.3, 127.3, 128.9, 129.0, 129.3, 129.8, 133.8, 151.1, 170.2, 170.8, 171.4, 171.5; positive ion FAB-MS *m*/*e* 772.3 (MH^+).

DansylNH-Cys-Val-Ile-Met-OH (2). The protected tetrapeptide **9** (100 mg, 0.13 mmol) was dissolved in DMF (5.0 mL) and H_2O (0.29 mL) under argon and then cooled to 0 °C. Sodium carbonate (0.5 M, 0.29 mL, 0.14 mmol) was added, followed by sodium hydroxide (0.5 M, 0.29 mL, 0.14 mmol), and the reaction mixture was stirred for 1 h at 0 °C and 15 min at room temperature. The solution was then treated with dithiothreitol (80 mg, 0.52 mmol) and stirred overnight (in the dark). The solution was treated with trifluoroacetic acid (30 μL), filtered, and purified by reversed-phase HPLC using a gradient of 25%

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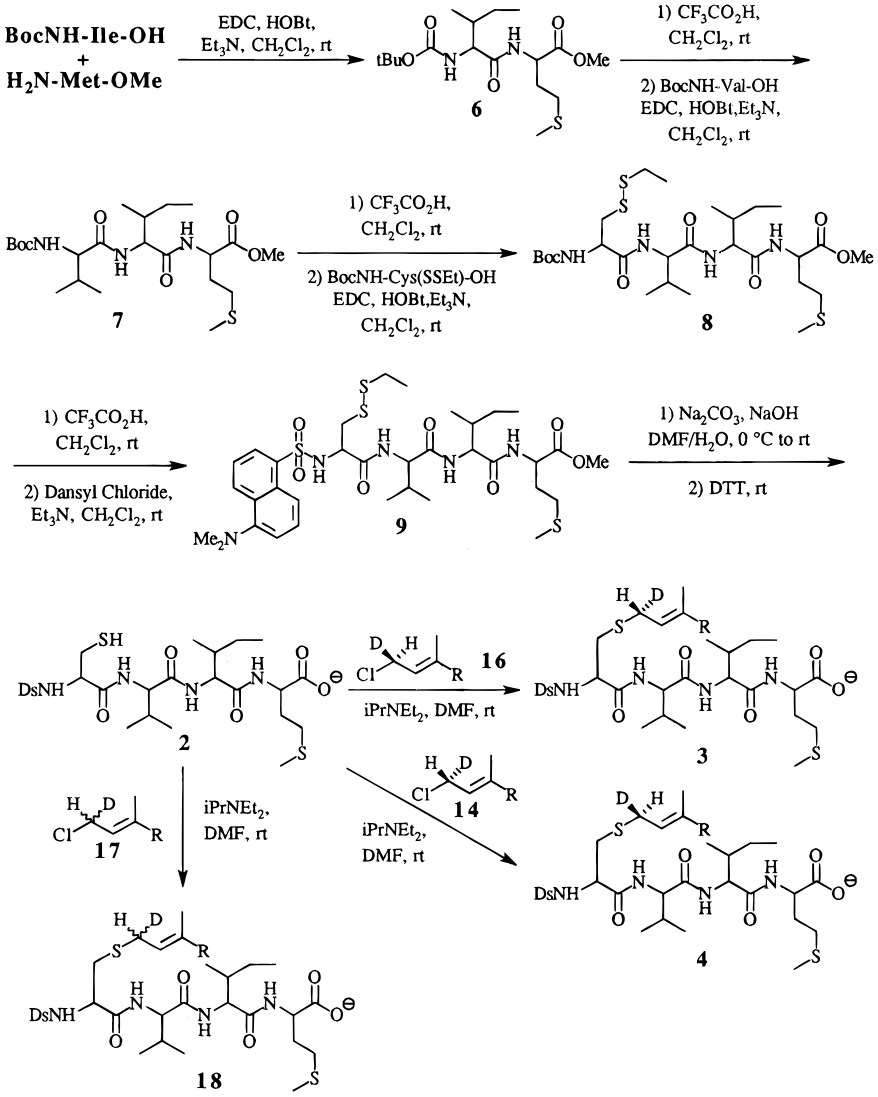
Scheme 2



B to 100% B over 30 min (A, 0.1% TFA/H_2O; B, 0.1% TFA/CH_3CN; column, Waters Deltapak C_{18} 25 mm × 100 mm Radial Pak cartridge; flow rate, 5 mL/min). Concentration (high vaccum rotovap) of the fraction eluting at 17 min afforded 50 mg (55%) of the deprotected, dansylated tetrapeptide **2**: ^{1}H NMR (300 MHz, DMF-d_7) δ 0.78 (6H, d, Val CH_3), 0.84 (3H, t, 1 Ile CH_3), 0.95 (3H, d, 1 Ile CH_3), 2.06 (2H, t, Met γ CH_2), 2.10 (3H, s, $-SCH_3$), 2.59 (2H, m, Cys β CH_2), 2.72 (2H, m), 2.90 (6H, s, dansyl $-N(CH_3)_2$), 4.25 (2H, m), 4.37 (1H, t), 4.56 (1H, m, Met α CH), 7.32 (1H, d, naphthyl H), 7.65 (2H, app q, naphthyl H), 7.78 (1H, d, amide H), 8.08 (1H, d, amide H), 8.23 (1H, d, amide H), 8.30 (2H, m, naphthyl H), 8.49 (1H, d, amide H), 8.58 (1H, d, naphthyl H); ^{13}C NMR (75.4 MHz, DMF-d_7) δ 10.9, 14.6, 15.4, 17.7, 19.1, 24.8, 27.7, 31.0, 31.6, 37.4, 45.1, 51.5, 57.5, 58.5, 59.0, 115.5, 119.9, 123.7, 128.2, 129.1, 129.9, 130.2, 152.1, 162.6, 169.6, 170.9, 171.6, 173.5; positive ion FAB-MS *m/e* 697.8 (MH^+).

DansylNH-Cys([1-^{2}H]-farnesyl)-Val-Ile-Met-OH (18). The farnesylation was carried out as previously described by Pompliano for farnesylation with farnesyl bromide.[19,25] The deprotected tetrapeptide **2** (17.5 mg, 0.025 mmol) was dissolved in DMF (1.0 mL) under argon, and then *N*,*N*-diisopropylethylamine (13 μL, 0.075 mmol) was added. Racemic [1-^{2}H]farnesyl chloride (**17**) (7 μL, 0.030 mmol) was then added, and the reaction mixture was stirred for 1 h at room temperature. The solution was then directly purified by reversed-phase HPLC using a gradient of 50% B to 100% B over 30 min (A, 0.025% TFA/H_2O; B, CH_3CN; column, Waters μBondapak C_{18} 25 mm × 100 mm Radial Pak cartridge; flow rate, 5 mL/min). Concentration (Speedvac) of the fraction eluting at 33 min afforded 6.0 mg (27%) of the farnesylated tetrapeptide **18**. The low yield is due in part to the lower reactivity of farnesyl chloride versus the bromide. The diastereomerically enriched farnesylated tetrapeptides **3** and **4** were prepared in the same manner, starting from tetrapeptide **2** and enantiomerically enriched farnesyl chlorides **16** and **14** (respectively): ^{1}H NMR (500 MHz, DMSO-d_6) δ 0.62 (6H, two d, Val CH_3), 0.76 (3H, t, 1 Ile CH_3), 0.81 (3H, d, 1 Ile CH_3), 1.39 (3H, s, farnesyl-CH_3), 1.52 (3H, s, farnesyl-CH_3), 1.54 (3H, s, farnesyl-CH_3), 1.62 (3H, s, farnesyl-CH_3), 1.91 (2H, m, Met ∂ CH_2), 1.99 (3H, s, $-SCH_3$), 2.79 (6H, s, dansyl $-N(CH_3)_2$), 2.815 (1H, app t, farnesyl-H_1; see text), 3.95 (1H, m), 4.08 (1H, t), 4.11 (1H, app q), 4.24 (1H, m, Met α CH), 4.85 (1H, d, $J \approx 8.0$ Hz, farnesyl-H_2; see text), 5.00 and 5.04 (2H, two t, farnesyl-H_6 and H_{10}), 7.20 (1H, d, naphthyl H), 7.54 (2H, m, naphthyl H), 8.15 (1H, d, naphthyl H), 8.27 (1H, d, naphthyl H), 8.41 (1H, d, naphthyl H); positive ion FAB-MS *m/e* 905.5 (MH^+).

(1*S*)-[1-^{2}H]Farnesol (13). The required [1-^{2}H]farnesal starting material was prepared as described below (see Scheme 3). (E,E)-Farnesal (**10**) (1.10 g, 4.99 mmol) and cerium(III) chloride (1.90 g; 5.1 mmol) were dissolved in CH_3OD (5 mL). This mixture was cooled to 0 °C, and $NaBD_4$ (209 mg, 4.99 mmol) was added in one portion. The reaction mixture was stirred for 15 min at room temperature, neutralized with ~5 mL of 10% HCl, and then extracted with CH_2Cl_2. The organic layer was washed with saturated aqueous NaCl and dried ($MgSO_4$). Flash column purification (20% EtOAc/hexane) afforded 729 mg of [1-^{2}H]farnesol (**11**) (82%). The resulting [1-^{2}H]farnesol was then dissolved in hexane (HPLC grade dried over 4 Å sieves, 100 mL) and stirred with MnO_2 (8.0 g) for 1 h under argon in the dark.

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Filtration followed by concentration afforded the desired [1-^{2}H]farnesal (**12**) which was used directly in the next reaction. Proton NMR analysis indicated 93% deuterium incorporation.[26]

(*E*,*E*)-[1-^{2}H]Farnesal (**12**) (400 mg, 1.80 mmol) and (*R*)-pinanyl-9-BBN (Aldrich, 92% ee; 0.5 M in THF, 4.32 mL, 2.20 mmol) were stirred under argon at room temperature for 4 h. Acetaldehyde (0.5 mL) was added to quench the reaction, and after 30 min the solution was concentrated (rotary evaporation followed by high vacuum). The residue was dissolved in THF (2.0 mL) and treated with 3 N NaOH (1.5 mL) and 30% H_2O_2 (1.5 mL). After 3 h at room temperature, the reaction mixture was extracted with ether (4 × 15 mL), and the combined extracts were dried ($MgSO_4$). Flash chromatography (20% EtOAc/hexane) afforded 399 mg (98%) of the desired alcohol **13**. Proton NMR analysis using the chiral shift reagent Eu(hfc)$_3$[27] indicated that the enantiomeric excess of the sample was 71% (82% corrected for deuterium incorporation). Reduction of [1-^{2}H]farnesal (**12**) with the enantiomeric reducing reagent (*S*)-pinanyl-9-BBN in the same manner afforded the enantiomeric (1*R*)-[1-^{2}H]farnesol (**15**) in 77% enantiomeric excess (89% corrected for deuterium incorporation).

(1*R*)-[1-^{2}H]Farnesyl Chloride (14). *N*-Chlorosuccinimide (144 mg, 1.08 mmol) was dissolved in 2.0 mL of CH_2Cl_2 (distilled from CaH_2), and the resulting solution was cooled to −30 °C in an acetonitrile/dry ice bath. Dimethyl sulfide (67 mg, 1.08 mmol) was added dropwise by a syringe. The mixture was briefly warmed to 0 °C before it was cooled to −40 °C. A solution of (1*S*)-[1-^{2}H]farnesol (**13**) (200 mg, 0.90 mmol) in 1 mL of CH_2Cl_2 was added dropwise by a syringe to the milky white suspension. The reaction mixture was warmed to 0 °C and stirred at that temperature for 2 h. After being stirred at room temperature for 15 min, the clear solution was washed with 2 mL of saturated NaCl. The aqueous layer was extracted with hexanes (2 × 5 mL). The combined organic layers were dried over $MgSO_4$ and concentrated (rotovap, then high vaccum) to give 159 mg (73%) of **14** as a yellow oil: ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.60 (6H, s, 2CH_3), 1.68 (3H, s, CH_3), 1.73 (3H, s, CH_3), 2.09 (8H, m, C_4, C_5, C_8 and C_9-CH_2), 4.08 (1H, d, H_1), 5.09 (2H, m, H_6 and H_{10}), 5.43 (1H, d, H_2).

(1*S*)-[1-^{2}H]Farnesyl Chloride (16). Using the same procedure as for **14**, (1*R*)-[1-^{2}H]farnesol (**15**) was converted into **16** in 70% yield.

[1-^{2}H]Farnesyl Chloride (17). Using same procedure as for **14**, racemic [1-^{2}H] farnesol (**11**) was converted into **17** in 82% yield.

(1*S*)-[1-^{2}H]Farnesyl Diphosphate (1). The diphosphorylation procedure of Poulter and co-workers was followed.[28,29] Tris(tetrabutylammonium) hydrogen pyrophosphate (1.19 g, 1.32 mmol) was dissolved in acetonitrile (5.0 mL, distilled from P_2O_5), and farnesyl chloride (**14**) (159 mg, 0.66 mmol) was added to the resulting milky white suspension. The mixture was stirred at room temperature for 2 h, and the solvent was removed by rotary evaporation. The residue was dissolved in 1 mL of ion exchange buffer, and the resulting clear solution was loaded onto a 2 × 10 cm column of Dowex AG 50w-X8 (100−200 mesh) cation exchange resin (ammonium form) and eluted with 60 mL of 0.025 M NH_4HCO_3 in 2% (v/v) iPrOH/H_2O. The eluant was lyophilized, the solid was dissolved in 2 mL of 0.05 M NH_4HCO_3, and the clear solution was transferred to a centrifuge tube. Ten mL of 1:1 CH_3CN/iPrOH was added, and the contents were mixed throughly, during which time a white precipitate formed. The suspension was cleared by centrifugation for 10 min. The supernatant solution was removed with a pipet, and the process was repeated three times. The combined supernatants were concentrated by rotary evaporation, and the residue was dissolved in a minimum amount of chromatography buffer (1:2:1) H_2O/iPrOH/CH_3CN, 0.05 M NH_4HCO_3) and loaded onto a 2 × 18 cm cellulose flash column. Fractions were analyzed on cellulose TLC plates eluted with chromatography buffer and visualized with sulfosalicylic acid−ferric chloride. Concentration and lyophilization gave 168 mg (63%) of **1** as a white solid. The deuterated FPP analogs can also be conveniently purified using the recently described HPLC method of Zhang and Poulter:[30] ^{1}H NMR (300 MHz, D_2O/ND_4-OD) δ 1.59 (6H, s, 2 CH_3), 1.66 (3H, s, CH_3), 2.08 (8H, m, C_4, C_5, C_8 and C_9 CH_2), 4.44 (1H, d, H_1), 5.17 (2H, m, H_6 and H_{10}), 5.44 (1H, d, H_2); ^{31}P NMR (121 MHz, D_2O) δ ppm −10.15 (d, J_{pp} = 21.4 Hz), −7.72 (d, J_{pp} = 21.4 Hz).

(1*R*)-[1-^{2}H]Farnesyl Diphosphate (5). Using the same procedure as for **1**, **5** was obtained from farnesyl chloride (**16**) in 74% yield.

Enzymatic Hydrolysis of (1*S*)-[1-^{2}H]Farnesyl Diphosphate (1). (1*S*)-[1-^{2}H]Farnesyl diphosphate (**1**) (52 mg, 0.13 mmol) was dissolved in 2.30 mL of H_2O. To this solution was added 0.2 mL of 0.2 M lysine buffer (pH 10.4), 5 μL of 1.0 M magnesium chloride, and 0.46 mg (20 units) of *E. coli* alkaline phosphatase (Sigma). This mixture was incubated at 37 °C for 7 h and then extracted four times with 5 mL of CH_2Cl_2. The combined organic layers were dried over anhydrous sodium sulfate. Solvent was removed by rotary evaporation, and 20 mg (79%) of crude farnesol **13** was obtained. Proton NMR analysis (using the chiral shift reagent Eu(hfc)$_3$)[27] of a sample purified by flash chromatography indicated that the enantiomeric excess of the (1*S*)-[1-^{2}H]farnesol (**13**) obtained was 64%.

Enzymatic Hydrolysis of (1*R*)-[1-^{2}H]Farnesyl Diphosphate (5). Following the same procedure described above, (1R)-[1-^{2}H]-farnesol **15** with 57% ee was obtained.

Analysis of Enzymatic Reactions by HPLC and Isolation of the Deuterated Farnesylated Peptides. Each individual reaction containing (1*S*)-[1-^{2}H]-FPP (**1**) (144 nmol), the dansylated tetrapeptide **2** (72 nmol), and recombinant human PFTase (the truncated form $\beta\alpha_{met\ 39}$;[9] 0.28 nmol) in 1.0 mL PFTase assay buffer (50 mM Hepes, 5 mM $MgCl_2$, 10 μM $ZnCl_2$, 5 mM DTT, pH 7.5, containing 2% v/v DMF) was incubated at 30 °C for 8 h. The reaction was monitored by removing aliquots at regular intervals, quenching them (by addition of an equal volume of CH_3CN), and analyzing for product by reversed-phase HPLC. Chromatography was performed on a Vydac 4.6 × 250 mm C_8 column eluted at 1.0 mL/min, and the products were detected by UV at 254 and 330 nm. A gradient of 35% B to 100% B over 300 min was used (solvent A: 0.025% TFA/H_2O; solvent B: 0.020% TFA/CH_3CN). The nonfarnesylated peptide eluted at 4.4 min, and the farnesylated product eluted at 17.4 min. The amount of farnesylated peptide was determined using a standard curve established with known amounts of the synthetic farnesylated peptide **18**. This analysis indicated that 35−50 nmol of peptide was produced in each reaction. The 17 min HPLC fractions from several reactions were collected, pooled, frozen, lyophilized, and then analyzed by 500 MHz ^{1}H-NMR. The enantiomeric (1*R*)-[1-^{2}H]-FPP (**5**) was condensed with the dansylated tetrapeptide **2** and isolated in the same manner.

Results and Discussion

Our experimental strategy involved the synthesis of both isomers of stereospecifically deuterated FPP, their conversion to product with recombinant PFTase, and the analysis of the resulting deuterated farnesylated peptides by ^{1}H-NMR. We have recently shown that the diastereotopic C-1 farnesyl protons in a dipeptide model system can be distinguished by 500 MHz ^{1}H-NMR.[31] The syntheses of both stereoisomers of [1-^{2}H]-FPP are shown in Scheme 3. The stereochemical center was established by reduction of [1-^{2}H]farnesal (**12**) using Midland's method to give (1*S*)- and (1*R*)-[1-^{2}H]farnesol (**13** and **15**, respectively).[31−33] The stereochemical assignments of **13** and **15** were based on the well-established stereoselectivity of the pinanyl-9-BBN reagent.[34] The enantiomeric excess was lower

(26) The oxidation of benzylic alcohols by MnO_2 is subject to a very large primary isotope effect: Goldman, I. M. *J. Org. Chem.* **1969**, *34*, 3289−3295.

(27) McCreary, M. D.; Lewis, D. W.; Wernick, D. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1974**, *96*, 1038−1054.

(28) Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768−4779.

(29) Woodside, A. B.; Huang, Z.; Poulter, C. D. *Org. Synth.* **1987**, *66*, 211−219.

(30) Zhang, D.; Poulter, C. D. *Anal. Biochem.* **1993**, *213*, 356−361.

(31) Gibbs, R. A.; Mu, Y. Q.; Wang, F. *Bioorg. Med. Chem. Lett.* **1993**, *3*, 281−284. Our previous ^{1}H-NMR studies on this deuterated farnesylated *N*-benzoyldipeptide model system indicated that the signal for the C-1 farnesyl proton appeared as two doublets at 3.3 ppm (see also ref 42). The upfield shift of the C-1 signal seen with peptides **3** and **4** may be due to deshielding by the naphthyl ring of the dansyl group.

(32) Midland, M. M.; Greer, S.; Tramontano, A.; Zderic, S. A. *J. Am. Chem. Soc.* **1979**, *101*, 2352−2355.

(33) Midland, M. M. *Chem. Rev.* **1989**, *89*, 1553−1561.

(34) Prabhakaran, P. C.; Gould, S. J.; Orr, G. R.; Coward, J. K. *J. Am. Chem. Soc.* **1988**, *110*, 5779−5784.

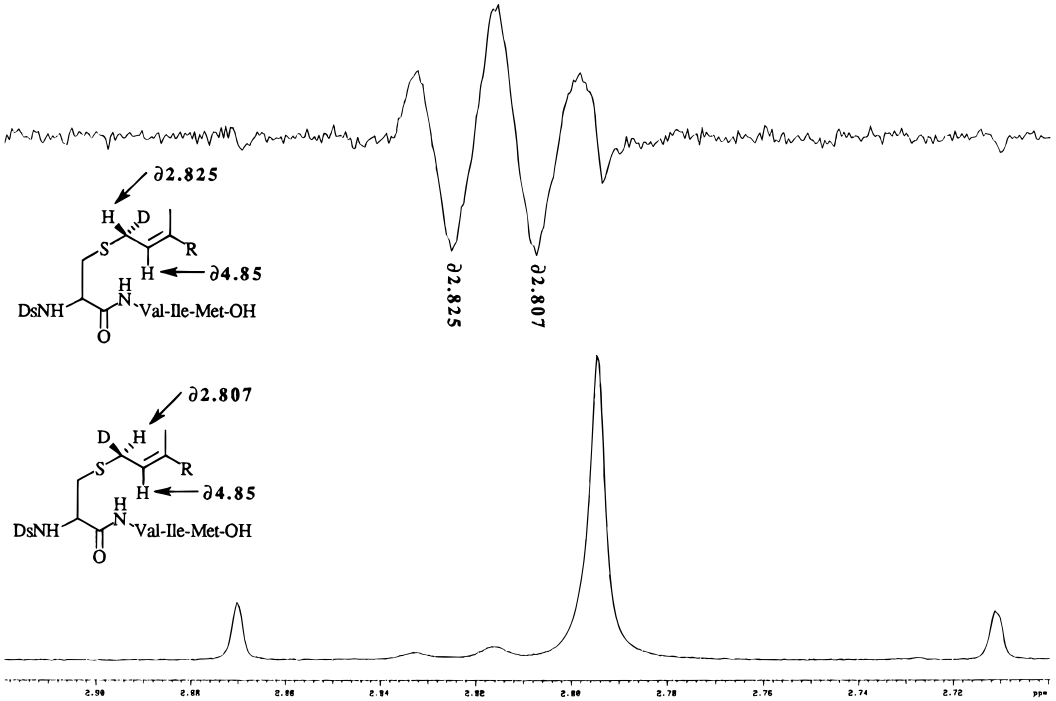
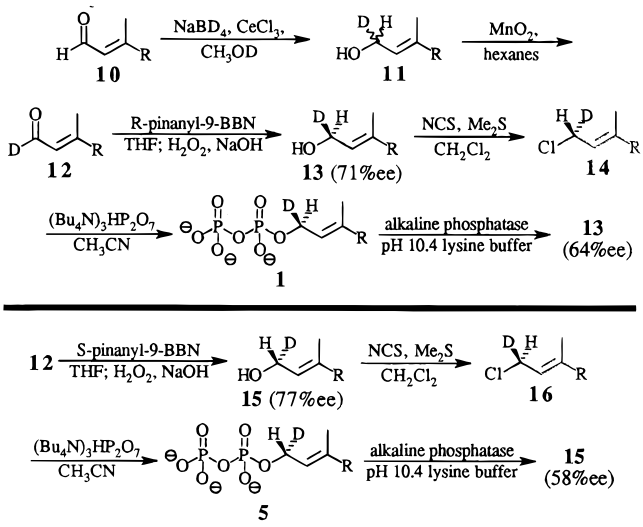


Figure 1. ^{1}H NMR spectrum (499.845 MHz; an expansion from 2.92 to 2.70 ppm) of the nonstereospecifically deuterated farnesylated peptide **18** in DMSO-d_6. The spectrum is referenced to the peak at 2.49 ppm due to DMSO-d_5. The lower trace is the standard 1D spectrum, while the upper trace is the result of a decoupling difference experiment with irradiation at 4.85 ppm (see text). The positive peaks represent the coupled spectrum and the negative peaks represent the decoupled spectrum. The chemical shift assignments are made on the basis of the results presented in Figure 2.

Scheme 3



than usually obtained, due to incomplete deuterium incorporation into **12**. The alcohols were then transformed to the desired pyrophosphates **1** and **5** using the two-step method of Poulter and co-workers.[28] The expected net retention of stereochemistry was confirmed by alkaline phosphate hydrolysis to give the starting alcohol. The slight loss of stereochemical integrity seen in the diphosphate products is probably due to racemization of the unstable allylic chloride intermediates.[28]

The tetrapeptide dansylNH-Cys-Val-Ile-Met-OH (**2**), which mimics the carboxyl terminus of the human K-Ras protein, was chosen as the peptide substrate for the farnesylation reaction, and was prepared as shown in Scheme 2.[35] The nonstereospecifically deuterated farnesylated peptide **18** was prepared by alkylating **2** with racemic [1-^{2}H]-farnesyl chloride **17**. The 500 MHz ^{1}H-NMR spectrum indicated that the C-1 proton of the farnesyl moiety appeared at 2.82 ppm. Unfortunately, the expected differences in the chemical shifts of the diasterotopic C-1 protons were thus partially obscured by the singlet at 2.79 ppm due to the dansyl dimethylamino moiety (Figure 1). The C-1 multiplet was then revealed via a decoupling difference experiment. The C-2 farnesyl proton signal at 4.85 ppm was irradiated, and the resulting decoupled spectrum was subtracted from the nonirradiated spectrum. This resulted in the pattern shown in the upper part of Figure 1. Analysis indicates that the signal in the coupled spectrum (the positive peaks) is due to an apparent triplet resulting from two overlapping doublets.[31] Decoupling by irradiation at 4.85 ppm then led to two singlets at 2.825 and 2.807 ppm (the negative peaks).

The diasteromerically enriched deuterated farnesylated peptides **3** and **4** were synthesized by coupling peptide **2** with farnesyl chlorides **16** and **14**, respectively. Decoupling difference experiments on these two samples indicated that the *pro* 1*S* proton appears downfield at 2.825 ppm (Figure 2a) and the *pro* 1*R* proton appears upfield at 2.807 ppm (Figure 2b). These stereochemical assignments are based on the important assumption that the alkylation proceeds via an S_N2 reaction with inversion of configuration at the farnesyl center. Despite a significant loss of stereochemical integrity in the production of **3** (but not **4**), this is a reasonable assumption considering that (a) the thiolate of peptide **2** should be a powerful nucleophile in the dipolar aprotic solvent DMF and (b) the observation of inversion of configuration in the formation of other alkyl sulfides by similar reactions.[36−41]

(35) It is well established that PFTase can utilize short CAAX motif tetrapeptides as substrates: Reiss, Y.; Goldstein, J. L.; Sebra, M. C.; Casey, P. J.; Brown, M. S. *Cell* **1990**, *62*, 81−88. Specifically, Pompliano and co-workers have previously reported that short dansylated CAAX peptides are substrates for human PFTase.[25]

(36) Woodard, R. W.; Mascaro, L.; Horhammer, R.; Eisenstein, S.; Floss, H. G. *J. Am. Chem. Soc.* **1980**, *102*, 6314−6318.

(37) Woodard, R. W.; Tsai, M.-D.; Floss, H. G.; Crooks, P. A.; Coward, J. K. *J. Biol. Chem.* **1980**, *255*, 9124−9127.

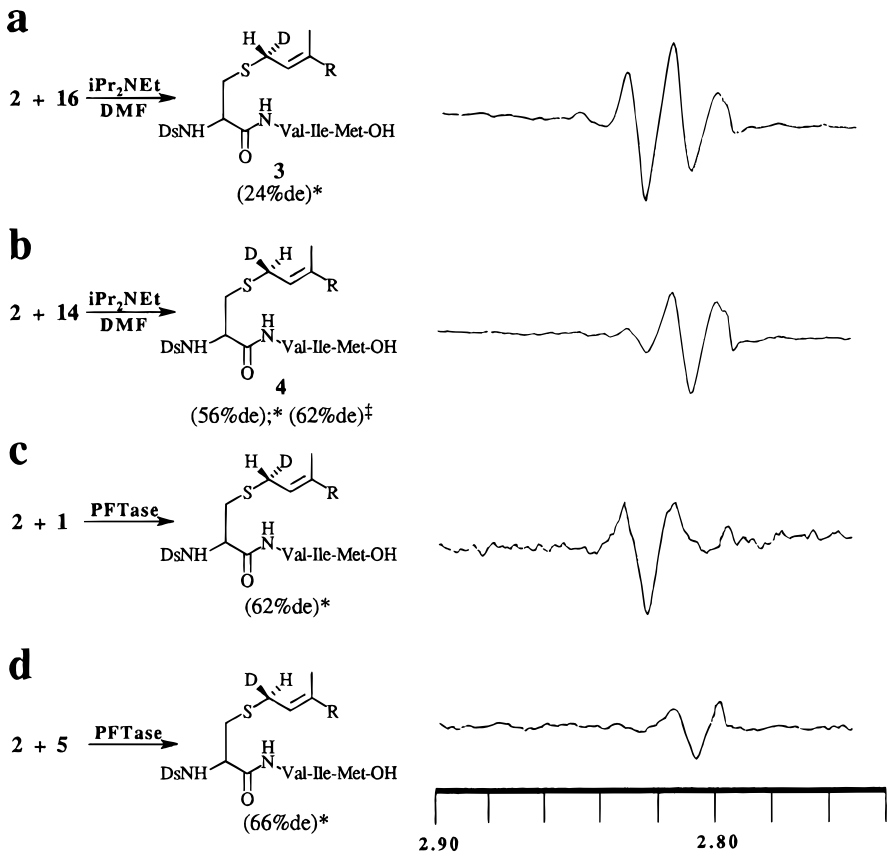


Figure 2. Decoupling difference spectra for synthetic and enzymatically prepared deuterated farnesylated peptides. (a) Synthetic (1*R*)-[1-^{2}H]-farnesyl peptide **3**, prepared from (1*S*)-[1-^{2}H]farnesyl chloride (**16**) and peptide **2**. (b) Synthetic (1*S*)-[1-^{2}H]farnesyl peptide **4**, prepared from (1*R*)-[1-^{2}H]farnesyl chloride (**14**) and peptide **2**. (c) The farnesylated peptide product isolated from the PFTase-catalyzed reaction of (1*S*)-[1-^{2}H]-FPP (**1**) and **2**. (d) The farnesylated peptide product isolated from the PFTase-catalyzed reaction of (1*R*)-[1-^{2}H]-FPP (**5**) and **2**. *Diasteromeric excess estimated from cut-and-weigh integration of the negative peaks in the decoupling difference spectra. ‡Diasteromeric excess calculated from a computer simulation of the decoupling difference spectra.

With the labeled substrates synthesized and the analytical method established, the stage was set for determining the stereochemical course of PFTase. Incubation of (1*S*)-[1-^{2}H]-FPP (**1**) and peptide **2** with recombinant human PFTase[9] as described in the Experimental Section produced the farnesylated tetrapeptide product, which was isolated by reversed-phase HPLC. The farnesylated products from several one mL enzymatic reactions were combined and analyzed by NMR. As shown in Figure 2c, this experiment clearly demonstrated that the enzymatic reaction occured with predominant inversion of configuration. Incubation of (1*R*)-[1-^{2}H]-FPP (**5**) with **2** and PFTase provided the opposite isomer of the prenylated peptide, providing confirmation of the stereochemical course of the reaction (Figure 2d). This represents the first time that sufficient amounts of the prenylated peptide production of a mammalian PFTase has been isolated to determine its structure by NMR. Thus, the identity of the ^{1}H-NMR spectrum of the chemically farnesylated and enzymatically farnesylated peptides directly confirms the structure of the prenylcysteine moiety produced by mammalian PFTase.[42]

One mechanistic question not addressed above concerns the possibility that the inversion observed in the transfer of the farnesyl group is accompanied by a small amount of racemization. While clean inversion or retention of configuration during an enzyme-catalyzed alkyl transfer is clearly more likely, there is precedent for loss of cryptic stereochemical integrity during an enzymatic reaction.[22] Unfortunately, the small amount of biosynthetic peptides **3** and **4** obtained from the enzymatic reactions (~0.4 mg from several combined enzymatic reactions) and the need to use the decoupling difference NMR method prevented the simple integration of the signals for the diastereotopic protons. Despite these difficulties, *estimates* of the diasteromeric excess (de) of the four peptides shown in Figure 2 were obtained by cut-and-weigh integration of the negative peaks in the decoupling difference spectra. Coupling of (1*S*)-[1-^{2}H]-FPP (**1**) (64% ee) with peptide **2** gave farnesylated peptide **3** with an estimated de of 62% (Figure 2c). Coupling of (1*R*)-[1-^{2}H]-FPP (**5**) (58% ee) with peptide **2** gave farnesylated peptide **4** with an estimated de of 66% (Figure 2d). While we cannot eliminate the possibility that a small amount of racemization accompanies the observed inversion of stereochemistry, the de's obtained are reasonably consistent with the expected values for complete inversion. Furthermore, in the case of the synthetic peptide **4** (Figure 2b), we have found that the estimated de value of 56% is in good agreement with a value of 62% obtained by computer simulation of the decoupling difference spectrum, which confirms the validity of the cut-and-weigh integration method.[43]

The classical stereochemical experiments of Popjak and Cornforth established that FPP synthase carries out its prenyl

(38) Orr, G. R.; Danz, D. W.; Pontoni, G.; Prabhakaran, P. C.; Gould, S. J.; Coward, J. K. *J. Am. Chem. Soc.* **1988**, *110*, 5791−5799.

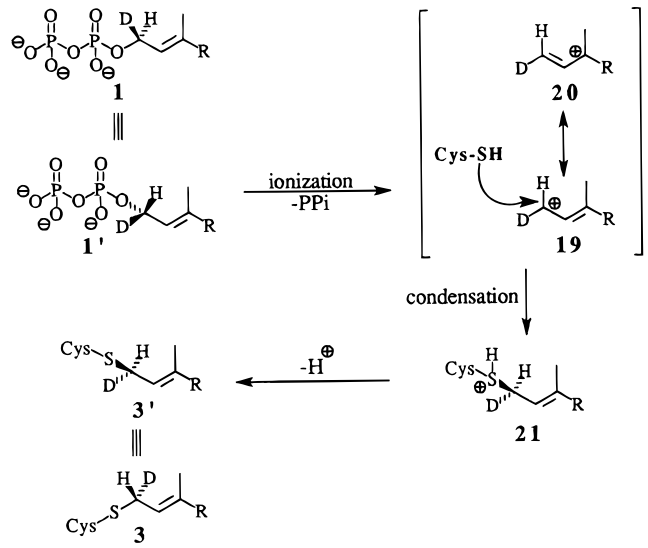
(39) Guo, J.; Wu, Y. Q.; Rattendi, D.; Bacchi, C. J.; Woster, P. M. *J. Med. Chem.* **1995**, *38*, 1770−1777.

(40) Wu, Y. Q.; Woster, P. M. *Bioorg. Med. Chem.* **1993**, *1*, 349−360.

(41) Parent, P.; Leborgne, F.; Lellouche, J. P.; Beaucourt, J. P.; Vanhove, A. *J. Labelled Compd. Radiopharm.* **1990**, *28*, 633−644 and references cited therein.

(42) Previously, the structure of the farnesylcysteine moiety produced by yeast PFTase in the formation of yeast **a**-factor was confirmed by ^{1}H-NMR: Anderegg, R. J.; Betz, R.; Carr, S. A.; Crabb, J. W.; Duntze, W. *J. Biol. Chem.* **1988**, *263*, 18236−18240.

Scheme 4



transfer reaction with inversion of configuration.[44] Later, Poulter and co-workers demonstrated with fluorinated substrates that FPP synthase utilizes an electrophilic, ionization−condensation−elimination mechanism.[45,46] The observed inversion in the FPP synthase reaction results from a combination of the rotational barrier about an allylic cation and enforcement of the stereochemical course by the enzyme active site, rather than the operation of an S_N2 reaction. The same mechanistic picture has emerged for the prenyl transfer reaction catalyzed by dimethallyltryptophan synthase.[22,47] The stereochemical experiments presented in this paper, together with the work of Dolence and Poulter on fluorinated FPP analogs,[19] indicate that the mechanistic course of PFTase is similar to the prototypical prenyltransferase FPP synthase, although the electrophilic character of the PFTase reaction is significantly lower due to the potent sulfur nucleophile. Thus, the mechanism shown in Scheme 4 is consistent with our stereochemical results, the kinetic results of Dolence and Poulter, and the mechanistic precedent set by FPP synthase and dimethallyltryptophan synthase. However, note that the stereochemical results can also accommodate reaction via an S_N2 mechanism.[48]

Acknowledgment. This work was supported by the American Cancer Society (IN-162), the PhRMA Foundation, a Rumble Fellowship from WSU (to Y.Q.M.), and the NIH (CA 67292). We thank Dr. Mohammed Ksebati (Department of Chemistry, WSU) for performing the NMR experiments, Drs. Jackson Gibbs and David Pompliano (Merck) for helpful discussions, and Dr. Kenneth Koblan (Merck) for assistance in preparing recombinant human PFTase. We also thank Professors James Coward (University of Michigan), Patrick Woster (WSU), and Stephen Benkovic (Penn State University) for helpful comments on the manuscript.

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(43) Note that if partial but significant racemization occurred during the PFTase reaction, then the diasteromeric excess of the biosynthetic **4** must be less than the 62% calculated for the synthetic sample of **3**, since both are derived from the same sample of farnesyl chloride **14**. In other words, this would mean that the minor negative peak in Figure 2c (at 2.807 ppm) would be larger than the minor negative peak in Figure 2b (at 2.825 ppm). Despite the significant noise level in Figure 2c, this does not appear to be the case, as such a peak would be more clearly visible above the noise. Thus, although we are only able to provide estimates of the diasteromeric excess of the biosynthetic farnesylated peptides, visual inspection of the spectra bolsters our confidence that the enzymatic transfer is accompanied by little if any racemization.

(44) Popjak, G.; Cornforth, J. W. *Biochem. J.* **1966**, *101*, 553−568.

(45) Poulter, C. D.; Rilling, H. C. *Acc. Chem. Res.* **1978**, *11*, 307−313.

(46) Poulter, C. D.; Wiggins, P. L.; Le, A. T. *J. Am. Chem. Soc.* **1981**, *103*, 3926−3927.

(47) Gebler, J. C.; Woodside, A. B.; Poulter, C. D. *J. Am. Chem. Soc.* **1992**, *114*, 7354−7360.

(48) It has been demonstrated by the Bristol-Meyers Squibb group that a bisubstrate adduct analog is a very potent inhibitor of PFTase: Patel, D. V.; Gordon, E. M.; Schmidt, R. J.; Weller, H. N.; Young, M. G.; Zahler, R.; Barbacid, M.; Carboni, J. M.; Gullo-Brown, J. L.; Hunihan, L.; Ricca, C.; Robinson, S.; Seizinger, B. R.; Tuomari, A. V.; Manne, V. *J. Med. Chem.* **1995**, *38*, 435−442. Previously, this has been taken as an indication that an enzymatic reaction proceeds via an S_N2-like transition state.[38]