# Stereochemical Analysis of the Reaction Catalyzed by Yeast Protein Farnesyltransferase

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Protein farnesyltransferase (PFTase) catalyzes the nucleophilic substitution reaction between farnesyl diphosphate (FPP, **1**) and a protein-derived cysteine residue to form a thioether linkage and is the target for inhibitors of Ras farnesylation.[1] This relatively new class of inhibitors takes advantage of the fact that inhibition of this process in a variety of mutant Ras-induced cancer models arrests the growth of tumor cells.[2] We are interested in studying the mechanism of the PFTase-catalyzed reaction as well as those used by other prenyltransferases to gain mechanistic information that may be useful in inhibitor design. Stereochemical experiments are useful in mechanistic studies because they can often be used to differentiate between associative, dissociative, and covalent intermediate reaction pathways.[3] Recent elegant studies by Mu et al. have demonstrated that human PFTase-catalyzes thioether bond formation with predominantly inversion of configuration.[4] We have been interested in defining the stereochemical course of the yeast PFTase and other prenyltransferases, including geranylgeranyltransferases. Here, we describe the stereochemical analysis of the yeast PFTase-catalyzed reaction using a method that consists of carboxypeptidase degradation of a farnesylated peptide product followed by $^1$H NMR analysis. This approach should provide a means for studying the stereochemistry of reactions catalyzed by other prenyltransferases that circumvents the problem of spectral overlap previously noted by Gibbs and co-workers.[4b]

Our approach for analyzing the stereochemistry of PFTase involved the use of $^1$H NMR to probe the stereochemistry at the C1 farnesyl center. In prenylated peptides and proteins, the C1 farnesyl protons are in close proximity to the chiral center of the alkylated cysteine and are thus diastereotopic. In simple model compounds such as *N*-acetyl-*S*-farnesyl cysteine, this can be easily observed in a 500 MHz $^1$H NMR spectrum.[5] Unfortunately, similar studies performed with larger prenylated peptides (greater than four residues) are more problematic; spectra from these more complex molecules are characterized by severe spectral overlap in the region of interest and the C1 protons become indistinguishable. Efforts to obtain better results by altering the NMR solvent, changing the temperature, or by using chemical shift reagents were not sucessful. To avoid these problems, we elected to farnesylate the peptide *N*-dansyl-GCVIA (**2**) using PFTase and then enzymatically degrade this compound to a smaller molecule as outlined in Scheme 1.[6] Thus, reaction between FPP (**1**) and **2** produced the farnesylated peptide (**3**) that was degraded with carboxypeptidase Y and purified by reversed-phase HPLC to yield **4**. Compound **4** was also prepared by a chemical route (see Scheme 1); L-cysteine was alkylated with farnesyl chloride[7] (**6**) followed by DCC coupling of the resulting cysteine adduct with *N*-dansyl-Gly and subsequent reversed-phase HPLC purification. The enzymatically and chemically synthesized compounds (**4**) were identical by $^1$H NMR and FAB-MS analysis. These studies demonstrated that the C1 farnesyl protons of **4** were distinguishable by $^1$H NMR and that sufficient quantities of this material could be obtained using the carboxypeptidase degradation method.

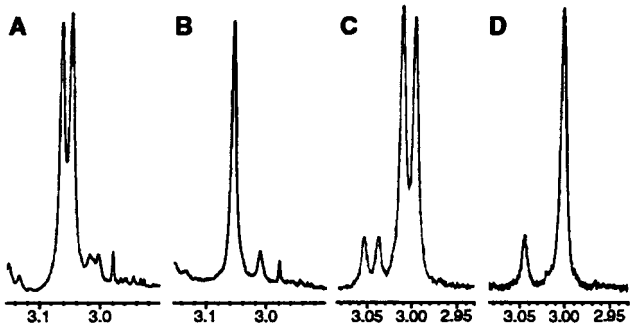


**Figure 1.** 500 MHz $^1$H NMR spectra of *N*-dansyl-Gly-Cys(*S*-[1-$^2$H]farnesyl)-OH (**4**) prepared by chemical synthesis and enzymatic synthesis with PFTase. (A) Spectrum of (α*R*,1*S*)-[1-$^2$H]-**4** obtained via chemical synthesis. (B) Spectrum of (α*R*,1*S*)-[1-$^2$H]-**4** obtained via chemical synthesis decoupled at 5.1 ppm. (C) Spectrum of [1-$^2$H]-**4** obtained via enzymatic synthesis. (D) Spectrum of [1-$^2$H]-**4** obtained via enzymatic synthesis decoupled at 5.1 ppm.

For the stereochemical experiments, (*S*)-[1-$^2$H]farnesol [(*S*)-[1-$^2$H]-**5**] was synthesized by oxidation of farnesol to methyl farnesoate,[8] reduction with Li$^2$H$_4$, oxidation with $MnO_2$ to [1-$^2$H]farnesal and reduction with (*R*)-alpine borane.[9] The route described here via methyl farnesoate allows the preparation of [1-$^2$H]farnesal with nearly quantitative deuterium incorporation, which greatly simplifies NMR studies. Mosher ester analysis of **5** indicated an enantiomeric purity of >95% ee.[10] To determine the chemical shifts of the C1 farnesyl protons in **4**, this compound was prepared in diastereomerically enriched form (75% de) using the route shown in Scheme 1. Since the conversion of **5** to the chloride **6** and the subsequent alkylation of L-Cys both proceed with inversion of configuration, the absolute stereochemistry at the C1 farnesyl position of **4** prepared from (*S*)-[1-$^2$H]-**5** was assigned the *S* configuration. This in turn allowed the chemical shifts of the C1 farnesyl protons in the $^1$H NMR spectrum of the chemically synthesized sample of **4** (Figure 1A,B) to be assigned. Given the stereochemical argument presented above, the large downfield singlet at

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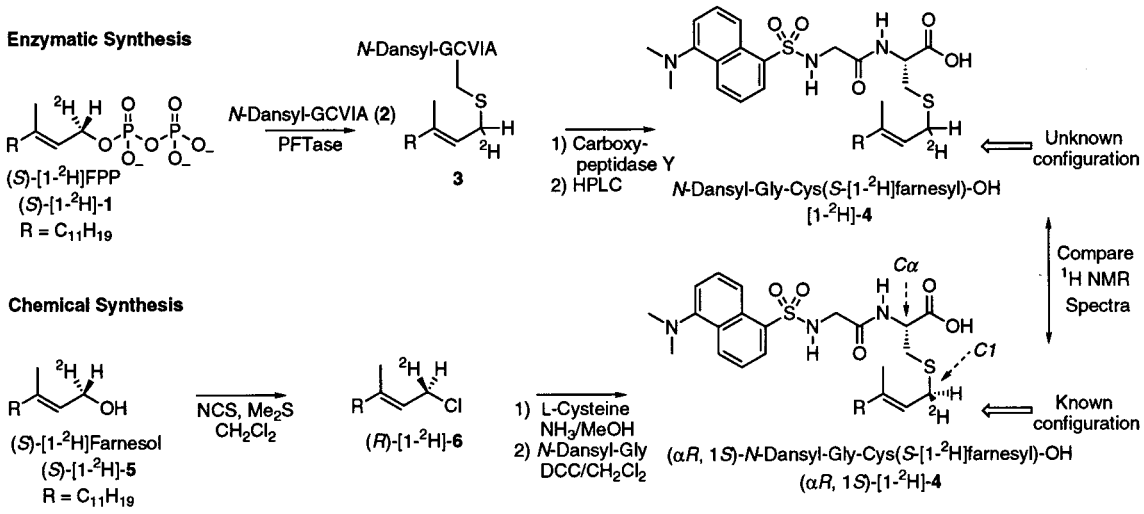
**Scheme 1**




3.056 ppm in the decoupled spectrum (Figure 1B) was assigned to be from the (α*R*,1*S*) diastereomer, while the singlet at 3.011 ppm was assigned to originate from the (α*R*,1*R*) diastereomer.[11]

To analyze the stereochemistry of the PFTase-catalyzed reaction, enantiomerically enriched **1** was required. This was synthesized from (*S*)-[1-$^2$H]-**5** by conversion to the corresponding chloride, (*R*)-[1-$^2$H]-**6**, followed by displacement with $[(n\text{-Bu})_4\text{N}]_3\text{HP}_2\text{O}_7$.[12] The stereochemistry of the yeast PFTase-catalyzed reaction was then analyzed as outlined in Scheme 1. Farnesylation was performed by incubating *N*-dansyl-GCVIA (**2**) with (*S*)-[1-$^2$H]-**1** in the presence of PFTase at 30 °C for 3 h. Carboxypeptidase Y was then added and allowed to react at room temperature for an additional 1 h. The sample was desalted, partially purified, and concentrated using a Sep-Pak cartridge. Final purification was carried out by reversed-phase HPLC. $^1$H NMR analysis of [1-$^2$H]-**4** prepared from (*S*)-[1-$^2$H]-**1** via the PFTase/carboxypeptidase Y route showed two doublets near 3.0 ppm in the $^1$H NMR spectrum as illustrated in Figure 1C. The major singlet in the decoupled spectrum (Figure 1D) at 3.002 ppm corresponds to the (α*R*,1*R*) diastereomer of [1-$^2$H]-**4** whose configuration at the C1 farnesyl center is *R*, while the smaller singlet centered near 3.044 ppm corresponds to the (α*R*,1*S*) diastereomer. Since the configuration at the C1 farnesyl position in the sample of enzymatically derived [1-$^2$H]-**4** is primarily *R* while the configuration at this position in the starting material (**1**) was mainly *S*, it is clear that the reaction catalyzed by yeast PFTase proceeds predominantly with inversion of configuration. Inspection of the $^1$H NMR spectra in Figures 1C,D reveals small amounts of the (α*R*,1*S*) diastereomer. Integration of the $^1$H NMR spectrum shown in Figure 1D indicates that the two diastereomers of [1-$^2$H]-**4** are present in a 1:5.7 ratio (70% de). This mixture of diastereomers could result either by racemization in the enzyme-catalyzed reaction between **1** and **2** or by racemization in the course of the chemical synthesis of **1** from **5**. The enantiomeric purity of the (*S*)-[1-$^2$H]-**1** obtained as described above was found to be 69% ee (1:5.6 ratio of enantiomers) as determined by enzymatic dephosphorylation followed by Mosher ester analysis of the resulting (*S*)-[1-$^2$H]-**5**; thus, some racemization occurred in the synthesis of **1**. Since the diastereomeric excess of [1-$^2$H]-**4**, obtained from the enzymatic route is equal (within 1.5%) to the enantiomeric purity of the starting material [(*S*)-[1-$^2$H]-**1**], we conclude that no racemization occurred in the enzyme-catalyzed reaction between **1** and **2**.

The stereochemical results described here indicate complete inversion of configuration and suggest an associative mechanism for the reaction catalyzed by PFTase. These results are inconsistent with a mechanism that involves the formation of a covalent intermediate between C1 of FPP (**1**) and PFTase. However, the data reported here cannot be used to rule out a dissociative mechanism involving a tight ion pair whose facial accessibility is controlled by the enzyme; additional experiments will be necessary to distinguish between possible associative or dissociative reaction mechanisms.[13] Nevertheless, the approach described in this study, which consists of carboxypeptidase degradation of a farnesylated peptide product followed by $^1$H NMR analysis, yields clear evidence for inversion of configuration in the yeast PFTase-catalyzed reaction and should be applicable to stereochemical studies of other prenyltransferases.

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**Supporting Information Available:** Characterization data for the compounds used in this study, descriptions of the procedures used to perform the stereochemical experiments, and NMR spectra of [1-$^2$H]-**4** obtained by enzymatic synthesis (13 pages).

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(11) The two stereogenic centers specified in this abbreviation are the Cys Cα (α) and the farnesyl C1 (1). In NMR spectra of samples of [1-$^2$H]-**4**, the exact chemical shifts of the C1 farnesyl protons as well as those from the dansyl moiety appear to be dependent on the concentration of the sample and on the amount of $H_2O$ present. However, in all spectra obtained, the C1 farnesyl protons of the (α*R*,1*S*) diastereomer appeared downfield relative to those from the (α*R*,1*R*) diastereomer, making determination of the stereochemical course of the enzyme-catalyzed reaction possible using this NMR strategy.

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