# Selective Modification of CaaX Peptides with *ortho*-Substituted Anilinogeranyl Lipids by Protein Farnesyl Transferase: Competitive Substrates and Potent Inhibitors from a Library of Farnesyl Diphosphate Analogues†

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ABSTRACT: Protein farnesyl transferase (FTase) catalyzes transfer of a 15-carbon farnesyl group from farnesyl diphosphate (FPP) to a conserved cysteine in the C-terminal $Ca_1a_2X$ motif of a range of proteins ("C" refers to the cysteine, "a" to any aliphatic amino acid, and "X" to any amino acid), and the lipid chain interacts with, and forms part of, the $Ca_1a_2X$ peptide binding site. Here, we employed a library of anilinogeranyl diphosphate (AGPP) derivatives to examine whether altering the interacting surface between the two substrates could be exploited to generate $Ca_1a_2X$ peptide selective FPP analogues. Analysis of transfer kinetics to dansyl-GCVLS peptide revealed that AGPP analogues with substituents smaller than or equal in size to a thiomethyl group supported FTase function, while analogues with larger substituents did not. Analogues with small *meta*-substitutions on the aniline ring such as iodo and cyano increased reactivity with dansyl-GCVLS and provided analogues that were effective FPP competitors. Other analogues with *ortho*-substitutions on the aniline were potent dansyl-GCVLS modification FTase inhibitors ($K_i$ in the 2.4−18 nM range). Both *meta*- and *para*-trifluoromethoxy-AGPP are transferred to dansyl-GCVLS while the *ortho*-substituted isomer was a potent farnesyl transferase inhibitor (FTI) with an inhibition constant $K_i = 3.0$ nM. In contrast, *ortho*-trifluoromethoxy-AGPP was efficiently transferred to dansyl-GCVIM. Competition for dansyl-GCVLS and dansyl-GCVIM peptides by FPP and *ortho*-trifluoromethoxy-AGPP gave both analogue and farnesyl modified dansyl-GCVIM but only farnesylated dansyl-GCVLS. We provide evidence that competitive modification of dansyl-GCVIM by *ortho*-trifluoromethoxy-AGPP stems from a prechemical step discrimination between the competing peptides by the FTase−analogue complex. These results show that subtle changes engineered into the isoprenoid structure can alter the reactivity and FPP competitiveness of the analogues, which may be important for the development of prenylated protein function inhibitors.

Farnesyltransferase (FTase[1]) and geranylgeranyltransferase type I (GGTase-I) catalyzes the first and obligatory step in a series of ordered posttranslational modifications that direct protein membrane localization (*1*−*4*). Protein farnesylation is important for the function of numerous proteins involved in signal transduction including the oncoprotein Ras (*5*). FTase and GGTase-I catalyze respective lipid transfer from farnesyl diphosphate (FPP, **1**) or geranylgeranyl diphosphate (GGPP) to a conserved Cys residue four amino acids from the C-terminus of target proteins (*6*, *7*). Protein substrates for both prenyltransferases contain a $Ca_1a_2X$ group at their C-terminus, where C is a conserved Cys residue, $a_1$ and $a_2$ are often aliphatic amino acids, and X is frequently M, Q, or S for FTase and L or F for GGTase-I (*8*, *9*). The prenyltransferase $Ca_1a_2X$ substrate specificities overlap, where some canonical GGTase-I substrates can be farnesylated by FTase, and vice versa (*10*, *11*). The $Ca_1a_2X$ sequence is sufficient for prenyltransferase recognition, and small peptides corresponding to this sequence often mimic the reactivity of full length proteins (*12*, *13*).

Prenylation of the oncoprotein Ras is obligatory for both membrane localization and cellular transformation (*14*). Mutations in Ras that result in constitutive activation are transforming in a wide variety of cell types and have been found in 30% of all cancers (*15*−*17*). Numerous other C-terminal $Ca_1a_2X$ proteins have been implicated in oncogenesis and tumor progression (*18*, *19*), spurring development

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[1] Abbreviations: FTase, protein farnesyltransferase; FPP, farnesyl diphosphate; GGTase-I; protein geranylgeranyltransferase type 1, GPP; geranyl diphosphate, GGPP; geranylgeranyl diphosphate, FTI, protein farnesyltransferase inhibitor; CaaX, tetrapeptide sequence cysteine−aliphatic amino acid−aliphatic amino acid−X (serine, glutamine, or methionine for FTase); dns, dansylated; RP-HPLC, reverse-phase high-performance liquid chromatography; H-Ras, Harvey-Ras; K-Ras, Kirsten-Ras; AGPP, 8-anilinogeranyl diphosphate; DTT, dithiothreitol; $^{app}k_{cat}$, apparent turnover number; $^{app}K_m^{peptide}$, apparent Michaelis−Menten constant for peptide; $^{app}K_m^{FPP}$, apparent Michaelis−Menten constant for FPP.

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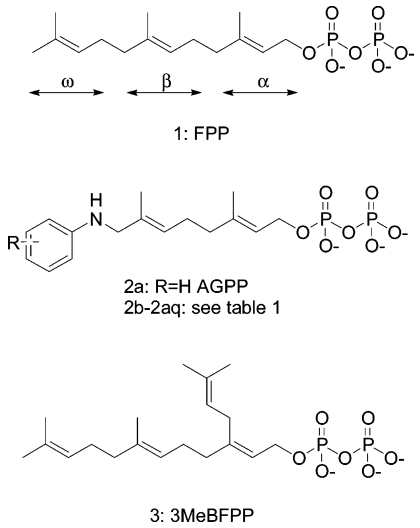


FIGURE 1: Isoprenoid structures.

of both FTase and GGTase-I inhibitors (*20*−*22*). Several FTase inhibitors (FTIs) are currently in clinical trials for the treatment of cancer (*21*−*23*). However, the overall response rate in patients has been less than hoped for (*20*). One possible explanation for the lack of FTI clinical efficacy is the process of alternative prenylation where some FTase substrates can become geranylgeranylated by GGTase-I when FTase activity is limiting (*20*, *24*−*26*). K-Ras is the most prevalent mutated Ras isoform found in human cancers, and K-Ras transformed cells are often resistant to FTI treatment (*27*). The K-Ras $Ca_1a_2X$ sequence (CVIM) is normally farnesylated, but in the presence of an FTI can be alternatively geranylgeranylated by GGTase-I (*28*).

Farnesylation alone is insufficient to promote the biological function of Ras, as subsequent downstream steps are needed for efficient membrane localization (*29*−*31*). Proteins that undergo prenylation and subsequent modifications have a high affinity for membranes and the modified C-terminus may serve as a recognition motif for specific protein−protein interactions (*32*−*34*). Over 30 proteins are known FTase substrates (*8*), and as many as 700 proteins in the human genome contain a C-terminal $Ca_1a_2X$ box (*35*). The $Ca_1a_2X$ sequence of a protein is often unique to a single protein or to small subsets of proteins (*35*). A potential mechanism for inhibiting prenylated protein function while circumventing competitive geranylgeranylation is to modify FTase target proteins with alternative lipids incapable of supporting normal prenyl group function (*36*−*38*).

With emerging evidence for the importance of alternative prenylation in FTI evasion (*28*, *39*) there is a clear need to develop agents to target this process. Isoprenoid analogues of FPP have been used to study the physical interactions between the lipid, FTase, and the $Ca_1a_2X$ peptide (*40*−*42*). The analogue, 8-anilinogeranyl diphosphate (AGPP, **2a**, Figure 1), contains an aniline moiety in the place of the $\omega$-terminal isoprene of FPP. AGPP is transferable to $Ca_1a_2X$ substrates with apparent steady-state kinetics nearly identical to FPP, and the aniline moiety appears to act as an isostere for the FPP terminal isoprene (*40*, *43*). AGPP has been used to probe the endogenous modification of proteins by FTase and is competitive with FPP *in vitro* and in cell culture (*43*, *44*). However, when the AGPP aniline is replaced with a *p*-$NO_2$-aniline **2j** or a pentafluoroaniline, the analogue

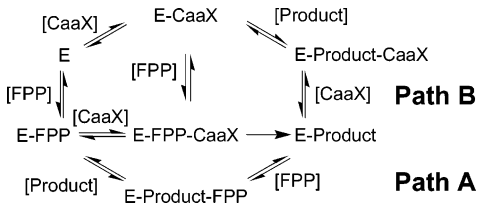


FIGURE 2: FTase reaction mechanism showing two pathways. Path A represents FPP stimulated product release, and path B represents peptide stimulated product release. E is the FTase enzyme, E-FPP is the FTase·FPP complex, E·FPP·CaaX is the FTase·FPP·CaaX peptide complex, E-Product is the FTase bound product complex, E-Product-FPP is the FTase bound to both FPP and the reaction product, E-CaaX is the peptide bound FTase inhibitory complex, and E-Product-CaaX is the peptide bound enzyme product complex. This figure is identical to Scheme 2 in the companion paper (*65*). Reprinted with permission from ref 65. Copyright 2007 American Chemical Society.

dependent steady-state transfer efficiency is substantially reduced (*40*).

The structural features that give efficiently transferable analogues are not clear, as the FTase reaction mechanism is unexpectedly complex and subject to peptide substrate inhibition (Figure 2) (*1*, *8*). The minimal kinetic scheme for FTase proceeds through a functionally ordered mechanism in which FPP first associates with the enzyme followed by protein substrate binding to the E·FPP complex to form the ternary E·FPP·$Ca_1a_2X$ complex (*45*). Product release is the rate determining step ($k_{cat}$) for the FTase reaction (*45*, *46*), and is greatly enhanced by binding of either a new FPP or $Ca_1a_2X$ peptide substrate (*47*). X-ray crystallographic analysis shows that the lipid chain of FPP forms a significant portion of the $Ca_1a_2X$ binding site with the third isoprene in direct contact with the $a_2$ and X side chains of the substrate (*48*, *49*). Additionally, FTase is highly selective for its natural substrate FPP which binds with a 5 nM affinity, while the smaller 10-carbon GPP is a poor substrate and the 20-carbon GGPP is a nanomolar competitive inhibitor (*50*). Pre-steady-state kinetic studies have shown that FPP association with the free enzyme enhances $Ca_1a_2X$ peptide affinity up to 70-fold (*51*). The interaction between the $Ca_1a_2X$ motif and the isoprenoid suggests that changes in the structure of FPP may be analogous to amino acid mutations in the active site of the enzyme. Gibbs and co-workers report that transfer of the FPP analogue 3-MeBFPP **3** (Figure 1) by FTase depends on the $Ca_1a_2X$ substrate sequence (*41*). In particular, 3-MeBFPP **3** is a good alternate substrate for a number of $Ca_1a_2X$ peptides but reacts very slowly with, and inhibits the farnesylation of, the RhoB CKVL $Ca_1a_2X$ sequence. These observations suggest that $Ca_1a_2X$ motif reactivity may be altered by changes in the structure of the isoprenoid donor, and that suitably modified FPP analogues may be active as $Ca_1a_2X$ selective FTIs or as $Ca_1a_2X$ selective alternative substrates for FTase (*41*).

Several studies have examined lipid features that influence the efficiency of isoprenoid transfer to $Ca_1a_2X$ peptides by FTase (*38*, *40*−*42*, *52*−*54*). These studies have focused on how the length of the isoprenoid affected transfer kinetics (*52*), replacing the terminal isoprene with aryl substituents (*40*, *42*, *43*, *55*), and altering the steric demands and electronic properties of the isoprenoid branched methyl groups (*38*, *41*, *52*, *56*, *57*). Despite these efforts, there is only limited information available on lipid structural features

that allow transfer by FTase. On the basis of kinetic and molecular modeling analysis, we have previously shown that an aniline group is isosteric with the terminal isoprene moiety of FPP and that some modification to the *para*-position resulted in transferable analogues (*40*). In those studies we examined a small set of molecules to provide an initial analysis of factors responsible for substrate selectivity. Here we have employed a library of FPP analogues to systematically examine how transfer to $Ca_1a_2X$ peptides by FTase depends on the size and shape of the $\omega$-terminus of the lipid.

Understanding the molecular requirements for enzyme substrate specificity is a key component of designing drugs that are selective toward one particular enzyme and one substrate over another. In order to enable this process, we utilized a library of AGPP analogues to examine how changes in the isoprenoid structure altered FTase catalyzed transfer of the lipid to a range of $Ca_1a_2X$ sequences. We found that the efficiency of lipid transfer was highly dependent on both the shape and size of the analogue. The apparent catalytic efficiency ($k_{cat}/K_m^{peptide}$) for transfer of several analogues to a dns-GCVLS (H-Ras $Ca_1a_2X$ sequence) peptide was greater than that for the natural substrate FPP. Additionally, a series of analogues with *ortho*-substitutions on the aniline ring were found to be farnesyl transferase inhibitors (FTI) with inhibition constants ($K_i$) lower than the apparent $K_m$ of FPP ($^{app}K_m^{FPP} = 46$ nM) (*40*). A number of analogues were found to effectively compete with FPP for FTase catalyzed modification of dns-GCVLS. The position and size of the aniline ring substitution have a major affect on the ability of the analogue to compete with FPP. In particular, small *meta*-substitutions on the aniline ring increase reactivity with dns-GCVLS and provided analogues that competed with FPP as well as, or better than, the biologically utilized parent molecule AGPP. Building on these observations, we tested the ability of *ortho*-substituted AGPP analogues to act as substrates with other $Ca_1a_2X$ peptides. We found that several analogues were both potent inhibitors of dns-GCVLS modification and excellent substrates with the K-Ras4B dns-GCVIM peptide. Several of the analogues enhanced the selectivity of FTase for dns-GCVIM over other $Ca_1a_2X$ sequences. In particular, we show that *o*-$CF_3$O-AGPP **2z** selectively modifies dns-GCVIM in the presence of dns-GCVLS and FPP. Selective modification of FTase target substrates is the first step to designing $Ca_1a_2X$ sequence selective molecules that can be used to inhibit the function of specific cellular proteins. These results show that subtle changes engineered into the isoprenoid structure can alter the reactivity and FPP competitiveness of the analogues, which may be important for the development of prenylated protein function inhibitors.

## EXPERIMENTAL PROCEDURES

*General.* RP-HPLC was performed on an Agilent 1100 HPLC system equipped with a microplate autosampler, diode array, and fluorescence detector. HPLC analysis utilized a Microsorb C18 column with 0.01% TFA in water (A) and 0.01% TFA $CH_3CN$ (B) as the mobile phase. *N*-Dansyl-$GCa_1a_2X$ peptides were purchased from peptidogenics. Spectrofluorometric analyses were performed in 96-well flat bottom, nonbinding surface, black polystyrene plates (Corning, excitation wavelength, 340 nm; emission wavelength 505 nm with a 10 nm cutoff) using a SpectraMax GEMINI XPS fluorescence well-plate reader. Absorbance readings were determined using a Cary UV/vis spectrophotometer. All assays were performed at minimum in triplicate where the average values are reported with a one standard of deviation error.

*Farnesyl Diphosphate Analogues.* Analogues **2a**−**aq** were prepared on solid support or in solution as previously described by Subramanian et al. (*58*) and Chehade et al. (*40*) FPP **1** was synthesized as described by Davisson et al. (*59*).

*Steady-State Peptide Kinetics.* The kinetic constants $^{app}k_{cat}$, $^{app}K_m^{peptide}$, and apparent $k_{cat}/K_m^{peptide}$ for transfer of isoprenoids **1**, **2a**−**aq** by FTase to peptide were determined in triplicate using a continuous spectrofluorometric assay originally developed by Pompliano et al. (*60*) and modified for a 96-well plate format as described (*65*).

*RP-HPLC Product Studies.* Reactions were prepared as above and stopped by addition of 50 $\mu$L of isopropyl alcohol and acetic acid (4:1). The 96-well plate was then placed in a RP-HPLC system, and 100 $\mu$L of the reaction mixture was loaded onto an analytical C18 column and then eluted with a linear gradient of 10% to 100% $CH_3CN$ in water over 20 min; the eluant contained 0.01% (v/v) TFA. The elution profile was monitored for dansyl fluorescence (excitation wavelength, 340 nm monitored at the emission wavelength 505 nm) and for dansyl absorption (340 nm). Analogue-modified peptides had longer retention times than the corresponding unmodified peptides due to an increase in hydrophobicity.

*FPP Competition with Analogues.* Reactions to measure the competition between analogues and FPP for modification of peptide were prepared as for steady-state kinetics described above, except the dns-$GCa_1a_2X$ peptide concentration was 3 $\mu$M and both FPP and the analogue were included at 6.7 $\mu$M each. The reactions were initiated with the addition of FTase (20 nM final FTase concentration) and analyzed spectrofluorometrically as described above. The reactions were stopped after 2 h by the addition of 20 $\mu$L of a 1:4 mixture of acetic acid and isopropanol followed by HPLC analysis as described above. All reactions went to completion. The dansyl absorption (340 nm) for each of the product peaks in the chromatogram was integrated and the product ratio calculated from $I_{analogue}/I_{FPP}$ where $I_{analogue}$ is the integral of the analogue modified peptide and $I_{FPP}$ is the integral of the farnesylated peptide.

*FTase Inhibition Screen.* Reactions to screen for inhibition of FTase catalyzed transfer of FPP to peptide were prepared as for steady-state kinetics described above, except the dns-$GCa_1a_2X$ peptide concentration was 3 $\mu$M and either FPP alone or FPP and the nonsubstrate analogue were included at 16 $\mu$M each. The reactions were initiated with the addition of FTase (20 nM final FTase concentration) and analyzed spectrofluorometrically as described above. The rate of FPP modification was determined based on the rate of fluorescence increase over time with and without the analogue present. The percent (%) FTase inhibition for the analogues was calculated using eq 1,

$$\% \text{ inhibition} = (1 - (R_{FPP}/R_I)) \times 100 \qquad (1)$$

wherein $R_{FPP}$ is the rate of the dns-$GCa_1a_2X$ reaction with FPP alone and $R_I$ is the rate of the reaction with the nontransferable analogue present.

*$K_i$ Determination.* The inhibition constant $K_i$ for the nontransferable analogues was determined using reactions to measure steady-state kinetics described above with the following modifications: Each FPP analogue was assayed in triplicate wells where the peptide concentration was 3 $\mu$M dns-GCVLS, FPP concentration was 16 $\mu$M, and four concentrations of the nontransferable analogues between 0.016 and 16 $\mu$M were used. The inhibitor concentration giving 50% inhibition ($IC_{50}$) for each analogue was determined by fitting the velocity from each reaction to the three parameter Hill function given in eq 2,

$$V/V_0 = (aI^b)/(IC_{50}{}^b + [I]^b) \quad (2)$$

wherein $V_0$ is the velocity of the uninhibited reaction; $V$ is the velocity of the analogue inhibited reaction; $I$ is the concentration of the nontransferable analogue; and $a$ and $b$ are coefficients for Hill function fitting. The $K_i$ for each analogue was then calculated from the corresponding $IC_{50}$ using eq 3,

$$K_i = IC_{50}/(1 + ([FPP]/{}^{app}K_m{}^{FPP})) \quad (3)$$

where [FPP] = 16 $\mu$M and ${}^{app}K_m{}^{FPP}$ = 46 nM (*40*).

*Peptide Competition for Modification by One Lipid Diphosphate.* Reactions to measure the competition between two peptides for modification by FPP or analogue diphosphate were prepared as for steady-state kinetics described above, except both dns-G$Ca_1a_2X$ peptide concentrations were 3 $\mu$M and the lipid diphosphate was 16 $\mu$M. The reactions were initiated with the addition of FTase (20 nM final FTase concentration) and analyzed spectrofluorometrically as described above. The reactions were stopped after 1 h by the addition of 20 $\mu$L of a 1:4 mixture of acetic acid and isopropanol followed by RP-HPLC analysis as described above. Peptide starting material and products were identified by comparing retention times of observed peaks with dansyl fluorescence of authentic standards. The dansyl absorption (340 nm) for each of the product and unmodified peptide (if any) peaks in the chromatogram was integrated and the product ratio calculated from $(I_{Amod}/I_{Atot})/(I_{Bmod}/I_{Btot})$, where $I_{Amod}$ is the integral of the modified peptide A, $I_{Atot}$ is the integral of the modified peptide A plus the unmodified peptide A, $I_{Bmod}$ is the integral of the modified peptide B, and $I_{Btot}$ is the integral of the modified peptide B plus the unmodified peptide B.

*Sequential dns-GCVIM Peptide Addition Experiment To Relieve Inhibition of dns-GCVLS Farnesylation by o-$CF_3$O-AGPP.* A sequential peptide addition experiment was performed to determine if the inhibition of dns-GCVLS farnesylation by *o*-$CF_3$O-AGPP **2z** was irreversible. Reactions were prepared as for steady-state kinetics described above, except with 16 $\mu$M FPP, 16 $\mu$M *o*-$CF_3$O-AGPP **2z**, and 3 $\mu$M *N*-dansyl-GCVLS. The reactions were initiated with the addition of FTase (20 nM final FTase concentration) and analyzed spectrofluorometrically for 600 s as described above. At 600 s, dns-GCVIM to a final concentration of 3 $\mu$M was added and the fluorescence emission monitored for an additional 600 s.

*Competition between dns-GCVLS and dns-GCVIM for Modification by FPP and o-$CF_3$O-AGPP.* Reactions to measure the competition between two peptides for modification by FPP and analogue diphosphate were prepared as for steady-state kinetics described above, except with 16 $\mu$M FPP, 16 $\mu$M *o*-$CF_3$O-AGPP **2z**, 3 $\mu$M dns-GCVLS, and 3 $\mu$M dns-GCVIM. The reactions were initiated with the addition of FTase (20 nM final FTase concentration) and analyzed spectrofluorometrically as described above. The reactions were stopped prior to consumption of more than 50% of either peptide by the addition of 20 $\mu$L of a 1:4 mixture of acetic acid and isopropanol followed by RP-HPLC analysis and calculation of product ratios as described above.

## RESULTS

*$Ca_1a_2X$ Peptide Reactivity Depends on the AGPP Aniline Moiety Substituent Size and Position.* In order to determine how systematic changes to the isoprenoid terminus affect reactivity with a FTase target peptide, we measured the steady-state transfer kinetics of a dansylated-GCVLS (dns-GCVLS) pentapeptide with a library of structurally diverse AGPP analogues (Table 1). The dns-GCVLS peptide corresponds to the $Ca_1a_2X$ sequence of H-Ras, and the steady-state transfer kinetics of both H-Ras and the dns-GCVLS peptide have been extensively characterized (*13*, *41*, *51*). Utilizing a continuous fluorescence assay (*60*) we found that the kinetic parameters ${}^{app}k_{cat}$, ${}^{app}K_m{}^{peptide}$, and apparent $k_{cat}/K_m{}^{peptide}$ for dns-GCVLS reaction with FPP were identical to those previously reported for both dns-GCVLS peptide and full length H-Ras protein (*13*, *41*). Using this method, we also found that the steady-state kinetic parameters for the reaction between the dns-GCVIM (K-Ras4B) $Ca_1a_2X$ sequence and FPP were identical to those previously published (Figure 3) (*41*).

Over half of the members of the 43 molecule AGPP library **2a**−**aq** were transferred to the dns-GCVLS peptide by FTase (Table 1). Analogues **2ac**−**2aq** with functional groups equal in size to or larger than the thiomethyl group were poorly transferred to the dns-GCVLS peptide, independent of the aniline ring substitution position. Conspicuous exceptions to this generalization are the *o*-I-AGPP (**2q**), *p*-Et-AGPP (**2y**), and *o*-$CF_3$O-AGPP (**2z**) analogues which showed very little reactivity with dns-GCVLS. Analogues with fluorine substituents at either the *meta* **2c** or *ortho* **2b** positions, reacted with an apparent catalytic efficiency (apparent $k_{cat}/K_m{}^{peptide}$) nearly identical to that of FPP or AGPP. Surprisingly, the catalytic efficiency of the *p*-F-AGPP **2d** analogue with the dns-GCVLS peptide was increased relative to FPP. The highly electronegative fluorine substituent is isosteric with hydrogen but has very different electronic properties. When the functional group on the aniline ring was smaller than a thiomethyl group (**2ac**, **2ad**), the reactivity of the molecules was dependent on both the substituent position and size. Of particular interest is the behavior of the *ortho*-, *meta*- and *para*-isomers of the I-AGPP (**2q**−**s**) and $CF_3$O-AGPP (**2z**−**ab**) analogues. For dns-GCVLS, the ${}^{app}K_m{}^{peptide}$ for the efficient FTase substrates *m*-I-AGPP **2r** and the *p*-I-AGPP **2s** are similar, while the *o*-I-AGPP **2t** is transferred very poorly and a similar trend is observed for the *o*-, *m*-, *p*-$CF_3$O-AGPP (**2z**−**ab**) isomers. Reaction with *o*-I-AGPP **2q** or *o*-$CF_3$O-AGPP (**2z**) resulted in very little product formation (<4%) even in the presence of enzyme concentrations well above what was required for complete modification of the dns-GCVLS peptide with the corresponding *meta*- and *para*-isomers.

Table 1: Steady-State Kinetic Properties of Isoprenoids for Reaction with dns-GCVLS

| R-group and position on aniline ring[a] | compd no. | $^{app}k_{cat}$[b] ($s^{-1}$) $\times 10^{-2}$ | $^{app}K_m^{dns-GCVLS}$ [b] ($\mu$M) | apparent $k_{cat}/K_m^{dns-GCVLS}$ [b] ($\mu M^{-1} \cdot s^{-1}$) $\times 10^{-2}$ |
|---|---|---|---|---|
| FPP | **1** | 14 ± 2 | 0.8 ± 0.1 | 18 ± 3 |
| H | **2a** | 12 ± 2 | 0.5 ± 0.1 | 24 ± 6 |
| *o*-F | **2b** | 15 ± 3 | 0.9 ± 0.2 | 16 ± 1 |
| *m*-F | **2c** | 10 ± 1 | 0.34 ± 0.08 | 26 ± 3 |
| *p*-F | **2d** | 9.5 ± 0.4 | 0.26 ± 0.04 | 35 ± 4 |
| *o*-Cl | **2e** | 10 ± 1 | 0.8 ± 0.2 | 10.3 ± 0.3 |
| *m*-Cl | **2f** | 13 ± 2 | 1.0 ± 0.2 | 13 ± 3 |
| *p*-Cl | **2g** | 30 ± 10 | 1.9 ± 0.8 | 18 ± 1 |
| *m*-CN | **2h** | 13 ± 1 | 1.0 ± 0.1 | 11.5 ± 0.3 |
| *p*-CN | **2i** | 70 ± 3 | 10.2 ± 0.6 | 6.8 ± 0.1 |
| *p*-$NO_2$ | **2j** | 160 ± 20 | 15 ± 2 | 10.7 ± 0.2 |
| *o*-Br | **2k** | 13 ± 1 | 1.0 ± 0.2 | 13 ± 3 |
| *p*-Br | **2l** | 27 ± 8 | 1.7 ± 0.5 | 16 ± 2 |
| *o*-Me | **2m** | 16.6 ± 0.9 | 4.0 ± 0.4 | 4.2 ± 0.5 |
| *m*-Me | **2n** | 6.9 ± 0.5 | 0.17 ± 0.06 | 38 ± 8 |
| *p*-Me | **2o** | 60 ± 20 | 4 ± 1 | 16 ± 1 |
| *o*-$CF_3$ | **2p** | 30 ± 10 | >10 | 0.9 ± 0.1 |
| *o*-I | **2q** | slow[d] | 8 ± 3 | ND[e] |
| *m*-I | **2r** | 24 ± 2 | 0.9 ± 0.1 | 26 ± 3 |
| *p*-I | **2s** | 17 ± 3 | 1.4 ± 0.4 | 12 ± 4 |
| *o*-MeO | **2t** | 8.8 ± 0.9 | 0.9 ± 0.2 | 9.3 ± 0.6 |
| *m*-MeO | **2u** | 53 ± 5 | 4.3 ± 0.6 | 12 ± 2 |
| *p*-MeO | **2v** | 140 ± 40 | 30 ± 10 | 5 ± 2 |
| *o*-Et | **2w** | 17 ± 2 | 7 ± 1 | 2.4 ± 0.6 |
| *m*-Et | **2x** | 8 ± 2 | 3.8 ± 0.8 | 2.1 ± 0.1 |
| *p*-Et | **2y** | NR[c] | NR | NR |
| *o*-$CF_3O$ | **2z** | slow | >10 | ND |
| *m*-$CF_3O$ | **2aa** | 40 ± 10 | 2.3 ± 0.6 | 19 ± 1 |
| *p*-$CF_3O$ | **2ab** | 27 ± 6 | 3.1 ± 0.9 | 8.7 ± 0.3 |
| *o*-MeS | **2ac** | NR | NR | NR |
| *p*-MeS | **2ad** | NR | NR | NR |
| *o*-EtO | **2ae** | NR | NR | NR |
| *m*-EtO | **2af** | NR | NR | NR |
| *p*-EtO | **2ag** | NR | NR | NR |
| *o*-iPr | **2ah** | NR | NR | NR |
| *p*-iPr | **2ai** | NR | NR | NR |
| *o*-Ph | **2aj** | NR | NR | NR |
| *p*-Ph | **2ak** | NR | NR | NR |
| *o*-PhO | **2al** | NR | NR | NR |
| *m*-PhO | **2am** | NR | NR | NR |
| *p*-PhO | **2an** | NR | NR | NR |
| *o*$PhCH_2$ | **2ao** | NR | NR | NR |
| *m*$PhCH_2$ | **2ap** | NR | NR | NR |
| *p*$PhCH_2$ | **2aq** | NR | NR | NR |

[a] The analogues are listed in order of increasing substituent surface area **2a** to **2aq**. [b] $^{app}k_{cat}$, $^{app}K_m^{peptide}$, and apparent $k_{cat}/K_m^{peptide}$ were determined using a Michaelis−Menten analysis described in the Experimental Procedures. [c] NR indicates no reaction. [d] Slow indicates that some reaction did occur, but insufficient to characterize. [e] ND indicates not determined due to the inability to measure $^{app}k_{cat}$.

The $^{app}K_m^{peptide}$ for transferable *meta*-substituted analogues was confined to a relatively narrow range from 0.17 $\mu$M for *m*-Me-AGPP **2n** to 4.3 $\mu$M for *m*-MeO-AGPP **2u**. The $^{app}K_m^{peptide}$ for the transferable *meta*-substituted analogues was also roughly correlated ($R = 0.7$) with substituent size (data not shown). This is in contrast to the behavior of the transferable *para*-substituted analogues which showed a larger range of $^{app}K_m^{peptide}$ and a lower correlation with substituent size ($R = 0.5$). The *ortho*-substituted analogues showed the lowest correlation between substituent size and $^{app}K_m^{peptide}$ ($R = 0.4$). Particularly striking is the difference in $^{app}k_{cat}$ and $^{app}K_m^{peptide}$ between *m*-MeO-AGPP **2u** and the less efficiently transferred *p*-MeO-AGPP **2v**. In addition, *p*-Et-AGPP **2y** is not transferred while the corresponding *ortho*- and *meta*- isomers are. Previous studies have shown that transferable *para*-substituted AGPP analogues (*40*) are likely to have the *para*-substituent nestled in a slot on the floor of the FTase active site. The abrupt transition to nonreactive analogues for the *meta*- and *para*-substituents larger than trifluoromethoxy suggests that there is a maximum size of isoprenoid that can be productively accommodated in the active site with dns-GCVLS as the cosubstrate. In addition, the narrow range of $^{app}K_m^{peptide}$ for the *meta*-substituents relative to the *para*-substituents suggests that substituents can be more readily accommodated in the *meta*-position.

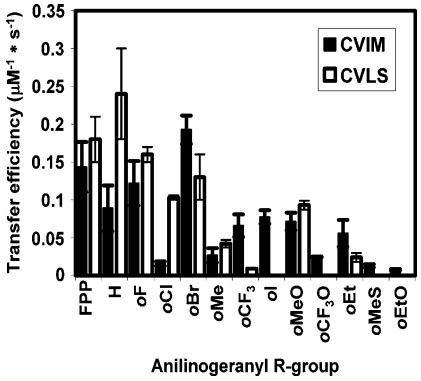


FIGURE 3: Peptide transfer efficiency (apparent $k_{cat}/K_m^{peptide}$) for dns-GCVIM and dns-GCVLS peptides from reaction containing 6.7 $\mu$M isoprenoid donor and varying concentrations of peptide in Tris-HCl buffer (pH = 7.4) plus reducing agent and detergent. Note that the transfer efficiency varies depending on the peptide target.

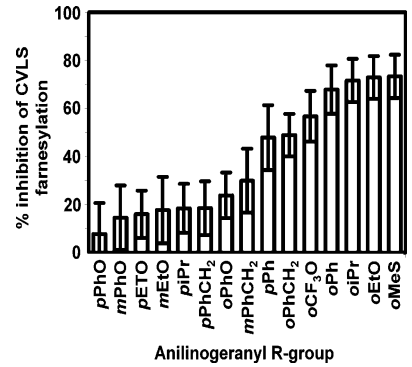


FIGURE 4: AGPP analogue inhibition of FTase catalyzed FPP transfer to peptide. Inhibition of FTase catalyzed FPP transfer to dns-GCVLS by individual analogues was measured as described in the Experimental Procedures. Data are expressed as the ratio of the rate of reactions containing equimolar (16 $\mu$M) analogue and FPP to the reaction rate with FPP alone (16 $\mu$M). Note that the *ortho*-substituted analogues are the most potent inhibitors.

*Nonsubstrate ortho-Substituted Analogues Are Potent FTase Inhibitors of dns-GCVLS Modification.* We screened the nonsubstrate analogues for their ability to inhibit dns-GCVLS peptide farnesylation and found that a number were FTIs. Transferable analogues were not included in the screen as they are competitive inhibitors by definition. Comparing the reaction rate of 1:1 analogue−FPP competition mixtures to the rate of FPP alone (Figure 4), we found that the *ortho*-isomers of the nontransferable analogues more effectively decreased the rate of farnesylation relative to the corresponding *meta*- and *para*-isomers (cf. Figure 4, *o*-, *m*-, *p*-$PhCH_2$-AGPP **2ao**−**aq**). The FTase inhibition constant ($K_i$) of the

Table 2: FTase Inhibition of dns-GCVLS Farnesylation by *ortho*-Substituted AGPP Analogues

| R-group on aniline ring | compd no. | $K_i$ (nM)[a] |
|---|---|---|
| *o*-$CF_3O$ | 2z | $3.0 \pm 0.3$ |
| *o*-MeS | **2ac** | $2.4 \pm 0.2$ |
| *o*-EtO | **2ae** | $12 \pm 1$ |
| *o*-iPr | **2ah** | $4.4 \pm 0.2$ |
| *o*-Ph | **2aj** | $3.6 \pm 0.4$ |
| *o*-PhO | **2al** | $> 40$ |
| *o*-$PhCH_2$ | **2ao** | $18 \pm 1$ |

[a] $K_i$ values were calculated from competition reaction rates for 3 $\mu$M dansyl-GCVLS peptide, with 16 $\mu$M FPP and analogue concentration between 0.016 and 16 $\mu$M. Note that the $K_i$ of the *ortho*-substituted AGPP analogues is lower than the $K_m$ of FPP (46 nM) (*40*).

most active *ortho*-substituted nonsubstrate analogues was determined and found to be below the apparent $K_m$ of FPP ($^{app}K_m^{FPP} = 46$ nM (*40*, *43*)) (Table 2). The $K_i$ for four of the analogues are in the nanomolar range, comparable to a number of previously reported FTIs (*61*).

*AGPP Analogue Substituent Size Affects Reactivity of CaaX Sequence Targets.* The intimate contacts between the two substrates in the FTase active site suggest that analogue size and shape may influence $Ca_1a_2X$ peptide selectivity. The reactivity of the *ortho*-substituted analogues with dns-GCVLS is more restricted and variable than the *meta*- and *para*-isomers (Table 1) opening the possibility of additional discrimination in reaction with other $Ca_1a_2X$ peptides. The reactivity of the K-Ras $Ca_1a_2X$ sequence dns-GCVIM relative to dns-GCVLS with FPP and 16 *ortho*-substituted AGPP analogues is shown in Figure 3 and Table 3. More of the *ortho*-substituted analogues react with dns-GCVIM than with dns-GCVLS, and analogues that are reactive with both peptides have different catalytic efficiencies with each sequence. With FPP, AGPP, *o*-F-AGPP **2c**, *o*-Cl-AGPP **2e**, *o*-Me-AGPP **2m**, or *o*-MeO-AGPP **2t**, as the isoprenoid donors, the FTase catalytic efficiency for dns-GCVLS was equal to or higher than the catalytic efficiency for dns-GCVIM. However, the catalytic efficiency of the reaction with dns-GCVIM was higher than dns-GCVLS with the *o*-$CF_3$-AGPP **2p**, *o*-Br-AGPP **2k**, *o*-I-AGPP **2q**, and *o*-Et-AGPP **2w** analogues. Notably, dns-GCVIM was modified by the *o*-$CF_3O$-AGPP **2z**, *o*-MeS-AGPP **2ac**, and *o*-EtO-AGPP **2ae** analogues which are poor substrates or nonsubstrates for, and indeed are potent FTIs with, dns-GCVLS (Table 3). The large *o*-iPr-AGPP **2ah**, *o*-Ph-AGPP **2aj**, *o*-PhO-AGPP **2al**, and *o*-$PhCH_2$-AGPP **2ao** analogues were not substrates with either peptide. These results indicate that the reactivity of the analogues is also highly dependent on the identity of the $Ca_1a_2X$ target sequence.

To further investigate the relationship of FTase reactivity to isoprenoid donor and peptide target structure, we measured the steady-state $^{app}K_m^{peptide}$ of four $Ca_1a_2X$ peptides and the 16 *ortho*-substituted AGPP analogues (Table 3). Analogues were deemed reactive if the $^{app}K_m^{peptide}$ was less than 8 $\mu$M. The peptide $Ca_1a_2X$ sequences correspond to the H-Ras (dns-GCVLS), K-Ras (dns-GCVIM), the DNAJ homologue RDJ-2 (dns-GCAHQ), and the centromere protein, CENP-F (dns-GCKVQ). Analogues that were not substrates for these peptides were further screened for their ability to inhibit dns-GCAHQ and dns-GCVIM modification (Figure 5, Table 3). The largest number of *ortho*-substituted analogues reacted with the K-Ras dns-GCVIM peptide, followed by the H-Ras dns-GCVLS, dns-GCKVQ, and dns-GCAHQ sequences. We found that the reactivity of each *ortho*-substituted analogue fell into four categories of substrate/inhibition activity that depend on both the size of the substituent and the sequence of the $Ca_1a_2X$ substrate. Twelve of the analogues tested (classes 1−3, Table 3) were substrates with some or all of the peptides, but their activity depended on the target $Ca_1a_2X$ sequence. The fourth class of analogues was unreactive with any of the four peptides. Peptides with lower $^{app}K_m^{peptide}$ with FPP ($^{app}K_m^{peptide}{}_{FPP}$) were able to react with more analogues than peptides with higher $^{app}K_m^{peptide}{}_{FPP}$. Except for *o*-MeO-AGPP **2t**, the reactivity of the analogues with the peptides was correlated with the analogue substituent size and negatively correlated with $^{app}K_m^{peptide}{}_{FPP}$. The upper substituent size limit for transferable analogues was also negatively correlated with $^{app}K_m^{peptide}{}_{FPP}$. For example, *o*-Br-AGPP **2k** was the largest transferable analogue for dns-GCAHQ, while the substantially bulkier *o*-EtO-AGPP **2ae** was transferred to dns-GCVIM. Analogues in the first category have small functional groups (*o*-MeO-AGPP **2t** excepted), were reactive with all peptides tested, and had an $^{app}K_m^{peptide}$ similar to those of FPP and AGPP (Table 3). Category 2 analogues were reactive with dns-GCVIM and dns-GCVLS but had a lower $^{app}K_m^{peptide}$ for dns-GCVIM. Analogues in the third category reacted very poorly with peptides other than dns-GCVIM and also potently inhibited the farnesylation of dns-GCVLS and dns-GCAHQ (Figure 5, Table 2). The fourth category contained the largest analogues which were not substrates with any of the peptides, and three of these analogues (*o*-Ph-AGPP **2aj**, *o*-iPr-AGPP **2ah**, and *o*-$PhCH_2$-AGPP **2ao**) were able to inhibit the farnesylation of the dns-GCVLS, dns-GCVIM, and dns-GCAHQ peptides (Figures 4, 5, Table 2).

Previous X-ray crystallographic analysis showed that the X-residue binding site of the FTase $Ca_1a_2X$ peptide substrate is partially occluded by the terminal phenyl ring of a nontransferable FPP analogue where the $\omega$-isoprene was replaced by a benzophenone moiety (*42*). The benzophenone analogue used in that study is similar in size to the largest benzylanilino (**2ao**−**aq**) and phenoxyanilino analogues (**2al**−**an**) used here. Our observations that analogues with substituents larger than a thiomethyl group were not substrates for FTase as well as the very high 30 $\mu$M $^{app}K_m^{peptide}$ and high $^{app}k_{cat}$ values for the poorly transferred to dns-GCVLS, *p*-MeO-AGPP **2o** supports the idea that analogues not much larger than FPP can interfere with productive peptide substrate binding by occluding the peptide binding site. In contrast, an AGPP derivative where the $\omega$-terminal isoprene was replaced by the fluorescent 7-nitrobenzo[1,2,5]oxadiazol-4-ylamino (NBD) group was transferred by FTase to K-Ras (dns-GCVIM $Ca_1a_2X$ sequence) (*62*). This analogue has a *para*-nitro group and the small oxadiazole ring fused to the *ortho*- and *meta*-positions of the aniline. Consistent with our observation that the dns-GCVIM sequence reacts with the widest range of substituents (Table 3), transfer of the NBD analogue suggests that there is sufficient room in the active site for molecules simultaneously bearing small substituents in the *ortho*-, *meta*- and *para*-positions. These results indicated that FTase substrate selectivity depends both on the size of the $\omega$-aniline moiety and on the structure of the $Ca_1a_2X$ substrate.

Table 3: Steady-State Kinetic Parameter Apparent $K_m^{peptide}$ for Transfer of Isoprenoids to dns-GCVLS, dns-GCAHQ, dns-GCKVQ, and dns-GCVIM

| class[c] | R | compd no. | $^{app}K_m^{dns-GCAHQ}$ | $^{app}K_m^{dns-GCKVQ}$ | $^{app}K_m^{dns-GCVLS}$ | $^{app}K_m^{dns-GCVIM}$ |
|---|---|---|---|---|---|---|
| 1 | FPP | **1** | 2.0 ± 0.3 | 1.5 ± 0.3 | 0.8 ± 0.1 | 0.30 ± 0.06 |
| 1 | H | **2a** | 2.4 ± 0.5 | 1.7 ± 0.2 | 0.5 ± 0.1 | 0.18 ± 0.06 |
| 1 | *o*-F | **2b** | 3.3 ± 0.4 | 0.19 ± 0.07 | 0.9 ± 0.2 | 0.23 ± 0.05 |
| 1 | *o*-Cl | **2e** | 1.9 ± 0.1 | 1.9 ± 0.2 | 0.8 ± 0.2 | 1.8 ± 0.3 |
| 1 | *o*-Br | **2k** | 5.3 ± 0.9 | 1.7 ± 0.2 | 1.0 ± 0.2 | 0.11 ± 0.01 |
| 2 | *o*-Me | **2 m** | >10 | >10 | 4.0 ± 0.4 | 0.3 ± 0.1 |
| 2[b] | *o*-$CF_3$ | **2p** | >10 | >10 | >10 | 1.0 ± 0.2 |
| 2 | *o*-I | **2q** | >10 | >10 | 8 ± 3 | 1.1 ± 0.1 |
| 1 | *o*-MeO | **2t** | >10 | 2.9 ± 0.2 | 0.9 ± 0.2 | 0.7 ± 0.1 |
| 2 | *o*-Et | **2w** | >10 | >10 | 7 ± 1 | 3.6 ± 0.9 |
| 3 | *o*-$CF_3O$ | **2z** | >10 | >10 | >10 | 2.76 ± 0.08 |
| 3 | *o*-MeS | **2ac** | NR[a] | >10 | >10 | 4.4 ± 0.6 |
| 3 | *o*-EtO | **2ae** | NR | >10 | NR | 4.0 ± 0.6 |
| 4 | *o*-iPr | **2ah** | NR | NR | NR | >10 |
| 4 | *o*-Ph | **2aj** | NR | NR | NR | NR |
| 4 | *o*-PhO | **2al** | NR | NR | NR | NR |
| 4 | *o*-$PhCH_2$ | **2ao** | NR | NR | NR | NR |

[a] NR indicates that no reaction was observed. [b] Did not effectively inhibit farnesylation of dns-GCVLS. [c] Analogue classification described in text. Class 1 have $^{app}K_m^{peptide}$ similar to FPP, class 2 showed significant preference with respect to the $^{app}K_m^{peptide}$ for one of the peptides, class 3 inhibited farnesylation of the other peptide but were substrates with dns-GCVIM, and class 4 inhibited farnesylation of all the peptides. [d] From Table 1.

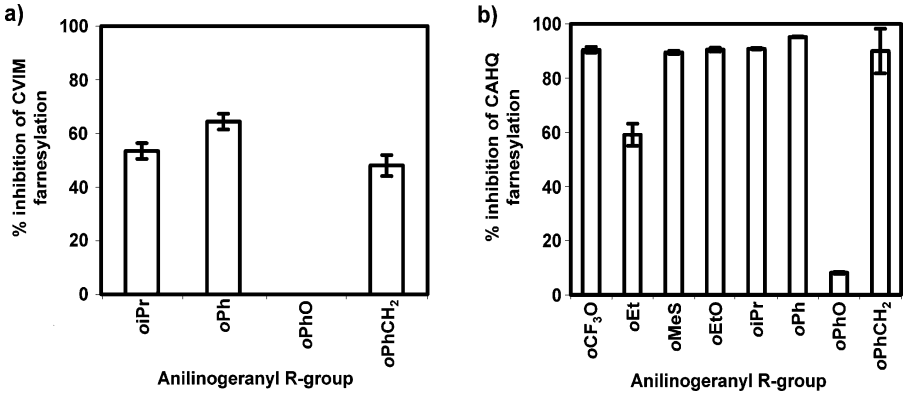


FIGURE 5: Peptide farnesylation inhibition by the analogues with (a) dns-GCVIM or (b) dns-GCAHQ as the isoprenoid acceptor. Inhibition is reported as percent inhibition based on the rate of a reaction containing only FPP relative to reactions containing a 1:1 mixture of FPP and the analogue with 3 $\mu$M peptide.

*FTase Peptide Selectivity Is Dependent on Both the $a_2$ and X-Residues of the $Ca_1a_2X$ Motif.* X-ray crystallographic studies show that the $\omega$-terminal isoprene of FPP interacts directly with the $a_2$- and X-residues of the target $Ca_1a_2X$ peptide. The $Ca_1a_2X$ peptide backbone conformation in the FTase active site depends on the X-residue identity which may affect the rate of the chemical step by altering the population of reactive and inactive forms of the isoprenoid in the active site (*48*) (*46*, *63*). The van der Waals contacts between the two substrates suggest that peptide reactivity with isoprenoid diphosphates will also be sensitive to the structure of the $a_2$-side-chain. The dependence of isoprenoid diphosphate reactivity on the $Ca_1a_2X$ peptide sequence is evident from inspection of the data in Table 3. Both the dns-GCAHQ and dns-GCKVQ peptides end in glutamine but react with a different subset of AGPP analogues, as well as having different $^{app}K_m^{peptide}$ for reaction with FPP. However, interpretation of the effect of the $a_2$-residues on peptide selectivity for the isoprenoids is complicated by the different dns-GCAHQ and dns-GCKVQ $a_1$-residues.

The H-Ras dns-GCVLS and K-Ras dns-GCVIM sequences have identical $a_1$-residues, similar isomeric, bulky, and hydrophobic $a_2$-residues, but different X-residues. The apparent catalytic efficiency ($k_{cat}/K_m^{peptide}$) of dns-GCVIM and dns-GCVLS with FPP are very similar (Figure 3), and catalytic efficiency has been proposed to be the relevant parameter for determination of substrate selectivity (*41*). Additionally, Fierke and co-workers have demonstrated that reactivity (apparent $k_{cat}/K_m^{peptide}$), not peptide substrate affinity, is the primary determinant of substrate specificity in FTase for FPP and a series of dns-TKCVIX peptides with different X-residues (*64*). However, the product ratio from competition experiments between dns-GCVIM and dns-GCVLS with FPP is 3:1 farnesylated-dns-GCVIM to farnesylated-dns-GCVLS, much more similar to the ratio of $^{app}K_m^{peptide}$ (2.7) than the ratio of apparent $k_{cat}/K_m^{peptide}$ (0.8), suggesting that the $^{app}K_m^{peptide}$ for reaction with isoprenoids is a more useful parameter to describe substrate selectivity (see companion paper (*65*)). The $^{app}K_m^{peptide}$ for reaction with FPP and *ortho*-substituted AGPP analogues are different for dns-GCVLS and dns-GCVIM (Table 3). However, it was not clear to what extent the peptide selectivity of FPP and the AGPP analogues depended on the identity of the $a_2$- and X-residues.

The similarity of the first three residues in the dns-GCVLS and dns-GCVIM peptides suggested that exchanging the $a_2$-

Table 4: Steady-State Kinetic Parameter Apparent $K_m^{peptide}$ for Transfer of Isoprenoids to dns-GCVLS, dns-GCVIS, dns-GCVIM, and dns-GCVLM

| | | H-Ras | | K-Ras | |
|---|---|---|---|---|---|
| R | compd no. | $^{app}K_m^{dns-GCVLS}$ ($\mu$M) | $^{app}K_m^{dns-GCVIS}$ ($\mu$M) | $^{app}K_m^{dns-GCVIM}$ ($\mu$M) | $^{app}K_m^{dns-GCVLM}$ ($\mu$M) |
| FPP | **1** | $0.8 \pm 0.1$ | $0.22 \pm 0.04$ | $0.30 \pm 0.06$ | $0.09 \pm 0.01$ |
| *o*-I | **2q** | $8 \pm 3$ | >10 | $1.1 \pm 0.1$ | $1.5 \pm 0.3$ |
| *o*-Et | **2w** | $7 \pm 1$ | $5 \pm 1$ | $3.6 \pm 0.9$ | $1.4 \pm 0.3$ |
| *o*-$CF_3O$ | **2z** | >10 | >10 | $2.76 \pm 0.08$ | $4.4 \pm 0.4$ |
| *o*-MeS | **2ac** | >10 | >10 | $4.4 \pm 0.6$ | $8 \pm 2$ |

residues to generate the dns-GCVIS and dns-GCVLM $Ca_1a_2X$ sequences would allow us to probe how the $a_2$- and X-residues affected reactivity. The $^{app}K_m^{peptide}$ for the dns-GCVIS and dns-GCVLM peptides with FPP and four *ortho*-substituted analogues was measured and compared with the results for dns-GCVLS and dns-GCVIM (Table 4). When both dns-GCVLS and dns-GCVIM $a_2$-residues were exchanged, there was a greater than 3-fold decrease in the $^{app}K_m^{peptide}$ with FPP as the cosubstrate. The decrease in $^{app}K_m^{peptide}$ (dns-GCVLS > dns-GCVIM $\cong$ dns-GCVIS > dns-GCVLM) indicates that the very small structural difference between the isomeric $a_2$-residues can strongly influence the peptide reactivity. The class of isoprenoid analogue (Table 3) that was reactive remained primarily dependent on the identity of the X-residue, with peptides ending in methionine reacting with more *ortho*-substituted analogues than the peptides ending in serine. The dependence of peptide specificity for reaction with isoprenoids on the $a_2$-, X-, and possibly the $a_1$-residues suggests that FPP analogues may be found that can selectively modify only a single $Ca_1a_2X$ sequence.

*AGPP Analogue Competition with FPP for dns-GCVLS Modification Depends on Isoprenoid Structure.* AGPP is competitive with FPP for FTase catalyzed modification of proteins both *in vitro* and in cell culture (*43*, *44*). The relative efficiency of peptide modification by an alternative isoprenoid substrate over FPP can be determined from the ratio of products formed in competition experiments between the transferable AGPP analogues, FPP and peptide. Product ratios from competition reactions between a subset of transferable AGPP analogues and FPP for modification of the dns-GCVLS peptide were measured by integration of the dansyl peak absorbance of the RP-HPLC separated products (Figure 6). Reaction of 1:1 ratios of AGPP and FPP with limiting dns-GCVLS peptide resulted in a 2:1 ratio of farnesyl to anilinogeranyl modified dns-GCVLS. The 2:1 product ratio was significantly different from the approximately 1.2:1 ratio predicted from the ratio of the previously reported apparent steady-state selectivity factors and apparent isoprenoid $K_m$ values ($^{app}k_{cat}/^{app}K_m^{AGPP} = 1.8 \pm 0.1\ \mu M^{-1}\cdot s^{-1}$, $^{app}K_m^{AGPP} = 46 \pm 3$ nM, $^{app}k_{cat}/^{app}K_m^{FPP} = 2.2 \pm 0.1\ \mu M^{-1}\cdot s^{-1}$, $^{app}K_m^{FPP} = 46 \pm 2$ nM) (*40*, *43*). These results suggest that the apparent selectivity factor ($^{app}k_{cat}/^{app}K_m^{AGPP}$) and isoprenoid $^{app}K_m$ are not useful for accurately predicting the ability of isoprenoid diphosphates to compete with each other for peptide modification. The complexity of the FTase mechanism implies that the apparent selectivity factor apparent $k_{cat}/K_m^{peptide}$ only predicts reactivity for competition reactions where either isoprenoid diphosphate or peptide binding is solely responsible for stimulating product release (Figure 2). However, steady-state conditions where only one mechanism of product release dominates are not easily achieved, and in practice, both mechanisms are likely to operate concurrently.

We found that all of the analogues tested for FPP competitiveness, with the exception of *o*-$CF_3O$-AGPP **2z**, were utilized to some extent by FTase in the presence of FPP (Figure 6). A number of analogues were as reactive as FPP, giving product ratios near 1:1. An even larger number of compounds were as competitive as the parent molecule AGPP. Surprisingly, *o*-F-AGPP **2b** was considerably more reactive than FPP in the competition reaction. With the exception of *o*-F-AGPP **2b**, the *meta*-substituted analogues were the most competitive followed by the *ortho*- and *para*-substituted analogues. For example, the catalytic efficiencies of *m*-CN-AGPP **2h** and *p*-CN-AGPP **2j** differ by a factor of 2 and are only 1.5- and 3-fold higher than FPP (Table 1). However, *m*-CN-AGPP **2h** effectively competed with FPP for modification of dns-GCVLS to give a nearly 1:1 product ratio, while *p*-CN-AGPP **2i** competed much more poorly, giving a 1:10 ratio of analogue to farnesyl modified products. These results parallel the improved dns-GCVLS transfer efficiency of the *meta*-substituted analogues (Table 1). These results indicate that the structure of the AGPP analogues is a significant factor in the transferability and competitiveness of the isoprenoid.

*AGPP Analogue Competition with FPP Is Also Dependent on CaaX Acceptor Sequence.* The variation seen in $^{app}K_m^{peptide}$ for the different peptides and competitiveness with FPP for dns-GCVLS modification for the *ortho*-substituted analogues

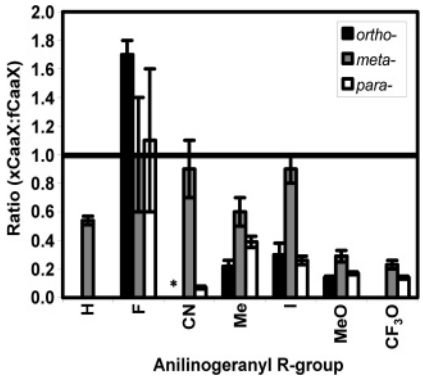


FIGURE 6: FPP competition with select analogues for modification of dns-GCVLS. Competition reactions between FPP and individual analogues were carried out as described under Experimental Procedures. Products from reactions of 3 $\mu$M peptide, 6.7 $\mu$M analogue, and 6.7 $\mu$M FPP were separated by RP-HPLC and quantified by integration of dansyl absorbance at 340 nm. Due to confounding absorption at 340 nm by the *meta*- and *para*-CN-AGPP, analogue product formation was estimated from the difference between the input peptide and the amount of farnesylated product. Ratios were calculated from the integrated absorbance of analogue and FPP modified peptide products. (*) *ortho*-CN-AGPP was not prepared.

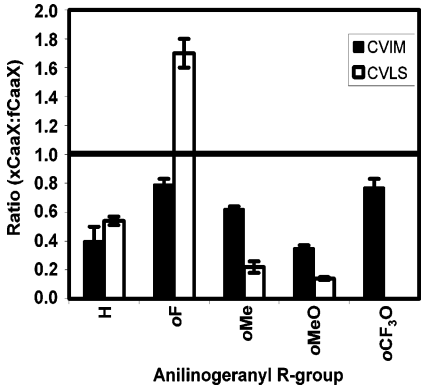


FIGURE 7: FPP competition is peptide and isoprenoid dependent. Ratio of products from competition reactions containing 6.7 $\mu$M FPP, 6.7 $\mu$M analogue, and 3 $\mu$M either dns-GCVIM or dns-GCVLS as the isoprenoid acceptor substrate. Ratios are reported as ratio of analogue modified to farnesylated peptide. Note that *o*-CF3O-AGPP **2j** is competitive with FPP only when dns-GCVIM is the target.

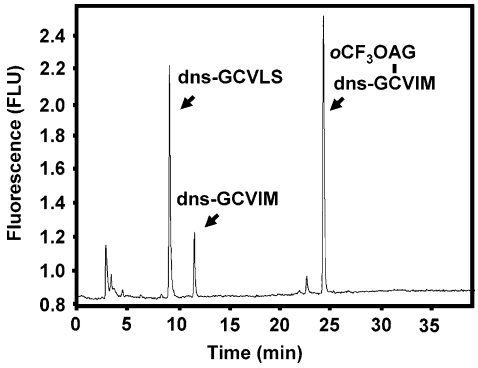


FIGURE 8: Selective modification of the peptide dns-GCVIM by *o*-$CF_3O$-AGPP **2j**. Peaks corresponding to products from the fluorescence detected RP-HPLC chromatogram for the reaction between dns-GCVIM (1.5 $\mu$M), dns-GCVLS (1.5 $\mu$M), *o*-$CF_3O$-AGPP **2j** (16 $\mu$M), and FTase are indicated. The reaction products were quantified using the dansyl group absorbance. Similar results were obtained with 3 $\mu$M concentrations of both peptides.

reinforces the idea that FTase peptide selectivity may depend on both isoprenoid structure and $Ca_1a_2X$ sequence. We found that the product ratio from reactions of a 1:1 mixture of AGPP and four *ortho*-substituted analogues with FPP and limiting dns-GCVIM peptide was different from that of dns-GCVLS and dependent on the analogue structure (Figure 7). The parent AGPP competed with FPP with similar effectiveness when either dns-GCVLS or dns-GCVIM was the peptide target (Figure 7). Interestingly, the *o*-$CF_3O$-AGPP **2z**, *o*-Me-AGPP **2m**, and *o*-MeO-AGPP **2t** analogues competed more effectively with FPP for dns-GCVIM compared with dns-GCVLS, while *o*-F-AGPP **2b** competed less efficiently.

*o-$CF_3O$-AGPP and o-MeS-AGPP Are Highly Selective for the dns-GCVIM Peptide.* The analogues *o*-$CF_3O$-AGPP **2z** and *o*-MeS-AGPP **2ac** were only reactive with dns-GCVIM and inhibited farnesylation of the other peptides examined (Tables 2 and 3, Figures 3, 4, 5). These results indicated that the analogues might selectively react with dns-GCVIM in the presence of other peptides. Analysis of competition reactions between peptide pairs dns-GCVIM and dns-GCVLS or dns-GCVIM and dns-GCAHQ with either *o*-$CF_3O$-AGPP **2z** or *o*-MeS-AGPP **2ac** by RP-HPLC showed that only the dns-GCVIM peptide was modified at the detection limits of the assay (<5%). A representative dansyl fluorescence detected chromatogram for *o*-$CF_3O$-AGPP **2z** is shown in Figure 8. These observations are consistent with the finding that the dns-GCVLS peptide is not a good cosubstrate for the *o*-$CF_3O$-AGPP **2z** or *o*-MeS-AGPP **2ac** analogues. The competition reaction rate of *o*-$CF_3O$-AGPP **2z** with dns-GCVIM and dns-GCVLS was the same as that of *o*-$CF_3O$-AGPP **2z** with dns-GCVIM alone, indicating that the dns-GCVLS had very little effect on the rate of dns-GCVIM modification. Reaction of dns-GCVIM with *o*-$CF_3O$-AGPP **2z** was not inhibited by dns-GCVLS concentrations (15 $\mu$M) up to five times that of dns-GCVIM (3 $\mu$M) nor was any modified dns-GCVLS product observed (data not shown). Gibbs and co-workers reported that slow reactivity of 3-MeBFPP **3** with dns-GCKVL is relieved by the addition of dns-GCVLS (*41*). Unfortunately, the products resulting from the reaction of 3-MeBFPP **3**, dns-GCKVL, and dns-GCVLS were not determined (*41*). A similar experiment was performed to determine if dns-GCVIM could relieve FTase inhibition by the *o*-$CF_3O$-AGPP **2z** analogue. dns-GCVLS, FPP, *o*-$CF_3O$-AGPP **2z**, and FTase were combined and allowed to react for 10 min, during which time only a negligible increase in fluorescence intensity was observed. dns-GCVIM was then added to the reaction mixture and the fluorescence intensity immediately increased to the rate previously observed for reaction of dns-GCVIM with FPP and *o*-$CF_3O$-AGPP **2z**. The relief of FTase inhibition by the dns-GCVIM peptide indicated that the *o*-$CF_3O$-AGPP·dns-GCVLS noncovalent complex or the *o*-$CF_3O$-AGPP modified dns-GCVLS product was not bound to FTase irreversibly. Inhibition of dns-GCVLS or dns-GCAHQ farnesylation by *o*-$CF_3O$-AGPP **2z** is therefore possibly due to simple occupation of the active site by the analogue. FTase binds isoprenoid diphosphates tightly, and the enzyme·FPP complex is highly committed to catalysis, suggesting that any FPP bound to the enzyme will rapidly react with dns-GCVLS or dns-GCAHQ (*50*, *51*). The rate determining step in the FTase reaction is product release, which is normally enhanced by binding of additional substrate molecules. Any product release stimulated by *o*-$CF_3O$-AGPP **2z** binding would inhibit dns-GCVLS or dns-GCAHQ farnesylation by forming an FTase·*o*-$CF_3O$-AGPP complex unreactive with dns-GCVLS or dns-GCAHQ. Therefore, addition of a second, reactive peptide should relieve *o*-$CF_3O$-AGPP **2z** inhibition of FTase by allowing the active site to be cleared. However, it was not clear from this experiment what products were formed and whether dns-GCVLS farnesylation was still inhibited after the addition of the dns-GCVIM peptide.

*o-$CF_3O$-AGPP Inhibits dns-GCVLS and dns-GCAHQ Farnesylation Only When dns-GCVIM Is Not Present.* The separate observations that *o*-$CF_3O$-AGPP **2z** reacts poorly with and inhibits both dns-GCVLS and dns-GCAHQ farnesylation, as well as efficiently modifying dns-GCVIM in the presence of dns-GCVLS, dns-GCAHQ, or FPP, suggests that the analogue may be dns-GCVIM $Ca_1a_2X$ sequence selective. A four substrate FTase competition reaction between FPP, *o*-$CF_3O$-AGPP **2z**, dns-GCVIM, and dns-GCVLS or dns-GCAHQ was performed and stopped before complete consumption of either peptide. Analysis of the reaction mixture by RP-HPLC showed that both peptides in each reaction mixture were farnesylated, but that only the dns-

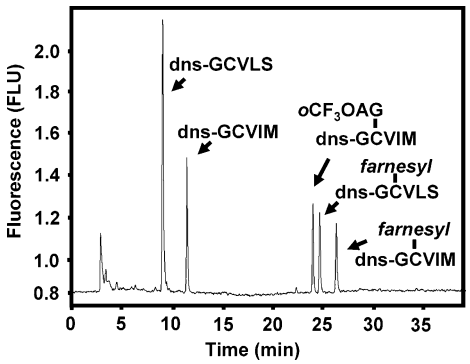


FIGURE 9: dns-GCVLS farnesylation inhibition by *o*-$CF_3$O-AGPP **2j** is relieved by the addition of the catalytically competent peptide substrate dns-GCVIM. Peaks corresponding to products from the fluorescence detected RP-HPLC chromatogram for the reaction between dns-GCVIM (1.5 $\mu$M), dns-GCVLS (1.5 $\mu$M), *o*-$CF_3$O-AGPP **2j** (16 $\mu$M), FPP (16 $\mu$M), and FTase are indicated. The reaction products were quantified using the dansyl group absorbance. Similar results were obtained with 3 $\mu$M concentrations of both peptides.

GCVIM peptide was modified with *o*-$CF_3$O-AGPP **2z** at the detection limits of the assay (<5%) (Figure 9). As noted above, the ratio of farnesylated-dns-GCVIM to farnesylated-dns-GCVLS was 3:1 in a dns-GCVIM/dns-GCVLS competition reaction with FPP. Interestingly, the ratio of farnesylated-dns-GCVIM to farnesylated-dns-GCVLS to *o*-$CF_3$O-AG modified dns-GCVIM was 1:1:1 in the four component reaction. The ratio of farnesylated-dns-GCVIM to farnesylated-dns-GCAHQ to *o*-$CF_3$O-AG modified dns-GCVIM was 2:1:1 in the four component reaction. These results indicate that the presence of the catalytically competent dns-GCVIM peptide in the four substrate reaction relieved inhibition of dns-GCVLS and dns-GCAHQ farnesylation. Importantly, *o*-$CF_3$O-AGPP **2z** only inhibited farnesylation of dns-GCVLS or dns-GCAHQ when a catalytically competent peptide was not present. These results demonstrate the selective modification of a single $Ca_1a_2X$ sequence in the presence of another by an FPP analogue and indicate that FTase inhibition by an analogue that is unreactive with one peptide can be relieved by addition of a second, reactive peptide to form isoprenoid modified product(s).

## DISCUSSION

We examined the effect of altered isoprenoid structure on FTase catalyzed transfer to $Ca_1a_2X$ peptides and found that relatively small structural changes in the analogues altered the peptide substrate selectivity and the product distribution from competition reactions with FPP and various combinations of peptides. Most notably, the *o*-$CF_3$O-AGPP **2z** analogue reacts selectively with dns-GCVIM in the presence of alternative $Ca_1a_2X$ sequences and FPP. The limited reactivity of *o*-$CF_3$O-AGPP **2z** provides insight into the mechanism of FTase substrate selectivity. Product release is the rate determining step of the FTase reaction mechanism for reactions between FPP and dns-GCVLS (Figure 2), and both FPP (path A) and the $Ca_1a_2X$ peptide (path B) are effective at stimulating product release from the E·product complex (*47*). The very slow reaction between dns-GCVLS peptide and *o*-$CF_3$O-AGPP **2z** substrates suggests that neither is effective at stimulating product release from the FTase·*o*-$CF_3$O-AG-dns-GCVLS complex. Alternatively, the reaction is slow because dns-GCVLS is unable to bind the FTase·*o*-$CF_3$O-AGPP complex in a productive fashion. Addition of FPP does not stimulate formation of *o*-$CF_3$O-AG modified dns-GCVLS. Only inclusion of the catalytically competent dns-GCVIM peptide leads to relief of *o*-$CF_3$O-AGPP **2z** inhibition of dns-GCVLS farnesylation with concomitant formation of *o*-$CF_3$O-AG modified dns-GCVIM product. The very slow reaction of dns-GCVLS with *o*-$CF_3$O-AGPP **2z** is consistent with the inability of either the dns-GCVLS peptide, FPP, or *o*-$CF_3$O-AGPP **2z** to stimulate *o*-$CF_3$O-AG modified dns-GCVLS release. The observed product distributions from the various three and four component competition reactions are also consistent with a prechemical step discrimination between the dns-GCVIM and dns-GCVLS peptides by the E·*o*-$CF_3$O-AGPP complex. In either case, FTase inhibition would be relieved by the addition of a more competent peptide to stimulate product release or to react with *o*-$CF_3$O-AGPP **2z**. If there is no discrimination between dns-GCVIM and dns-GCVLS prior to the chemical step, addition of dns-GCVIM to the dns-GCVLS, FPP, and *o*-$CF_3$O-AGPP **2z** reaction would be expected to lead to an increase in *o*-$CF_3$O-AG-dns-GCVLS formation. However, no difference in the amount of *o*-$CF_3$O-AG modified dns-GCVLS was observed in the four component reaction relative to products from the reaction of dns-GCVLS, FPP, and *o*-$CF_3$O-AGPP **2z**. The selective reactivity of *o*-$CF_3$O-AGPP **2z** suggests that interference with peptide binding may not be the only mechanism of farnesyl transferase inhibition. X-ray crystallographic analysis shows that the isoprenoid C-1 must move 5.4 Å toward the $Ca_1a_2X$ cysteine thiolate in the enzyme·isoprenoid·$Ca_1a_2X$ complex in order to form the product thioether bond (*46*). The position of the $\omega$-isoprenoid unit of the farnesylated product is unchanged relative to its position in the enzyme·isoprenoid·$Ca_1a_2X$ complex. However, translocation of C-1 is accompanied by a 180° rotation of the second isoprenoid unit with respect to the $\omega$-isoprene of the lipid chain (*46*). Therefore, $Ca_1a_2X$ peptide dependent steric interactions between *ortho*-substituents on the aniline ring and the second isoprene may interfere with the bond rotations in the prenyl group required to achieve a reactive conformation. These results indicate that the observed substrate discrimination is most likely due to a prechemical step discrimination between the dns-GCVIM and dns-GCVLS peptides by the E·*o*-$CF_3$O-AGPP complex.

Based on the observation that 3-MeBFPP **3** reacts slowly with and inhibits the farnesylation of the CKVL petptide, Gibbs and co-workers have proposed that suitable FPP analogues may be able to selectively inhibit farnesylation of one $Ca_1a_2X$ sequence in the presence of others (*41*). Our results suggest that it will be very difficult to design a transferable isoprenoid diphosphate that acts as a $Ca_1a_2X$ selective FTI. Isoprenoid diphosphate analogues acting as competitive inhibitors can only inhibit farnesylation of a particular peptide when it is in the active site of FTase, either in a noncovalent complex or as a tightly bound product. In analogy with the behavior of *o*-$CF_3$O-AGPP **2z** described above, the very slow reaction between dns-GCKVL peptide and 3-MeBFPP **3** suggests that neither substrate is effective at stimulating product release from the FTase·3-MeBF-dns-GCKVL complex or because dns-GCKVL is unable to bind the FTase·3-MeBFPP complex in a productive fashion. In light of the results presented here, the dramatic stimulation

of the reaction between dns-GCKVL peptide and 3-MeBFPP **3** by dns-GCVLS suggests that the second peptide relieves inhibition by promoting the release of the 3-MeBF-dns-GCKVL product to give the E·dns-GCVLS complex (path B, Figure 2), and/or reacts with the isoprenoid diphosphate to form 3-MeBF-dns-GCVLS. In either case, the dns-GCVLS clears the 3-MeBFPP **3** analogue from the FTase active site, providing an opportunity for FPP to bind the enzyme. Once the FTase·FPP complex has been formed, it becomes possible to farnesylate the CKVL peptide, providing it can outcompete any other available $Ca_1a_2X$ peptides. This scheme is likely to be operational for any transferable isoprenoid diphosphate, except if the analogue were able to completely outcompete FPP for binding to FTase.

Gibbs and co-workers also proposed that "it may be possible to design or discover FPP analogues that allow for the selective modification of only a single $Ca_1a_2X$ box protein in a cell" (*41*). Indeed, a number of AGPP analogues presented here have properties that suggest that selective modification of a single $Ca_1a_2X$ motif with an unnatural analogue is possible. It may be possible to selectively modify a target peptide with an alternative ligand if the analogue is sufficiently unreactive with other $Ca_1a_2X$ peptides and can outcompete the endogenous levels of FPP. Recently, Krzysiak et al. reported on the CaaX peptide substrate selectivity of a class of FPP analogues structurally different from those described here. Interestingly, they also found little clear correlation between reactivity and the structures of the analogues and peptides. Further modification of the AGPP structure may lead to analogues that have enhanced selectivity for other $Ca_1a_2X$ sequences as well as increased reactivity in competition with FPP.

The results reported here indicate that analogue selectivity, competitiveness, and reactivity depend both on small changes in structure of the $\omega$-isoprene of FPP and on the target $Ca_1a_2X$ sequence. For example, analogues with an *ortho*-substituent approximately the size of a thiomethyl or trifluoromethoxy group are selective for the dns-GCVIM $Ca_1a_2X$ sequence and small *meta*-substituents such as cyano provide analogues competitive with FPP for modification of dns-GCVLS. We have recently shown that transferable analogues of FPP with reduced lipid hydrophobicity block H-Ras function in a *Xenopus* oocyte model system (*41*). Taken together, these results are a step toward chemical tools to prepare pharmacological dominant negative phenotypes based on selective modification of $Ca_1a_2X$ boxes with FPP analogues that impair normal protein function. Such analogues could provide a level of specificity to inhibition of prenyl group function that is not possible using currently available FTIs.

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