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Farnesyl Diphosphate Analogues with ω-Bioorthogonal Azide and Alkyne Functional Groups for Protein Farnesyl Transferase-Catalyzed Ligation Reactions

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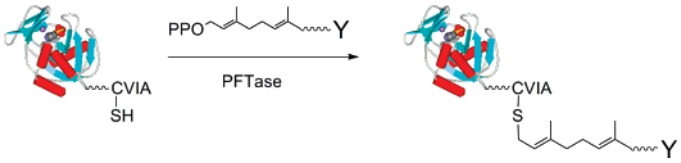

Eleven farnesyl diphosphate analogues, which contained ω-azide or alkyne substituents suitable for bioorthogonal Staudinger and Huisgen [3 + 2] cycloaddition coupling reactions, were synthesized. The analogues were evaluated as substrates for the alkylation of peptide cosubstrates by yeast protein farnesyl transferase. Five of the diphosphates were good alternative substrates for farnesyl diphosphate (FPP). Steady-state kinetic constants were measured for the active compounds, and the products were characterized by HPLC and LC-MS. Two of the analogues gave steady-state kinetic parameters (k_{cat} and K_m) very similar to those of the natural substrate.

Introduction

Post-translational modification of proteins to append isoprenoid chains to enhance their association with membranes is required in a variety of important biological processes, including signal transduction pathways controlling cell growth and differentiation, cytoskeletal rearrangement, membrane rearrangement during cellular division, vision, and vesicular transport. Approximately 1% of mammalian proteins is modified at a C-terminal cysteine residue by C_{15} farnesyl or C_{20} geranylgeranyl groups.[1] Among the prenylated proteins that have been identified are nuclear lamins, the γ-subunit of heterotrimeric small G proteins such as transducin, the Ras super family of small G proteins, and enzymes such as protein tyrosine phosphatases, inositol polyphosphatases, and phospholipase A.

Several prenylated proteins are implicated in human diseases. Ras proteins function as an on/off switch to regulate a variety of cellular functions, including proliferation. In approximately 30% of human cancers, mutations in Ras compromise its ability to hydrolyze GTP to GDP, and the persistently active GTP-bound protein contributes to the development of cancer.[2,3] Ras proteins are farnesylated on the cysteine sulfur of their C-terminal CAAX box, where A is often an aliphatic residue and X is typically either serine or methionine (alanine, glutamine, threonine, and, in certain cases, leucine can also serve as the X residue). Farnesyl transferase inhibitors have been advanced to clinical trials as anticancer agents.[4,5]

Recent reports suggest that the nuclear blebbing seen in cells from patients with Hutchinson−Gilford progeria syndrome (HGPS) results from a mutation that prevents maturation of lamin A by blocking proteolytic cleavage of a farnesylated C-terminal 15 amino acid peptide.[6,7] Protein prenylation has also been identified as a target for antiparasitic agents.[8,9] Protein

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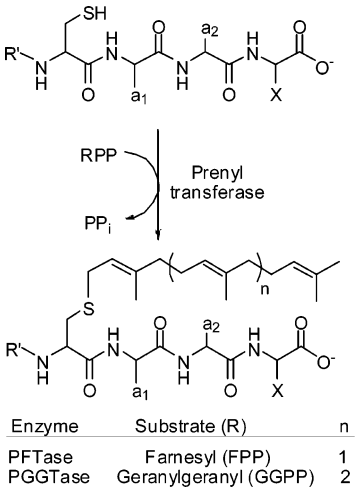
farnesyl transferase (PFTase) inhibitors have shown a high potency against the parasites responsible for malaria (*Plasmodium falciparum*)[10,11] and the Chagas' disease parasite *Trypanosoma cruzi*.[12,13]

The modification reactions are catalyzed by three different protein prenyl transferases: PFTase (EC 2.5.1.58), protein geranylgeranyltransferase-I (PGGTase-I, EC 2.5.1.59), and Rab (a Ras-like protein) geranylgeranyltransferase (RabGGTase or PGGTase-II, EC 2.5.1.60). The closely related PFTase and PGGTase-I transfer prenyl groups from prenyl diphosphates to proteins containing a C-terminal CAAX motif (also known as a CAAX box), where C is cysteine, A is usually a small aliphatic amino acid, and X can be a variety of amino acids (Figure 1).



Enzyme	Substrate (R)	n
PFTase	Farnesyl (FPP)	1
PGGTase	Geranylgeranyl (GGPP)	2

FIGURE 1. Reactions catalyzed by PFTase and PGGTase-I.

The X residue determines whether a farnesyl (X = A, S, C, M, Q) or a geranylgeranyl (X = L, F) is added.[14]

The rational design of farnesyl diphosphate (FPP) analogues for PFTase with specific functions has been facilitated by the X-ray crystal structures for rat[15] and human[16] PFTases. Spielmann and co-workers reported that incorporation of an aniline moiety at the location of the ω-isoprene unit of FPP resulted in a transferable analogue.[17] Recently, successful incorporation of this aniline-geranyl diphosphate (GPP) (AGPP) analogue was monitored in HEK-293 cells by the use of antibodies raised against the FPP analogue.[18] Photoaffinity analogues of FPP incorporating a benzophenone moiety or a functionalized aniline moiety were developed by the Distefano and Spielmann groups, respectively.[19−21] FPP analogues appended with fluorescent anthranilate esters were developed by Waldmann and co-workers.[22,23] Wiemer and co-workers reported the synthesis of N-alkylated derivatives of GPP as fluorescent labels that are resistant to esterases.[24] Those compounds were subsequently effectively transferred to peptides and proteins. Distefano and co-workers recently reported that alkynyl ether derivatives of GPP are alternative substrates that can be tethered to other biomolecules after farnesylation.[25] Prestwich and Liu synthesized a conjugated geranylgeranyl diphosphate (GGPP) derivative ($\Delta\Delta$GG) with a conjugated olefinic fluorophore.[26]

Incorporation of a bioorthogonal functional group[27,28] into the FPP structure provides a technique for modifying proteins for subsequent tethering and analysis. We focused our attention on the Staudinger ligation and the Cu(I)-catalyzed Huisgen cycloaddition (click reaction), both of which have been used in vivo. A version of the Staudinger ligation, introduced by Bertozzi and Saxon,[29] involves intramolecular trapping of a phosphine/azide adduct eventually to give a stable amide linkage. The click ligation, introduced by Sharpless and coworkers,[30,31] is a Cu(I)-catalyzed [2 + 3] cycloaddition reaction between azide and terminal alkyne to produce a 1,2,3-triazole. We recently reported that proteins derivatized with suitably functionalized analogues of FPP could be readily immobilized on glass slides.[32] Related approaches were recently reported from the laboratories of Distefano and coworkers[33] and Zhao and coworkers[34] We now report the synthesis of a family of azido- and alkyne-substituted FPP analogues and their ability to function as alternative substrates for yeast PFTase.

Results and Discussion

Synthesis. We designed a series of azido and alkyne analogues as reagents with which to modify proteins for Huisgen and Staudinger ligations (Figure 2). All of the analogues have

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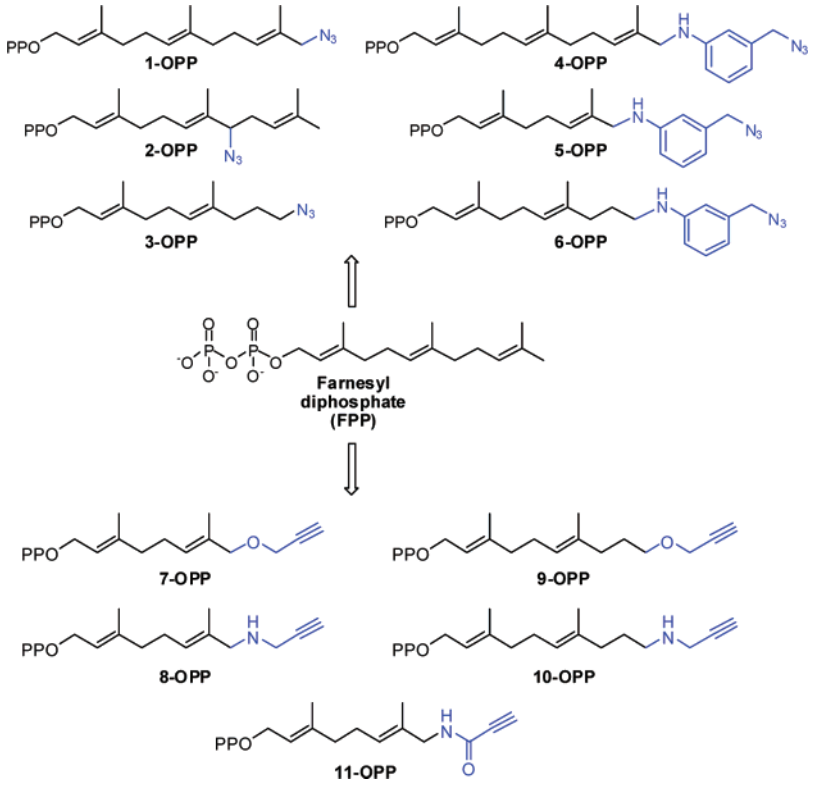
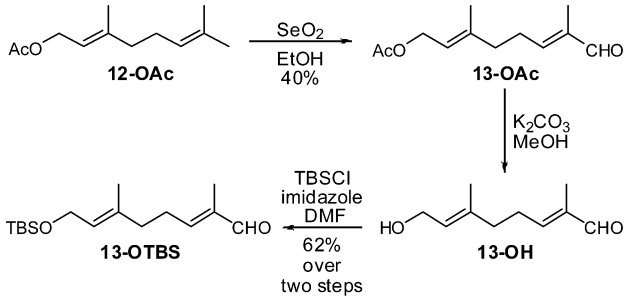


FIGURE 2. Structures of azido and alkyne FPP analogues.

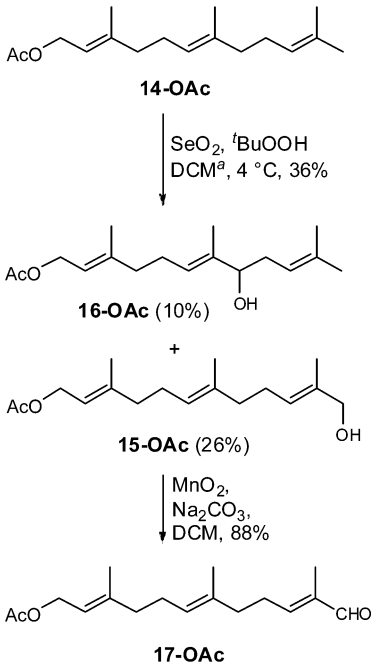
SCHEME 1. Synthesis of Aldehydes 13-OAc and 13-OTBS



a trisubstituted allylic diphosphate moiety in the first isoprene unit, a requirement for all prenyl transferase reactions. Azido-substituted analogues **1-OPP** and **4-OPP** contain a farnesyl unit, while **3-OPP**, **5-OPP**, and **6-OPP** contain a geranyl unit. Alkyne-substituted analogues include **7-OPP** and **9-OPP** with a propargyl ether moiety and **8-OPP** and **10-OPP** with propargyl amine. **11-OPP** contains an amide-linked alkyne unit. Analogues **1-OPP**, **2-OPP**, and **4-OPP** are derivatives of FPP, while the remaining compounds are derivatives of GPP substituted at the *E*-ω-methyl group. In general, the analogues were prepared from geranyl or farnesyl acetate by oxidation of the isoprenoid chain followed by subsequent modification of the oxidized carbon.

Aldehyde **13-OAc** was obtained from geranyl acetate (**12-OAc**) by oxidation with SeO_2/EtOH followed by hydrolysis as shown in Scheme 1.[26] Protection of the hydroxyl group with TBSCl gave **13-OTBS**. A similar oxidation of farnesyl acetate (**14-OAc**) by ^{t}BuOOH/SeO_2 gave 8-hydroxy- and 12-hydroxy-farnesyl acetate (**15-OAc** and **16-OAc**, respectively) in an overall yield of 40% along with 33% of unreacted farnesyl acetate (Scheme 2).[35,36] Alcohol **15-OAc** was oxidized to aldehyde **17-OAc** by treatment with MnO_2 under basic conditions that minimized isomerization of the ω-double bond.[37] Treatment of **14-OAc** with NBS followed by K_2CO_3/MeOH gave the ω-epoxide but hydrolyzed the acetate group, which

SCHEME 2. Synthesis of Alcohols 15-OAc and 16-OAc and Aldehyde 17-OAc



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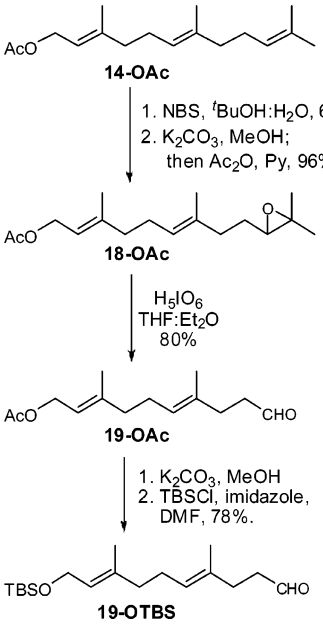
SCHEME 3. Synthesis of Aldehydes 19-OAc and 19-OTBS

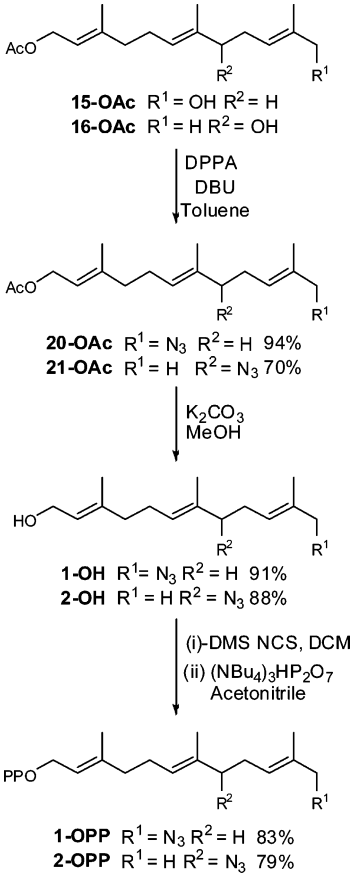


was subsequently reintroduced under standard conditions.[38] Epoxy acetate **18-OAc** was treated with periodic acid[39] to give aldehyde **19-OAc**. This aldehyde was converted to silyl-protected **19-OTBS** by hydrolysis and treatment with TBS chloride (Scheme 3).

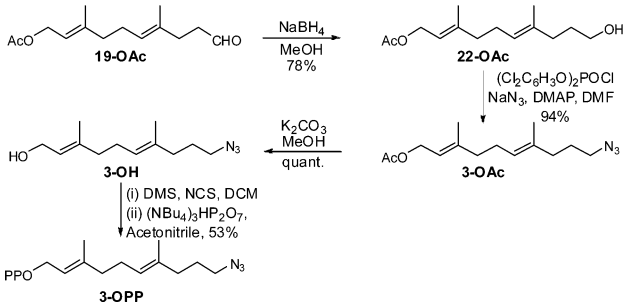
Syntheses of azido analogues **1-OPP** and **2-OPP** are outlined in Scheme 4. Allylic alcohols **15-OAc** and **16-OAc** were converted to the corresponding azides **20-OAc** and **21-OAc**, respectively, by treatment with diphenylphosphorylazidate (DPPA) according to the procedure of Thompson and co-workers.[40] The acetate group was then hydrolyzed, and the resulting alcohols were phosphorylated as described by Davisson et al.[41,42] to give diphosphates **1-OPP** and **2-OPP**. **1-OPP** and **2-OPP** were obtained as 1:1 and 2:1 mixtures of their allylic isomers, respectively. Allylic azides undergo a 1,3 rearrangement at room temperature,[43] and isomerization is seen when azides are synthesized from allylic alcohols.[44,45]

The synthesis of azido analogue **3-OPP** is shown in Scheme 5. Aldehyde **19-OAc** was reduced with sodium borohydride to give alcohol **22-OAc**. When **22-OAc** was treated with DPPA, the phosphate intermediate was not displaced by azide. We then resorted to Hu's method,[46] which uses a more reactive 2,4-dichlorophenylphosphate leaving group. When alcohol **22-OAc** was treated with bis-(2,4-dichlorophenyl)phosphoryl chloride, DMAP, and sodium azide, compound **3-OAc** was obtained in high yield. The acetate was hydrolyzed with K_2CO_3, and alcohol **3-OH** was phosphorylated as described for **1-OPP**.

SCHEME 4. Synthesis of Azide Analogues 1-OPP and 2-OPP



SCHEME 5. Synthesis of Azide Analogue 3-OPP



Benzyl azido analogues **4-OPP**, **5-OPP**, and **6-OPP** were prepared by the four-step sequence shown in Scheme 6. Reductive amination of aldehydes **19-OAc**, **17-OAc**, and **13-OAc** by treatment with 3-aminobenzyl alcohol and sodium triacetoxyborohydride[21,24,47,48] gave good yields of amino alcohols **23-OAc**, **24-OAc**, and **25-OAc**, respectively. The benzylic hydroxyl groups were displaced by azide using the Thompson procedure,[40] followed by acetate hydrolysis and phosphorylation to provide diphosphates **4-OPP**, **5-OPP**, and **6-OPP**. Diphosphates **5-OPP** and **6-OPP** were purified by chromatography on cellulose and characterized. Diphosphate **4-OPP** was unstable under these conditions. While we were able to obtain an exact mass for the compound by HRMS, NMR spectra were not suitable for characterization.

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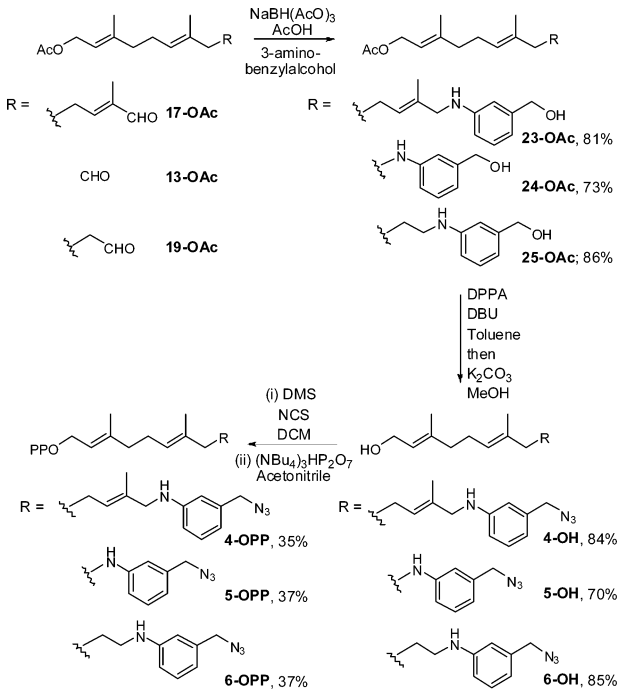
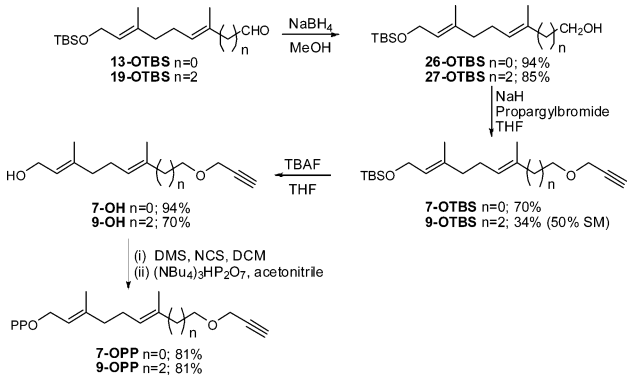
SCHEME 6. Synthesis of Azide Analogues 4-OPP, 5-OPP, and 6-OPP



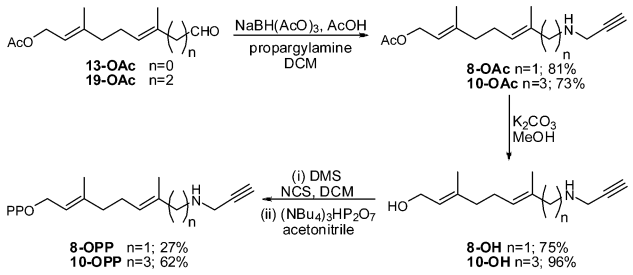
SCHEME 7. Synthesis of Alkynyl Analogues 7-OPP and 9-OPP



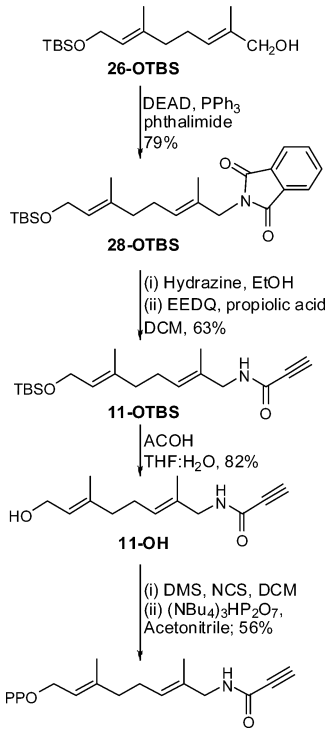
Propargyl ethers **7-OPP** and **9-OPP** were synthesized from aldehydes **13-OTBS** and **19-OTBS** as outlined in Scheme 7. The aldehydes were reduced with sodium borohydride to give alcohols **26-OTBS** and **27-OTBS**, respectively. Propargyl ether **7-OTBS** was prepared by treatment with sodium hydride followed by the addition of propargyl bromide. The same procedure gave low yields of **9-OTBS** from **27-OTBS**. After several attempts, the best yield of the propargyl ether was obtained when potassium hydride was used to deprotonate the alcohol. Half of the starting alcohol was recovered despite using rigorously anhydrous conditions and longer reaction times. The TBS groups were then removed with tetrabutylammonium fluoride (TBAF), and the resulting alcohols were phosphorylated as described for the azido analogues to give ethers **7-OPP** and **9-OPP**.

Propargyl amines **8-OPP** and 1**0-OPP** were prepared by reductive amination of aldehydes **13-OAc** and **19-OAc** with propargyl amine using conditions described previously for **23-OAc**, **24-OAc**, and **25-OAc** (Scheme 8). Hydrolysis of the acetate groups, followed by phosphorylation of the resulting

SCHEME 8. Synthesis of Alkynyl Analogues 8-OPP and 10-OPP



SCHEME 9. Synthesis of Alkynyl Analogue 11-OPP



alcohols, gave modest to good yields of diphosphates **8-OPP** and **10-OPP**.

Amide analogue **11-OPP** was synthesized from alcohol **26-OTBS** as outlined in Scheme 9. The hydroxyl group was replaced with a phthalimide moiety using Mitsunobu conditions to give **28-OTBS**,[49,50] which was then treated with hydrazine hydrate followed by propiolic acid in the presence of 1,2-dihydro-2-ethoxy-1-quinolinecarboxylic acid (EEDQ)[51] to give **11-OTBS**. The TBS group was removed with acetic acid in THF/H_2O, and the resulting alcohol was phosphorylated to give **11-OPP** in moderate yield.

Kinetic Studies with Yeast PFTase. Recombinant yeast PFTase from an *Escherichia coli* clone was purified by affinity chromatography on a Ni-NTA column as previously reported.[52] Details of the enzymatic assays are provided in the Supporting

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TABLE 1. Steady-State Kinetic Parameters of Active Substrates

compound	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($s^{-1}\,\mu M^{-1}$)	$(k_{cat}/K_m)_{rel}$[a]
FPP	1.31 (0.04	1.71 (0.04	0.77	1
1-OPP	0.45 (0.01[b]	2.12 (0.15	0.21	0.28
3-OPP	1.10 (0.05	0.67 (0.10	1.6	2.1
5-OPP	0.35 (0.01	1.05 (0.22	0.33	0.43
6-OPP	0.48 (0.01	4.23 (0.47	0.11	0.15
9-OPP	1.06 ± 0.03	1.90 ± 0.22	0.55	0.72

[a] V_{rel} refers to k_{cat}/K_m with respect to FPP. [b] Rate corresponds to the incorporation of both isomers as was shown by HPLC.

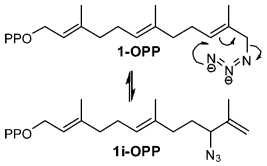
Information. Preliminary studies indicated that analogues **1-OPP**, **3-OPP**, **5-OPP**, **6-OPP**, and **9-OPP** were good to excellent alternative substrates for the enzyme. The other analogues were less active and were not characterized further.

Steady-state kinetic parameters for the most active analogues with dansyl-GCVIA (Dn-GCVIA) as a cosubstrate are summarized in Table 1. Two other groups[34,53] reported that **1-OPP** was a substrate for PFTase, although a full set of kinetic parameters was not presented. We found that the V_{max} value for the 1:1 mixture of **1-OPP** and its allylic isomer (**1i-OPP**, Scheme 10) was lower than for FPP, although their K_m values are similar. The rapid interconversion of the ω-allylic azides (see Scheme 10) prevents us from obtaining individual kinetic constants for the two isomers. The terminal alkyl azide, **3-OPP**, is an excellent substrate with a k_{cat} value almost equal to that of FPP and a K_m value that is substantially lower, resulting in a 2.1-fold enhancement in catalytic efficiency (k_{cat}/K_m) over the natural substrate. Clearly, the linear carbon chain in **3-OPP** terminated by an azide moiety is a good surrogate for the ω-prenyl unit in FPP. A similar observation was recently reported by Nguyen et. al for an azidopropyl ether analogue of FPP.[54]

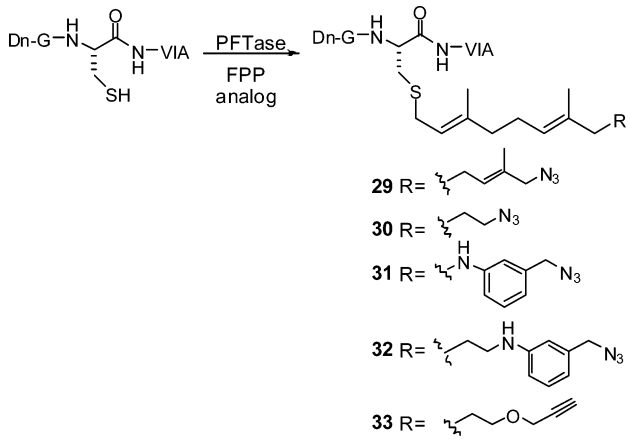
Our results for **5-OPP** are similar to those reported by the Spielmann group for a related p-substituted nitroaniline derivative with rat PFTase, where $(k_{cat}/K_m)_{rel}$ was 0.42.[21] The catalytic efficiency of analogue **6-OPP**, with a substantially larger volume in the region of the ω-isoprene unit than **3-OPP**, was 14-fold lower as the result of both a smaller k_{cat} and a larger K_m. Although phenyl groups are used as surrogates for the prenyl moiety,[55] we found that the analogues with linear side chains were better alternative substrates. Analogue **9-OPP** was the only propargyl derivative with a good kinetic profile, with a catalytic efficiency of 72% of FPP.

Analogues **2-OPP**, **4-OPP**, **7-OPP**, **8-OPP**, **10-OPP**, and **11-OPP** were substantially less reactive or inactive with yeast PFTase in our assay. Most likely, these compounds are less compatible with the active site of the enzyme because of branching along the chain or bulk in the chain at the location of the ω-isoprenoid unit in FPP.[15,16,56] We were somewhat surprised that propargyl ether **7-OPP** derived from a geranyl scaffold was a poor substrate in view of a previous report that related that benzyl ethers were good analogues for human PFTase.[55] Recently, Distefano and coworkers used **7-OPP** as a substrate to tag proteins, although the rate of farnesylation by the analogue in their assay was poor (~0.08 of the rate with FPP under the same conditions).[25] The lack of activity of propargylamino analogues **8-OPP** and **10-OPP** is understandable given the poor behavior of **7-OPP** and the presence of a charged amino group in the isoprenoid chain at the pH of the assay. Both compounds share the same structure except for the heteroatom. Propiolamide analogue **11-OPP** is also not active. In addition to being constructed on a geranyl scaffold, the rigidity imposed by the conjugation of the propiolamide may be incompatible with the active site of PFTase. The same behavior has been seen observed for a pentaene analogue of FPP that is not a good substrate for PFTase.[57] On the other hand, the α-azido acetamide analogue reported by Waldmann and coworkers,[54] which does not have restricted rotation, is transferred 37 times more slower that the natural substrate, suggesting that the alkylamide moiety is also not optimal.

SCHEME 10. 1-OPP Azide Equilibrium



SCHEME 11. Farnesylation of DansylGCVIA with FPP Analogues



Characterization of Prenylated Peptides by HPLC and LC-MS. The products from prenylation of Dn-GCVIA by each of our five active analogues were established using procedures similar to those previously reported (see Supporting Information).[58] The modified peptides (see Scheme 11) were characterized by HPLC and LC-MS. In each case, the HPLC retention time and the mass of the prenylated peptide (Table 2) were

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TABLE 2. HPLC Retention Times for Dn-GCVIA and Dn-GC(Far)VIA Peptides and LC-MS Molecular Ions for the Products

compound	retention time (min)	LC-MS ($M^+ + H$)
Dn-GCVIA	4.9	695.3
29	19.3	940.5
30	17.5	900.3
31	14.4	977.4
32	14.8	1005.5
33	16.46	913.6

consistent with alkylation of the cysteine residue in Dn-GCVIA by the appropriate analogue. Interestingly, the LC-MS trace for **29** had two closely spaced peaks with the same mass. This suggests that both regioisomers of **1-OPP** were incorporated into the peptide or that a single isomer, which subsequently rearranged to its allylic isomer, was incorporated.[53]

Conclusion

A series of 11 FPP analogues containing biologically orthogonal functional groups as substrates for the prenylation of cysteine residues in C-terminal CAAX recognition sequences by yeast PFTase was synthesized. Five of these were alternative substrates. Four of the alternate substrates were azides (**1-OPP**, **3-OPP**, **5-OPP**, and **6-OPP**), and one was an alkyne (**9-OPP**). Two of the compounds, azide **3-OPP** and alkyne **9-OPP**, were excellent alternative substrates with respective catalytic efficiencies (*V*/*K*) that were 210 and 72% of *V*/*K* for FPP. We used **3-OPP** and **9-OPP** to regioselectively modify recombinant versions of green fluorescent protein and glutathione *S*-transferase that contained a genetically engineered C-terminal CAAX recognition motif.[32] The modified proteins were subsequently covalently attached to glass slides derivatized with complementary functional groups using azide/alkyne cycloaddition and the Staudinger ligation. This technology offers a promise for the attachment of any soluble protein with a C-terminal CAAX motif to a wide variety of substrates.

Experimental Section

Acetoxydecadienyl Alcohol 22-OAc. $NaBH_4$ (120 mg, 3.17 mmol) was added in small portions to a solution of aldehyde **19-OAc** (630 mg, 2.65 mmol) in MeOH (30 mL) at −10 °C. The mixture was allowed to stir at −10 °C for 2 h before ice-cold water (50 mL) was added. The solvent was removed at reduced pressure. The aqueous residue was saturated with solid NaCl and extracted with ether (4 × 15 mL). The combined ether extracts were dried over Na_2SO_4, and the solvent was removed. The residue was chromatographed to give 495 mg (78%) of a colorless oil; ^{1}H NMR ($CDCl_3$, δ): 5.34 (d, J = 7.2 Hz, 1H); 5.13 (d, J = 6.3 Hz, 1H); 4.59 (d, J = 6.9 Hz, 2H); 3.63 (t, J = 6.3 Hz, 2H); 2.20−2.00 (m, 6H); 2.06 (s, 3H); 1.70 (s, 3H); 1.65 (m, 2H); 1.61 (s, 3H); ^{13}C NMR ($CDCl_3$, δ): 171.4, 142.2, 135.3, 124.2, 118.6, 62.7, 61.6, 39.6, 36.0, 30.8, 26.1, 21.2, 16.5, 16.0; IR (neat): 3444, 2938, 2883, 1738, 1235. HRMS (CI, M + H^+) Calcd for $C_{14}H_{25}O_3$ 241.1804; found 241.1800.

Acetoxydecadienyl Azide 3-OAc. Sodium azide (974 mg, 14.98 equiv), DMAP (687 mg, 5.62 equiv), and bis-(2,4-dichlorophenyl)-chlorophosphonate (1.98 g, 4.87 equiv) were added to a stirred solution of alcohol **22-OAc** (900 mg, 3.75 equiv) in DMF (20 mL) at room temperature (rt). The reaction mixture was allowed to stir overnight at rt before 50 mL of ethyl ether and 50 mL of brine were added. The layers were allowed to separate. The aqueous layer was diluted with 100 mL of brine and extracted with ethyl ether (2 × 50 mL). The combined ether extracts were washed with water and dried over $MgSO_4$. The solution was filtered and concentrated, and the residue was chromatographed to give 933 mg (94%) of **3-OAc** as a colorless oil; ^{1}H NMR ($CDCl_3$, δ): 5.34 (t, J = 7.2 Hz, 1H), 5.13 (t, J = 6.3 Hz, 1H), 4.58 (d, J = 7.2 Hz, 2H), 3.23 (t, J = 6.6 Hz, 2H), 2.16−2.00 (m, 6H), 2.06 (s, 3H), 1.70 (s, 3H), 1.68 (m, 2H), 1.60 (s, 3H). ^{13}C NMR ($CDCl_3$, δ): 171.3, 142.1, 134.0, 125.1, 118.6, 61.5, 51.0, 39.5, 36.6, 27.1, 26.2, 21.2, 16.6, 16.0; IR (neat): 2940, 2098, 1741, 1232. HRMS (CI, M + H^+ − N_2) Calcd for $C_{14}H_{24}NO_2$ 238.1807; found 238.1812.

Decadienol Azide 3-OH. Following the general procedure, acetate **3-OAc** (1.04 g, 3.92 mmol) was treated with K_2CO_3 (1.63 g, 11.76 mmol) in 40 mL of MeOH. Workup followed by purification via column chromatography gave 876 mg (100%) of **3-OH** as a light yellow oil; ^{1}H NMR ($CDCl_3$, δ): 5.41 (dt, J_1 = 6.9 Hz, J_2 = 0.9 Hz, 1H); 5.14 (t, J = 6.6 Hz, 1H); 4.16 (d, J = 6.6 Hz, 1H); 3.23 (t, J = 6.9 Hz, 1H); 2.20−2.00 (m, 6H); 1.7 (m, 2H); 1.68 (s, 3H); 1.60 (s, 3H); ^{13}C NMR ($CDCl_3$, δ): 139.4, 133.8, 125.2, 123.6, 59.3, 50.9, 39.5, 36.5, 26.9, 26.2, 16.3, 15.9; IR (neat): 3339, 2938, 2877, 2100, 1448, 1291, 1258. HRMS (CI, M + H^+ − N_2) Calcd for $C_{12}H_{22}NO$ 196.1701; found 196.1683.

Decadienyl Azide Diphosphate 3-OPP. Using the standard phosphorylation protocol, alcohol **3-OH** (124 mg, 0.56 mmol) was converted to the corresponding chloride using NCS (83 mg, 0.62 mmol) and dimethylsulfide (49 μL, 0.67 mmol). The chloride was then treated with $(NBu_4)_3HP_2O_7 \cdot 3H_2O$ (1.65 g, 1.68 mmol), and the product was purified as described to give 127 mg (53%) of a white solid; ^{1}H NMR (D_2O, δ): 5.40 (t, J = 7.2 Hz, 1H); 5.19 (t, J = 6.6 Hz, 1H); 4.40 (t, J = 6.6 Hz, 2H); 3.22 (t, J = 6.9 Hz, 2H); 2.20−1.90 (m, 6H); 1.66 (s, 3H); 1.62 (m, 2H); 1.56 (s, 3H); ^{13}C NMR (D_2O, δ): δ 142.4, 134.7, 125.6, 120.7 (J = 9.0 Hz), 62.8 (J = 5.0 Hz), 51.1, 39.7, 36.8, 27.1, 26.6, 16.3, 15.8; ^{31}P NMR (D_2O, δ): 6.59 (d, J = 22.6 Hz, 1P), −10.15 (d, J = 22.6 Hz, 1P); HRMS (CI) Calcd for $C_{12}H_{22}N_3O_7P_2$ 382.0938, found 382.0937.

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Supporting Information Available: General methods; experimental protocols for the synthesis of diphosphates **2-OPP**−**11-OPP** including all intermediates; ^{1}H, ^{13}C, and ^{31}P NMR spectra for **2-OPP**, **3-OPP**, **5-OPP**, **6-OPP**, and **9-OPP**−**11-OPP**, including intermediates, and intermediates for **4-OPP**; protocols for enzymatic assays and protocols; and chromatogram and spectra for HPLC and LC-MS analyses. This material is available free of charge via the Internet at http://pubs.acs.org.

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