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

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Abstract

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 alkylation of peptide co-substrates by yeast protein farnesyl transferase. Five of the diphosphates were good alternative substrates for FPP. Steady-state kinetic constants were measured for the active compounds, and the products were characterized by HPLC and LC-Mass. Two of the analogues gave steady state kinetic parameters (k_{cat} and K_M) very similar to those of the natural substrate.

INTRODUCTION

Posttranslational 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 are modified at a C-terminal cysteine residue by C₁₅ farnesyl or C₂₀ 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). Farnesyltransferase 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 high potency against the parasites responsible for malaria (*P. falciparum*)^{10,11} and the Chagas' disease parasite *T. 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). 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 FPP analogues for PFTase with specific functions has been facilitated by the X-ray crystal structures for the rat¹⁵, and human¹⁶ PFTases. Spielmann and coworkers reported that incorporation of an aniline moiety at the place of the ω -isoprene unit of FPP resulted in a transferable analogue.¹⁷ Recently, successful incorporation of this aniline-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 Distefano and Spielmann groups respectively.^{19,20,21} FPP analogues appended with fluorescent anthranilate esters were developed by Waldmann and coworkers.^{22,23} Wiemer and coworkers reported 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 coworkers recently reported that alkynyl ether derivatives of GPP are alternative substrates which can be tethered to other biomolecules after farnesylation.²⁵ Prestwich and coworkers synthesized a conjugated 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,²⁹ involves intramolecular trapping of a phosphine/azide adduct eventually to give a stable amide linkage. The "click" ligation, introduced by Sharpless^{30,31} is a Cu(I) catalyzed [2.3] cycloaddition reaction between an 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 Distefano³³ and Zhao.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 a trisubstituted allylic diphosphate moiety in the first isoprene unit, a requirement for all prenyltransferase 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 $t\text{BuOOH}/\text{SeO}_2$ gave 8-hydroxy- and 12-hydroxyfarnesyl 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 $\text{K}_2\text{CO}_3/\text{MeOH}$ gave the ω -epoxide but hydrolyzed the acetate group, which was subsequently reintroduced under standard conditions.³⁸ Epoxy-acetate **18-OAc** was treated with periodic acid³⁹ 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 diphenylphosphorylazide (DPPA) according to the procedure of Thompson and coworkers.⁴⁰ The acetate group was then hydrolyzed, and the resulting alcohols were phosphorylated as described by Davisson and coworkers^{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 diphenylphosphorylazide, the phosphate intermediate was not displaced by azide. We then resorted to Lu'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**.

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.

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 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 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 **10-OPP** were prepared by reductive amination of aldehydes **13-OAc** and **19-OAc** with propargyl amine using conditions described above for **23-OAc**, **24-OAc** and **25-OAc** (Scheme 8). Hydrolysis of the acetate groups, followed by phosphorylation of the resulting 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:H₂O, 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 Supporting 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 co-substrate are summarized in Table 1. Two other groups^{34,53} reported that **1-OPP** is a substrate for PFTase, although a full set of kinetic parameters were not presented. We found that $V_{\max}$ for the 1:1 mixture of **1-OPP** and its allylic isomer (**1i-OPP**, Scheme 10) is lower than for FPP, although their K_M s are similar. The rapid interconversion of the ω -allylic azides (see Scheme 10) prevents us from obtaining the individual kinetic constants for the two isomers. The terminal alkyl azide, **3-OPP**, is an excellent substrate with a k_{cat} almost equal to that of FPP and a K_M 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 analog 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 $(k_{\text{cat}}/K_M)_{\text{rel}}$ 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**, is 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 are 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 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).²⁶ 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. 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,⁵⁴ which does not have restricted rotation, is transferred 37 times more slowly than the natural substrate, suggesting that alkylamide moiety is also not optimal.

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) are consistent with alkylation of the cysteine residue in Dn-GCVIA by the appropriate analogue. Interestingly, 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 a single isomer, which subsequently rearranged to its allylic isomer, was incorporated.⁵³

CONCLUSIONS

A series of eleven FPP analogues containing biologically orthogonal functional groups as substrates for prenylation of cysteine residues in C-terminal CaaX recognition sequences by yeast PFTase was synthesized. Five of these were alternative substrates. Four of 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**, are excellent alternative substrates with respective catalytic efficiencies (V/K) that are 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 promise for attachment of any soluble protein with a C-terminal CaaX motif to a wide variety of substrates.

Experimental Section

Acetoxystyryl alcohol **22-OAc**

NaBH₄ (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. 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 Na₂SO₄ and solvent was removed. The residue was chromatographed to give 495 mg (78 %) of a colorless oil; ¹H NMR (CDCl₃, 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); ¹³C NMR (CDCl₃, 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 C₁₄H₂₅O₃ 241.1804; found 241.1800.

Acetoxystyryl 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 rt. The reaction mixture was allowed

to stir overnight at rt before 50 mL 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 MgSO₄. The solution was filtered, concentrated and the residue was chromatographed to give 933 mg (94 %) of **3-OAc** as a colorless oil; ¹H NMR (CDCl₃, 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). ¹³C NMR (CDCl₃, 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⁺-N₂) Calcd for C₁₄H₂₄NO₂ 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 K₂CO₃ (1.63 g, 11.76 mmol) in 40 mL of MeOH. Work-up followed by purification *via* column chromatography gave 876 mg (100 %) of **3-OH** as a light yellow oil; ¹H NMR (CDCl₃, 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); ¹³C NMR (CDCl₃, 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⁺-N₂) Calcd for C₁₂H₂₂NO 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 DMS (49 μL, 0.67 mmol). The chloride was then treated with (NBu₄)₃HP₂O₇·3H₂O (1.65 g, 1.68 mmol), and the product was purified as described to give 127 mg (53 %) of a white solid; ¹H NMR (D₂O, 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); ¹³C NMR (D₂O, 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; ³¹P NMR (D₂O, δ): 6.59 (d, *J* = 22.6 Hz, 1P), -10.15 (d, *J* = 22.6 Hz, 1P); HRMS (CI) Calcd for C₁₂H₂₂N₃O₇P₂ 382.0938, found 382.0937.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgment

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

Figure 2.
Structures of azido and alkyne FPP analogues

Scheme 1.
Synthesis of aldehyde **13-OAc** and **13-OTBS**

a Abbreviation: DCM - Dichloromethane;
b 33% farnesyl acetate recovered

Scheme 2.
Synthesis of alcohols **15-OAc** and **16-OAc** and aldehyde **17-OAc**

Scheme 3.
Synthesis of aldehydes **19-OAc** and **19-OTBS**

^a Abbreviation : DPPA - Diphenyl phosphoryl azide;
 DMS - Dimethyl sulfide

Scheme 4.
 Synthesis of azide analogues **1-OPP** and **2-OPP**

^a Abbreviation : DMAP - N,N -Dimethylaminopyridine; DCM - Dichloromethane
 DMS - Dimethyl sulfide

Scheme 5.
Synthesis of azide analogue **3-OPP**

^a Abbreviation : DPPA - Diphenylphosphorylazide; DMS - Dimethyl sulfide

Scheme 6.
Synthesis of azide analogues **4-OPP**, **5-OPP** and **6-OPP**

Scheme 7.
 Synthesis of alkynyl analogues **7-OPP** and **9-OPP**

Scheme 8.
 Synthesis of alkynyl analogues **8-OPP** and **10-OPP**

^a Abbreviation: DEAD - Diethylazodicarboxylate;
EEDQ - 1,2-dihydro-2-ethoxy-1-quinolinecarboxylic acid

Scheme 9.
Synthesis of alkyne analogue **11-OPP**

Scheme 10.
1-OPP azide equilibrium

Table 1
Steady-State Kinetic Parameters of Active Substrates

Compound	k_{cat} (s^{-1})	K_{M} (μM)	$k_{\text{cat}}/K_{\text{M}}$ ($\text{s}^{-1} \mu\text{M}^{-1}$)	$(k_{\text{cat}}/K_{\text{M}})_{\text{rel}}^a$
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 $k_{\text{cat}}/K_{\text{M}}$ with respect to FPP

^b Rate corresponds to the incorporation of both isomers as was shown by HPLC.

Table 2

HPLC Retention times for Dn-GCVIA and Dn-GC(Far)VIA peptides and LC-Mass molecular ions for the products.

Compound	Retention time (min)	LC-Mass(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