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Synthesis and Evaluation of 3- and 7-Substituted Geranylgeranyl Pyrophosphate Analogs

Michelle Maynor, Sarah A. Scott, Emily L. Rickert, and Richard A. Gibbs

Department of Medicinal Chemistry and Molecular Pharmacology, School of Pharmacy and Pharmaceutical Sciences, Purdue University, West Lafayette, Indiana 47907

Abstract

Protein prenyl transferases have been a focus of anti-cancer drug discovery in recent years due to their roles in posttranslational modification of small GTP binding proteins. Attention is now turning to the development of GGTase I inhibitors. Here, we present the synthesis and biological evaluation of four GGPP analogs versus mammalian GGTase I and the discovery that 7-allyl GGPP is a surprisingly efficient GGTase I substrate.

Protein prenylation occurs on many important mammalian proteins. These proteins are recognized by prenyl transferases via a C-terminal motif called the “CaaX box”, where cysteine is denoted by C, “a” is typically an aliphatic amino acid, and “X” represents a small subset of residues. Protein farnesyl transferase (FTase) catalyzes the transfer of a 15-carbon isoprenoid, and protein geranylgeranyltransferase I (GGTase I) catalyzes the transfer of a 20 carbon-isoprenoid.¹ The X amino acid of the CaaX box plays a key role in determining enzyme recognition; FTase CaaX motifs usually terminate in Ser, Met, or Gln, whereas GGTase I motifs usually end in Leu. CaaX proteins use their farnesyl or geranylgeranyl groups as lipid anchors to associate with membranes, or as lipid handles for recognition by other proteins.² Protein geranylgeranylation by GGTase I and the subsequent associated modifications are summarized in Figure 1.

Because many essential proteins require prenylation, inhibiting prenyl transferases to mislocalize proteins was recognized as an intriguing drug strategy. Farnesyl transferase inhibitors (FTIs) were originally developed as anti-cancer agents based on a straightforward paradigm; Ras, a signaling protein, is mutated in ~15–30% of human cancers to a continually activated form.³

Ras is farnesylated by FTase; thus, preventing farnesylation should prevent Ras from signaling.⁴ Although this approach was elegant in its simplicity, complications arose when K-Ras4B, the main oncogenic form of Ras, was found to be alternatively geranylgeranylated by GGTase I in the presence of FTIs.⁵ This problem, together with a growing appreciation of the key role of the Ras-related, geranylgeranylated small monomeric GTPases in cancer, has prompted interest in developing GGTase I inhibitors (GGTIs).⁶

It was hypothesized initially that GGTase I was a more risky anti-cancer drug target in part because there are more mono-geranylgeranylated proteins than farnesylated proteins. However, the number of farnesylated and geranylgeranylated proteins is roughly the same.⁷ More strikingly, a very recent study demonstrates that GGTase I knockout cells remained viable

after loss of GGTase I function.⁸ In the corresponding mouse model, GGTase I-deficient mice with K-Ras-driven lung cancer had fewer tumors and lived longer than mice with full GGTase I activity.⁸ This key study provides strong support for GGTase I as an anti-cancer drug target, and for further investigation into the basic biology of geranylgeranylated proteins.

Significantly less work has been performed on GGTIs than on FTIs, but several strategies have been investigated. Peptidomimetics have been developed based on the CaaX box,⁹ and in particular CaaL-based 3-aryl-piperazinones inhibit GGTase I in a selective and potent manner in cells.¹⁰ Small molecule GGTIs have also been discovered utilizing combinatorial library approaches,¹¹ including GGTI-DU40, which shows efficacy in cellular tumor model systems.¹² GGPP-based inhibitors have been designed with modifications of the pyrophosphate group,^{13,14} and the isoprenoid moiety.¹⁵ Depletion of cellular GGPP pools, either indirectly via statin treatment,¹⁶ or directly via GGPP synthase inhibition,¹⁷ represents an alternative strategy for blocking protein geranylgeranylation.

GGTase I and FTase are very similar, possessing identical α subunits and ~30% sequence homology in the β subunit.¹ Thus, we hope to apply our knowledge of FTase and FPP analogs to the targeting of GGTase I. Previously, we have examined a diverse set of FPP-based compounds versus mammalian FTase.¹⁸ Our focus has been on substitutions at the 3 and 7 positions on FPP, to determine what modifications are tolerated by the FPP binding pocket of FTase as alternative substrates and what modifications result in enzyme inhibition.¹⁹

Compounds **1b–1e**, shown in Figure 2, have proved particularly instructive. 3-vFPP (**1b**) is an efficient alternative FTase substrate with a K_M value of 156 nM.¹⁹ While 3-*al*FPP (**1c**) only differs by one carbon from 3-vFPP (**1b**), it is not recognized as a substrate and is strictly an inhibitor with an IC_{50} value of 189 nM.¹⁹ Several 7-substituted compounds, including 7-vFPP (**1d**) and 7-*al*FPP (**1e**),²⁰ are alternative substrates for FTase, but ones that exhibit unusual selective substrate behavior.²¹

Four analogous GGPP derivatives - 3-vGGPP, 3- *al*GGPP, 7-vGGPP, and 7-*al*GGPP (**2b–e**, Figure 2) - have now been targeted for evaluation against GGTase I to gain further insight into GGTase I isoprenoid SAR. The four corresponding FPP analogs have provided us with valuable information on the isoprenoid selectivity of FTase, and are useful biological tools.²² We believed that if the four GGPP compounds showed similar biochemical profiles to their FPP counterparts, then we would be able to utilize our knowledge of FPP SAR with FTase to develop GGPP-based GGTIs.

The syntheses of 3-*al*GGPP and 3-vGGPP have been previously reported.¹⁹ The GGPP analogs modified at the 7-position were synthesized using a similar approach to that developed for 7-substituted FPP analogs,²⁰ utilizing the appropriate starting material (**3a** or **3b**). The synthetic route is shown in Scheme 1. Farnesol derivatives **3a** and **3b** were generated via the vinyl triflate method as described previously.¹⁹ Alcohols **3a** and **3b** were converted to the corresponding bromides by addition of carbon tetrabromide and triphenylphosphine. The resulting bromides were coupled with the dianion of ethyl acetoacetate to afford the modified β -keto esters **4a** and **4b**. β -keto esters **4a** and **4b** were converted to vinyl triflates **5a** and **5b**.

For the 7-allyl GGPP compound, triflate **5b** was subjected to a Stille coupling reaction with tetramethyltin to result in modified ester **6b**. Subsequent diisobutylaluminum hydride reduction and chlorination yielded chloride **8b**, which was carried on directly to the next reaction. Tris (tetra-*n*-butylammonium)hydrogen pyrophosphate and chloride **8b** were combined, solvent was removed by rotary evaporation, followed by ion-exchange chromatography, and cellulose purification to give 7-allyl GGPP (**9b**).

For 7-vinyl GGPP, an alternative approach was taken, due to our lack of success with the Stille methylation procedure in this case. Triflate **5a** was subjected to a copper mediated Grignard

reaction²³ to give ester **6a**. DIBAL-H reduction and chlorination gave chloride **8a**, which was carried on directly to the next reaction without further purification. 7-Vinyl GGPP (**9a**) was generated from **8a** using the same conditions used for 7-allyl GGPP. The purity and identity of the key intermediates was confirmed through analysis of their proton NMR spectra and comparison to the previously reported 7-substituted farnesyl derivatives.²⁰ A minimal amount (~6%) of isomerization is seen with the 7-vinyl substituted intermediates **4a–6a**, where a small ($\text{H}_2\text{C}=\text{CH}$, dd, ~6.2 ppm) vinyl proton peak is observed next to the primary peak at ~6.5 ppm ($\text{H}_2\text{C}=\text{CH}$, dd). This result is presumably due to isomerization during the coupling of the doubly allylic bromide derived from **3a** with the acetoacetate dianion to give **4a**. The corresponding 7-allyl derivatives, compounds **4b–6b**, exhibit no discernible isomerization from their proton NMR spectra.

The four GGPP analogs, 3-vGGPP, 3-alGGPP, 7-vGGPP, and 7-alGGPP, were evaluated against GGTase I in a modified version of the previously published FTase biochemical continuous fluorescent assay.²⁴ The minimum substrate for GGTase I is a tetrapeptide identical to the CaaX box of an existing protein substrate. By using pentapeptides of the generic form dansyl-GCaaX, we can monitor protein prenylation by observing the change in fluorescence of the dansyl group that results from protein prenylation.²⁴ The dansyl fluorophore is useful as the fluorescent tag because the increased hydrophobic environment resulting from protein prenylation causes an observable increase in fluorescence and a blue shift.²⁵ For all assays, the excitation wavelength used was 340 nm, and emission was monitored at 500 nm. The inhibitor assay mixture consisted of assay buffer (52 mM Tris-HCl pH=7.0, 5.8 mM DTT, 12 mM MgCl_2 , and 12 μM ZnCl_2), 0.25% detergent (0.4% *n*-dodecyl β -D-maltoside in 52 mM Tris-HCl, pH=7.0), 0.7 μM dansyl-GCVLL, 1.35 μM GGPP, varying analog concentrations (0.1–5.0 μM) and 0.02 μM GGTase I.²⁶ The reaction is initiated by addition of enzyme, and change in fluorescent intensity is monitored over 300 s. For substrate K_M determination, the same assay protocol is followed, but GGPP is omitted from the assay mixture. Substrate data is expressed as a V/S plot and fit to the Michaelis-Menten equation to determine K_M and V_{MAX} . Inhibitor data is expressed as a V/S plot and the IC_{50} is determined. Biochemical data for the four analogs are given in Table 1. All GGPP analogs were also evaluated by HPLC to confirm substrate activity via product formation.¹⁹

Previously, we have found that 3-vGGPP and 3-alGGPP were modestly effective inhibitors of the incorporation of tritiated GGPP into a peptide substrate by GGTase I.¹⁹ However, this assay did not distinguish between inhibitor and substrate behavior. Using the current assay, 3-vGGPP was determined to be a poor co-substrate with dansyl-GCVLL, having a K_M value of 1.75 μM , and exhibited no inhibition of GGTase I. 3-alGGPP is a modest inhibitor of geranylgeranylation of dansyl-GCVLL by GGTase I, with an IC_{50} value of 846 nM, and exhibits no substrate behavior. The overall biochemical pattern seen with these two analogs was thus similar to that seen with their respective FPP counterparts, although the GGPP analogs bound much more poorly to GGTase I than would be expected from the FTase results.

7-vGGPP is a modest inhibitor of geranylgeranylation of CVLL by GGTase I, with an IC_{50} value of 1 μM . In sharp contrast, 7-alGGPP is an excellent co-substrate, having a K_M value of 27 nM, which is within an order of magnitude of the natural substrate, GGPP ($K_M = 3$ nM).²⁴ Note that neither 7-vinyl GGPP nor 7-allyl GGPP exhibits any significant binding to mammalian FTase (Table 1). It is intriguing that the 7-substituted GGPP compounds exhibit strikingly different behavior compared to each other, and to that seen with their FPP counterparts and FTase.¹⁹

Recently, 7-substituted FPP analogs were evaluated qualitatively against CaaX boxes of several key FTase substrates²¹, to further explore our discovery that FPP analogs can alter FTase peptide substrate selectivity.²⁷ 7-vFPP was found to be a good co-substrate with the

majority of CaaX boxes tested.²¹ 7-*al*FPP was found to be an excellent co-substrate with CVIM (K-Ras4B) but an inhibitor of farnesylation with all other tested CaaX boxes.²⁷ Currently, we are working towards screening our GGPP compounds versus diverse CaaL peptides, in a similar manner as described above.

In conclusion, four GGPP analogs were synthesized and evaluated for biological activity against mammalian GGTase I. These four compounds help provide insight into the GGPP binding pocket of GGTase I. It is surprising that the isoprenoid SAR for GGTase I is different than that for FTase, and thus we cannot reliably draw parallels between the two. The discovery that 7-*al*GGPP is a highly effective alternative substrate suggests that it and other GGPP analogs may be valuable tools to selectively modify geranylgeranylated proteins and thus to investigate their biological function.²

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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1a: FPP; R^1 =Me, R^2 =Me

1b: 3-vFPP; R^1 =vinyl, R^2 =Me

1c: 3-alFPP; R^1 =allyl, R^2 =Me

1d: 7-vFPP; R^1 =Me, R^2 =vinyl

1e: 7-alFPP; R^1 =Me, R^2 =allyl

2a: GGPP; R^1 =Me, R^2 =Me

2b: 3-vGGPP; R^1 =vinyl, R^2 =Me

2c: 3-alGGPP; R^1 =allyl, R^2 =Me

2d: 7-vGGPP; R^1 =Me, R^2 =vinyl

2e: 7-alGGPP; R^1 =Me, R^2 =allyl

Figure 2.

Previously studied FPP analogs, and the corresponding GGPP analogs evaluated in this study.

Scheme 1.
Synthesis of 7-substituted GGPP analogs.

Table 1

Abilities of 3- and 7-substituted GGPP analogs to act as inhibitors or co-substrates of mammalian GGTase I.

Analogue	IC ₅₀ (nM) GGTase I	K _M (nM) GGTase I	IC ₅₀ (nM) FTase
3- ν GGPP	nd	1750	715 ¹⁹
3-alGGPP	846	nd	453 ¹⁹
7- ν GGPP	1000	nd	>5000
7-alGGPP	nd	27	>5000