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Exploration of GGTase-I substrate requirements. Part 1. Synthesis & biochemical evaluation of novel aryl-modified geranylgeranyl diphosphate analogs

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Abstract

Protein geranylgeranylation is a type of post-translational modification that aids in the localization of proteins to the plasma membrane where they elicit cellular signals. To better understand the isoprenoid requirements of GGTase-I, a series of aryl-modified geranylgeranyl diphosphate analogs were synthesized and screened against mammalian GGTase-I. Of our seven-member library of compounds, six analogs proved to be substrates of GGTase-I, with **6d** having a $k_{\text{rel}} = 1.93$ when compared to GGPP ($k_{\text{rel}} = 1.0$).

Graphical Abstract

Keywords

Geranylgeranylation; Aryl-modified GGPP analogs; GGTase-I; Post-translational modification; Protein Prenylation

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Supplementary Material

Supplementary material has been submitted as a Word document and includes a description of the assay used, experimental procedures, and ¹H NMR, ¹³C NMR, ³¹P NMR data for new compounds synthesized.

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Many proteins undergo prenylation, a type of posttranslational modification, which localizes the proteins to the plasma membrane.^{1,2} Proteins such as small Ras and Rho GTPase superfamilies, nuclear lamins, and the kinesin motor proteins require prenylation in order to become biologically functional.³

Protein prenylation occurs on a cysteine four residues from the C-terminus. Prenylated proteins contain a C-terminal “CaaX box” sequences, where ‘C’ denotes cysteine, ‘a’ is typically an aliphatic amino acid, and ‘X’ represents a small subset of amino acid residues.⁴ This tetrapeptidic sequence allows recognition by prenyl transferase enzymes located in the cytosol and subsequent enzymatic catalysis to form a thioether bond between the Cys residue of the CaaX box and isoprenyl lipids.⁵ There are two CaaX prenyltransferases in mammalian cells: 1) farnesyl transferase (FTase) catalyzes the covalent attachment of a 15-carbon farnesyl isoprenoid (farnesyl pyrophosphate, FPP) and 2) geranylgeranyl transferase-I (GGTase-I) catalyzes the attachment of a 20-carbon geranylgeranyl isoprenoid (geranylgeranyl pyrophosphate, GGPP) to cysteine (Figure 1). After covalent attachment of the isoprenoid(s), the protein relocates to the endoplasmic reticulum where it undergoes proteolytic cleavage of the “-aaX” residues by the endoprotease Ras-converting enzyme-1 (Rce-1) followed by methyl-esterification by isoprenylcysteine carboxyl methyl transferase (Icmt). Upon completion of these modifications, the newly isoprenylated protein can be anchored in the plasma membrane and regulate various cellular functions.⁶

Estimations approximate that 0.5–2% of all mammalian proteins are prenylated; however, roughly only 60 proteins have been identified thus far.^{7,8} Of the known prenylated proteins, many exhibit a plethora of cellular functions including cell signaling, cell mobility, cell division, organelle structure, and vascularization. Thus, targeting protein prenylation may prove to be a potential treatment not only for cancer but for a wide variety of other diseases as well.^{9–15}

Many FTase studies have stemmed from structural investigations of FTase by the Beese group. Their work unveiled a hydrophobic binding pocket rich with aromatic amino acid residues such as Tyr, Trp, and Phe.^{16–18} This sparked many researchers, our group included, to explore the possibility of pi-pi stacking interactions between these aromatic amino acids and FPP analogs containing aromatic motifs.^{3,19–21} While these aryl-modifications have been greatly explored in relation to FTase, little has been done to investigate these modifications in relation to GGTase-I binding ability.

Previously, our laboratory has concentrated on generating GGPP analogs containing substitutions at the 3 and/or 7 positions of GGPP.^{22,23} Some of these analogs have been shown to act as efficient substrates of GGTase-I while others have high nanomolar IC₅₀ values. In order to investigate greater structural diversity in GGPP analogs, we synthesized a series of aryl ω -modified GGPP analogs. Some of the aromatic residues (W102, Y361) in FTase correspond to non-aromatic residues in GGTase-I (T49, Y361, respectively) in order to allow for a more spacious binding pocket to accommodate the longer isoprene chain of GGPP; however, structural studies have revealed that GGTase-I does have a hydrophobic binding pocket bountiful with aromatic residues.^{5,24} The potential of aryl-containing GGPP

analogues to participate in pi-pi stacking interactions with the aromatic amino acid residues of the GGTase-I binding pocket prompted us to synthesize and evaluate a small library of aryl-modified GGPP analogues.

When considering which analogues to evaluate, our goal was to select analogues that best mimicked the terminal isoprene unit. The two analogues that best simulate the isoprene unit both contain methyl-substituted benzene rings (Figure 1). Analogues **6a** and **6b** also aligned well with GGPP, and though they lack the additional CH₃ of **6d-e**, both of these analogues provide the double bond of the terminal isoprene unit. Comparing the catalytic efficiency of analogues **6a-b** with **6d-e** may provide great insight into whether methyl substitution of the aromatic ring is beneficial. Analogue **6c** was included to determine if hydrophobic bulk would be sufficient for GGTase-I catalysis or, as we hypothesize, if aromaticity would be more beneficial. Compound **6f** was chosen to evaluate the effects of electronics on the aromatic ring on catalytic efficiency.

Recently, our lab has synthesized a potent Icmt inhibitor designated “TAB.”²⁵ This methyltransferase accepts both farnesylated and geranylgeranylated proteins that have been proteolyzed by Rce1 as substrates for methylation. Although a crystal structure has yet to be determined, it stands to reason that Icmt and the CaaX prenyltransferases have similar prenyl-binding pockets. Therefore, we wished to evaluate the corresponding diphosphate, **15**, as a potential inhibitor of GGTase-I to test this hypothesis and gain more insight into prenyl substrate binding requirements.

The synthesis of the aryl-modified GGPP analogues was designed in such a way that all compounds could be generated from a common intermediate, **3**. Additionally, the availability of a wide variety of commercially available Grignard reagents and benzylic/phenylic halides in addition to the ease of introduction of the aryl-motifs motivated us to explore this synthetic route. To begin the synthesis, THP-protected farnesol (**1**) underwent oxidation in the presence of SeO₂ followed by a NaBH₄ reduction to generate alcohol **2**.²⁶⁻²⁸ Next, diethyl chlorophosphate is subjected to a displacement reaction in the presence of **2** and DIEA to generate diethyl phosphate **3** in 74% yield. There were a few advantages of choosing this type of intermediate. One advantage to using diethyl phosphate **3** is that it can be stored for longer periods of time than the corresponding allylic halides which are unstable and easily degrade. More so, the corresponding allylic halides generally undergo Grignard displacement reactions to give a mixture of S_N2 and S_N2' products usually in fairly equal quantities and isolations of one isomer are not facile.²⁹ Thus, with common intermediate **3** in hand, a similar method as Snyder & Treitler was employed and a variety of Grignard reagents could be utilized in an S_N2 displacement reaction to generate the aryl-modified analogues **4a-f**.³⁰ These analogues were first deprotected using PPTS in EtOH to generate alcohols **5a-f** and then converted into the corresponding pyrophosphates (**6a-f**) utilizing the method of Davisson *et al.*^{31,32}

The synthesis of the “TAB-pyrophosphate” **15** was accomplished according to the procedure of Bergman *et al.*²⁵ It began with the conversion of 4-bromobut-1-yne (**7**) to alcohol **8** using Negishi's zirconium-catalyzed asymmetric carbo-alumination (ZACA) reaction.³³ Next, alcohol **8** was THP-protected using a standard procedure to generate compound **9**. The

second half of the molecule was generated by subjecting biphenyliodide **10** to the 1-(trimethylsilyl)-1-propyne (**11**) anion followed by TMS deprotection with TBAF to afford alkyne **12**. Alcohol **9** was then converted into the corresponding azide *in situ* by displacement of the primary bromide with sodium azide. Utilizing standard Cu(I) mediated conditions, biphenyl alkyne **12** was then “clicked” with the freshly generated azide resulting in a 1,4-disubstituted 1,2,3-triazole, **13**.^{34–37} These analogs were first deprotected using PPTS in EtOH to generate alcohol **14**. Halogenations of triazole-containing compounds via standard Corey-Kim conditions using NCS has been revealed to be problematic in the past. Thus, alcohol **14** was first converted into the mesylate and then converted into the corresponding pyrophosphate, **15**, utilizing the method of Davisson *et al.*^{31,32}

Previously, our laboratory and others have shown that aryl-modified FPP analogs can behave as substrates or inhibitors of FTase with various CaaX-peptides. Thus, we aimed to explore these modifications when applied to GGTase-I. Preliminary evaluation of the biochemical activity of our aryl-modified GGPP analogs (**6a–f** & **15**) was achieved utilizing an *in vitro* continuous spectrofluorometric assay with GGTase-I and the co-substrate CaaX-peptide, dansyl-GCVLL. (Figure 2). The six analogs displaying increased fluorescence were further evaluated and their k_{cat}/K_M values were calculated according to a published protocol (Table 1).³⁸ The compound that did not show any substrate activity, compound **15**, was based on an Icmt inhibitor recently synthesized in our laboratory. Our original motive for synthesizing this compound was to determine if this isoprene-mimic could also inhibit GGTase-I. Lack of reactivity was confirmed via HPLC to ensure analog **15** was indeed a non-substrate. Further studies have revealed that compound **15** is not an inhibitor of GGTase-I.

Analog **6a**, which is the same overall length as the endogenous ligand, and **6b**, which is one methylene unit shorter, display similar catalytic efficiency *in vitro* ($k_{rel} = 0.46$ and 0.61 , respectively). It is evident that chain length plays a less crucial role in substrate binding and catalysis than originally hypothesized. However, in accordance with our original hypothesis, adding a methyl substituent on the benzene ring of **6a** and **6b** to mimic the terminal isoprene increased substrate activity (**6d** and **6e**, respectively). In fact, analog **6d** has a $k_{rel} = 1.93$, which is nearly twice that of the natural substrate GGPP. This suggests the methyl substituent in the terminal isoprene unit may have a more significant contribution to reactivity than chain length.

The results of the remaining two analogs, **6c** and **6f**, were interesting. We had hypothesized that our aromatic compounds would have the added benefit of being able to participate in additional favorable interactions with the binding site (such as pi-pi stacking). Thus, we believed the aromatic compounds would display a greater degree of activity than a non-aromatic counter part due to the large number of aromatic residues in the binding pocket of GGTase-I. This proved to be true in the case of analog **6c** ($k_{rel} = 0.48$) versus **6b** ($k_{rel} = 0.61$); the primary difference being the lack of aromaticity in **6c**. However, the difference in efficiency is smaller than one would expect, suggesting hydrophobicity contributes to binding and efficient turnover.

Due to its small size, high electronegativity, and unique chemical reactivity, fluorine is becoming more and more common place in medicinal chemistry and drug discovery. In fact,

fluorine's unique nature has been linked to enhancing binding interactions, changing physical properties (lipophilicity and/or solubility), metabolic stability, and selective reactivities.³⁹ We hypothesized that a fluorinated aryl-GGPP compound could be beneficial to enzyme activity. As fluorine may enhance binding of analog **6f** to GGTase-I, product release could occur very slowly, thereby decreasing catalytic efficiency. This scaffold may serve well as an inhibitor template to generate a tight-binding, slow-releasing GGPP analog to specifically target GGTase-I.

Replacing the terminal ω -isoprene unit with an aryl motif was well tolerated by the enzyme; however, the number of methylene spacers between the aryl group and the γ -isoprene unit was important. By adding a methyl-group to the homobenzyl group (**6d**) to mimic the isoprene unit, we generated a compound with a $k_{\text{rel}} \approx 2$ when compared to GGPP ($k_{\text{rel}} = 1$). We speculate that the longer carbon chain allows the aryl-moiety to be positioned in a more favorable area of the exit groove allowing for higher enzyme turnover. When the number of methylene spacers was decreased by one (**6b** and **6c**), there was diminished substrate activity, although these compounds still retained some ability to be turned over by GGTase-I. One possible reason for this observation could be that the shorter aryl-analogs bind in such a way that the pyrophosphate head group is further away from the catalytic Zn^{2+} ion in the binding pocket (i.e. bound deeper within the pocket). Therefore, coordination to the zinc ion would be attenuated and in return the catalytic ability of the enzyme to transfer the isoprenoid chain to the peptide would be diminished.

The cyclohexyl analog (**6c**) also displayed substrate activity, albeit, attenuated. This suggests that the added aromaticity of our compounds was beneficial for substrate activity. An alternative explanation could lie in the differences in flexibility and/or structure between the two motifs and the product release of the prenylated peptide. In order for product release from the enzyme, the isoprenoid group must rotate into an exit groove of the enzyme followed by the binding of a new molecule of the pyrophosphate analog. The cyclohexyl motif is a much more flexible and non-planar group as opposed to the phenyl group which is much more rigid and planar. Therefore, it can be speculated that the lack of planarity of the cyclohexyl group hinders it binding to the enzyme and/or ability to shift into the exit groove of the enzyme.

This work served as a preliminary study of the isoprene requirements and their effect on GGTase-I activity. Previously, similar analogs were utilized to investigate protein farnesylation; however, those compounds took advantage of amine and ether linkages to install the terminal aryl-motif. Our goal was to synthesize compounds containing completely carbon backbones while simultaneously incorporating an aryl-motif. A library of aryl-modified GGPP compounds was synthesized and evaluated as co-substrates with dansyl-GCVLL in a fluorescence-based assay. Interestingly, the majority of our compounds displayed *in vitro* biochemical activity and provided us with interesting insights into GGTase-I isoprene binding and catalysis which potentially will aid researchers in the development of chemical probes to investigate protein prenylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Ras protein prenylation pathways. Box: Overlay of GGPP (black) and aryl-modified analogs (red) is depicted.

Figure 2.

Preliminary screen of substrate activity represented in relative fluorescence increase (RFI) of aryl-modified GGPP analogs (5 μ M) versus GGPP (+ control) with 5 μ M dansyl-GCVLL and 50 nM GGTase-I at 2 hrs with GGPP (2hr) being normalized to 1.0. Error bars represent mean $\pm$ SD (n = 3).

Scheme 1.

Synthesis of Aryl-Modified GGPP analogs. (a) i. DHP, PPTS, DCM; ii. SeO₂, t-BuOOH, salicylic acid, DCM; iii. NaBH₄, EtOH (37% - 3 steps); (b) DIEA, (EtO)₂POCl, Et₂O (74%); (c) R-MgX, THF, 22 hr; (d) PPTS, EtOH, 70°C; (e) NCS, DMS, DCM, 2.5 hr.; (f) (NBu₄)₃HP₂O₇, ACN, 3 hr.

Scheme 2.

Synthesis of "TAB" pyrophosphate. (a) Me_3Al , Cp_2ZrCl_2 , DCM, 0°C , 18 hr then $(\text{CH}_2\text{O})_n$, 3 hr (83%); (b) PPTS, DHP, DCM (79%); (c) i. TMS-propyne, $n\text{-BuLi}$, THF, -78°C ; ii. K_2CO_3 , MeOH, 12 hr (36% - 2 Steps); (d) NaN_3 , $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, Sodium ascorbate, DMF, 55°C (20%); (e) PPTS, EtOH, 70°C (85%); (f) MsCl , DMAP, DCM, 2.5 hrs; (g) $(\text{NBu}_4)_3\text{HP}_2\text{O}_7$, ACN, 3 hr (89%).

Table 1

Evaluation of aryl-modified GGPP analog substrate ability versus GGTase-I with dansyl-GCVLL.

Compound	$k_{\text{cat}}/K_{\text{M}}$ ($\text{M}^{-1}\text{s}^{-1}$)	k_{rel}^a
6a	1.73×10^4	0.46
6b	2.30×10^4	0.61
6c	1.80×10^4	0.48
6d	7.30×10^4	1.93
6e	3.10×10^4	0.82
6f ^b	1.0×10^4	0.26
15	non-substrate	non-substrate
GGPP	3.78×10^4	1

^a k_{rel} = relative rate in preliminary screen, with $k_{\text{cat}}/K_{\text{M}}$ of GGPP = 1.0^b $k_{\text{cat}}/K_{\text{M}}$ for **6f** is estimated with an upper limit for K_{M} .