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Didehydrogeranylgeranyl (ΔΔGG): A Fluorescent Probe for Protein Prenylation

Xiao-hui Liu and Glenn D. Prestwich*

Department of Medicinal Chemistry and the Center for Cell Signaling, 30 South 2000 East, Room 201, The University of Utah, Salt Lake City, Utah 84112-5820

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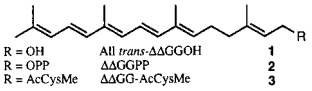
Protein prenylation involves the covalent attachment of a farnesyl or geranylgeranyl moiety via a thioether linkage to the cysteine residue of a C-terminal CAAX motif. This posttranslational modification of small G proteins is required for cellular trafficking, membrane association, and formation of protein-protein signaling complexes that trigger a wide variety of cellular responses.[1] Specifically, the GTPase farnesylated Ras is essential for normal cell growth and regulation of differentiation.[2] Oncogenic H-Ras is well-known for its Akt-1 activation and transforming ability.[3] Geranylgeranylated Rho GTPases play key roles in the reorganization of the actin cytoskeleton and the control of gene transcription,[4] while bisgeranylgeranylated Rab GTPases regulate vesicular trafficking and exocytosis.[5] Recently, farnesylation and geranylgeranylation have emerged as important new targets in cancer therapy[6] (Chart 1).

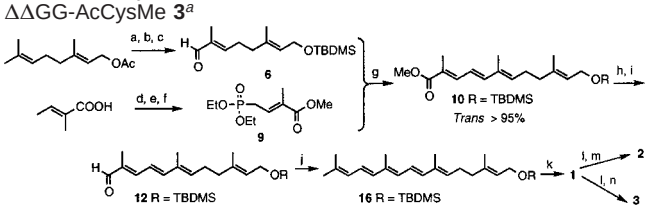
We describe herein the first intrinsically fluorescent analogue of the geranylgeranyl moiety, and we demonstrate its utility in cell-based and in vitro protein−ligand interactions. The design of the parent compound, (2*E*,6*E*,8*E*,10*E*,12*E*,14*E*)-geranyl-geraniol (all-*trans*-ΔΔGGOH **1**), its diphosphate (ΔΔGGPP **2**), and its *N*-acetyl cysteine methyl ester adduct (ΔΔGG-AcCysMe **3**) required stereoselective introduction of 8*E*- and 12*E*-alkene bonds to produce a conjugated pentaene. The pentaene was predicted to fluoresce by analogy with the naturally occurring pentaene, *trans*-retinol (vitamin A_1).[7] Importantly, the intrinsic fluorophore of ΔΔGGOH **1** does not add additional steric bulk, although pentaene will be more rigid than GGOH. Crystallographic and NMR data support an extended conformation for isoprenoid chains, both in solution and when bound to PFTase or RhoGDI.[8] Moreover, yeast protein geranylgeranyltransferase (PGGTase-I) was capable of transferring isoprenoid analogues to substrate peptides or proteins.[9] We hypothesized that the ΔΔGG moiety would substitute on a molecular level for the geranylgeranyl group for both biochemical and physiological functions. In particular, we anticipated that ΔΔGG-modified peptides would provide new ligands for in vitro assays, and that ΔΔGG derivatives could be delivered intracellularly to study protein translocation and protein−protein interactions of signaling complexes in living cells.

The primary challenge in preparing all *trans*-ΔΔGGOH was the stereoselective installation of the 8*E* and 12*E* double bonds. Scheme 1 illustrates the synthetic route to compounds **1**, **2**, and **3**, beginning with selective oxidation of geranyl acetate with selenium dioxide. Attempted selective oxidation of the geranyl TBDMS ether resulted in lower yield and poor selectivity.[10] The 8*E*-alkene was introduced using a Wittig−Horner reagent, allylic diethylphosphonate **9**.[11] Moreover, if the proper isomeric allylic diethylphosphonate **9** in the mixed isomers was 1.2 equiv of the geranyl-TBDMS aldehyde **6**, then the 8*E*-alkene **10** was obtained with over 95% geometric purity. The remaining 5% 8*Z*-isomer was readily removed by SiO_2 separation of either the alcohol **11** or aldehyde **12** isomers.

Chart 1. Structure of ΔΔGGOH **1**, ΔΔGGPP **2**, and ΔΔGG-AcCysMe **3**.



Scheme 1. Synthesis of ΔΔGGOH **1**, ΔΔGGPP **2**, and ΔΔGG-AcCysMe **3**[a]



[a] (a) SeO_2; (b) KOH; (c) TBDMS-Cl, Et_3N; (d) CH_3OH, conc. H_2SO_4; (e) NBS; (f) $P(OEt)_3$; (g) *n*-BuLi, 0 °C; (h) $LiAlH_4$; (i) PCC; (j) *n*-BuLi, **15**; (k) TBAF (l) Ph_3P, CBr_4; (m) $(n\text{-}Bu_4N)_3HP_2O_7$; (n) AcCysMe, $Zn(OAc)_2$, H_2O.

Reports on the stereoselective synthesis of *trans* di-substituted alkenes using isoprenyl-substituted phosphorus reagents are scarce.[12] Strong bases such as *t*-BuOK, or NaOMe, which are required to ionize Wittig−Horner reagents without electron-withdrawing substitutes, would quickly decompose the sensitive aldehyde **12**, even at −78 °C. On the basis of the mechanism of the Wittig reaction, we reasoned that a smaller phosphine ligand would minimize the steric interaction with the two alkyl groups, R_1 and R_2,[13] giving rise to the desired *E*-isomer as the dominant product (see Supporting Information). Three isoprenyl trialkyl phosphites (diphenyl **13**, dibutyl **14**, and diethyl **15**) were employed at both −78 and 0 °C. At 0 °C, **13** and **14** gave 64 and 85% *E*-isomer **16**, respectively. At −78 °C, **14** and **15** afforded **16** with 91 and >99% selectivity. It appeared that the Wittig reaction was subject to both steric and kinetic control, since the lower temperature resulted in a slower collapse of the intermediate and also amplified the effect of energy difference between the two transition states.[14]

All-*trans*-ΔΔGGOH **1** showed blue fluorescence in methanol, with λ_{ex} = 310 nm and λ_{em} = 410 nm ($\epsilon_{310} = 2.4 \times 10^4$ M^{-1} cm^{-1}), but was only weakly fluorescent in aqueous solution. This environmental sensitivity was confirmed by titration (see Supporting Information). The fluorescence intensity at 410 nm was quenched 4-fold when the percentage of water reached 50% (v/v). The relative quantum yield (Φ) in methanol was 0.0075 using *trans*-retinol (Φ = 0.0298, with λ_{ex} = 355 nm) as the standard;[7,15] retinol was selected for having solubility and spectral properties similar to those of **1**.

The ability of ΔΔGGPP **2** to serve as a substrate for yeast PGGTase-I was measured using a continuous fluorescence method.[16] All-*trans*-ΔΔGGPP **2** had a K_M value of 0.33 μM (3.45 μM for

* Address for correspondence. Telephone: 801 585-9051. Fax: 801 585-9053. E-mail: gprestwich@deans.pharm.utah.edu.

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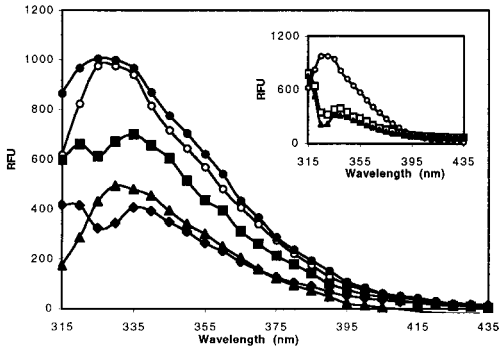

Figure 1. Titration of RhoGDI (5.62 μM) with ΔΔGG-AcCysMe (○ 0 μM, ■ 5.62 μM, ▲ 16.9 μM, ◆ 56.2 μM), AcCysMe (● 48 μM). Inset: Titration with GG-AcCysMe (□ 16.9 μM, ▲ 56.2 μM).

GGPP), and the prenyl-transfer efficiency was 60% relative to that for GGPP.

To test the interaction of the ΔΔGG moiety with signaling proteins that bind geranylgeranylated peptides, we prepared ΔΔGG-AcCysMe **3** as a fluorescent ligand.[17] This is the minimum unit recognized by the binding domain of proteins that bind the prenylated C-terminal Cys methyl ester. The fluorescence spectrum of ΔΔGG-AcCysMe **3** (10 mM Tris-Cl pH 7.5) showed $\lambda_{ex} = 360$ nm and $\lambda_{em} = 460$ nm. The 50 nm red-shift in fluorescence excitation and emission further demonstrated the environmental sensitivity of the ΔΔGG fluorophore in this peptide adduct relative to the free alcohol **1**. We examined the binding of ΔΔGG-AcCysMe to the recombinant Rho GTPase dissociation inhibitor (RhoGDI).[18] In cell cytosol, RhoGDI forms a complex with geranylgeranylated Rho (GDP bound), which, in addition to slowing the rate of GDP dissociation from the Rho, has been shown to solubilize the GTPase from subcellular membranes.[19] Therefore, RhoGDI regulates vesicular trafficking between donor membranes to acceptor membranes.

The binding of ΔΔGG-AcCysMe **3** to RhoGDI[20] was determined through the quenching of the intrinsic fluorescence of the tryptophan residues (Trp192, Trp194, and Trp202) in a hydrophobic binding pocket (Figure 1).[21] Thus, when RhoGDI was titrated with ΔΔGG-AcCysMe **3** or GG-AcCysMe (Figure 1, inset), but not with non-prenylated AcCysMe, a significant reduction in fluorescence was observed within 5 min. A red-shift (10−15 nm) of the Trp fluorescence indicated that water was involved in this binding interaction. After 1 h, the fluorescence of RhoGDI had completely recovered, further suggesting that quenching was due to dynamic changes in the Trp microenvironments.[21]

To measure the binding constant of ΔΔGG-AcCysMe **3** to RhoGDI, fluorescence anisotropy was employed. Binding of rhodamine-labeled geranylgeranyl cysteine methyl ester (GG-RhodCysMe) to RhoGDI showed increasing polarization as the concentration of RhoGDI in the assay buffer increased. A dissociation constant (K_D) was determined to be 2.45 ± 0.14 μM by curve fitting, in close agreement with the reported value.[18] Competitive binding was measured for ΔΔGG-AcCysMe **3**, and curve fitting gave a K_D value of 15.1 ± 1.2 μM. This indicated that the more rigid ΔΔGG fluorophore had approximately 6-fold lower affinity compared with the geranylgeranyl moiety, equivalent to the displacement obtained with Farn-AcCysMe.

In conclusion, the in vitro reaction of ΔΔGGPP **2** with yeast PGGTase-I and the binding between ΔΔGG-AcCysMe **3** and RhoGDI demonstrate the potential of the ΔΔGG moiety as a novel fluorophore for studying the interaction and subcellular localization of prenylated small GTPase proteins in signaling complexes. Cell-based fluorescence assays[22] to monitor the time course of changes in subcellular distribution of ΔΔGG-modified proteins will be presented in due course.

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Supporting Information Available: Preparative procedures and analytical data for **1**, **2**, and **3**. Protocols for expression and purification of PGGTase-I and RhoGDI; enzymatic, fluorescence quenching, fluorescence polarization assay procedures. Equations to calculate the quantum yield and for fitting of fluorescence polarization data (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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