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Didehydrofarnesyl diphosphate: an intrinsically fluorescent inhibitor of protein farnesyltransferase☆

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Abstract—Didehydrofarnesyl diphosphate (ΔΔFPP), a fluorescent pentaene analogue of farnesyl diphosphate (FPP), was synthesized using stereoselective Wittig reactions. Although ΔΔFPP was not an alternative substrate for yeast protein farnesyltransferase (FTase), the fluorescent analogue was a potent competitive inhibitor with a K_i value of 8.8 μM (K_m (FPP) = 27 μM).

Post-translational modification of proteins with isoprenyl groups is required for signaling proteins. Prenylated proteins include the small GTPases in the Ras, Rho, and Rab families, heterotrimeric G proteins, and cGMP phosphodiesterase and nuclear lamin protein.[1–7] In particular, small GTPases play important roles in cell proliferation, signal transduction, and malignant transformation. For example, farnesylated H-, N-, K_A-, and K_B-Ras are mutated in 30% of human cancers.[8] Geranylgeranylated GTPases such as RhoA, Rac1, Cdc42, and TC10 promote tumorigenesis and metastasis, and are required for Ras-induced transformation.[9] Each of these mature small GTPases have farnesyl or geranylgeranyl moieties linked to a C-terminal cysteine methyl ester via a thioether bond. Three carboxyl terminal motifs specify the type of prenylation: CA_1A_2X, CC, and CAC, where A is an aliphatic amino acid and X is any amino acid. Generally, the CA_1A_2X (X = C, S, Q, A, M, T, H, V, N, F, G, I) sequence is a substrate of farnesylation unless X = L, I, V, in which case, geranylgeranylation results. The CC and CAC sequence are substrates for bis-geranylgeranylation and are found exclusively in the Rab family of small GTPases.[10]

Three protein prenyltransferases have been distinguished: protein farnesyltransferase (FTase), protein geranylgeranyltransferase type I (GGTase-I), and protein geranylgeranyltransferase type II (GGTase-II). These heterodimeric prenyltransferases contain one α and one β subunit; both subunits are required for the prenylation reaction. However, the β subunit contains the substrate-binding pocket.[11–19] Among these proteins, FTase is the most well studied. The availability of cloned and expressed enzymes, point mutants,[11] kinetic studies,[20–22] and the three-dimensional structure from X-ray crystallography,[12–15] have afforded a significant understanding of the structure and function of this mammalian zinc metalloenzyme.[23,24] A plethora of inhibitors have been developed and evaluated in clinical trials as potential anti-cancer candidates.[25–33]

Previously, we synthesized didehydrogeranylgeraniol diphosphate (ΔΔGGPP) and determined that this fluorescent pentaene analogue of GGPP was an efficient alternative substrate for GGTase-I.[34] Moreover, ΔΔGG-cysteine derivatives were found to bind tightly to prenyl binding proteins such as guanine nucleotide dissociation protein (GDI). We describe herein the synthesis and biological activity of didehydrofarnesyl diphosphate (ΔΔFPP), the corresponding fluorescent pentaene analogue of FPP.

The synthesis of ΔΔFPP (**12**) is outlined in Scheme 1. We first attempted to make isoprenyl-TBDMS aldehyde **6** using the SeO_2 oxidation of 3-methyl-butyl-2-enyl acetic acid, analogous to our method used for the corresponding didehydrogeranylgeranyl precursor.[34] We believe this reaction gave a lower yield (18%) than previously for the isoprenylogous geranyl acetic acid ester (39%) based upon the rationale shown in Scheme 2. A

Keywords: Farnesol; Geranylgeraniol; Pentaene; Stereoselective Wittig; FTase; GGTase-I; Inhibitor; Protein prenylation.

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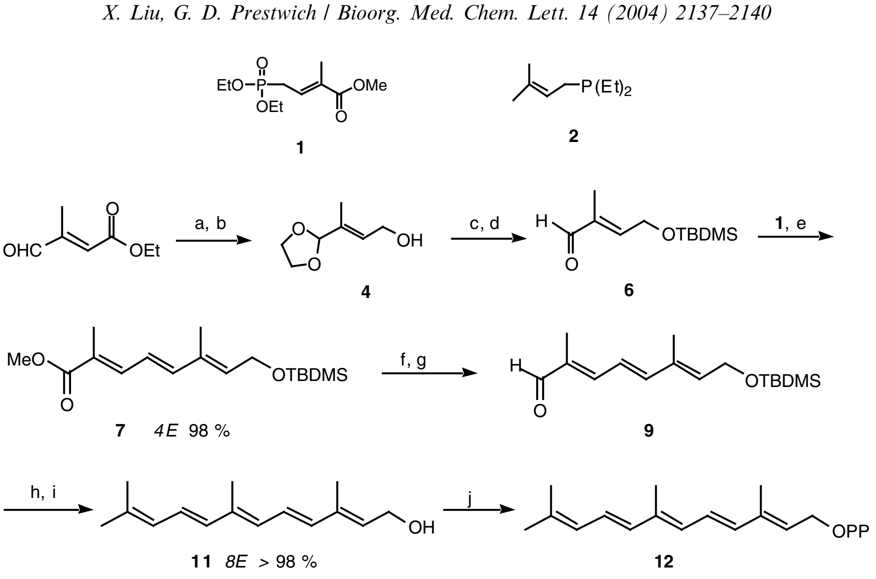
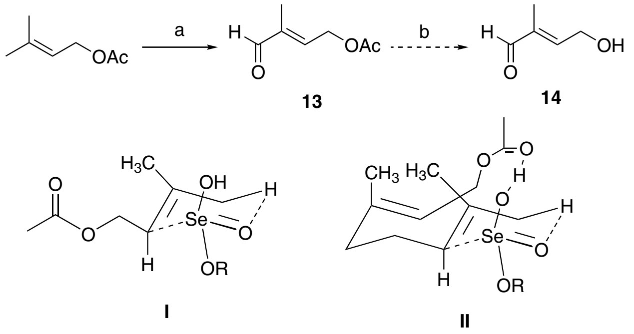


Scheme 1. Synthesis of ΔΔFPP. Reagents and conditions: (a) ethylene glycol, PPTS, 89%; (b) $LiAlH_4$, ether, 59%; (c) TBDMSCl, imidazole, 57%; (d) *p*-TsOH, acetone, 92%; (e) **1**, *n*-BuLi, 0 °C, 70%; (f) $LiAlH_4$/ether, 70%; (g) MnO_2, Na_2CO_3, 30%; (h) **2**, *n*-BuLi, THF, 54%; (i) TBAF, 34%; (j) (n-Bu_4N)H_2PO_4, CH_3CN.



Scheme 2. Attempted preparation of 3-methyl-butyl-2-enyl acetate and six-electron cyclic transition state for SeO_2 oxidation (R = ethyl). Reagents and conditions: (a) SeO_2, 95% ethanol; (b) KOH, 50% EtOH. (I) Proposed transition state for 3-methyl-butyl-2-enyl acetate. (II) Proposed transition state for geranyl acetate.

six-electron transition state mechanism is generally accepted for this *E*-methyl selective oxidation. The nature of the protecting groups greatly affect the stability of transition state and the product yield.[35–37] In the transition state of 3-methyl-butyl-2-enyl acetic acid ester (Scheme 2, I), the acetyl group is probably unable to form hydrogen bonds that can form for the geranyl acetic acid ester (Scheme 2, II). Second, subsequent hydrolysis with KOH/EtOH affords the labile aldehyde **14** that can be quickly degraded under basic reaction conditions.

Isoprenyl-TBDMS aldehyde **6** was prepared according to Torrado's method as outlined in Scheme 1.[38] Next, the Wittig–Horner reaction of isoprenyl-TBDMS aldehyde **6** with allylic diethylphosphonate **1** installed the 4*E* double bond with >98% stereoselectivity to give ester **7** (Scheme 1). Subsequent reduction and oxidation gave the C-1 protected aldehyde **9** ready for the second Wittig reaction. Following coupling of aldehyde **9** with isoprenyl diethyl phosphite **2** at −78 °C, a fluorescent compound TBDMS ether was obtained with >98% 8*E*-selectivity for the newly introduced olefinic bond.[34] Removal of the TBDMS gave the fluorescent pentaenol, ΔΔFOH **11**.

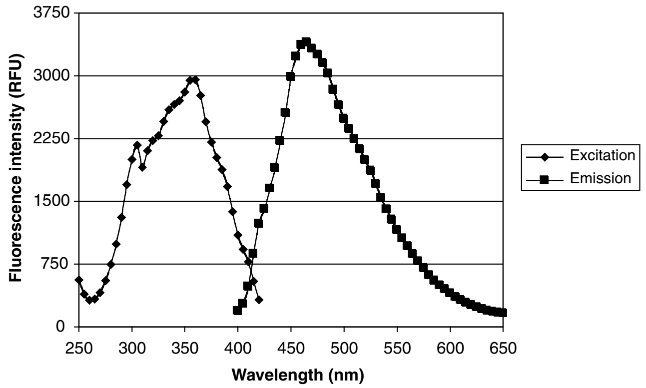


Figure 1. Fluorescence spectra of ΔΔFOH: $\lambda_{ex} = 360\,nm$, $\lambda_{em} = 465\,nm$.

Didehydrofarnesol (ΔΔFOH) displayed a bright blue fluorescence in methanol with $\lambda_{ex} = 360\,nm$ and $\lambda_{em} = 465\,nm$ (Fig. 1). Its fluorescence was moderately environmentally sensitive; when 30% (v/v) of water was titrated into a methanol solution, the fluorescence intensity of **11** at 465 nm was quenched to 50% of the original value (Fig. 2).

General methods to make lipid diphosphates involve converting an alcohol to a halide or a *p*-methylsulfonate followed by substitution with tris(tetra-*n*-butylammonium)hydrogen pyrophosphate.[39] However, attempts to activate ΔΔFOH with numerous reagents, such as PBr_3/pentane,[39] TsCl/Et_3N,[39] LiBr/$(CH_3)_3SiCl$,[40] LiCl/CH_3SO_2Cl,[41] $(CH_3)_3SiBr$,[42] or HMPS/PTB,[43] all failed. In some basic conditions, bis(didehydrofarnesyl)ether was obtained. Obviously, production of the highly

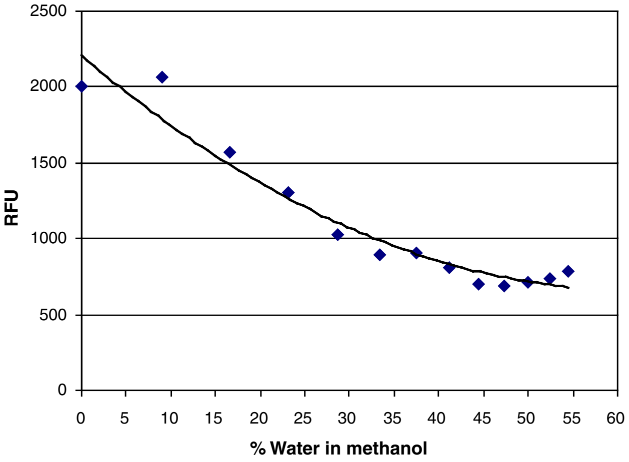


Figure 2. Quenching the fluorescence of ΔΔFOH (methanol) with water.

reactive pentene–allylic intermediate was contraindicated. Therefore, a milder, more S_N2-like protocol using tetra-*n*-butylammonium dihydrogen phosphate as the direct phosphorylation reagent was employed together with HPLC purification to give the desired product, ΔΔFPP.[44]

Protein FTase assays were conducted using a continuous fluorescence assay based on the large fluorescence enhancement that accompanies farnesylation of dansyl-GCVIM.[45,46] Surprisingly, no obvious fluorescence enhancement was observed, demonstrating that ΔΔFPP was not an alternative substrate for the enzyme. This contrasts with the situation for ΔΔGGPP, which had a K_m within a factor of two of the natural substrate GGPP for yeast GGTase-I.[34] However, ΔΔFPP **12** was found to be a potent competitive inhibitor of yeast FTase (Fig. 3). The dissociation constant K_i was 8.8 μM, indicating a threefold higher affinity relative to the natural substrate FPP ($K_m = 27$ μM). The pentaene analogue **12** is a rigid, linear, and electron-rich species. Interestingly, the three-dimensional structure revealed that the binding site of FPP in FTase is not a very straight pocket.[15] Taken together, we expect that the high affinity of **12** as a competitive inhibitor is most likely due to the Van der Waals interaction between the pentaene and hydrophobic and electron-rich aromatic amino acid residues from FTase. This conclusion is substantiated by the observation in the crystal structure of FTase that the substrate-binding site is a hydrophobic pocket surrounded by Trp and Tyr amino acids.[15] Future studies could employ the use of fluorescence resonance energy transfer (FRET) and fluorescent polarization (FP) together with the fluorescent inhibitor ΔΔFPP **12** to identify high-affinity competitive binding inhibitors of FTase.[47]

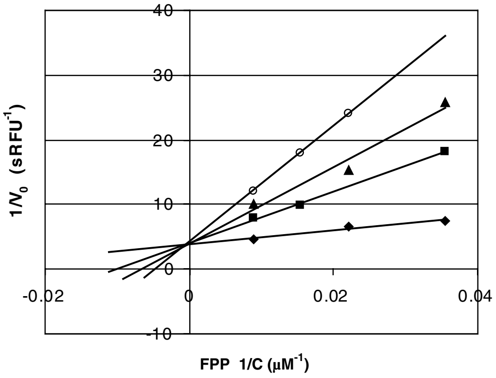


Figure 3. Inhibition of FTase by ΔΔFPP. Reaction mixtures contained 8.2 μM of dansyl-GCVIM, and from 28.2 to 113.2 μM of FPP, and 3 μL FTase. Concentrations of ΔΔFPP: (◆) 0, (■) 21.5, (▲) 43, (○) 64.5 μM.

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