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Synthesis and Biochemical Evaluation of 3,7-Disubstituted Farnesyl Diphosphate Analogues

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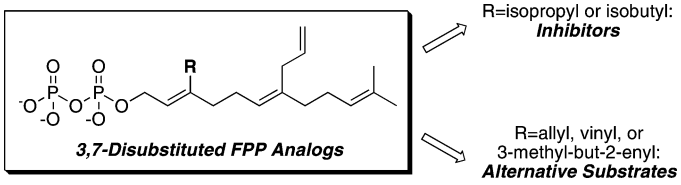

Farnesyl diphosphate (FPP) analogues have proven to be both potent inhibitors of protein-farnesyltransferase (FTase) and valuable probes for the investigation of the function of prenylated proteins. Previously, we have demonstrated that certain 3-substituted and 7-substituted FPP analogues can act as inhibitors of FTase, while others are effective alternative substrates. We have now utilized our vinyl triflate-mediated route to synthesize the first seven FPP variants bearing substituents in both the 3- and 7-positions of the isoprene unit. Despite their exceptional steric bulk with respect to FPP itself, six of the seven analogues bind to FTase. Two of the analogues are potent inhibitors of the enzyme, but a more striking finding is that three FPP variants (**4a**, **4b**, and **4f**) are efficient alternative substrates for FTase.

Introduction

The mevalonate pathway has attracted intense biological[1] and medicinal[2] interest over the past 25 years. The statins, HMG-CoA reductase inhibitors[3] that inhibit the first committed step in the mevalonate pathway and thus act as cholesterol-lowering agents, are one of the most important classes of pharmaceutical agents. Farnesyl diphosphate (FPP, **1**, Scheme 1), a 15-carbon isoprenoid, occupies a key branch point in the mevalonate pathway. The primary route for FPP metabolism in mammalian cells is its conversion into squalene by the enzyme squalene synthase. Squalene is then transformed by a series of enzymatic steps to cholesterol. FPP is converted in the cell to other important isoprenoids, such as dolichol and ubiquinone, which are utilized in protein glycosylation and electron transport, respectively. In plants and many other organisms, FPP is transformed into a vast array of sesquiterpenes, which play diverse biological roles,[4] through the action of sesquiterpene cyclases.[1,5,6]

More recently, it was established that FPP also plays an additional crucial role in eukaryotic cells. It is utilized by the enzyme protein-farnesyl transferase (FTase)[7,8] as the source of a farnesyl moiety that is attached to the cysteine sulfhydryl on the ras oncogenes and proto-oncogenes. Ras, and numerous other proteins that bear a carboxyl-terminal Cys-aaX-OH sequence, where X = methionine, serine, or glutamine (Scheme 1), are modified with a farnesyl moiety. This farnesylation event and the subsequent proteolysis and carboxymethylation modifications serve to increase the hydrophobicity of Ras proteins, target them to the plasma membrane, and are required for their biological activity.[9,10] It is now estimated that >60 human proteins, including many proteins important in signaling and the maintenance of nuclear morphology, are farnesylated.[11,12] Ras proteins must be farnesylated to exert their biological action. Since mutant forms of Ras proteins are involved in numerous

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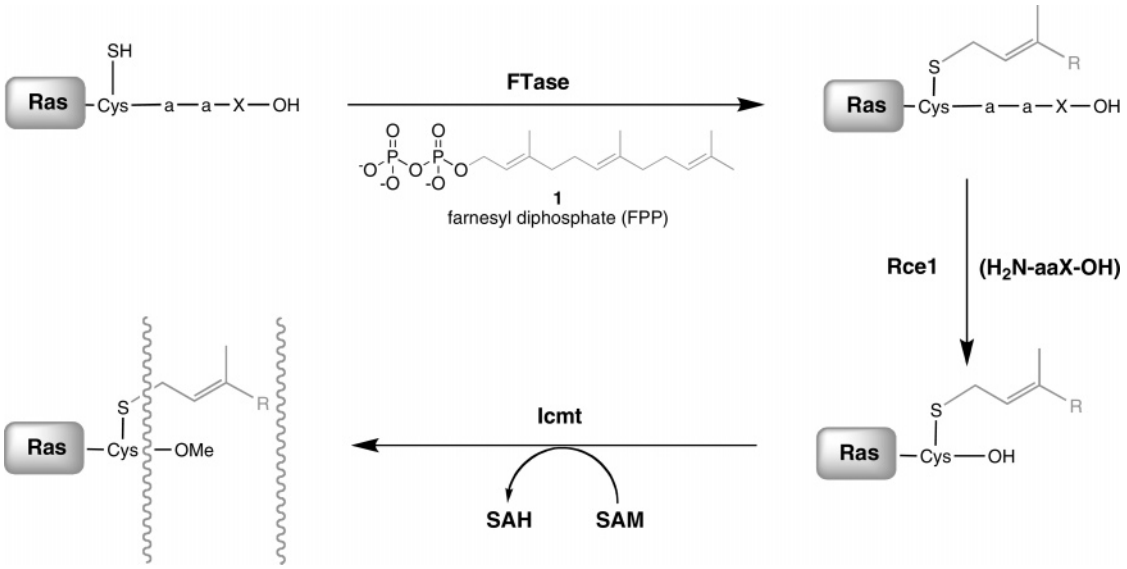
SCHEME 1



human carcinomas,[13] the development of FTase inhibitors as anticancer agents has been an area of intense pharmaceutical interest.[14] Certain FTase inhibitors are in advanced clinical trials as anticancer agents,[14] and are also undergoing clinical investigation for the treatment of progeria.[15,16]

The bulk of the success with regard to the development of FTase inhibitors has been achieved with small molecules derived from compound screening efforts.[17,18] However, studies in this laboratory have demonstrated that FPP analogues such as 3-allylFPP (**2**, Figure 1) are nanomolar FTase inhibitors,[19,20] and can exhibit cellular activity through prodrug variants.[21] In addition, studies in our laboratory[19,22−25] and the Spielmann laboratory[26−28] have demonstrated that diverse FPP analogues can be utilized by FTase to prenylate peptide and protein substrates. The ability of FTase to accept modified FPP analogues has been utilized to provide insight into the mechanism of this enzyme.[22,24,29,30] Moreover, the ability of FTase to accept alternative FPP substrates has been utilized by other groups to incorporate photoaffinity labels,[28,31] affinity tags,[32,33] and fluorophores[34] into CaaX-bearing proteins. In summary, FPP analogues are exceptionally valuable tools to investigate both the mechanism of protein prenylation and the biological consequences of this key post-translational modification. Thus, there is a continuing need to explore the limits of the isoprenoid substrate specificity of FTase.

Previous studies in our laboratory have developed a vinyl triflate-based route to isoprenoids[22] that enabled the facile synthesis of both 3-substituted isoprenoids such as 3-allylFPP (**2**, Figure 1)[19] and geometric isomers of FPP.[25,35] More recently, we have extended our synthetic route to the preparation of 7-substituted FPP analogues (e.g., 7-allylFPP, **3**, Figure 1).[36] The nature of the synthetic route allows for its straightforward extension to the preparation of FPP analogues modified in both the 3- and 7-positions. Such analogues are of interest as a test

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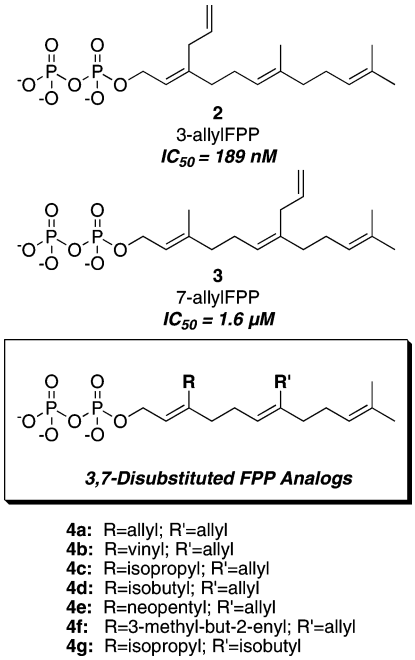

FIGURE 1. Structures of 3-substituted, 7-substituted, and 3,7-disubstituted FPP analogues, with previously determined IC_{50} values for **2**[19] and **3**.[36]

of the ability of the FTase active site to accommodate very bulky isoprenoids. We had observed that certain substituents in the 3-position and the 7-position led to FPP analogues that were potent FTase inhibitors; specifically, both 3-allylFPP and 7-allylFPP are FTase inhibitors. It is possible that a combination of inhibitory features would lead to a synergistic enhancement of potency. Herein we report the preparation of seven 3,7-disubstituted FPP analogues (**4a**−**g**, Figure 1), and their biochemical evaluation as FTase substrates and inhibitors.

Results

The key feature of our synthetic route to substituted isoprenoids is the generation, in a stereocontrolled fashion, of isoprenoid vinyl triflates from β-ketoester precursors.[22] From these intermediates, we then utilize the diversity of coupling reactions leading from vinyl triflates to trisubstituted olefins. In the synthesis of 7-substituted FPP analogues, 7-substituted vinyl triflates such as **5** (Scheme 2) were prepared via a linear, iterative synthetic protocol.[36] Stille coupling of **5** with tetramethyltin provided ester **6**, which was then transformed in three steps into 7-allylFPP **3**. It was realized that **5** could serve as a branch point for the synthesis of numerous analogues of the FTase inhibitor **3**, through coupling reactions of **5** with various organometallic partners to give **7**. In our hands, Stille couplings are preferred to introduce vinyl and allyl **R** groups into vinyl triflates,[22] while copper-catalyzed couplings with Grignard reagents are preferred to introduce alkyl moieties.[37] We have utilized **5** as a starting material to prepare six FPP analogues (**4a**−**f**, Figure 1), via **7a**−**f** (Scheme 3). In addition, to evaluate a more sterically demanding analogue we prepared diphosphate **4g**, bearing branched alkyl chains in both the 3 and 7 positions, via the route shown in Scheme 4.

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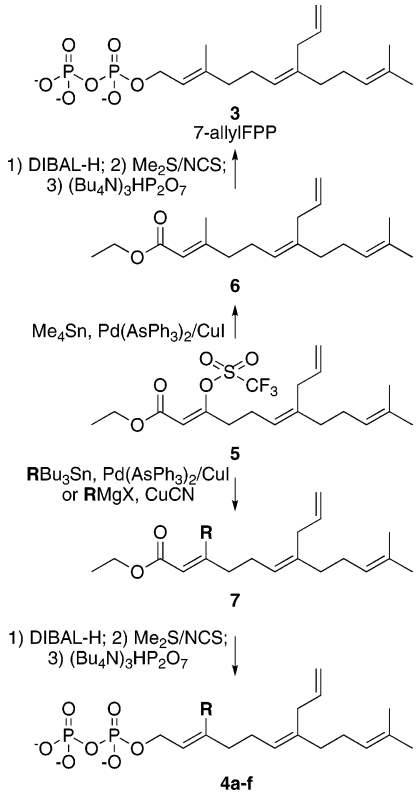
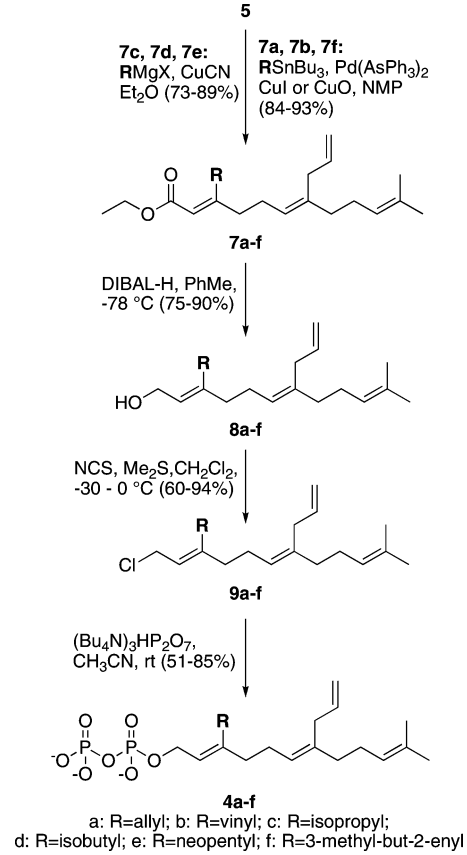
SCHEME 2

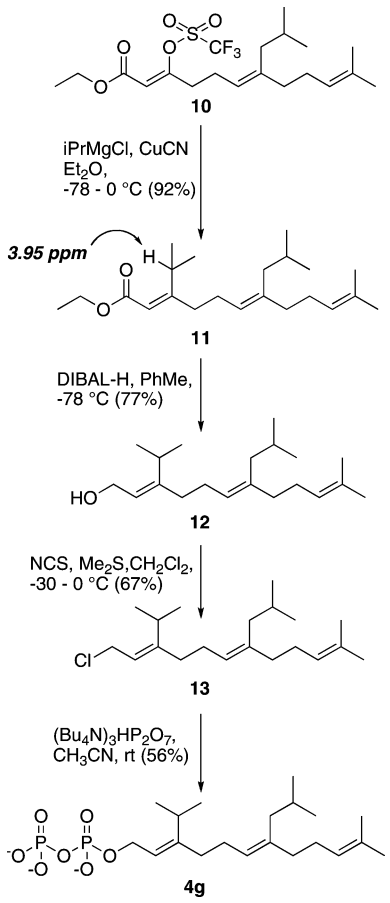


SCHEME 3



Copper cyanide-mediated coupling of vinyl triflate **5** (prepared as previously described[36]) with isopropyl, isobutyl, and neopentyl Grignard reagents afforded esters **7c**, **7d**, and **7e**,

SCHEME 4



respectively, in good yield (73−89%; Scheme 3).[37] A modified Stille coupling (using CuI or CuO as a cocatalyst) of **5** with vinyltributyltin, allyltributyltin, or tributyl(3-methyl-but-2-enyl)-tin led to **7a**, **7b**, and **7f**, respectively, in very good yield (84−93%; Scheme 3).[36] All six esters were reduced to the corresponding farnesol derivatives (**8a**−**f**) by using DIBAL-H in toluene. Conversion of the alcohols to the corresponding allylic chlorides using Corey−Kim conditions proceeded smoothly; due to their instability, **9a**−**f** were not purified but were used directly in the final diphosphorylation step, following the protocol of Davisson et al.,[38] to give the six desired FPP analogues **4a**−**f**. The crude diphosphates were purified by cellulose flash chromatography followed by ion exchange, and then characterized by ^{1}H NMR, ^{31}P NMR, and high-resolution ESI-MS.

The synthesis of the dialkyl diphosphate **4g** proceeded in a very similar manner to that of the six 7-allylFPP derivatives, but starting from vinyl triflate **10**[36] as illustrated in Scheme 4. Copper cyanide-mediated coupling of **10** with isopropyl magnesium chloride afforded ester **11** in excellent yield (92%; Scheme 4).[37] One unusual characteristic of ester **11** is the exceptional downfield shift of the signal for the indicated proton in the isopropyl moiety, which appears at 3.95 ppm. This shift, which is presumably due to the deshielding effect of the carbonyl oxygen, is similar to one seen previously in the ester intermediate in the synthesis of 3-vinylFPP[22,39] but is even more striking in its magnitude. Ester **11** was reduced to the corresponding farnesol derivative **12** by using DIBAL-H in toluene. Conversion of the farnesol analogue to the corresponding allylic chloride **13**, followed by diphosphorylation, gave the desired FPP analogue **4g**.

The FTase substrate activity of FPP analogues can be measured through a fluorescence assay[40] in a 96-well plate format,[23,41,42] with a dansylated pentapeptide that mimics the CaaX box of H-Ras. Briefly, 3 μM Dansyl-GlyCysValLeu-Ser-OH, 3 or 9 μM FPP analogue, and buffer are combined, farnesylation is initiated with recombinant mammalian FTase (0.05 μM), and the increase in fluorescence intensity is measured at 30 min (335 nm excitation, 485 and 535 nm emission). To confirm that the increase in fluorescence seen in the spectrofluorimetric assay was due to the alternative prenylation of the peptide, HPLC analysis was performed for each FPP analogue reaction.

The results of the substrate screen for the seven disubstituted FPP analogues (Supporting Information, Figure S1) are strikingly bimodal: four analogues exhibit little (**4c**) or no ability (**4d**, **4e**, and **4g**) to act as alternative FTase substrates. However, the other three analogues (**4a**, **4b**, and **4f**) are essentially equivalent to the natural substrate FPP in their ability to act as FTase cosubstrates with Dansyl-GlyCysValLeuSer-OH (dn-GCVLS). It is noteworthy that all three analogues that are good substrates bear unsaturated substituents in both the 3- and 7-positions. The results of the fluorescence-based screen were confirmed by HPLC analysis (Supporting Information, Figure S4); under the conditions utilized, with **4a**, **4b**, and **4f** dn-GCVLS was completely converted to prenylated peptide product, while no prenylated peptide product was seen with **4d**, **4e**, and **4g**. The 3-isopropyl-7-allyl analogue **4c** exhibited a small amount of product formation after 1 h, but the bulk of the dn-GCVLS substrate remained. The four analogues that were not efficient substrates were then evaluated further as FTase inhibitors, using the 96-well plate assay (Supporting Information, Figure S2). This evaluation demonstrated that **4c** and **4d** are both effective inhibitors, with estimated IC_{50} values less than 1 μM. The very bulky 3-isopropyl-7-isobutyl derivative **4g** exhibits mixed behavior—modest inhibition at 3 μM concentration, but potentially substrate behavior at 9 μM. We have observed similar mixed inhibitor/substrate behavior with other FPP analogues.[24,37] Of the seven analogues evaluated, only the 3-neopentyl-7-allyl derivative **4e** exhibits no appreciable interaction with the enzyme.

The preliminary results described above require more detailed examination of the kinetic behavior of the six analogues that are bound to FTase. The K_M and k_{cat}/K_M values for the three effective substrates **4a**, **4b**, and **4f** were determined, along with the IC_{50} values for the three apparent inhibitors (**4c**, **4d**, and **4g**). The results are summarized in Table 1, and the kinetic plots are presented in the Supporting Information (Figure S3). Note that **4c** exhibits tight binding behavior, and also very slow but measurable turnover. Consistent with the preliminary screening results, **4a**, **4b**, and **4f** are effective alternative substrates, with k_{cat}/K_M values within a factor of 4 of FPP, despite their steric bulk. Analogues **4c** and **4d** are potent inhibitors of FTase, although not as effective as 3-*tert*-butylFPP, one of the most

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TABLE 1. Determination of Kinetic Constants for 4a−g in a 96-Well Plate Format[g]

	K_M^{app} (nM)[a]	k_{cat}/K_M(rel)[c]	IC_{50}
1: FPP	28 ± 21[b]	1.0	nd
2: 3-allylFPP	nd	nd	119[d]
3-*tert*-butylFPP	nd	nd	4[e] (31[d])
3: 7-allylFPP	nd	nd	1600[d]
4a: 3,7-diallylFPP	51 ± 33	0.33	nd
4b: 3-vinyl-7-allylFPP	68 ± 65	0.27	nd
4c: 3-isopropyl-7-allylFPP	nd	nd	41
4d: 3-isobutyl-7-allylFPP	nd	nd	20
4e: 3-neopentyl-7-allylFPP	nd	nd	nd
4f: 3-(3-methylbut-2-enyl)-7-allylFPP	∼120	∼0.28	nd
4g: 3-isopropyl-7-isobutylFPP	nd	nd	<10[f]

[a] Apparent isoprenoid K_M values. [b] K_M value for FPP determined by using the method described in the Experimental Section; our previously determined K_m value for FPP, using the standard continuous spectrofluorimetric assay,[40,43] was 107 nM.[19] [c] Relative apparent k_{cat}/K_M values, with the value for FPP (0.178 μM^{-1} s^{-1}) set at 1.0. [d] IC_{50} values previously determined for 3-allylFPP,[19] 3-*tert*-butylFPP,[19] and 7-allylFPP.[36] [e] IC_{50} value determined for 3-*tert*-butylFPP, using the assay procedure described in the Experimental Section. [f] Estimated IC_{50} value for the inhibitory phase seen with **4g**; see the text for further description. [g] See the Experimental Section for the Experimental Protocol.

potent isoprenoid-based FTase inhibitors previously reported.[19] Analogue **4g** exhibits unusual, biphasic behavior. Addition of 10 nM **4g** leads to >50% percent inhibition of FTase, but no further inhibition is seen with addition of up to 500 nM **4g**.

Discussion

One of the key objectives of our study was to delineate the tolerance of the FPP binding pocket of FTase for diphosphate analogues modified in the first and second isoprene units. Our results indicate that the enzyme can accept surprisingly bulky isoprenoid derivatives—only one of the seven analogues exhibited no ability to bind to FTase. This compound (**4e**) bears a neopentyl moiety in the 3-position, and we have previously demonstrated that 3-neopentylFPP itself binds poorly to FTase ($IC_{50} \approx 6.2$ μM),[37] whereas the other 3-substituents examined led to FPP analogues that bound with nanomolar affinity to FTase.[19,24,37] Given the large volume of the FTase active site, it is perhaps not surprising that bulky isoprenoids such as **4d** bind to and inhibit FTase. However, the ability of three of these analogues to act as FTase substrates is somewhat surprising, in view of the unique mechanism utilized by FTase.[7,8] As illustrated by the elegant structural studies of the Beese group,[44] the displacement of the prenylated peptide from the enzyme is assisted through the binding of a second FPP molecule, leading to a crystallographically determined product-FPP-FTase complex, where the isoprenoid unit of the product has undergone a large rearrangement into an exit groove on the enzyme. The ability of three of the 3,7-disubstituted analogues to act as very efficient substrates for FTase places useful constraints on any models for the unusual rearrangement exhibited by the prenylated peptide product in the FTase active site.[45,46]

A second objective of the project was to utilize the ability to combine isoprenoid substituents with the previously existing SAR for 3- and 7-substituted isoprenoids to generate potentially more potent FTase inhibitors. One might expect that combining the 3-allyl substituent present in **2**—a potent FTase inhibitor—with the 7-allyl substituent present in **3**—a modest FTase inhibitor—would lead to a more potent compound. This was clearly not the case—instead, the 3,7-diallylFPP analogue **4a** is a very effective alternative substrate. Currently, we have no explanation for the ability of **4a**, **4b**, and **4f** to act as potent substrates, while other closely related substrates are inhibitors. Note that FTase has a complex, multistep reaction mechanism. We have recently reported an extensive study of the interaction of 7-substituted isoprenoid analogues with different peptide analogues, and this study clearly demonstrated that substrate ability of the peptide/isoprenoid pairs could not be predicted through evaluation of relative sizes or any other simple experimental model.[23] Spielmann and co-workers have recently reported on peptide/isoprenoid interactions with FTase, and have also demonstrated that the factors that govern substrate versus inhibitory interactions are quite subtle and complex.[47,48] A more detailed understanding of this phenomenon, and also the unusual biphasic behavior of **4g**, will require more detailed kinetic, and perhaps crystallographic analysis of the binding of isoprenoid analogues to FTase.

Regardless of the rationale for their activity, the development of efficient alternative isoprenoid substrates may be as valuable as the determination of more potent FTase inhibitors. We have demonstrated that certain FPP analogues exhibit potentially useful selective substrate behavior.[23,24] In addition, the Spielmann laboratory has demonstrated that the introduction of more polar farnesyl moieties onto Ras proteins can interfere with their biological activity through a loss of membrane targeting.[49] Moreover, it is now well-established that the prenyl moiety on proteins can act as a "handle" for interaction with other proteins,[50] and in particular, it has been demonstrated that the farnesyl moieties of Ras proteins play a more complex role in their biological activity than simple membrane attachment.[51] It would be interesting to determine if the introduction of an unnatural bulky prenyl moiety, such as the 3,7-diallylfarnesyl group, onto Ras could modulate its biological activity.

Experimental Section

(2*Z*,6*Z*)-Ethyl 3,7-Diallyl-11-methyldodeca-2,6,10-trienoate (7a). General procedure for the Stille coupling reaction: A suspension of Ph_3As (16.2 mg, 0.05 mmol), $Pd(II)Cl_2(PhCN)_2$ (12.7 mg, 0.03 mmol), and CuI (6.29 mg, 0.03 mmol) in 1.0 mL of NMP was stirred under an argon atmosphere at 70−75 °C. A solution of triflate **5** (140 mg, 0.33 mmol)[36] in 0.5 mL of NMP was added, followed by allyltributyltin (163.9 mg, 0.49 mmol), and the reaction mixture was stirred at the same temperature for 15 h. It was then cooled and taken up in ethyl acetate (25 mL), and washed with aqueous KF solution (2 × 20 mL) and water (2 × 20 mL), then the water layer was back extracted with ethyl acetate (2 × 30 mL).

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The combined organic layers were dried over $MgSO_4$, and after concentration purified by flash chromatography (2% ethyl acetate/hexanes). Yield 97 mg (93%); ^{1}H NMR ($CDCl_3$) δ 1.24 (t, J = 7.18 Hz, 3H), 1.54 (s, 3H), 1.57 (s, 3H), 1.92−1.99 (m, 6H), 2.26 (br s, 2H), 2.69 (d, J = 6.50 Hz, 2H), 3.34 (d, J = 6.71 Hz, 2H), 4.11 (d, J = 7.10 Hz, 2H), 4.89−5.12 (m, 6H), 5.64 (m, 3H); MS EI (*m*/*z*) 316 (M^+); HRMS calcd for $C_{21}H_{32}O_2$ 316.2404, found 316.2403. Anal. Calcd for $C_{21}H_{32}O_2$: C, 79.69; H, 10.19. Found: C, 79.91; H, 10.02.

(2*Z*,6*Z*)-Ethyl 7-Allyl-3-isopropyl-11-methyldodeca-2,6,10-trienoate (7c). General procedure for the CuCN-catalyzed Grignard coupling reaction: To a stirred suspension of CuCN (79 mg, 0.88 mmol) in 5 mL of dry ether was added isopropyl magnesium bromide (3 M in ether, 0.25 mL, 0.75 mmol) dropwise at −78 °C, and the resulting suspension was then stirred at 0 °C for 5 min. The suspension was recooled to −78 °C and a solution of triflate **5** (107 mg, 0.25 mmol) in 5 mL of ether was added dropwise. After 2.5 h at −78 °C, the reaction mixture was quenched with saturated aqueous NH_4Cl. The aqueous layer was extracted with ethyl acetate (3 × 20 mL), and the combined organic layers were dried with $MgSO_4$ and concentrated. The viscous oil thus obtained was purified by flash chromatography with 3% ethyl acetate/hexanes as an eluent. Yield 89%; ^{1}H NMR ($CDCl_3$) δ 1.02 (d, J = 6.92 Hz, 6H), 1.24 (t, J = 7.25 Hz, 3H), 1.57 (s, 3H), 1.65 (s, 3H), 1.97−2.12 (m, 8H), 2.77 (d, J = 6.25 Hz, 2H), 3.79 (s, 1H), 4.11 (q, J = 7.13 Hz, 2H), 4.94−5.18 (m, 4H), 5.55 (s, 1H), 5.69 (m, 1H); ^{13}C NMR ($CDCl_3$) δ 14.3, 17.6, 20.6, 25.6, 26.5, 26.7, 29.4, 31.6, 34.9, 36.9, 59.4, 114.1, 115.1, 124.2, 124.6, 131.4, 136.1, 137.6, 166.5, 168.3; GC MS (retention time 8.79 min) CI (*m*/*z*) 319 (M^+ + H), 273, 245.

(2*Z*,6*Z*)-3,7-Diallyl-11-methyldodeca-2,6,10-trien-1-ol (8a). General procedure for the DIBAL-H reduction of esters: A solution of ester **7a** (95 mg, 0.3 mmol) in toluene (5 mL) was cooled to −78 °C, and diisobutylaluminum hydride (1.5 M in toluene, 149.4 mg, 1.05 mmol) was added. The reaction mixture was stirred at the same temperature for 1.0 h. The reaction mixture was then quenched with 30 mL of aqueous potassium sodium tartrate solution and the aqueous phase was extracted with ethyl acetate (3 × 20 mL). The combined organic layers were washed with brine (35 mL) and dried ($MgSO_4$). Filtration and concentration followed by flash chromatography (12% ethyl acetate/hexanes) gave 61.5 mg (75%) of the desired alcohol **8a**. ^{1}H NMR ($CDCl_3$) δ 1.52 (s, 3H), 1.61 (s, 3H), 1.93−2.15 (m, 8H), 2.17 (br s, 1H), 2.71 (d, J = 6.40 Hz, 2H), 2.77 (d, 2H), 4.09 (d, J = 7.08 Hz, 2H), 4.93−5.02 (m, 6H), 5.45 (t, J = 6.70 Hz, 1H), 5.61 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 17.6, 25.6, 26.2, 26.7, 34.8, 35.0, 37.0, 59.0, 115.0, 115.1, 115.5, 124.2, 124.7, 125.1, 131.3, 136.0, 136.3, 137.0, 140.9; MS CI (*m*/*z*) 275 (M^+ + H).

(4*Z*,7*Z*)-8-Allyl-4-(2-chloroethylidene)-12-methyltrideca-1,7,-11-triene (9a). General procedure for the conversion of alcohols to chlorides: *N*-Chlorosuccinimide (NCS; 43.8 mg, 0.33 mmol) was dissolved in 2.5 mL of dichloromethane, and the resulting solution was cooled to −35 °C. To this solution was added 20.4 mg of dimethyl sulfide (0.33 mmol) dropwise. The reaction mixture was warmed to 0 °C, and maintained at that temperature for 5 min, then it was recooled to −40 °C, and a solution of **8a** (60 mg, 0.22 mmol) in 2 mL of dichloromethane was added dropwise. The reaction was stirred at 0 °C for 2.5 h, and solvent was then removed. Standard aqueous workup afforded the desired chloride, which was sufficiently pure for direct use in the next reaction. Yield 60%; ^{1}H NMR ($CDCl_3$) δ 1.54 (s, 3H), 1.58 (s, 3H), 1.97−2.13 (m, 8H), 2.74 (d, J = 6.0 Hz, 2H), 2.89 (d, J = 6.0 Hz, 2H), 4.09 (d, J = 7.08 Hz, 2H), 4.95−5.16 (m, 6H), 5.50 (t, 1H), 5.76 (m, 2H); ^{13}C NMR ($CDCl_3$) δ 17.7, 25.7, 26.0, 26.7, 34.8, 34.9, 37.0, 40.6, 115.1, 116.1, 121.7, 124.3, 124.8, 131.4, 135.2, 136.2, 137.3, 143.7; GC MS (retention time 8.205 min) EI (*m*/*z*) 292 (M^+), 294 (M^+ + 2), 257.

(2*Z*,6*Z*)-7-Allyl-3-isopropyl-11-methyldodeca-2,6,10-trien-1-diphosphate (4a). General procedure for the synthesis of diphosphates: To a suspension of tris(tetrabutylammonium)-hydrogen pyrophosphate (258.5 mg, 0.59 mmol) in 3.0 mL of acetonitrile was added a solution of **9a** (41 mg, 0.15 mmol) in 2 mL of acetonitrile dropwise. The reaction mixture was stirred at room temperature for 3.0 h, and solvent was then removed by rotary evaporation at 35 °C. The residue was dissolved in deionized water, and resulting solution was passed through a 2 × 8 cm Dowex AG 50 × 8 ion exchange column, using NH_4HCO_3 buffer (350 mg of NH_4HCO_3, 500 mL of H_2O, 10 mL of isopropanol) as an eluant. A two-column volume of the eluant (120 mL) was collected and lyophilyzed for 10−12 h. The residue thus obtained was redissolved in deionized water and purified by cellulose flash columm chromatography [eluant buffer: isopropanol:acetonitrile:H_2O (500:250:250 mL), NH_4HCO_3 (4.0 g)] as an eluant. Fractions 10−20 contained the product, as demonstrated by cellulose TLC detection (visualized with sulfosalicylic acid (1% in ethanol:water 3:2 v/v), and ferric chloride (0.2% in ethanol:water 4:1 v/v) solution). These fractions were concentrated by speedvac to remove the eluent buffer, which afforded the diphosphate **4a** as an amorphous, flocculent white solid. Yield 59%; ^{1}H NMR (D_2O) δ 1.32 (s, 3H), 1.39 (s, 3H), 1.79 (m, 8H), 2.54−2.64 (m, 4H), 4.27 (br s, 2H), 4.97 (m, 6H), 5.28 (br s, 1H), 5.52 (m, 2H); ^{31}P NMR (100 MHz, D_2O) δ −6.02 (d, J = 20 Hz), −8.89 (d, J = 20 Hz); MS ESI (*m*/*z*) 433 (M^+ − H); HRMS, ESI (negative) calcd for $C_{19}H_{31}P_2O_7$ 433.1705, found 433.1702.

FTase Spectrofluorimetric Assay Procedures. Screening procedure: Farnesylation of dansylated-CaaX peptides is evaluated with an established fluorescence assay based protocol,[40,43] scaled to a 96-well plate format in the same manner as for the GGTase I fluorescence assay,[41] as we have recently described.[23] Assays are run in black 96-well plates in buffer composed of 52 mM Tris at pH 7.5 with 5.8 μM DTT, 12 μM $MgCl_2$, and 12 mM $ZnCl_2$. FTase is maintained in 20 mM Tris at pH 7.0 with 1 μM dithiothreitol (DTT) and 5 mM $ZnCl_2$; FPP analogues are maintained in 25 mM NH_4HCO_3. Reactions were performed in triplicate utilizing 3,7-substituted FPP analogues or FPP at 1, 3, and 9 μM Dansyl-GlyCysValLeuSer-OH (3 μM) and 50 nM FTase. The total reaction volume is 200 μL. Enzyme reactions were initiated with 10 μL of 1 μM recombinant mammalian FTase[52] and the resulting increase in fluorescence from prenylation was measured at 0, 30, and 60 min on a fluorimetric plate reader (excitation 335 nm, emission 485 and 535 nm).

Kinetic procedure: The K_m and IC_{50} values for the 3,7-substituted FPP analogues were determined by using the standard fluorescence assay for FTase activity.[40,43] We have modified the kinetic assay to a 96-well plate format incorporating the features of our single cuvette assay into our screening assay,[23] in a similar manner to that recently described by Spielmann and colleagues.[47,48] Measurements were performed in triplicate with 1 mM dansyl-GCVLS, 1 μM FPP (for IC_{50} determination only), varied concentrations of 3,7-substituted analogue, and 10 nM FTase in 52 mM Tris at pH 7.5 with 5.8 μM DTT, 12 μM $MgCl_2$, 12 mM $ZnCl_2$, and 0.005% dodecylmaltoside buffer, dnGCVLS, FPP (for IC_{50}), and FPP analogue to the 96-well plates (Nunc F96 Microwell Plates, black). After a 30 min incubation, reactions were initiated with 20 μL of 100 nM recombinant rat FTase[52] for a final reaction volume of 200 μL. The peptide fluorescence was measured for 15 min on a Perkin-Elmer Fusion plate reader (excitation 335 nm, emission 485 nm). The initial velocities were obtained from the slope of the increase in fluorescence seen during the first minute of reaction. The velocities were plotted into Graphpad prism software and analyzed for either k_{cat}/K_M or IC_{50} values.

FTase HPLC Assay Procedure. The HPLC assays were performed in the same manner as the continuous spectrofluorimetric assay except that the reactions were quenched with acetonitrile at 0, 5, 30, or 60 min and then analyzed via HPLC with a C_8 reversed-

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phase column. Samples were eluted with a 20−100% acetonitrile/0.025% TFA(aq) gradient over 30 min (1 mL min^{-1}) and detected by absorbance (254 nm) and fluorescence (excitation 335 nm, emission 486 nm). The results of the 60 min timepoints (see Supporting Information, Figure S4) demonstrate that **4a**, **4f**, and **4g** are effective substrates, leading to complete conversion of dn-GCVLS to prenylated peptides, while **4c** is a poor substrate that leads to partial conversion of dn-GCVLS (retention times of prenylated peptide products: **4a**, 23.0 min; **4b**, 22.1 min; **4c**, 23.8; **4f**, 24.0 min). No prenylated peptide product was observed with **4d**, **4e**, or **4g**.

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Supporting Information Available: Additional synthetic experimental and spectral data, HPLC traces for the FTase-catalyzed coupling of **4b**−**g** with Dansyl-GCVLS, preliminary screening data for analogues with FTase, kinetic and inhibitory graphs used to determine K_m and IC_{50} values, and proton NMR spectra of esters **7b**−**f**, alcohols **8b**−**f**, chlorides **9b**−**f**, diphosphates **4b**−**f**, and compounds **11**−**13** amd **4g**. This material is available free of charge via the Internet at http://pubs.acs.org.

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