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Novel Limonene Phosphonate and Farnesyl Diphosphate Analogues: Design, Synthesis, and Evaluation as Potential Protein-Farnesyl Transferase Inhibitors

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Abstract—Limonene and its metabolite perillyl alcohol are naturally-occurring isoprenoids that block the growth of cancer cells both in vitro and in vivo. This cytostatic effect appears to be due, at least in part, to the fact that these compounds are weak yet selective and non-toxic inhibitors of protein prenylation. Protein-farnesyl transferase (FTase), the enzyme responsible for protein farnesylation, has become a key target for the rational design of cancer chemotherapeutic agents. Therefore, several α-hydroxy-phosphonate derivatives of limonene were designed and synthesized as potentially more potent FTase inhibitors. A noteworthy feature of the synthesis was the use of trimethylsilyl triflate as a mild, neutral deprotection method for the preparation of sensitive phosphonates from the corresponding *tert*-butyl phosphonate esters. Evaluation of these compounds demonstrates that they are exceptionally poor FTase inhibitors in vitro ($IC_{50} \geq 3$ mM) and they have no effect on protein farnesylation in cells. In contrast, farnesyl phosphonyl(methyl)phosphinate, a diphosphate-modified derivative of the natural substrate farnesyl diphosphate, is a very potent FTase inhibitor in vitro ($K_i = 23$ nM).

Introduction

The development of protein farnesylation inhibitors as potential novel cancer chemotherapeutic agents has been remarkably rapid. The initial evidence that proteins are modified with a mevalonate pathway intermediate was presented scarcely a decade ago. Protein farnesylation, the best-studied protein prenylation process, is carried out by the enzyme protein-farnesyl transferase (FTase), which recognizes the CAAX box (where A = aliphatic and X = Ser or Met) at the carboxyl terminus of a protein substrate and then attaches the farnesyl group from farnesyl diphosphate (FPP, Figure 1) to the free sulfhydryl of the cysteine residue.[1] Since the key signal transduction protein and oncogene product Ras is farnesylated, FTase has been the subject of intense research interest. Inhibitors of this enzyme block the action of mutant Ras proteins and halt the growth of *ras* transformed cells.[2–4] Significant progress has been made in the development of peptide-based FTase inhibitors, and some of these compounds have shown great promise in vivo as potential anti-cancer agents.[5] However, less work has been done on FPP-based FTase inhibitors,[3] and thus less is known regarding the specificity of FTase for its isoprenoid substrate.

Concurrent with the discovery of protein farnesylation was the observation that certain isoprenoid natural products are effective, at high micromolar to low millimolar levels, in blocking the growth of cancer cells in vitro and in vivo.[6,7] Particular interest has been focused on the dietary monoterpenes limonene and perillyl alcohol (Fig. 1), which are currently undergoing clinical trials as anticancer agents. These compounds have multiple modes of action in cells. They appear to act in part by inhibiting protein prenylation,[8] but there has been some controversy about the cellular relevance of this inhibition.[9–11] However, work by Gelb et al. clearly indicates that limonene, perillyl alcohol, perillic acid, and perillic acid methyl ester are low millimolar to micromolar inhibitors of mammalian and yeast FTase in vitro.[12] In particular, it was demonstrated that the methyl ester is a FPP-competitive inhibitor of yeast FTase. But the potency of these compounds is modest compared to many synthetic FTase inhibitors, which

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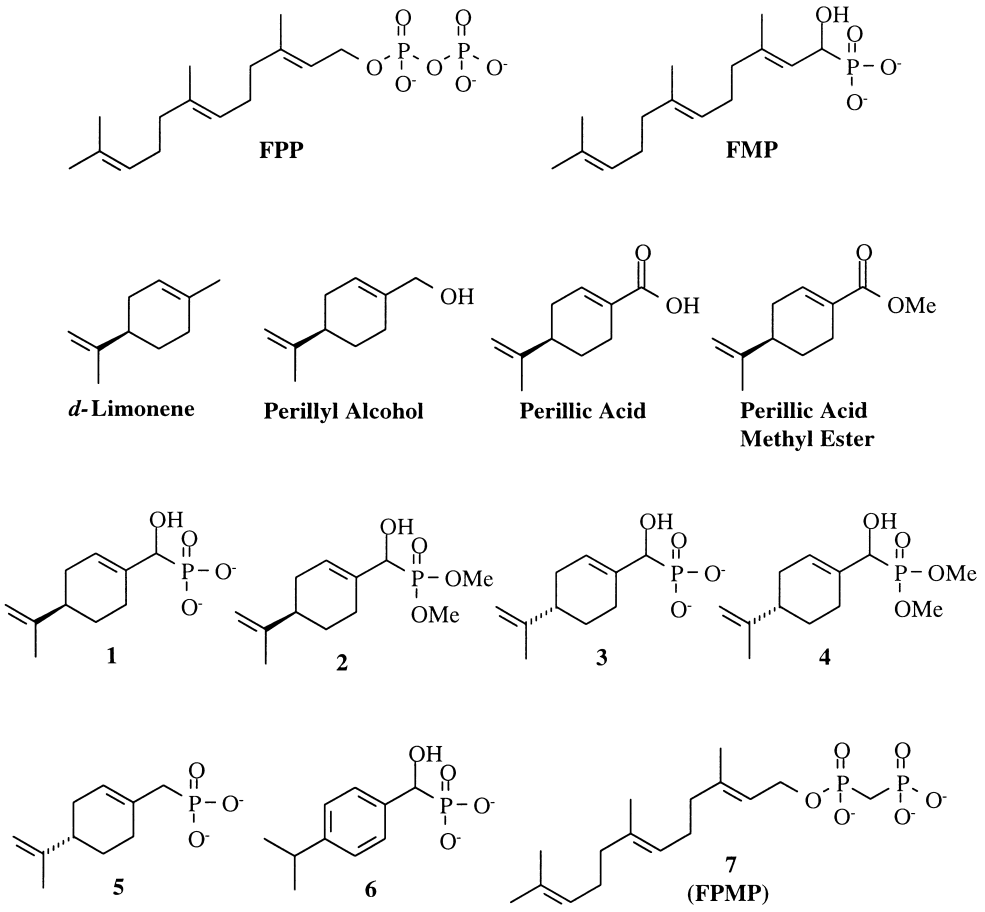

Figure 1.

are effective in the nanomolar or even subnanomolar range.[3] For example, the FPP derivative α-hydroxyfarnesylphosphonate (FMP, Figure 1), exhibits a K_i of 5 nM versus mammalian FTase.[13] Despite this in vitro potency, FMP exhibits poor selectivity and high toxicity in cellular assays for protein prenylation inhibition.[14] We hypothesized that combining the α-hydroxyphosphonate moiety of FMP with the cyclic monoterpene structure of limonene would generate compounds more potent than the natural products, yet potentially with the same cellular potency and selectivity. Thus we have synthesized compounds **1–4** and tested them in vitro and in situ as potential FTase inhibitors. The limonene phosphonate analogue **5**, along with the aromatic α-hydroxyphosphonate **6** were also prepared and tested. Although a number of limonene derivatives have been evaluated as inhibitors of cancer cell growth,[15] these are the first phosphonate analogues of limonene reported. Finally, we have evaluated the readily prepared FPP analogue FPMP **7** as an alternative diphosphonate modified FTase inhibitor.

Results

Synthesis

The primary targets of this project were the two (*R*)-hydroxyphosphonate analogues **1** and **2**, as these compounds have the same configuration as the biologically active natural products *d*-limonene and (*R*)-perillyl alcohol. However, (*R*)-perillyl alcohol and (*R*)-perillaldehyde, the starting materials needed to prepare **1** and **2**, were not readily available at the time this project was initiated. Therefore, the (*S*)-isomers **3** and **4** were initially targeted for synthesis in order to test and develop the synthetic methodology. This also provided an opportunity to evaluate the effect of isomerization on the biological activity of the phosphonates. If the monoterpenes bind to FTase as conformationally restricted analogues of the farnesyl moiety of FPP, then this isomerization may have a profound effect on the ability of the hydroxyphosphonates to inhibit the enzyme.

Initial attempts were made to prepare **3** and **4** from commercially available (*S*)-perillaldehyde **8** with the same procedure used by the Merck group to synthesize FMP from farnesal.[13] The triethylamine-catalyzed coupling of dimethyl phosphite with **8** proceeded smoothly to give **4**. However, deprotection of **4** to give **3** was unsuccessful. Using the original conditions of Pompliano et al. (TMSBr in the presence of triethylamine), the methyl protecting groups were removed but the terminal double bond of the isopropylidene side chain was also removed, possibly via addition of HBr to give the tertiary bromide. Deprotection attempts using a variety of other bases in place of triethylamine were also unsuccessful,[16]

as was the use of conditions that generate TMSBr in situ from TMSCl.[17] The lack of success was surprising and unanticipated, in view of the fact that other groups have successfully used methyl protecting groups for the synthesis of a variety of phosphonates.[18] However, it is known that perillyl alcohol and its derivatives are quite susceptible to acid-catalyzed rearrangement reactions.[19]

We next examined the synthesis and deprotection of the dibenzyl phosphonate limonene derivatives, specifically the compound leading to the limonene phosphonate **5**, as a wide variety of deprotection conditions are available to remove benzyl groups. Again, the preparation of the protected phosphonate was quite facile. Commercially available (*S*)-perillyl alcohol **9** was readily transformed to the chloride **10** using the Corey–Kim procedure (Fig. 2).[20] Coupling of **10** with the sodium salt of dibenzyl phosphinate afforded the desired dibenzyl phosphonate **11** in virtually quantitative yield. As with the dimethyl phosphonate **4**, treatment with TMSBr leads to removal of the protecting groups but also destruction of the isopropylidene double bond. A variety of hydrogenolysis conditions were used in an attempt to deprotect **11**, and all of them were unsuccessful, leading to reduction of one or both double bonds in **11** as well as removal of the benzyl groups. A very mild transfer hydrogenation protocol did afford the monobenzyl derivative **12**, but further treatment of **12** again led to reduction of the terminal double bond. Deprotection was also attempted using hydrolytic conditions. The dibenzyl ester was refluxed under basic conditions (1 M KOH), but this resulted in only partial hydrolysis after 5 h, and further or more vigorous treatment led to decomposition. Thus it appeared that a different protecting group that allowed for an alternative deprotection protocol would be needed to prepare the desired limonene phosphonates.

The *tert*-butyl ester protecting group has been extensively employed in peptide synthesis. It has the particular advantage in that it can be removed under acidic, but aprotic conditions, which contrast strongly with the conditions used for the deprotection for methyl and benzyl esters.[21,22] More recently, it has also been used as a protecting group in the synthesis of various phosphonates. In particular, Burke and co-workers have used the *tert*-butyl protecting group in the synthesis of aromatic, α-hydroxyphosphonate inhibitors of tyrosine kinases.[23] We therefore investigated the synthesis of *tert*-butyl limonene-derived α-hydroxyphosphonates and their deprotection.

The synthesis of the aromatic analogue **6** via the *tert*-butyl protected compound **14** was first attempted (Fig. 3). This compound was chosen as a model system closely analogous to the α-hydroxyphosphonates prepared by Burke, and also as an aromatic analogue of the hydroxyphosphonates **1** and **3**. Alumina-mediated coupling of 4-isopropylbenzaldehyde **13** with di-*tert*-butylphosphite afforded the protected α-hydroxyphosphonate **14**. Deprotection with trifluoroacetic acid/anisole afforded the desired aromatic phosphonate **6** in excellent yield. Next, the synthesis of **3** using the same procedure was attempted. Although preparation of **15** using alumina as a base was not successful, it was synthesized in modest yield from **8** using both sodium ethoxide and triethylamine as bases. Next, deprotection of **15** was attempted using TFA/anisole as for **14**. However, compound **3** was not obtained, and instead it appeared that the limonene skeleton had undergone acid-catalyzed double bond migration, possibly along with more profound skeletal rearrangements.[19] The replacement of anisole with the alternative scavenger triethylsilane did not change the results obtained with TFA.[19,22,24] However, the *tert*-butyl protecting groups

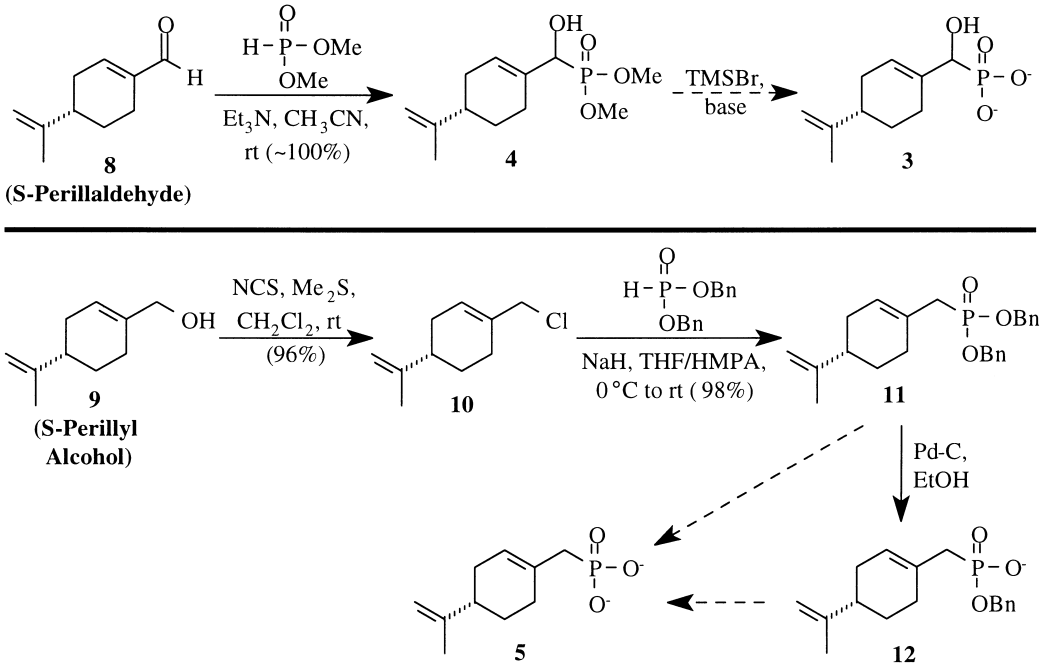

Figure 2.

were successfully and selectively removed from **15** using trimethylsilyl trifluoromethanesulfonate in the presence of 2,6-lutidene.[25] The limonene phosphonate **5** was also prepared in an analogous manner via deprotection of the *tert*-butyl phosphonate **16** (Fig. 3). Our results suggest that this mild, non-acidic method may provide a valuable alternative route for the preparation of sensitive phosphonate derivatives. Note that the synthesis of **16** required preformation of the di-*tert*-butylphosphinate anion with sodium hydride, prior to its coupling with allylic chloride **10**.

With the synthetic procedure developed, we next turned to the preparation of the (*R*)-isomers **1** and **2** (Fig. 4). Oxidation of the allylic alcohol with manganese dioxide smoothly afforded (*R*)-perillaldehyde **17**. The aldehyde **17** was coupled with dimethyl phosphinate, in the same manner as described for **4**, to give compound **2**. The *tert*-butylphosphonate **18** was prepared in the same manner as described above for **15**. Deprotection of **18** with trimethylsilyl trifluoromethanesulfonate afforded the desired hydroxyphosphonate **1**.

A different synthetic route was used to prepare the FPP analogue FPMP (**7**, Fig. 5). This procedure was developed by Poulter and co-workers to prepare the geranyl (C_{10}) analogue of **7**. Commercially available *trans,trans*-farnesyl chloride (**19**) was coupled with the *tris*-tetrabutylammonium salt **20** in acetonitrile. Ion-exchange chromatography followed by reversed phase HPLC

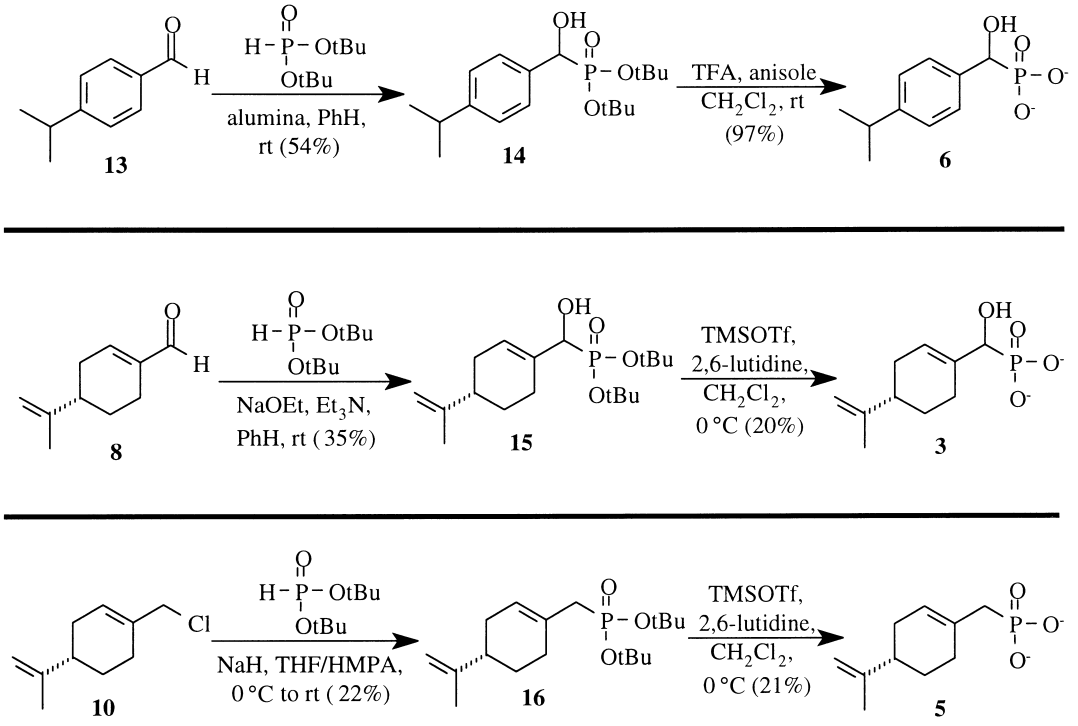

Figure 3.

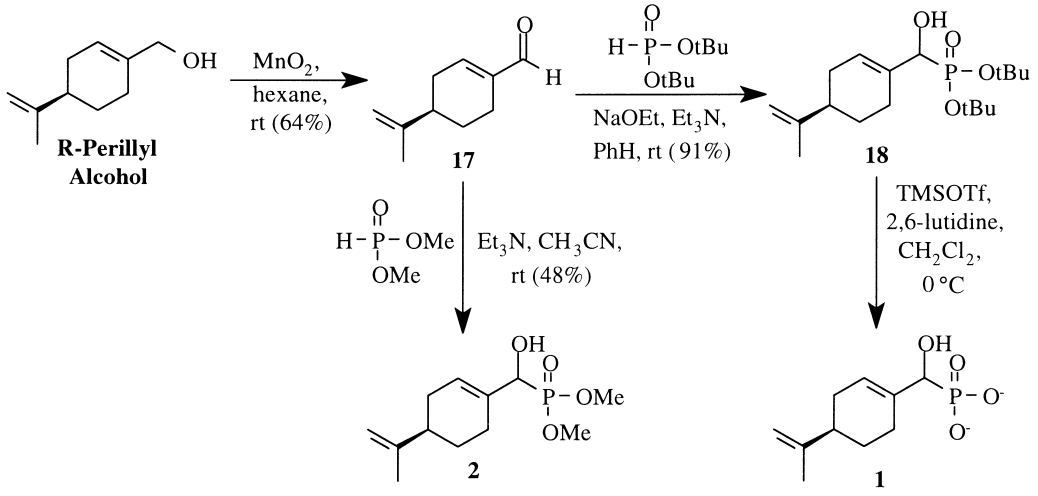

Figure 4.

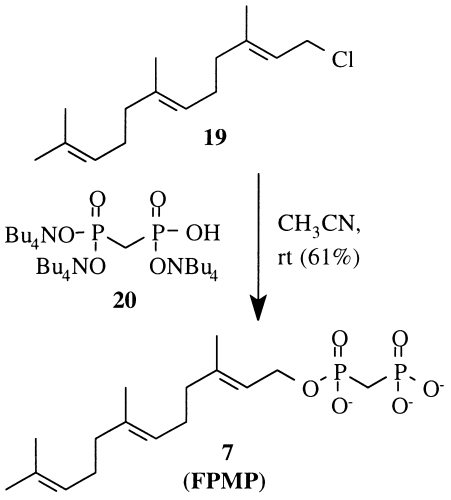

Figure 5.

purification afforded pure FPMP **7** in good yield. The HPLC retention time of **7** was similar to that of the natural product FPP, and its proton and phosphorous NMR spectra were consistent with those expected for FPMP.

Biological assays

The seven potential FTase inhibitors **1–7** shown in Figure 1 were tested for their ability to inhibit mammalian FTase in vitro using a scintillation proximity assay as previously described (Table 1).[26] Compounds **3–7** were also evaluated for their ability to block protein farnesylation in cells through a mobility shift assay, again using a previously developed procedure.[26] Surprisingly, **3–6** exhibited no significant inhibition of mFTase at 1 mM, and these compounds were not evaluated further in vitro. The (*R*)-isomers **1** and **2** were tested more extensively, and IC_{50} values of 3.0 mM and ~10 mM were determined. While the actual values may be lower due to solubility problems with the limonene analogues (particularly with the more hydrophobic compound **2**), it is clear that these compounds are several orders of magnitude less potent than FMP, which exhibits an IC_{50} of 30 nM under the same conditions. Despite their lack of potency versus isolated mFTase, compounds **3–7** were tested in a mobility shift assay to determine their ability to interfere with the farnesylation of Ras proteins in cells. At low micromolar concentrations, these compounds had no effect on the farnesylation of Ras, and at higher concentrations **4** and **5** exhibited nonspecific cellular toxicity. Even at these higher concentrations (100 μM), there was no indication of any effect on Ras protein farnesylation, consistent with the lack of in vitro potency.

Table 1 Biological assay of analogues[a]

Analogue	IC_{50} FTase	IC_{50} GGTase	K_i FTase	Mobility shift assay
1	3.0 mM			
2	~10 mM			
3	> 1.0 mM[b]			> 25 μM[c]
4	> 1.0 mM[b]			> 100 μM[d]
5	> 1.0 mM[b]			> 100 μM[d]
6	> 1.0 mM[b]			> 25 μM[c]
7	160 nm	14.2 μm	23 nm	> 30 μM[c]

[a]Conditions: FTase values were determined using recombinant mFTase in a scintillation proximity assay with tritiated FPP and the peptide Biotin-Aha-TKCVIM as substrates, in the same manner as previously described (see experimental section). GGTase I values were determined in a similar manner using recombinant mGGTase I, tritiated GGPP, and the peptide Biotin-Aha-TKCVIL. The mobility shift assays (determination of the ability of the analogue to block the prenylation of H-Ras in NIH3T3 rat fibroblasts) were performed in the same manner as previously described.
[b]At the indicated concentration, the compound had no effect on the rate of mFTase-catalyzed prenylation.
[c]At the indicated concentration, the analogue had no effect on the prenylation of Ras in cells.
[d]At the indicated concentration, the analogue had no effect on the prenylation of Ras cells, and exhibited non-specific toxicity.

The in vitro biological results obtained with the diphosphate-modified FPP analogue FPMP were strikingly different. This compound is a very potent FPP-based mFTase inhibitor, with an IC_{50} value only fivefold higher than that of FMP. This compound also exhibits good selectivity for mFTase versus mGGTase I, a potentially desirable feature for a therapeutically useful mFTase inhibitor.[3] As expected, this close structural mimic of the natural substrate FPP is an FPP-competitive inhibitor (Fig. 6). FPMP was also evaluated as a potential inhibitor of Ras farnesylation in cells, but it has no effect at 30 μM. This might be due to FPMP acting as an alternative substrate for mFTase, leading to farnesylation of Ras in the same manner as seen with the natural substrate FPP. However, preliminary evaluation of FPMP in an HPLC mFTase assay indicates that it is an exceptionally poor alternative substrate. No farnesylated peptide product was seen under conditions where ~5% of the product obtained with FPP would have been clearly observed. Note that Stremler and Poulter have demonstrated that the corresponding geranyl analogue of FPMP is a poor alternative substrate for FPP synthase.[27]

Discussion

The dietary monoterpenes limonene and perillyl alcohol (Fig. 1) are effective, at high micromolar to low millimolar levels, in blocking the growth of cancer cells in vitro and in vivo.[6,7] Particular interest has been focused on these compounds, which are currently undergoing clinical trials as anticancer agents. An initial report by Gould indicated that these compounds act as anticancer agents by inhibiting protein prenylation.[8] Further work by Gould in collaboration with Gelb, Tamanoi, and their groups demonstrates that limonene, perillyl alcohol, perillic acid, and perillic acid methyl ester are low millimolar to micromolar inhibitors of mammalian and yeast FTase in vitro.[12] In particular, these workers showed that the methyl ester is a FPP-competitive inhibitor of yeast FTase. These reports, in combination with the demonstrated in vitro mFTase inhibition seen with FMP, led us to synthesize and assay the phosphonate analogues **1–5**.

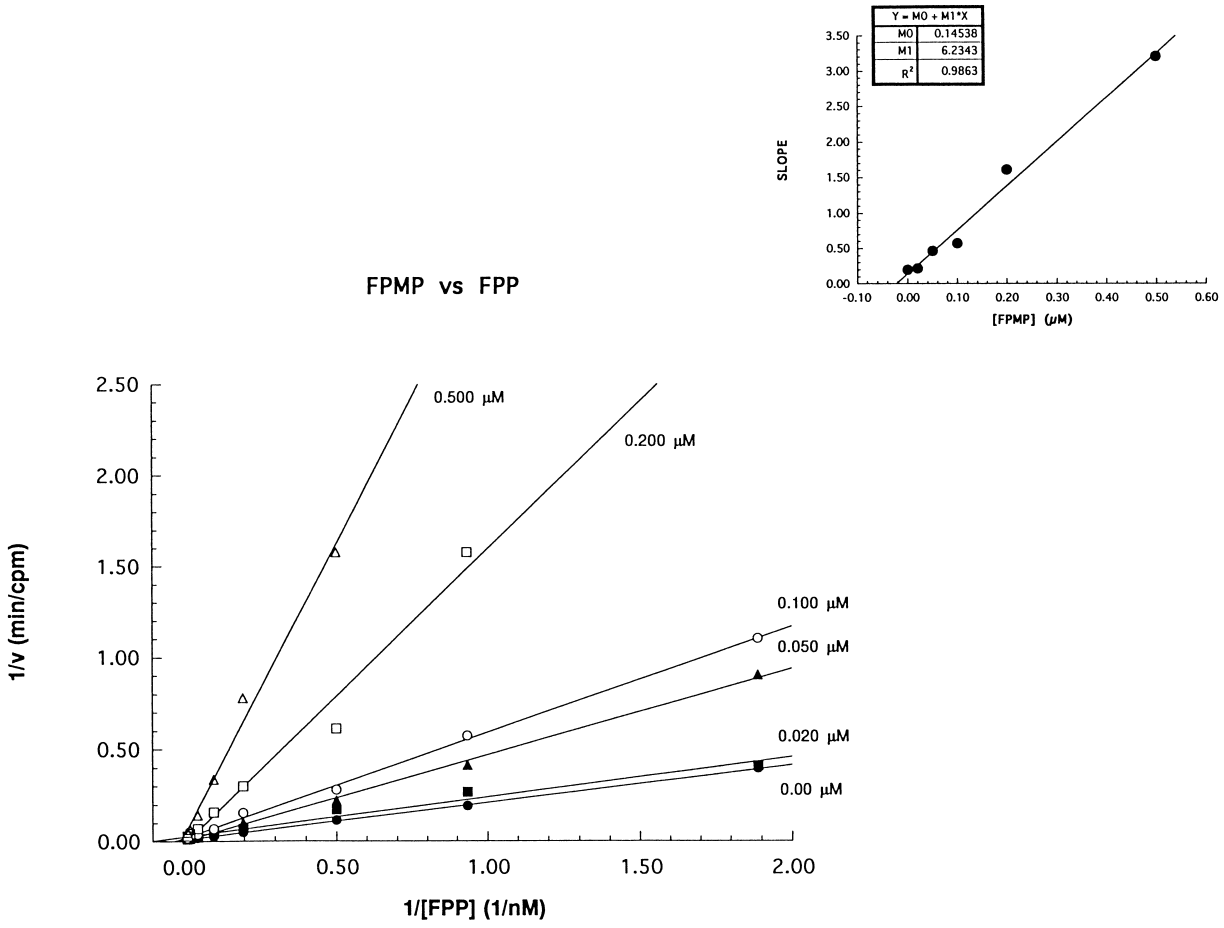

Figure 6. Lineweaver–Burk plot of competitive mFTase inhibition by FPMP (**7**). The values were obtained using the scintillation proximity assay as described in the experimental section, with the exception that the peptide concentration was maintained at 0.10 μM and the FPP concentration was varied. Enzyme rates were determined at 0, 0.02 μM, 0.05 μM, 0.10 μM, 0.20 μM, and 0.50 μM FPMP, as indicated on the figure.

However, it has become clear that limonene and its derived monoterpenes such as perillyl alcohol have multiple modes of action in cells. In particular, there has been some controversy about the cellular relevance of mFTase inhibition seen with limonene and its derivatives. Hohl and Lewis have found that perillyl alcohol, rather than blocking farnesylation, decreases total cellular Ras levels.[10] Two other groups have confirmed that the growth inhibition of transformed cells seen with monoterpenes is not due to blockage of Ras prenylation, and furthermore is not dependent on the mutational status of Ras in the limonene-treated cells.[9,11] Very recently, Gould and co-workers also confirmed that perillyl alcohol does not inhibit Ras farnesylation in cells, but reported that instead it interferes with protein geranylgeranylation in cells.[28] In view of these more recent reports, the lack of activity of **1–6** versus mFTase in vitro and in the mobility shift assay may not be surprising. Our results indicate that the inhibition of mFTase by perillyl alcohol and perillic acid methyl ester is not due to the ability of limonene to act as a conformationally restricted mimic of the farnesyl moiety of FPP.

In sharp contrast to the results seen with the limonene phosphonate analogues, FPMP (**7**) is a very potent mFTase inhibitor. This finding is consistent with previous reports that FMP and other farnesyl-bearing FPP analogues such as **21**[29] and **22**[30] are also potent inhibitors of this enzyme (Fig. 7). However, note that the oxygen at C_1 of the farnesyl structure appears to be a key recognition element that significantly increases binding of the analogue to the enzyme; compare the IC_{50} values for **22** and the 'all-carbon' analogue **23**[13] to **7**. This finding is also consistent with an active site model suggested by the Bristol–Meyers Squibb group, which proposes that this oxygen interacts with an acidic residue in the mFTase active site.[29]

The recently disclosed crystal structure of mFTase indicates a deep, hydrophobic pocket lined with aromatic residues, which apparently binds the farnesyl moiety of FPP in an extended conformation.[31] Such a binding site, which would provide many contacts with the hydrocarbon chain of FPP, could explain the relatively tight binding of all of the compounds shown in Figure 7 to mFTase. The structure of the putative FPP binding site may also provide a clue to the very weak inhibition seen with **1**. If the hydroxyphosphonate moiety of this compound binds to the zinc ion in the active site, then the crystal structure model indicates that the monoterpene portion of **1** would only be able to make limited

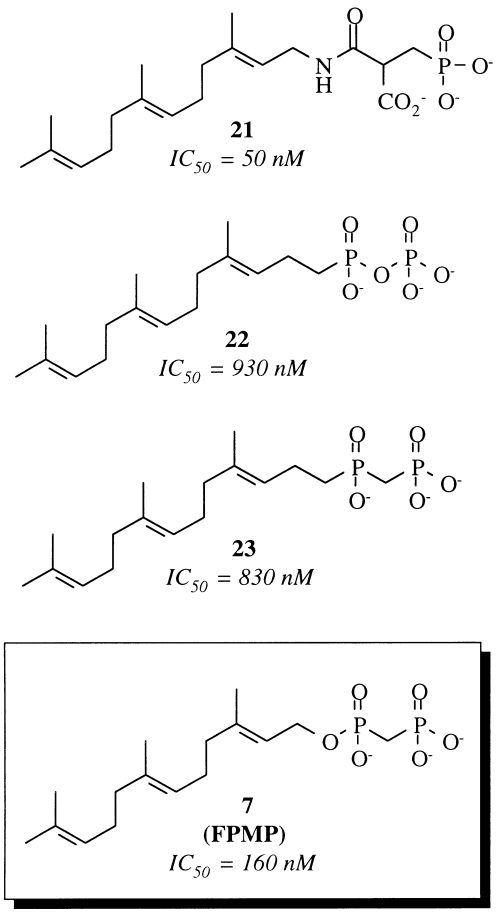

Figure 7.

contacts with the hydrophobic pocket. This interpretation implies that FPP-competitive inhibitors of mFTase must have hydrocarbon moieties that closely mimic the farnesyl chain in hydrophobic bulk, if not in actual structure. This is in fact the case with two recently disclosed series of potent, aromatic-derived, FPP-competitive mFTase inhibitors.[32,33,37]

Experimental

General

All moisture and/or sensitive reactions were conducted in flame or oven dried glassware under a dry argon atmosphere. Moisture sensitive reagents were transferred by syringe and introduced to the reaction vessel through rubber septum caps. All reaction contents were stirred magnetically. Solvents, organic and inorganic reagents were obtained from the following suppliers: Aldrich Chemical Company, Fisher Scientific, and VWR Scientific, and all reagents were used without further purification, unless otherwise stated. The following solvents were prepared anhydrous prior to use as follows: THF and DME were distilled from Na/benzophenone; CH_2Cl_2 was distilled from CaH_2; PhH was distilled from Na; CH_3CN was freshly distilled from phosphorous pentoxide; toluene, DMF, hexanes and diethyl ether were dried and stored over activated 3 Å molecular sieves. Brine refers to saturated aqueous sodium chloride. Normal phase flash column chromatography was performed with silica gel 60 (230–440 mesh, E. Merck). Thin-layer chromatography was conducted on precoated alumina plates (silica gel 60 F-254, E. Merck), and was visualized by ultraviolet illumination or treatment with 8% phosphomolybdic acid (PMA) in ethanol, followed by heating. Reversed phase HPLC was conducted on a Waters system with a Vydac pH-stable C_8 4.6×250 mm column, a U6K injector, two 501 pumps and a 490E multiwavelength UV detector. Melting points were taken on a MEL-TEMP II melting point apparatus using FLUKE 51 K/J thermometer and are uncorrected. Nuclear magnetic resonance spectra were recorded at either 300 MHz (^{1}H NMR), 76.5 MHz (^{13}C NMR) or 121 MHz (^{31}P NMR) on a General Electric QE-300 or GN-300 spectrometer. Chemical shifts are reported in parts per million (δ) downfield from tetramethylsilane (TMS) using an internal TMS standard. Multiplicities are indicated by the following symbols: (br) broad, (s) singlet, (d) doublet, (t) triplet, (q) quartet, (m) multiplet. Coupling constants (J) are reported in Hertz (Hz). Infrared spectra were recorded on a Nicolet 550 Magna-IR™ spectrometer and are reported in reciprocal centimeters (cm^{-1}). Mass spectra were recorded on a Kratos MS 80 RFA mass spectrometer at 70 eV. Microanalysis were performed by Galbraith Laboratories, Inc., Knoxville, TN, USA.

(*S*)-(*p*-Menth-1,8-dien-7-ol-7-yl) phosphonic acid, dimethyl ester (4). In a dry 25 mL round-bottom flask, 2.0 mL of CH_3CN, (*S*)-(−)-perillaldehyde **8** (151 mg, 0.16 mL, 1.0 mmol), Et_3N (0.28 mL, 2.0 mmol) and dimethyl phosphite (0.14 mL, 1.5 mmol) were combined. The solution was stirred for 24 h under Ar. The solvent was removed and the crude mixture was purified by reversed-phase silica gel flash chromatography (70% methanol/water) to give the desired compound as an oil (~350 mg, ~100%). ^{1}H NMR ($CDCl_3$) δ 1.25 (d, 1H), 1.74 (s, 3H), 3.79 (d, 6H), 4.37 (d, J = 12 Hz, 1H), 4.72 (br s, 1H), 5.93 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 20.70, 25.57, 25.97, 27.36, 27.52, 30.57, 40.69, 53.54, 70.66, 71.00, 72.77, 73.10, 108.72, 125.27, 126.02, 133.00, 133.46, 149.43; IR (NaCl) 3396, 2953, 2851, 2366, 1643, 1055 cm^{-1}; ^{31}P NMR ($CDCl_3$) δ 25.40, 25.45; HREIMS m/z calcd for $C_{12}H_{21}PO_4$, 260.1179 (M^+); found, 260.1181.

(*S*)-*p*-Menth-7-chloro-1,8-diene (10). In a 25 mL round-bottom flask, *n*-chlorosuccinimide (NCS; 588 mg, 4.4 mmol) was dissolved in 10 mL of CH_2Cl_2. The mixture was cooled to 0 °C and Me_2S (0.352 mL; 4.8 mmol) was added dropwise. The mixture was cooled to −20 °C and (*S*)-(−)-perillyl alcohol (**9**; 0.62 mL, 3.9 mmol) was slowly added. The solution was warmed to 0 °C, stirred for 1 h, quenched with 10 mL of cold brine, and dried over $MgSO_4$. The crude product was purified by flash chromatography on silica gel (10% EtOAc/hexane) to give a light yellow oil (654 mg, 96% yield). ^{1}H NMR ($CDCl_3$) δ 1.74 (s, 3H), 2.17 (br s, 4H), 4.01 (s, 2H), 4.72 (d, J = 6 Hz, 2H), 5.84 (br s 1H); IR (NaCl) 3089, 2945, 1643 cm^{-1}; HREIMS m/z calcd for $C_{10}H_{15}Cl$, 170.0862 (M^+); found, 170.0861.

(*S*)-(*p*-Menth-1,8-dien-7-yl) phosphonic acid, dibenzyl ester (11). In a 25 mL round-bottom flask, NaH (192 mg, 8.0 mmol, 60% oil dispersion) was suspended in 5 mL of dry THF. The flask was cooled to −10 °C and dibenzyl phosphite (1.44 mL, 6.5 mmol) was slowly added. After 15 min, the mixture was transferred to a flask cooled to 0 °C containing the chloride **10** (411 mg, 2.4 mmol) in 5 mL of THF and HMPA (0.35 mL, 2 mmol) over 10 min. The solution was stirred for 16 h at rt. THF was removed and the residue was then diluted with water and extracted with CCl_4. The organic layer was collected and washed with 5% citric acid, 5% $NaHCO_3$ and brine. The product was dried over $MgSO_4$ and solvent was removed. Mineral oil was removed by stirring the product in hexane at −78 °C under Ar and the supernatant was removed by pipette. The crude product was then purified by reversed-phase silica gel flash chromatography (90% methanol/water) to give a clear oil (940 mg, 98% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.71 (s, 3H), 2.52 (d, J = 21 Hz, 2H), 4.68 (d, J = 8 Hz, 2H), 4.98 (m, 4H), 5.56 (s, 1H), 7.34 (br s, 10H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 20.77, 27.69, 29.93, 30.88, 33.15, 34.32, 34.97, 36.13, 40.46, 67.24, 67.57, 108.62, 126.50, 126.92, 127.89, 128.28, 128.51, 129.90; IR (NaCl) 3064, 3038, 2919, 2425, 1643, 1455, 1268, 1013 cm^{-1}; ^{31}P NMR (121 MHz, $CDCl_3$) δ 29.35; MS-EI: 396.20.

(*S*)-(*p*-Menth-1,8-dien-7-yl) phosphonic acid, monobenzyl ester (12). In a dry 25 mL flask, 403 mg of the di-benzyl phosphonate **12** (1.02 mmol), 0.94 mL of cyclohexadiene (10 mmol), 796 mg of 10% Pd–C catalyst and 4 mL of dry ethanol were combined. The solution was stirred for 2 h under Ar. The solid was filtered through a plug of Celite and washed three times with ethanol. The solvent was removed to give an oily liquid (247 mg, 79% yield). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.70 (s, 3H), 2.48 (d, J = 21 Hz, 2H), 4.21 (narrow m, 1H), 4.68 (d, J = 4 Hz, 2H), 4.96 (m, 2H), 5.60 (narrow m, 1H), 7.34 (narrow m, 5H); IR (NaCl) 2327, 1643, 1497, 1457, 734 cm^{-1}.

[4-(Isopropyl)phenyl]-hydroxymethyl phosphonate, di-*tert*-butyl ester (14). In a 50 mL flask, 4-(isopropyl)-benzaldehyde (**13**, 1.52 mL, 10 mmol), di-*tert*-butyl phosphite (Lancaster Chemical Co.; 1.94 g, 10 mmol), and Woelm basic alumina oxide (4.0 g, activity I) were combined and stirred at room temperature overnight. The crude product was triturated with CH_2Cl_2/EtOH (9/1, 4×10 mL). The solvent was removed and the resultant oil was crystallized from petroleum ether to give the product as a light brown solid (mp 105–106 °C; 1.83 g, 54% yield). ^{1}H NMR ($CDCl_3$) δ 1.22 (d, 6H), 1.39 (d, 18H), 2.84 (m, 1H), 4.80 (dd, 1H), 7.18 (d, 2H), 7.34–7.37 (dd, 2H); ^{13}C NMR ($CDCl_3$) δ 24.02, 30.31, 33.83, 71.33, 73.48, 83.26, 125.92, 127.15, 134.75, 148.33; Elemental analysis: C–53.34% (calcd), 53.04% (exptl); H–7.22% (calcd), 6.85% (exptl); P–13.04% (calcd), 12.51% (exptl).

[(4-Isopropyl)phenyl[hydroxymethyl] phosphonic acid (6). In a 50 mL flask, **14** (500 mg, 1.5 mmol), TFA (10 mL), anisole (0.82 mL, 7.5 mmol), and CH_2Cl_2 (10 mL) were combined and stirred at room temperature for 45 min. The reaction was quenched with 10 mL of H_2O and the solvent was removed to yield a brownish oil. The oil was crystallized from MeOH/$CHCl_3$ to give a brownish fluffy solid (336 mg, 97%). ^{1}H NMR (CD_3OD) δ 1.23 (d, 6H), 2.89 (m, 1H), 4.83 (dd, 1H), 7.21 (d, 2H), 7.42 (d, 2H); ^{13}C NMR (CD_3OD) δ 24.6 (d), 35.34, 71.2 (d), 73.5 (d), 127.15, 128.80, 137.48, 149.68, 163.91; ^{31}P NMR ($(CD_3)_2SO$) δ 15.2; MSEI m/z 263.1.

(*S*)-(*p*-Menth-1,8-dien-7-ol-7-yl) phosphonic acid, di-*tert*-butyl ester (15). In a dry 50 mL flask, di-*tert*-butyl phosphite (583 mg, 3.0 mmol), (*S*)-(−)-perillaldehyde (**8**, 0.47 mL, 3 mmol), 4.0 mL of PhH and 0.40 mL of triethylamine were combined under Ar. A 0.07 M solution of sodium ethoxide (204 mg in 5.0 mL of ethanol) was slowly added to the mixture. The mixture was stirred for 15 min and 0.40 mL of triethylamine was added. After an additional 30 min the solvent was removed and the crude product was purified by flash chromatography (5% MeOH/$CHCl_3$) to yield a clear oil (362 mg, 35% yield). ^{1}H NMR ($CDCl_3$) δ 1.50 (s, 18 H), 1.79 (s, 3H), 2.58 (br s, 1H), 4.15 (d, 1H), 4.72 (s, 2H), 5.82 (s, 1H); ^{13}C NMR (75.4 MHz, $CDCl_3$) δ 20.81, 25.88 (d), 27.54, 30.50, 41.04, 72.9 (d), 74.95 (d), 82.9 (dd), 108.62, 123.7 (d), 124.4 (d), 134.0 (d), 149.89; ^{31}P NMR (121 MHz, $CDCl_3$) δ 15.68, 15.42; EIMS, 345.

(*S*)-(*p*-Menth-1,8-dien-7-ol-7-yl) phosphonic acid (3). In a dry 25 mL flask, di-*tert*-butyl phosphonate **15** (307 mg, 0.89 mmol) and 2,6-lutidine (0.31 mL, 2.68 mmol) were dissolved in 4 mL of CH_2Cl_2 under Ar. The flask was cooled to 0 °C and trimethylsilyl trifluoromethanesulfonate (TMSOTf; 0.40 mL, 2.0 mmol) was added. The reaction was stirred for 1 h. Solvent was then removed and the reaction was quenched with methanol. The crude product was washed twice with toluene and following concentration it was purified by reversed-phase HPLC using a program of 5 min of 100% A followed by a linear gradient of 100% A to 100% B over 30 min (A, 25 mM aqueous ammonium bicarbonate (pH 8.0); B, acetonitrile), with a flow rate of 1 mL (UV monitoring at 214 and 230 nm). The fractions containing the product were lyophilized to yield a white fluffy solid (47 mg, 20% yield). ^{1}H NMR (CD_3OD) δ 1.72 (s, 3H), 4.08 (d, 1H), 4.68 (narrow m, 2H), 5.77 (narrow m, 1H); ^{13}C NMR (CD_3OD) δ 20.98, 27.45 (d), 29.30, 32.16 (d), 42.81, 53.68, 54.85, 55.9 (d), 56.91, 109.03, 124.36, 137.85 (d), 151.65; ^{31}P NMR (CD_3OD) δ 20.25, 20.16.

(*S*)-(*p*-Menth-1,8-dien-7-yl) phosphonic acid, di-*tert*-butyl ester (16). In a 25 mL round-bottom flask, NaH (192 mg, 8 mmol, 60% oil dispersion) was suspended in 5 mL of THF. The flask was cooled to −10 °C and di-*tert*-butyl phosphite (777 mg, 4.0 mmol) was slowly added. After 15 min, the mixture was transferred to a flask cooled to 0 °C containing the chloride **10** (525 mg, 3.1 mmol) in 5 mL of THF and 0.35 mL of HMPA (2 mmol) over 10 min. The solution was stirred for 16 h at room temperature, concentrated, diluted with water, and extracted with CCl_4. The organic layer was washed with 5% citric acid, 5% $NaHCO_3$ and brine, dried over $MgSO_4$, and concentrated. The crude product was purified by flash chromatography (5% MeOH/$CHCl_3$) to yield **16** as a clear oil (218 mg, 22% yield). ^{1}H NMR

($CDCl_3$) δ 1.52 (s, 18H), 1.74 (s, 3H), 2.38 (d, 2H), 4.71 (s, 2H), 5.54 (s, 1H); ^{13}C NMR ($CDCl_3$) δ 21.1 (d), 29.23, 30.90, 32.22, 39.28, 41.18, 42.27, 83.76, 109.41, 126.64, 130.63, 151.00; ^{31}P NMR ($CDCl_3$) δ 20.31; HREIMS calcd for $C_{18}H_{33}O_3P$, 328.2167; found, 328.2177.

(*S*)-(*p*-Menth-1,8-dien-7-yl) phosphonic acid (5). In a dry 25 mL flask, di-*tert*-butyl phosphonate **16** (106 mg, 0.410 mmol) and 2,6-lutidine (0.143 mL, 1.23 mmol) were dissolved in 2 mL of CH_2Cl_2 under Ar. The flask was cooled to 0 °C and trimethylsilyl trifluoromethanesulfonate (TMSOTf; 0.18 mL, 0.92 mmol) was added. The reaction was stirred for 1 h, the solvent was removed and the reaction was quenched with methanol. The crude product was washed twice with toluene, concentrated, taken up in water, and converted to the ammonium salt by ion exchange. Lyophilization afforded the desired phosphonate as a white fluffy solid (21 mg, 21%). ^1H NMR (CD_3OD) δ 1.48 (m, 1H), 1.71 (s, 3H), 2.29 (d, 2H), 4.68 (narrow m, 2H), 5.52 (br s, 1H). ^{13}C NMR (CD_3OD) δ 21.17, 29.50, 31.35, 32.35, 38.62, 40.35, 42.50, 108.96, 123.80, 133.36, 151.72; ^{31}P NMR (CD_3OD) δ 25.68.

(*R*)-(*p*-Menth-1,8-dien-7-ol-7-yl) phosphonic acid, di-*tert*-butyl ester (18). In a dry 50 mL flask, (*R*)-perillyl alcohol (commercial material from Aldrich; 152 mg, 1.0 mmol) and MnO_2 (1.3 g, 15 mmol) were stirred in 22 mL of hexane for 2 h at room temperature. After stirring, the solution was filtered through Celite, which was then washed three times with ether. Concentration followed by flash chromatography (95% hexane/ethyl acetate) gave (*R*)-(−)-perillaldehyde **17** as a clear oily liquid (97 mg, 64% yield). ^1H NMR ($CDCl_3$) δ 1.76 (s, 3H), 4.74 (d, 2H), 6.83 (narrow m, 1H), 9.44 (s, 1H). In a dry 25 mL flask, di-*tert*-butyl phosphite (125 mg, 0.65 mmol), **17** (97 mg, 0.65 mmol), 1.5 mL of PhH and 0.15 mL of triethylamine were combined. A 0.5 M solution of sodium ethoxide (68 mg in 2.0 mL of ethanol) was slowly added to the mixture. The mixture was stirred for 15 min. Then 0.15 mL of triethylamine was added and the reaction was stirred for 30 min. The solvent was removed and the crude product was purified by flash chromatography (5% MeOH/$CHCl_3$) to yield a clear oil (201 mg, 91% yield). ^1H NMR ($CDCl_3$) δ 1.44 (s, 18H), 1.70 (s, 3H), 3.40 (m, 1H), 4.10 (m, 1H), 4.68 (app s, 2H), 5.79 (narrow m, 1H); ^{13}C NMR ($CDCl_3$) δ 20.70 (d), 25.85 (d), 27.56, 30.47, 41.00, 72.57 (d), 74.68 (d), 82.80, 108.55, 123.49 (d), 124.30 (d), 134.26 (d), 149.79.

(*R*)-(*p*-Menth-1,8-dien-7-ol-7-yl) phosphonic acid (1). The phosphonate **18** was converted to the ammonium salt as described above for **15** to yield **1** as a white fluffy solid. This compound exhibited the same NMR data as its enantiomer **3**. Elemental analysis: C–23.48% (calcd), 23.60% (exptl). H–4.90% (calcd), 4.75% (exptl). N–6.58% (calcd), 6.61% (exptl). P–3.93% (calcd), 4.17% (exptl).

(*R*)-(*p*-Menth-1,8-dien-7-ol-7-yl) phosphonic acid, dimethyl ester (2). In a dry 50 mL flask, (*R*)-perillyl alcohol (152 mg, 1.0 mmol) and MnO_2 (1.3 g, 15 mmol) were stirred in 22 mL of hexane for 2 h at room temperature. The solution was then filtered through Celite, which was washed three times with ether. Concentration gave crude (*R*)-(−)-perillaldehyde **17** as a clear oil; ^1H NMR indicated a ~5:1 mixture of the aldehyde and alcohol, which was used directly in the next reaction. In a dry 25 mL round-bottom flask, 2.0 mL of CH_3CN, crude **17** (~150 mg), Et_3N (0.28 mL, 2.0 mmol) and dimethyl phosphite (0.14 mL, 1.5 mmol) were combined. The solution was stirred for 24 h under Ar. The solvent was removed and the crude product was purified by flash chromatography (5% MeOH/$CHCl_3$) to yield a clear oil (126 mg, 48% yield from (*R*)-perillyl alcohol). The ^1H NMR spectrum of **2** was identical to that of the enantiomer **4**.

Farnesyl phosphonyl(methyl)phosphonate (FPMP, 7). Tris(tetrabutylammonium) hydrogen methyldiphosphate **20** (1.35 mmol, 548 mg; prepared as described by Poulter and co-workers[34] from methanediphosphonic acid (Alfa Chemical Co.)) was dissolved in CH_3CN (4.5 mL) under an argon atmosphere. Commercial all-*trans*-farnesyl chloride (**19**, 0.21 mmol, 50 mg) was added to the resulting solution and the reaction was stirred at rt for 2.5 h. The reaction mixture was then concentrated (rotary evaporation), the residue was dissolved in 3 mL of deionized water, and the resulting solution was passed through a 2×8 cm Dowex AG50×8 ion exchange column (NH_4^+ form). Lyophilization then yielded a pale white solid, which was dissolved in 2 mL of 25 mM ammonium bicarbonate. This resulting mixture was purified by reversed-phase HPLC using a program of 5 min of 100% A followed by a linear gradient of 100% A to 100% B over 30 min (A, 25 mM aqueous ammonium bicarbonate (pH 8.0); B, acetonitrile), with a flow rate of 1 mL. Under these conditions, FPMP exhibited a retention time of 20 min (UV monitoring at 214 and 230 nm). The fractions containing FPMP were collected, the acetonitrile was removed via rotary evaporation, and lyophilization afforded 48 mg (61%) of pure FPMP **7** as a white fluffy solid. ^1H NMR (D_2O) δ 1.62 (s, 6H), 1.69 (two s, 6H), 2.02 (t, 2H, P-CH_2-P, partially buried), 2.1 (m, 8H), 4.41 (app t, 2H), 5.19 (m, 2H), 5.45 (t, 1H); ^{31}P NMR (D_2O) δ 13.5 (d), 20.8 (d).

Prenyltransferase assays. FTase IC_{50} values were determined using recombinant mFTase[35] in a scintillation proximity assay (using streptavidin beads from Amersham) with tritiated FPP (specific activity 15–30 Ci/mmol, final concentration 0.12 μM) and the peptide Biotin-Aha-Thr-Lys-Cys-Val-Ile-Met-OH (final concentration 0.1 μM) as substrates, in the same manner as previously described.[36] The K_i values were determined using the same assay system with varying concentrations of tritiated FPP. GGTase I values were determined in a similar manner using recombinant mGGTase I, tritiated GGPP, and the peptide Biotin-Aha-Thr-Lys-Cys-Val-Ile-Leu-OH in a scintillation proximity assay.

Mobility shift assay. This procedure was performed as previously described from this group.[26] Western blotting

detection of the Ras protein used pan-Ras Ab-2 (Oncogene Science) and an anti-mouse HRP conjugate secondary antibody (Amersham). Blots were developed using ECL techniques (Amersham).

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37. **Note added in proof**: Recently it has been demonstrated that α-hydroxygeranylphosphonate, the shorter C_{10} derivative of FMP, is a much poorer inhibitor of FTase than FMP. This is in accord with our proposal that hydrophobic bulk plays a key role in the binding of farnesyl-based inhibitors to FTase: Hohl, R. J.; Lewis, K. A.; Cermak, D. M.; Wiemer, D. F. *Lipids* **1998**, *33*, 39.