Trichodiene Synthase: Synthesis and Inhibition Kinetics of 12-Fluoro-farnesylphosphonophosphate for Sesquiterpene Cyclases

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trans,trans-Farnesyl diphosphate (FPP) serves as a universal substrate for a large family of sesquiterpene cyclases that are responsible for biosynthesis of more than 300 structurally diverse sesquiterpenes in nature. A new FPP substrate analogue, 12-fluoro-farnesylphosphonophosphate (12-F-CH2PP), was synthesized in this paper for applications on kinetic and mechanistic studies of the enzyme family. Trichodiene synthase (TS), a sesquiterpene cyclase, catalyzes the conversion of trans,trans-farnesyl diphosphate (FPP) to trichodiene. 12-F-CH2PP was tested as a potential inhibitor of TS. Inactivation and inhibition kinetic experiments showed that 12-F-CH2PP was not a mechanism-based inactivator for TS; instead, a mixed-type reversible inhibition was observed with inhibition constants $K_{i1} = 2.33 \pm 0.50 \mu M$ and $K_{i2} = 25.80 \pm 7.70 \mu M$, values close to those previously determined for farnesyl-phosphonophosphate, $K_{i1} = 3.25 \mu M$ and $K_{i2} = 9.10 \mu M$. Although 12-F-CH2PP did not irreversibly inactivate TS, this new analogue serves as a potential active-site directed inactivator and mechanistic probe of other sesquiterpene cyclases and FPP-utilizing enzymes, which utilize FPP as a common acyclic substrate.

Keywords: Sesquiterpene cyclases; Enzyme kinetics; Terpenes; Biosynthesis; Enzyme inhibitors.

INTRODUCTION

Terpene synthases are responsible for more than 20,000 different terpene compounds identified to date in nature that act with extremely diverse roles in biology and human society.1-5 For example, some plants are able to secrete antimicrobial terpenes to defend against fungal and bacterial challenges; sesquiterpenes produced by various bacterial and fungal species exhibit potent antibacterial and antifungal properties; the diterpene retinal plays a key role in vision; the triterpene lanosterol serves as a biosynthetic precursor of cholesterol that modulates biological membrane properties; many plants emit a wide range of terpenes that attract beneficial insects.2a,6,7 In particular, sesquiterpene synthases (also known as cyclases) gather as a unique family that catalyzes the biosynthetic conversion of a universal substrate, farnesyl diphosphate (FPP), into more than 300 structurally diverse sesquiterpenes with different structures and stereochemistries.2b,8-9 Scheme I shows a characteristic pattern of biosynthetic pathways leading to the formation of various sesquiterpenes. As shown in Scheme I, the linear substrate FPP must undergo an ionic cleavage of the pyrophosphate and generate a reactive allylic carbocation species, which consequently trigger a series of the cyclization and rearrangement cascades that are finally quenched by a water molecule or deprotonation with an aid of an as yet unidentified nearby base. An increasing number (over about 200) of sesquiterpene synthase gene sequences have been reported in GenBank, and an extensive effort has been made to study their biochemical functions and mechanisms in detail.10,11 Recently, four x-ray structures of sesquiterpene synthases have been solved; trichodiene synthase from Fusarium sporotrichiodes,12 aristolochene synthase from Penicillium roqueforti,13 5-epi-aristolochene synthase from Nicotiana tabacum14 and pentalenene synthase from Streptomyces UC5319.15 A wealth of structural information reveals that, despite a distinct lack of overall sequence similarity (c.a. 6-15% sequence identity), the enzyme family shares the “terpenoid synthase fold”, a characteristic fold also shared by human

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squalene synthase and avian farnesyl synthase,\textsuperscript{16,17} and also concludes that the enzymes evolve with divergence from a common ancestor early in the evolution of terpene biosynthesis. This interesting finding has encouraged scientists to devote even more attention to future study to the issues of resolving the relationship between enzyme structure and the mechanistic pathway as illustrated in Scheme I, as well as locating the critical and however-not-yet identified base that quenches and terminates the cyclization cascade in members of the sequiterpene cyclase family.

Trichodiene synthase (TS) belongs to the family of sesquiterpene synthases and catalyzes the conversion of the trans\textsubscript{,}trans\textsubscript{-}farnesyl diphosphate (FPP, 1) to trichodiene 2, a parent hydrocarbon of the trichothecene family of antibiotics and mycotoxin.\textsuperscript{18} In the biosynthesis of about 100 different trichothecene toxins and antibiotics produced by many fungal species, the TS-catalyzed cyclization of FPP to trichodiene is the first committed step (Scheme II). The cyclase (synthase) from \textit{Fusarium sporotrichioides} has been purified to homogeneity, cloned and overexpressed in \textit{Escherichia coli}.\textsuperscript{19,20} As shown in Scheme III, FPP first undergoes ionization to generate the isomeric intermediate, (3\textsubscript{R})-nerolidyldiphosphate ((3\textsubscript{R})-NPP, 3), which can then adopt the correct conformation to allow the first cyclization to give the bisabolyl cation intermediate 4. The bisabolyl cation 4 undergoes a second electrophilic cyclization to generate a highly reactive carbocationic intermediate 5, which after a 1,4-hydride shift, two 1,2-methyl migration, and final deprotonation, gives rise to trichodiene 2.

Although the proposed mechanism has been supported by a wealth of previous studies,\textsuperscript{21} many questions remain to be answered by direct evidence about an exact molecular pattern or motion of the cyclization cascade taking place in the active site of the enzyme. TS catalyzes a com-
plex of reaction in a single active site. How does the enzyme accommodate a substrate which has to go through a substantial degree of rearrangement and also stabilize the carbocationic intermediate without losing control of specificity? Is there any protein conformational change involved and how does it contribute to the enzyme reaction? Is it possible that some of the reactions, e.g. rearrangements of reactive carbocation intermediates, occur spontaneously under the protection from the enzyme simply because of the “proper” molecular conformation of the intermediate itself? These questions also outline current, critical issues encountered by studies of sesquiterpene synthases in general, the catalytic mechanism of which requires further investigation. Recent x-ray structure studies of wild-type and mutant TS have shed substantial light on the structure-functional relationship of the enzyme, suggesting that the pyrophosphate binding to TS may trigger the enzyme conformational change associated with the initial catalysis of TS, and Mg$^{2+}$ ions may mediate a complex network of enzyme-pyrophosphate interactions.$^{22-24}$ The crystallization studies and previous mutagenesis studies also suggested that mutations of the proposed active-site amino acids responsible for the metal or pyrophosphate binding may perturb the initial protein conformational change and thus vary the volume of the TS active-site template. The mutations consequently cause premature quenchings of various carbocation intermediates in the pathway by unknown nearby bases in the active site so as to generate anomalous sesquiterpene products as observed in mutant TS like D101E and Y305 mutants.$^{22,23,25}$ Although the active-site bases responsible for the premature quenching have not yet been identified, recent modeling studies of TS with various reaction intermediates propose that some possible candidates, including D100, Y305, Y295 and pyrophosphate, might be involved.$^{26}$ However, identification of actual active-site bases still rely on further investigations for solid, experimental proof.

In this paper, we designed and synthesized a potential active-site directed inactivator of sesquiterpene synthases, 12-fluoro-farnesylphosphonophosphate (12-F-F-CH$_2$PP, 6). Using TS as our initial test with the synthesized compound, we intended to study sesquiterpene synthases with the following prospectives: (1) As illustrated in the TS reaction mechanism (Scheme III), it has been proposed that an amino-acid residue in the active site may act as a base to abstract a proton, catalyzing the final rearrangement of the highly reactive intermediate 5 to trichodiene 2. This critical base may also be responsible for the abstraction of the pro-

**Scheme III** Isomerization and cyclization of FPP (1) to Trichodiene (2)
tons in different positions of FPP to generate a mixture of anomalous sesquiterpene products in the reaction catalyzed by mutant trichodiene synthase D101E. It is worthwhile to design and synthesize a substrate analogue which may position a reactive electrophile close to this proposed basic residue. Irreversible inactivation resulting from the affinity labeling by the analogue may allow us to identify the active-site base. (2) It has been a challenging and interesting issue to design a mechanism-based inhibitor for sesquiterpene cyclases. There have been only a few reports of mechanism-based inhibitors of isoprenoid-utilizing enzymes and TS. We intended to carry out kinetic studies of TS using this substrate analogue to gain further mechanistic insight into the reaction catalyzed by TS, as well as other sesquiterpene synthases.

RESULTS

Design and Synthesis of 12-fluoro-farnesylphosphonophosphate (6)

Fluorine has become a common substitute of hydrogen atoms in pharmacologically active organic compounds. Many studies have used fluorine as a favorite choice for leaving group in enzyme inhibition and inactivation. The main reason may be at least twofold: (i) the van der Waals radius of F is similar to that of H. The substitution of H with F usually would not disturb proper positioning of a substrate analogue in an enzyme active site. (ii) F is a strong electron-withdrawing atom, which can greatly enhance electrophilicity of a fluorinated carbon so as to attract a nearby nucleophilic attack under the proximity effect in enzyme chemistry. This concept of principle has been practiced before and well documented.

To introduce a reactive electrophile to the allylic carbon near the proposed active-site base, we decided to replace the hydrogen with fluorine on C-12 of FPP in the synthesis of the inactivator. In our design of inactivation, the nearby enzyme base originally abstracting the proton may attack the fluorinated carbon of the inhibitor, displace the fluoride and then form a covalent bond with the inhibitor, provided the nearby base orients properly in space for nucleophilic substitution. This strategy is, indeed, supported by the fact that Poulter has demonstrated the mechanism-based inactivation of isopentyl diphosphate-dimethylallyl diphosphate (IPP-DMAPP) isomerase by 3-(fluoromethyl)-3-butenyl-diphosphate (FIPP). Using the same allylic fluoride as a reactive electrophile, Poulter et al. successfully observed the nucleophilic substitution of fluoride by the enzyme base at the allylic carbon of FPP.

Nevertheless, pre-steady-state kinetic analysis of TS has shown that the chemical conversion of FPP to trichodiene is relatively fast (15 s⁻¹ at 30 °C). Moreover, the overall cationic cyclization cascade to form trichodiene product is very fast (c.a. 200 s⁻¹ at 30 °C), and individual cyclization steps can be even much faster. At such a high turnover rate, there would be no sufficient time for the putative base to interact with and attack the inactivator before the final product is formed, especially when the base may not be a strong nucleophile. It is thus reasonable to propose that if one could slow or prevent the enzyme-catalyzed consumption of fluorinated FPP, the intended chemical modification by the proposed active-site base may be able to take place. As shown in the TS reaction pathway (Scheme III), FPP must first undergo the ionization of allylic diphosphate through the cleavage of a C-O bond for the future cyclization to trichodiene. To avoid this reaction, the bridging oxygen atom is thus replaced by a methylene group (CH₂) in the synthesis of the inactivator. Hence, the initial ionization is blocked and the downstream cyclization can not occur. Such an approach can allow sufficient time for the reactive, fluorinated electrophile to interact with the enzyme base, which may lead to nucleophilic substitution and enzyme inactivation.

Synthesis of 12-fluoro-farnesylphosphonophosphate 6 is summarized in Scheme IV. Sharpless oxidation using selenium dioxide on trans,trans-farnesyl acetate 7 gave a mixture of allylic alcohols from which 12-hydroxy-farnesyl acetate 8 was obtained by a two-step purification, involving silica gel flash chromatography followed by medium pressure liquid chromatography (MPLC). Mesylation of 8 with mesyl chloride (MsCl), followed by S₅₂ displacement with fluoride by treatment with tetra-(n-butyl)ammonium fluoride (TBAF) in THF afforded 12-fluoro-farnesyl acetate 10. Basic hydrolysis of 10 catalyzed by potassium carbonate (K₂CO₃) in wet methanol generated 12-fluorofarnesol 11. Reaction of 11 with PBr₃ in ether at 0 °C for 2-3 h afforded 12-fluoro-farnesyl bromide 12.

Corey and others have previously reported synthe-
sis of C-substituted methylenephosphonophosphate (i.e. farnesylphosphonophosphate). However, when the synthetic method was applied for the synthesis of 12-F-FCH₂PP, the allylic fluoride decomposed since the synthesis involved harsh conditions, i.e. 0.2N NaOH and Dowex-50H⁺. An alternative method was therefore pursued using a phosphorylating agent methyl methylphosphonomorpholidate developed by van Boom et al.

Reduction of 12 with methyl lithiomethylphosphonomorpholidate in dry THF, obtained in situ by reaction of 1 equiv. n-butyllithium with 13 at -78 °C, gave, after workup and purification by flash chromatography, homogeneous 14. Demethylation of 14 was achieved by the addition of trimethylsilyl bromide to 14 in dry acetonitrile at 20 °C for 1 h to generate 15. After evaporation of excess reactant and solvent, the silyl ether 15 was obtained in a sufficiently pure state. The TMS group was readily removed with tetra-(n-butyl)ammonium fluoride (TBAF) in dry dioxide to produce 16, which was used in the following steps without further purification. The final phosphorylation was accomplished by condensation of 16 with tri-(n-butyl)ammonium salt of phosphoric acid in dry pyridine at 20 °C for 2 days. The following two step purification with DEAE ion exchange chromatography, 4 °C; 2. CHP-20P reverse phase chromatography, 4 °C.

Scheme IV

Conditions: (a) SeO₂, 95% EtOH, 64 °C; (b) MsCl, Et₃N, Pentane/benzene (1/1), -10 °C; (c) TBAF, THF, rt, 14 h; (d) K₂CO₃, wet MeOH, rt, 5 h; (e) PBr₃, Et₂O, 0 °C, 3 h; (f) 13, n-BuLi, THF, -78 °C to rt; (g) TMSBr, MeCN, 20 °C, 1 h; (h) TBAF, dioxane, 20 °C, 1 h; (i) n-Bu₃NH⁺H₂PO₄⁻, pyridine, 20 °C, 2 days; (j) 1. DEAE ion exchange chromatography, 4 °C; 2. CHP-20P reverse phase chromatography, 4 °C.
tography and CHP-20P reverse phase chromatography gave the desired substrate analogue, 12-fluoro-farnesylphosphonophosphate 6, as a white powder. The identity of 6 was confirmed by $^1$H, $^{13}$C, $^{19}$F, and $^{31}$P NMR spectroscopy and FAB-Mass spectrometry. The $^{31}$P NMR spectrum showed the characteristic pattern (i.e. dd, $J_{P-P} = 25.6$ Hz) of the phosphonophosphate function.

**Attempted inactivation of trichodiene synthase by 12-fluoro-farnesylphosphonophosphate**

With the potential active-site directed inactivator 6 designed and synthesized, we first evaluated its ability to inactivate trichodiene synthase. When TS was treated with a range of concentrations of the fluoro-methylene analogue (5-90 $\mu$M), no appreciable inactivation was observed but thermal degradation of enzyme activity occurred at 30 °C. However, time-dependent loss of enzyme activity was observed at concentration-dependence and followed an apparent pseudo-first-order decay mechanism (data not shown). The rate constant for inactivation ($k_2$) at saturating levels of inhibitor and the inhibition constant ($K_I$) were determined to be 1.72 ± 0.57 min$^{-1}$ and 1.97 ± 0.73 mM, respectively. Notably, the calculated $K_I$ was nearly 6x the maximum concentration of 6 used in the inactivation experiments, indicating that this value is, at best, a lower limit. Even though a typical time-dependent inactivation mechanism was observed with the fluoro-methylene analogue, the fact that the calculated $K_I$ was in the mM range prompted us to determine the inhibition behavior of 6 as a reversible inhibitor of trichodiene synthase. Cane et al. reported that farnesylphosphonophosphate (FCH$_2$PP) was a mixed, noncompetitive inhibitor for TS with $K_{i1} = 3.25$ mM and $K_{i2} = 9.10$ mM. It seemed unlikely that the presence of the allylic fluoride in 6 could be responsible for a 200-600 fold increase in inhibition constant compared to FCH$_2$PP. Bertolino et al. has reported that a variety of detergent-like analogues are nonspecific inhibitors of squalene synthase. Alternatively, trace impurities in the sample of 6 might be responsible for the observed inactivation. Therefore, we decided to determine directly the inhibition constant for the inactivation by carrying out a simple reversible inhibition of TS by 6.

**Inhibition of trichodiene synthase by 12-fluoro-farnesylphosphonophosphate (6)**

Inhibition of TS by 6 was carried out in the same assay conditions to allow direct comparison with the kinetics data for the inactivation. The Lineweaver-Burk plots of 1/V versus 1/S (double reciprocal plots) for each inhibitor concentration (0-20 $\mu$M) are shown in Fig. 1. The inhibition constants were determined by direct fitting of the data (V, S and I) to the appropriate equations for competitive, mixed competitive, and uncompetitive inhibitions. The best fit of the data showed a mixed-type inhibition with $K_{i1} = 2.33$ ± 0.50 $\mu$M and $K_{i2} = 25.80$ ± 7.70 $\mu$M. As previously reported, FCH$_2$PP is also a mixed-type inhibitor and exhibited similar inhibition constants, $K_{i1} = 3.25$ $\mu$M and $K_{i2} = 9.10$ $\mu$M. Not only was the type of inhibition the same for both 12-F-FCH$_2$PP 6 and FCH$_2$PP, but also the competitive component of the inhibition ($K_{i1} = 2.33$ mM and 3.0 $\mu$M, respectively) was essentially the same. This result suggests that the enzyme can not distinguish these substrates, validating our proposal that the presence of a single fluoride should not alter the positioning of FCH$_2$PP in the active site. Most importantly, the observed inhibition constant ($K_{i2} = 2.33$ $\mu$M) for 12-F-FCH$_2$PP 6 differed from the calculated inactivation constant ($K_I = ca. 2$ mM) by a factor of 860. We therefore concluded that 12-F-FCH$_2$PP 6 is also a reversible inhibitor for TS and that the apparent inactivation was not caused by 6. Although the nature of the inactivation is still to be determined, it may have been caused by a nonspecific interaction of the inhibitor with enzyme or by a trace amount of unknown impurity, the presence of...
which may be pronounced when a large quantity of inhibitor is used.

**DISCUSSION**

The inhibition studies showed that 12-F-FCH$_2$PP 6 was not a mechanism-based inactivator for TS; instead, a mixed-type reversible inhibition was observed with inhibition constants close to those previously determined for FCH$_2$PP. However, the results have allowed us to draw possible conclusions for the lack of inactivation by 12-F-FCH$_2$PP, which have further led us to a better understanding of action and molecular recognition of TS.

1. Replacement of the allylic diphosphate ester oxygen with a methylene may in fact alter the precise positioning of FCH$_2$PP in the active site. The allylic methyl group (C12) that carries the fluorine atom may be displaced from favorable interaction for nucleophilic attack by the active-site base. Previous work on the structural requirements for substrate recognition by monoterpenoid cyclase has indicated that the principal binding determinant is the pyrophosphate moiety, with other domains of the substrate acting synergistically to enhance and assist in aligning the allylic system and pyrophosphate in order to promote the initial ionization step. The change in the precise alignment of pyrophosphate moiety in the diphosphate binding domain may disturb the correct positioning of lipophilic side chain of FPP in the hydrophobic domain of the active site. Such an effect has, in fact, been proposed to account for the generation of anomalous sesquiterpenes by the D101E mutant of TS accompanying changes in the pyrophosphate binding domain. It has been previously proposed that the hydrophobic FPP binding domain may be permissive enough for movement of the lipophilic chain.

2. The substitution of the methylene group may have three effects on the binding of the pyrophosphate moiety. First, there is an intrinsic difference in the free three-dimensional rotation of chemical bonds between C-CH$_2$-P and C-O-P. Second, the replaced ether oxygen of FPP may be involved in the hydrogen bonding to the pyrophosphate binding pocket. The possible H-bonding of this ester oxygen with a key active-site acid residue has previously been suggested by Biller based on studies of the inhibition of squalene synthase. Third, the pKa of the methylenediphosphate (CH$_3$PP) moiety is greater than the pyrophosphate (OPP) by 1.2 unit. The change in pKa may have a dramatic effect on the mode or strength of pyrophosphate binding because of the alteration in H-bonding donor-acceptor interaction.

3. A protein conformational change accompanying the cyclization reaction may be required to place the base in a favorable position to abstract the proton. Previous pre-steady-state kinetic analysis of the TS reaction suggested that a protein conformational change may be involved during the isomerization of FPP to the intermediate NPP. The base may only be positioned favorably for proton abstraction subsequent to NPP formation. It is possible that the proposed conformational change can not proceed when 12-F-FCH$_2$PP 6 is used as substrate because replacement of the ester oxygen with methylene blocks the initial isomerization. As a consequence, the inhibitor-enzyme complex may be in an FPP-bond conformation which is incapable of catalyzing the conversion of NPP to trichodiene.

4. The active-site nucleophilic base may be suitably positioned to abstract the proton from C-13 but may not be at a proper distance or in an appropriate orientation for the chemical SN2 displacement of the allylic fluoride from C-12 to take place.

**CONCLUSION**

In this paper, 12-F-FCH$_2$PP 6 was successfully synthesized and applied to probing the active site and mechanism of TS, a sesquiterpene synthase that serves as our ini-
tial test target. Despite the fact that 12-F-FCH₂PP 6 was not an irreversible inactivator for trichodiene synthase, this new FPP substrate analogue continues to serve as an important candidate for mechanism-based inactivation and probing the active site on other sesquiterpene cyclases (e.g. aristolochene synthase) or FPP-utilizing enzymes in general. Sesquiterpene synthases like TS catalyze a complex sequence of reactions at a single enzyme active site. A study of the mechanism utilized by TS may be very useful in understanding the mechanistic machinery used by other sesquiterpene cyclases. Studies of the inhibition on other sesquiterpene cyclases by 12-F-FCH₂PP are currently under way.

**EXPERIMENTAL**

**Materials and Methods**

Recombinant wild-type trichodiene synthase was purified from *E. coli* BL21(DE3)/pZW03 as previously described. General methods for spectroscopic analysis, protein purification and analysis, trichodiene synthase assay, and numerical analysis of the kinetic data have been previously described. Sources of reagents and chromatographic materials have also been described. [1-³H]FPP (78.6 μCi/μmol) was prepared by diluting [1-³H]FPP purchased from NEN DuPont with synthetic FPP, prepared as described. Nanopure water was obtained from a Barnstead Nanopure II water purification system and was used to prepare culture and assay media. All solvents for reactions were distilled prior to use. All reagents and buffer components used for enzyme assays and protein purification were of the highest quality commercially available. NMR spectra were obtained on a Bruker AM 400 spectrometer at 400.134 MHz for ¹H, 100.614 MHz for ¹³C, 376.308 MHz for ¹⁹F, and 162 MHz for ³¹P. H₃PO₄ (85%) and CFCl₃ were used as external reference for ³¹P and ¹⁹F NMR, respectively. High-resolution mass spectra were obtained on a Kratos MS80 instrument. All analytical TLC plates were visualized by spraying with a solution of p-anisaldehyde (3.5%), H₂SO₄ (3%), and ethanol (93.5%). Methyl methylphosphonomorfolide 13 was synthesized as previously described. All reactions were carried out under a positive pressure of dry argon, unless otherwise specified. Tetrahydrofuran (THF) and diethyl ether were distilled from sodium benzophenone prior to use. Acetonitrile, dichloromethane, dioxane, and pyridine were distilled over calcium hydride prior to use. Flash chromatography was performed on Merck Type 60 silica gel, 230-240 mesh. Kinetic incubations were performed in a GCA/Precision Scientific Thelco 184 constant temperature water bath. Nonlinear regression analysis of kinetic data was carried out using KaleidaGraph software (Synergy Software) or KINFIT simulation program (written by Professor Vernon Anderson).

**Synthesis of 12-fluoro-farnesyl methylphosphonomorfolide (14)**

Synthesis of 12-fluoro-farnesyl bromide 12 from farnesyl acetate 7 has been previously described. To a solution of methyl methylphosphonomorfolide 13 (209 mg, 1.16 mmol) in dry distilled THF (10 mL) at -78 °C was added n-butyllithium (2.4M in hexane, 582 μL) under argon. The mixture was stirred at -78 °C for 10 min before it was warmed to room temperature for 10 min. After being cooled down to -78 °C and stirred for another 15 min, the resulting mixture was added dropwise over 20 min via cannula to a solution of 12-fluoro-farnesyl bromide 12 (283 mg, 0.93 mmol) in dry THF (5 mL). The reaction mixture was stirred overnight at -78 °C under argon and was allowed to gradually warm to room temperature before being quenched by two drops of deionized water. Upon removal of the solvent, the resulting viscous mixture was added to a solution of saturated NaCl (20 mL) before extraction with EtOAc (20 mL × 3). The combined extracts were dried over MgSO₄, filtered, and concentrated to give a crude product. The crude mixture was purified by flash chromatography on silica gel (EtOAc, 2 cm × 6.5 inch) to give 235 mg (63%) of the desired pure product 14 as a viscous liquid, with unreacted starting material 12 recovered. Rf = 0.072 (EtOAc).

¹H NMR (CDCl₃, 250 MHz) δ 1.58 (3H, s), 1.61 (3H, s), 1.68 (3H, s), 1.76-2.36 (12H, m), 2.99-3.25 (4H, m), 3.50-3.65 (4H, m), 3.57 (3H, s), 4.65 (2H, d, J₉ₓF = 47.94 Hz), 5.07 (2H, m), 5.38-5.50 (1H, m); ¹³C NMR (CDCl₃, 100 MHz) δ 13.16, 15.94, 16.01, 20.78, 26.24, 26.52, 28.93, 30.41, 38.90, 39.55, 44.04, 67.36, 88.50; ³¹P NMR (D₂O, 162 MHz) δ 35.26 (1P, s); HRMS (EI) [M⁺] calcd for C₂₁H₃₇O₃FNP 401.2495, found: 401.2494.

**Synthesis of 12-fluoro-farnesylphosphonophosphate (6)**

Trimethylsilyl bromide (109 μL, 0.823 mmol) was...
added to a solution of 12-fluorofarnesyl phosphonomorpholide 14 (220 mg, 0.548 mmol) in dry distilled acetonitrile (1.1 mL). The resulting light brown mixture was stirred at 20 °C for 1 h under argon. After evaporation of excess solvent and TMSBr in vacuo for 2.5 h, the recovered product 15 was used directly in the next step without further purification. To the same flask was added dry, freshly distilled dioxane (1 mL) to dissolve 15. Tetra-(n-butyl)ammonium fluoride (1M in THF, 549 μL, 0.549 mmol) was then added to the above solution, and the resulting mixture was stirred at 20 °C for 1 h under argon. Upon complete removal of solvent in vacuo at room temperature overnight, the viscous mixture was then subjected to phosphorylation without future purification. Dry, freshly distilled pyridine (2 mL) was added to the same flask to dissolve 16 and was then removed by vacuum distillation. This procedure was repeated two times to completely remove residual moisture before another 2 mL of dry pyridine was added under argon. To the resulting solution was added a solution of tri-(n-butyl)ammonium phosphate (1.17M in dry pyridine, 1.40 mL), prepared as previously described. The resulting reaction mixture was stirred at 20 °C for 2 days under argon before complete removal of solvent in vacuo. After being dissolved in 15 mL of KHCO₃ (pH 7.02, adjusted by CO₂), the crude mixture was purified as potassium salt by DEAE-Sephadex (8 g) ion exchange chromatography at 4 °C with a gradient of H₂O (pH 7.0-7.5), followed by the removal of inorganic salts using CHP-20P resin (1.5 cm × 32.5 cm, Mitsubishi Chemical Industries) with a gradient of H₂O (pH 7.06, adjusted by HN₄OH) and acetonitrile. The purification, 12-F-FCH₂PP 6 was visualized on TLC with Rₜ of 0.37 using a solution of p-anisaldehyde (3.5%), H₂SO₄ (3%), and ethanol (93.5%). Pure 6 was obtained as a white tripotassium salt and stored at -78 °C for use in the inhibition study. ¹H NMR (D₂O, 400 MHz) δ 1.47 (3H, s), 1.50 (3H, s), 1.55 (3H, s), 1.82-2.16 (12H, m), 4.64 (2H, d, Jₙ-H = 47.75 Hz), 5.06 (1H, t, Jₙ-H = 6.89 Hz), 5.12 (1H, t, Jₙ-H = 7.07 Hz); ¹³C NMR (D₂O, 100 MHz) δ 13.31, 15.97, 22.28, 26.20, 26.55, 28.10, 29.44, 38.82, 39.57, 90.58, 125.25, 125.49, 131.99, 132.35, 136.78, 137.44; ¹⁹F NMR (D₂O, 376 MHz) δ 207.36 (t of m, Jₙ-H = 47 Hz); ³¹P NMR (D₂O, 162 MHz) δ 9.56 (1P, d, Jₚ-p = 25.6 Hz), δ 20.54 (1P, d, Jₚ-p = 25.6 Hz). FABMS (thioglyceral/KI) [M⁺+K⁺] calecd for C₁₀H₂₅O₅P₂FK₄ 550.9737, found: 550.9738.

Inactivation of trichodiene synthase by 12-fluorofarneslyphosphonophosphate (6)

Kinetic analysis of inactivation was measured using recombinant trichodiene synthase²⁰b in buffer T containing 5 mM MgCl₂, 10 mM Tris-HCl, and 15% glycerol. Pre-incubation of trichodiene synthase with varying concentrations of 12-F-FCH₂PP 6 was carried out at pH 7.03 to prevent possible basic hydrolysis of the allylic fluoride. Assay of residual enzyme activity was, however, measured at pH 7.8. Typically, a preincubation was initiated by addition of enzyme to a solution of inhibitor in buffer T at 30 °C. Portions of the preincubation mixture were removed at successive time intervals and diluted 65-fold into a solution of substrate [¹-³H]FPP (specific activity, 78.6 μCi/µmole, 2 µM) in buffer T at 30 °C to measure the remaining enzyme activity. 12-F-FCH₂PP 6 as a white tripotassium salt was dissolved in water (adjusted to pH 7.12 by NH₄HCO₃) immediately before mixing with buffer T (10 mM Tris-HCl, 5 mM Mg⁺², 15% glycerol, pH 7.03) to give an appropriate concentration in the preincubation with TS. To the preincubation solution containing varying concentrations of 12-F-FCH₂PP (120, 150, 198, and 269 µM) in buffer T (pH 7.03) at 30 °C was added trichodiene synthase, which has been held in an ice-bath (50 µL, 21 µM before dilution), to give a total volume of 0.5 mL. At successive time intervals, aliquots (8 µL) of the preincubation mixture were mixed with a solution containing [¹-³H]FPP (78.6 µCi/µmole, 2 µM) in buffer T (pH 7.8) at 30 °C to give a 65-fold dilution, and the mixture was assayed for the residual enzyme activity. After 10 min at 30 °C, the reaction was quenched by addition of EDTA (100 µM, pH 8.0). During each assay, the mixture was overlaid with hexane (500 µL) to prevent the loss of volatile [³H]trichodiene. The reaction mixture was extracted with hexane (1 mL × 2) and the hexane extract was passed through a silica gel pipette column to remove polar components. The hexane extract was then mixed with 5-mL of liquid scintillation cocktail (LSC, Opti-fluor) and subjected to liquid scintillation counting for 10 min to quantitate [³H]trichodiene corresponding to the remaining enzyme activity. All data points were measured in duplicate and each set of experiments was carried out two to three times. The rate constants for inaction (kₙact) for each concentration of 12-F-FCH₂PP 6 were determined by a semi-logarithmic plot of residual activity versus time (KaleidaGraph, Synergy software). The values of k₂ and
$K_i$ were estimated by extrapolating the values of $k_{inact}$ to saturating concentration of 12-F-FCH$_2$PP by fitting eq. 1 (KaleidaGraph).

$$k_{inact} = \frac{k_1}{K_1 + 1}$$  \hspace{1cm} (1)

**Inhibition of trichodiene synthase by 12-fluoro-farnesylphosphono phosphate (6)**

The inhibition of trichodiene synthase by 12-F-FCH$_2$PP 6 was assayed at 30 °C in buffer T (pH 7.03). In a typical experiment, six concentrations of [1-^3$H$]FPP (78.6 μCi/μmole, 2 μM) from 80 to 480 μM in buffer T (pH 7.03) were used. To each series was added an appropriate volume of 12-F-FCH$_2$PP stock solution to give a particular concentration of 12-F-FCH$_2$PP (0, 1, 3, 5, 10 or 20 μM) in a total volume of 480 μL. The resulting mixture was pre-equilibrated at 30 °C before the addition of trichodiene synthase (20 μL). The reaction proceeded at 30 °C for 10 min with an overlay of hexane (500 μL) and was assayed for production of [^3$H$]trichodiene after quenching with EDTA (100 μL). After passing through a silica gel pipette column, the hexane extract was mixed with liquid scintillation cocktail and subjected to liquid scintillation counting for 10 min. The resulting data ($V$, dpm) was plotted against various concentrations of substrate (S, μM), giving a typical hyperbolic curve for each concentration of 12-F-FCH$_2$PP (I, μM). The Lineweaver-Burk plots of $1/V$ versus $1/S$ for each inhibitor concentration (0-20 μM) suggested a mixed-noncompetitive inhibition for 12-F-FCH$_2$PP. The type of inhibitor and inhibition constants were actually determined by fitting the data ($V$, $S$, and $I$) with the KINFIT simulation program to equations 2, 3, 4, and 5, corresponding to mixed-type, competitive, uncompetitive, and noncompetitive inhibitions, respectively.

$$V = \frac{V_{max} \cdot S}{K_s(1+1/K_i) + S (1+1/K_s)}$$  \hspace{1cm} (2)

$$V = \frac{V_{max} \cdot S}{K_s(1+1/K_i) + S}$$  \hspace{1cm} (3)

$$V = \frac{V_{max} \cdot S}{(K_s + S)(1+1/K_i)}$$  \hspace{1cm} (4)

$$V = \frac{V_{max} \cdot S}{K_s + S(1+1/K_i)}$$  \hspace{1cm} (5)

**ACKNOWLEDGEMENT**

We are very grateful to Professor David E. Cane for carrying out the experiments in his laboratory at Brown University.

Received November 21, 2005.

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