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TRICHODIENE SYNTHASE: MECHANISM-BASED INHIBITION OF A SESQUITERPENE CYCLASE

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Abstract: The 10-cyclopropylidene analog of farnesyl diphosphate was shown to be a mechanism-based inhibitor of trichodiene synthase with an inactivation rate (k_{inact}) of 0.010 ± 0.0003 min^{-1} and an apparent K_I of 663 ± 75 nM. The presence of three anomalous sesquiterpene products detected in incubation mixtures indicate that the compound also serves as a substrate of the enzyme.

Sesquiterpene synthases comprise a versatile group of catalysts responsible for the formation of more than 300 distinct sesquiterpene carbon skeletons.[1] The versatility of these enzymes is demonstrated by the fact that each cyclase catalyzes the formation of a unique sesquiterpene product or group of products from a single common precursor, farnesyl diphosphate (FPP, **1**). Furthermore, the production of each cyclic product proceeds via a common mechanism initiated by ionization of the allylic diphosphate ester moiety followed by a series of electrophilic cyclizations, methyl or hydride migrations and final quenching of the positive charge by elimination of a proton or capture of water.[2] The ultimate structure and stereochemistry of the sesquiterpene product is a consequence of the precise folding conferred by each individual cyclase on the common substrate FPP. Through extensive isotopic labeling experiments, many of the mechanistic details of sesquiterpene formation have been elucidated[2,3] and the recently reported crystal structures of pentalenene synthase[4] and epi-aristolochene synthase[5] have provided the first glimpses of the active sites of sesquiterpene synthases. Intriguingly, in spite of the absence of any significant primary amino acid sequence similarity between the two enzymes, the two proteins have remarkably similar groups of helical folds and overall three-dimensional structure. In both cases the putative active sites are lined with a high proportion of aromatic amino acids which are thought to be ideally suited to the stabilization of the carbocation intermediates. In addition, a conserved cluster of aspartate residues appear to play a role in binding of the divalent Mg^{++} cation, which in turn helps to bind and orient the substrate and assist ionization by interaction with the pyrophosphate moiety. On the other hand, the identity and specific role of amino acid residues important for proper folding of the substrate or for mediating the final deprotonation step in each cyclization is completely unknown for not only these two synthases, but all other members of the cyclase class.

Trichodiene synthase, a sesquiterpene cyclase isolated from a variety of fungal sources,[6] catalyzes the cyclization of FPP to trichodiene (**2**), the parent hydrocarbon of the trichothecane family of antibiotics and mycotoxins. The enzyme from *Fusarium sporotrichioides* is a homodimer with subunits of 45 kDa[5d] and has been successfully overexpressed in *Escherichia coli*.[7] Extensive studies of the fate of stereospecifically labeled

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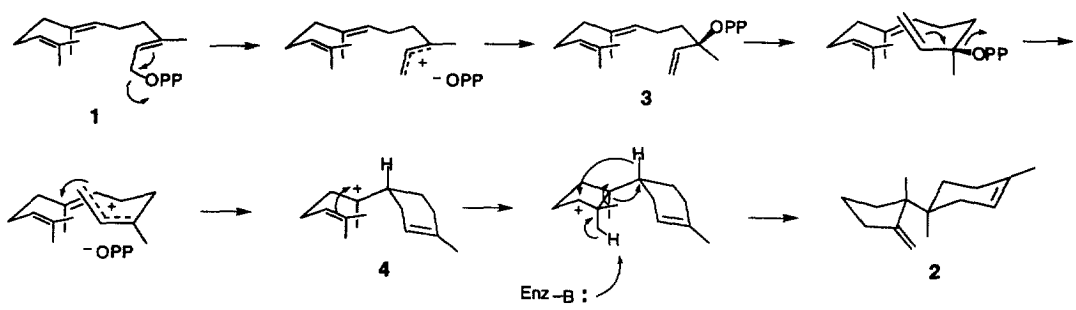
Scheme 1

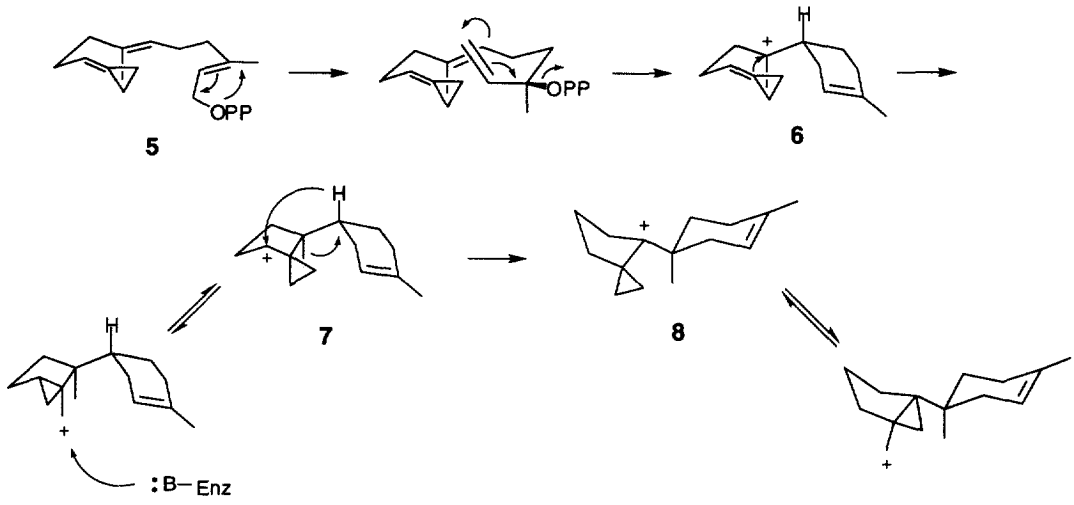


substrates have provided strong evidence for the mechanism of cyclization in which FPP undergoes an initial ionization and rearrangement to (3*R*)-nerolidyl diphosphate [(3*R*)-NPP, **3**][5a,8] (Scheme 1). Rotation of the 2,3-bond and subsequent ionization of the pyrophosphate group places the resultant allylic cation in a cisoid conformation allowing backside attack by the 6,7-double bond yielding the cyclic bisabolyl cation **4**. The formation of the cyclohexene ring is followed by a second cyclization, a hydride shift, two methyl migrations and a final elimination to yield trichodiene. Further support for this mechanism has come from experiments with substrate[9,10ab] and intermediate[9c] analogs as well as the ability of (3*R*)-NPP to act as a substrate for trichodiene synthase.[7,8]

Recent site-directed mutagenesis experiments on trichodiene synthase[11] have demonstrated the importance of the aspartates in the consensus sequence D^{100}DSKD and suggested an as yet undefined role for R^{304}YR and C^{190} in binding and catalysis. In order to identify additional catalytically important amino acid residues within the active site of the enzyme, we have been exploring the application of mechanism-based inactivators. In particular, we are interested in targeting the proposed active site base responsible for mediating the final deprotonation reaction from the position corresponding to C-12 of FPP to generate the product trichodiene (Scheme 1). To this end, we designed the FPP analog, 10-cyclopropylidene farnesyl diphosphate (CPFPP, **5**, Scheme 2) in which the terminal gem-dimethyl group of FPP is replaced by a cyclopropylidene moiety.[12] As an analog of FPP, CPFPP was expected to bind well to the active site and undergo the usual ionization, isomerization and cyclization to yield the bisabolyl cation analog **6**. Further cyclization of **6** would yield the cyclopropylcarbinyl intermediate **7**. Cations of this type are known to undergo extensive rearrangements through a cyclobutyl cation intermediate.[13] These rearrangements would delocalize the positive charge to regions of the active site which do not normally encounter carbocations and favor the capture of nearby nucleophilic amino acid side chains in the vicinity of C-12. Alternatively, **7** could undergo the normal hydride shift and methyl migration to generate a second cyclopropyl carbinyl cation species **8** that would be subject to a similar set of rearrangements.

CPFPP was synthesized from farnesyl acetate trisnoraldehyde[14] by Wittig reaction[15] with cyclopropyltriphenylphosphonium bromide[16] followed by deprotection of the alcohol, conversion to the allylic bromide and introduction of the diphosphate group by reaction with tris(tetra-*n*-butylammonium)pyrophosphate.[17]

Scheme 2



Incubations of recombinant *F. sporotrichioides* trichodiene synthase (13 nM) with increasing concentrations of CPFPP at 0 °C in Buffer T-BSA (10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 15% glycerol, 100 μg/mL bovine serum albumin) resulted in a pseudo-first-order, time-dependent inactivation of the enzyme as evidenced by the linear dependence of the log of % residual activity versus time (Figure 1). A replot of apparent inactivation rates (k_{app}) versus concentration of CPFPP followed by non-linear regression fitting demonstrates that the inactivation of the cyclase follows saturation kinetics with a maximum inactivation rate, k_{inact}, of 0.010 ± 0.0003 min^{-1} and a K_I of 663 ± 75 nM (Figure 1, Inset).[18] Coincubation with the substrate FPP effectively protected the cyclase from inactivation. Although the K_I for CPFPP inactivation compares favorably to the Michaelis constant for the natural substrate FPP (K_m = 90 nM), the k_{inact} is substantially slower than the corresponding steady state turnover rate (k_{cat} = 19 min^{-1}) for FPP. When the incubation time of CPFPP and the cyclase was extended to over 4 h, the fractional enzymatic activity remaining approached a limit which was proportional to the molar ratio of inhibitor to enzyme. This observation indicates that a branched pathway exists for the reaction of CPFPP and the enzyme in which an intermediate either inactivates the cyclase or derails yielding product and active enzyme. Thus, the process leading to inactivation comes to a halt as the inhibitor is turned over by the cyclase. Following the method of Knight and Waley,[19] a ratio of turnover to inactivation of 150:1 was determined by extrapolation from the plot of remaining fractional activity versus the molar ratio of CPFPP to enzyme (Figure 2). The ability of CPFPP to act as a substrate of trichodiene synthase was confirmed by the initial GCMS analysis of pentane extracts of incubation mixtures which revealed the presence of three hydrocarbon products with *m/z* 202. The observed molecular ion is consistent with the expected molecular weight of aborted cyclization products of CPFPP turnover. Extensive ultrafiltration of inactivated enzyme using a Centricon 30K exclusion membrane failed to restore activity to the enzyme.

The results presented above are consistent with mechanism-based inactivation of trichodiene synthase in a which a derailment intermediate is intercepted by a nucleophilic residue within the active site, leading to alkylation of the protein. Current studies are aimed at identifying the specific site of modification as well as identification of the anomalous products of CPFPP turnover. CPFPP should provide a useful tool for identifying catalytically important active site residues, not only within trichodiene synthase, but potentially other sesquiterpene synthases as well. The covalent nature of the inactivation process could also provide a powerful means to specifically label related cyclases for purification and subsequent molecular cloning.

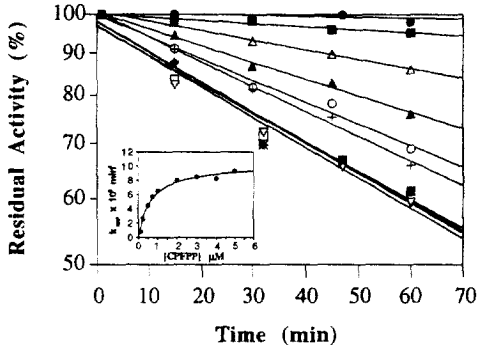


Figure 1. Kinetics of inactivation of trichodiene synthase by CPFPP. Enzyme (0.13 µM) was incubated at 0 °C with the following concentrations of CPFPP, 5: 0.0 (●), 0.10 (■), 0.25 (Δ), 0.50 (▲), 0.75 (O), 1.0 (+), 2.0 (□), 3.0 (▽), 4.0 (◆) and 5.0 (X) µM. Incubations (2 mL total volume) were carried out in Buffer T-BSA. At various time intervals, 10 µL aliquots were removed and serially diluted 100-fold. A 50 µL aliquot of this mixture was added to 450 µL of Buffer T containing 2 µM [1-^{3}H]-FPP (76 mCi/mmol) and assayed as previously described.[9] Semilog plot of residual % activity versus time. Inset: Plot of k_{app} versus [CPFPP].

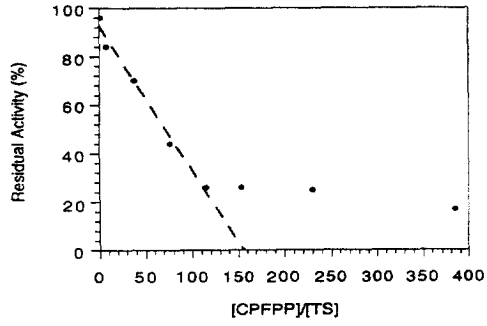


Figure 2: Titration of trichodiene synthase with CPFPP. Increasing amounts of inhibitor were incubated with a fixed amount of enzyme and the inactivation process was allowed to go to completion. The remaining activity was determined as described previously and a plot of percent residual activity versus the ratio of inhibitor to enzyme was constructed. At high ratios, the plot deviates from linearity most likely as a result of competitive inhibition between CPFPP and pyrophosphate product formed from CPFPP turnover. Extrapolation of the linear region of the plot to the abscissa yields the turnover number, i.e., the number of inhibitor molecules required for complete inactivation of the cyclase. Since the turnover number includes the one molecule which inactivates the enzyme, the partition ratio is one minus the turnover number.

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Chemistry

General Reagents obtained from Aldrich Chemical Company, St. Louis, MO and solvents obtained from Mallinckrodt Baker, Inc., Paris, KY. Farnesyl acetate trisnoraldehyde was prepared following the method of Cane et al.[14] Thin-layer chromatography (TLC) performed on Whatman aluminum-backed silica plates, 250 μM thickness and visualized using vanillin. Purifications performed by flash chromatography using silica gel (230–400 mesh) obtained from EM Science. All ^{1}H and ^{31}P NMR spectra obtained using Bruker instruments at either 400 or 250 MHz. Chemical shifts (δ) are reported in ppm relative to $CDCl_3$ or D_2O for ^{1}H NMR and phosphoric acid for ^{31}P NMR. Hex refers to hexane and EtOAc to ethyl acetate.

Synthesis of 10-cyclopropylidene farnesyl acetate. To a suspension of 5.14 mmol NaH (60% suspension in oil) in 17 mL of THF over nitrogen was added 5.14 mmol of dry cyclopropyltriphenylphosphonium bromide. After refluxing for 10 h, farnesyl acetate trisnoraldehyde (3.7 mmol in 4 mL of THF) was added to the dark orange mixture along with 0.5 mmol of tris[2-(2-methoxyethoxy)ethyl]amine (TDA-1). The mixture was refluxed for 6 h, quenched with water and extracted three times with diethyl ether. The combined extracts were washed successively with 40% methanol in half saturated NH_4Cl, water and saturated NaCl and dried over $MgSO_4$. Purification yielded the desired product in 48% yield. R_f = 0.36 (Hex/EtOAc, 10/1). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 5.71 (m, 1H), 5.32 (t, J = 7.2 Hz, 1H), 5.09 (t, J = 6.9 Hz, 1H), 4.57 (d, J = 7.1 Hz, 2H), 2.23 (m, 2H), 2.07 (m, 8H), 1.68 (s, 3H), 1.59 (s, 3H), 0.99 (s, 4H).

Synthesis of 10-cyclopropylidene farnesol. To 1.5 mmol of 10-cyclopropylidene farnesyl acetate in 20 mL of methanol was added 1.5 mmol of K_2CO_3 with stirring at 25 °C. After 1 h, saturated NaCl was added to the solution followed by extraction with hexane. The combined hexane extracts were washed with water and saturated NaCl and dried over $MgSO_4$. Purification yielded the desired alcohol in 97% yield. R_f = 0.14 (Hex/EtOAc, 10/1). ^{1}H NMR ($CDCl_3$, 400 MHz) δ 5.73 (m, 1H), 5.41 (tm, J = 7.0 Hz, 1H), 5.12 (tm, J = 7.0 Hz, 1H), 4.15 (d, J = 7.0 Hz, 2H), 2.26 (m, 2H), 2.10 (m, 3H), 2.03 (m, 3H), 1.68 (s, 3H), 1.61 (s, 3H), 1.27 (bs, 1H), 1.00 (s, 4H).

Synthesis of 10-cyclopropylidene farnesyl bromide. To a solution of 10-cyclopropylidene farnesol (0.62 mmol in 10 mL of dry CH_2Cl_2) over nitrogen at 0 °C was added 1.3 mmol of triphenylphosphine, 10 μL of 2,6-lutidine and 1.3 mmol of sublimed CBr_4. After stirring at 0 °C for 30 min, the solution volume was reduced to 2 mL by rotary evaporation followed by addition of 2 mL of hexane and cooling at –20 °C for several hours to precipitate triphenylphosphine oxide. After filtering through Celite, the solvent was removed in vacuo yielding the crude bromide which was used without further purification. The bromide was confirmed as the main product by TLC and ^{1}H NMR. R_f = 0.61 (Hex/EtOAc, 10/1). ^{1}H NMR ($CDCl_3$, 250 MHz) δ 5.71 (m, 1H), 5.51 (t, J = 8.4 Hz, 1H), 5.08 (m, 1H), 4.01 (d, J = 8.4 Hz, 2H), 2.26 (m, 2H), 2.03 (m, 6H), 1.71 (s, 3H), 1.59 (s, 3H), 1.00 (s, 4H).

Synthesis of 10-cyclopropylidene farnesyl diphosphate (5). The diphosphate was added to the crude bromide following the method of Davisson et al.[20] R_f = 0.53 (*n*-propanol/*n*-butanol/2 M NH_4OH, 5/2/3). ^{1}H NMR (D_2O, 400 MHz) δ 5.50 (m, 1H), 5.20 (t, J = 6.7, 1H), 4.98 (t, J = 5.9 Hz, 1H), 4.27 (t, J = 6.0 Hz, 2H), 2.25 (m, 2H), 2.10 (m, 3H), 2.03 (m, 3H), 1.69 (s, 3H), 1.62 (s, 3H), 1.05 (s, 4H). ^{31}P NMR (D_2O, 400 MHz) δ –5.57 (d, J = 55 Hz), –9.59 (d, J = 55 Hz).