Aristolochene Synthase. Mechanism-Based Inhibition of a Terpenoid Cyclase

David E. Cane* and Clifford Bryant

Department of Chemistry, Box H, Brown University
Providence, Rhode Island 02912

Received September 6, 1994

Aristolochene synthase catalyzes the cyclization of *trans,-trans*-farnesyl diphosphate (**1**, FPP) to the eremophilane-type sesquiterpene, (+)-aristolochene (**2**).[1] Extensive labeling studies have supported a cyclization mechanism in which FPP initially cyclizes to germacrene A (**3**) (Scheme 1). The latter intermediate is not released from the active site but undergoes reprotonation, leading to the bicyclic eudesmane cation (**4**), which itself is converted to **2** by successive hydride shift, methyl migration, and deprotonation.[1,2] Two distinct aristolochene synthases which appear to act by identical mechanisms have been isolated from fungal sources: the enzyme from *Aspergillus terreus* has a molecular weight of approximately 58 000,[3] while the *Penicillium roquefortii* cyclase, which has been purified to homogeneity, is an apparent monomer of subunit M_D 39 200.[4] The latter enzyme has been cloned[4] and overexpressed in *Escherichia coli*.[5]

Although some half-dozen cyclases catalyzing the formation of sesquiterpenes, monoterpenes, and diterpenes have now been cloned and sequenced,[6] little is known about the identity of the key amino acid residues which are present at the active site.[7] The development of mechanism-based inactivators for terpenoid cyclases is particularly challenging since these enzymes have evolved to handle highly reactive electrophilic species.[8] One strategy to overcome this problem is to use a substrate analog which, upon cyclization, could undergo rearrangement or delocalization to place positive charge in a region of the protein that does not normally encounter reactive electrophilic centers and that therefore would be susceptible to alkylation. Indeed, we have used such an approach and have recently reported mechanism-based inactivation of limonene synthase and related monoterpene synthases by the methylenecyclopropane analog of the normal substrate, geranyl diphosphate.[9] We now report that the vinyl analog of FPP, 12-methylidenefarnesyl diphosphate (**5**, VFPP), is an effective, mechanism-based inactivator of aristolochene synthase.[10]

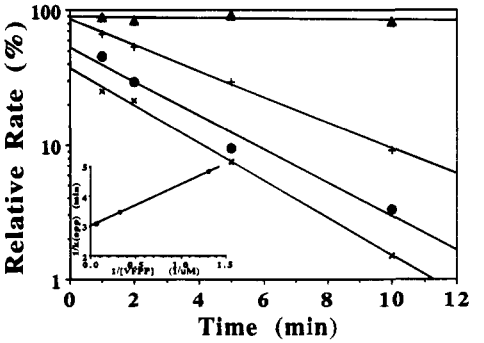
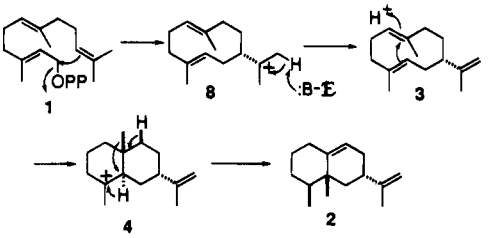
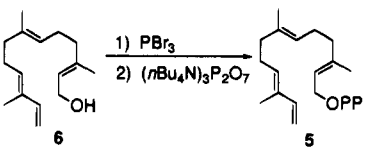


Figure 1. Kinetics of inactivation of aristolochene synthase by VFPP. Enzyme (0.16 μM) was incubated at 30 °C with the following concentrations of VFPP (**5**): 0.0 (▲), 0.77 (+), 3.06 (●), and 15.3 (×). Total incubation volume was 0.5 mL in Buffer T. At the indicated time intervals, 50 μL aliquots were withdrawn, diluted to a total volume of 0.5 mL in Buffer T containing 5.43 μM [1-^{3}H]FPP (71.7 mCi/mmol), and assayed for aristolochene synthase activity as previously described. Semilog plot of residual % activity versus time. (The lines corresponding to increasing concentrations of VFPP do not extrapolate to 100% activity at $t = 0$ min due to residual competitive inhibition after 10-fold dilution.) Inset: Plot of $1/k_{app}$ versus 1/[VFPP].

Scheme 1



Scheme 2



VFPP was readily prepared from the known alcohol **6**[11] by conversion to the corresponding allylic bromide and reaction with tris(tetra-*n*-butylammonium)pyrophosphate[12] (Scheme 2). Incubation of increasing concentrations of VFPP with recombinant *P. roquefortii* aristolochene synthase[5] (0.16 μM) at 30 °C in Buffer T (10 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 5 mM β-mercaptoethanol, 15% glycerol) resulted in pseudo-first-order, time-dependent inactivation of the cyclase, as evidenced by the linear dependence of the log of residual activity against time[13] (Figure 1). The rate of inactivation by VFPP was saturable, with a maximum rate of inactivation, k_{inact}, of 0.33 ± 0.01 min^{-1} ($t_{1/2} = 2.10$ min) at 0.16 μM protein and a K_I for VFPP of 0.46 ± 0.01 μM.[14] These values compare very favorably with the steady state kinetic parameters for the normal substrate FPP ($k_{cat} = 1.95 \pm 0.2$ min^{-1}, $K_m = 1.2 \pm 0.3$ μM).[5] Co-incubation with the normal substrate, FPP, afforded protection against inactivation. As expected, no inactivation occurred when the incubations were carried out in Buffer T containing 5 mM EDTA and lacking $MgCl_2$, consistent with the absolute depen-

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(4) Proctor, R. H.; Hohn, T. M. *J. Biol. Chem.* **1993**, *268*, 4543–4548.

(5) Cane, D. E.; Wu, Z.; Proctor, R. H.; Hohn, T. M. *Arch. Biochem. Biophys.* **1993**, *304*, 415–419.

(6) Cane, D. E. In *Genetics and Biochemistry of Antibiotic Production*; Vining, L. C.; Stuttard, C., Eds; Butterworth-Heinemann: Stoneham, MA, 1995; pp 633–655.

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(9) Croteau, R.; Alonso, W. R.; Koepp, A. E.; Shim, J. H.; Cane, D. E. *Arch. Biochem. Biophys.* **1993**, *307*, 397–404. Incubation of the corresponding methylenecyclopropane analog of FPP with aristolochene synthase led not to observable inactivation but only to simple competitive inhibition. (Cane, D. E.; Lamberson, C. R., unpublished observations.)

(10) Prestwich has recently reported that 29-methylidene-2,3-oxidosqualene is a mechanism-based inactivator of rat liver oxidosqualene cyclase and has determined the site of covalent modification in the inactivated enzyme. Abe, I.; Bai, M.; Xiao, X. Y.; Prestwich, G. D. *Biochem. Biophys. Res. Commun.* **1992**, *187*, 32–38. Abe, I.; Prestwich, G. D. *J. Biol. Chem.* **1994**, *269*, 802–804.

(11) Mori, K. *Agric. Biol. Chem.* **1972**, *36*, 2563–2567.

(12) Davisson, V. J.; Woodside, A. B.; Neal, T. R.; Stremler, K. E.; Muehlbacher, M.; Poulter, C. D. *J. Org. Chem.* **1986**, *51*, 4768–4779.

(13) Aristolochene synthase activity was assayed using 5 μM [1-^{3}H]-FPP as previously described.[5]

(14) A second set of incubations carried out at 0.16 μM aristolochene synthase and four concentrations of VFPP gave comparable values of k_{inact} (0.31 ± 0.03 min^{-1}) and K_I (0.46 ± 0.20 μM).

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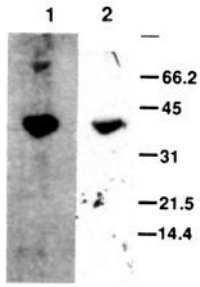
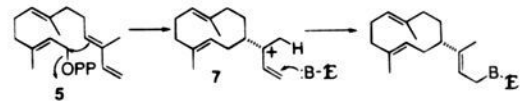


Figure 2. Labeling of aristolochene synthase by [1-^{3}H]VFPP (**5**). Lane 1, Coomassie blue-stained 12% SDS−PAGE gel of aristolochene synthase after treatment with [1-^{3}H]VFPP (**5**) and boiling with SDS buffer, as described in text. Lane 2, Radiofluorogram of SDS−PAGE gel. Migration of protein standards (molecular mass in kDa) is indicated. Aristolochene synthase migrates with an apparent M_r of 39 000.

dence of aristolochene synthase, as well as all other related cyclases, on a divalent metal cation.[7]

Gel filtration of the inactivated enzyme failed to restore aristolochene synthase activity. The covalent nature of the binding was established by incubation of 60 μM [1-^{3}H]VFPP (100.2 mCi/mmol)[15] with 30 μM aristolochene synthase in pH 7.4 Buffer T containing 10 mM $MgCl_2$ for 30 min at 30 °C, conditions sufficient to effect essentially complete inactivation. After gel filtration through Sephadex G-25 and dialysis, the recovered protein was lyophilized to dryness and then boiled for 3 min in SDS buffer prior to analysis by 12% SDS−PAGE. Staining with Coomassie blue confirmed the presence of a single protein of the expected M_r, while treatment of the gel with DMSO−2,5-diphenyloxazole and radiofluorography[17] revealed the presence of a single radioactive component with mobility identical to that of aristolochene synthase (Figure 2). In control experiments, [1-^{3}H]VFPP was separately incubated with boiled aristolochene synthase and bovine serum albumin. Analysis of the corresponding SDS−PAGE gels, either by radiofluorography or by excision of the relevant protein bands and direct liquid scintillation counting, failed to indicate any comigration of tritium activity with the protein, thereby confirming the specificity of the covalent modification of aristolochene synthase by VFPP. To determine the stoichiometry of binding, 24 μM [1-^{3}H]VFPP (71.4 mCi/mmol) was incubated with 1.2 μM aristolochene synthase in Buffer T for 30 min at 30 °C. Assay of an aliquot of the incubation mixture confirmed the loss of >98% of the original aristolochene synthase activity. The incubation mixture was dialyzed for 48 h against 5 mM Tris, pH 8.0, and then subjected to repeated ultrafiltration using a Centricon 10K exclusion membrane to give a final ratio of tritium activity to protein[18] which was calculated to correspond to 1.25 ± 0.15 equiv of inhibitor per mole of enzyme.[21]

Scheme 3



To determine the fate of the pyrophosphate moiety in **5** during inactivation, 37 μM [^{32}P]VFPP (10.5 mCi/mmol)[22] was incubated with 9.4 μM aristolochene synthase in Buffer T. Repeated ultrafiltration of the inactivated enzyme gave protein that retained less that 0.05 equiv of the [^{32}P]pyrophosphate per mole of enzyme compared to protein from a control incubation carried out in the absence of $MgCl_2$. The latter result is fully consistent with cleavage of the diphosphate ester during inactivation of aristolochene synthase by VFPP.

The above results are readily explained by a mechanism-based inactivation process in which initial ionization and cyclization of the FPP analog **5** by aristolochene synthase gives rise to the allylic cation species **7** (Scheme 3). The latter compound, which is an analog of the normal germacradienyl cation intermediate **8**, could react with the active site base or a nearby nucleophilic amino acid side chain. Experiments to establish the mechanism of inactivation and to determine the site of covalent protein modification are in progress. It is also anticipated that **5** may also prove to be useful in specific and irreversible labeling of the *A. terreus* aristolochene synthase, thereby providing a powerful tool for the purification and eventual molecular cloning of this related enzyme.

Acknowledgment. This work was supported by a grant from the NIH, GM30301. We would like to thank Mr. Ilgu Kang for assistance in the purification of recombinant aristolochene synthase and for carrying out the control experiments with bovine serum albumin and VFPP.

(15) [1-^{3}H]**6** was prepared by oxidation of **6** with MnO_2, followed by reduction with [^{3}H]$NaBH_4$, as previously described for the preparation of [1-^{3}H]FPP.[16]

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(18) Routine measurements of protein concentration were performed according to the method of Bradford[19] using commerical reagent (Bio-Rad) and bovine γ globulin as reference standard. For more accurate measurements, the results of the Bradford assay were corrected by division by a factor of 1.88. The correction factor was determined by carrying out Bradford assays in triplicate on aliquots of a standard solution of aristolochene synthase in deionized water and then directly weighing evaporated and thoroughly dried aliquots of the same solution. Measurement of A_{280} on the same solution gave an extinction coefficient of 38 775 $M^{-1}cm^{-1}$. The accuracy of the latter measurement was further corroborated by DTNB titrations,[20] which gave a ratio of 6.0 ± 0.4 Cys per subunit, in agreement with the six Cys residues predicted by the deduced amino acid sequence.[4]

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(21) In a second experiment, the inactivated protein was first passed through a column of Sephadex G-25, and the eluant was dialyzed for 48 h. Analysis of tritium activity and protein content gave a stoichiometry of 0.9 ± 0.1 equiv of inhibitor per subunit of protein.

(22) [^{32}P]VFPP was prepared from **6** in the usual manner using [^{32}P]-inorganic pyrophosphate.