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Prenyltransferase

KINETIC STUDIES OF THE 1′-4 COUPLING REACTION WITH AVIAN LIVER ENZYME*

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Prenyltransferase catalyzes the sequential, irreversible 1′-4 condensation of isopentenyl-PP with dimethylallyl-PP and geranyl-PP to yield farnesyl-PP. A kinetic study shows substrate inhibition by isopentenyl-PP at concentrations above 0.7 μM when the concentration of geranyl-PP is 1.0 μM or less as a result of binding by the homoallylic substrate to the allylic region of the active site. Inhibition studies were carried out with the products, farnesyl-PP and PP_i, and dead-end inhibitors 2-F-isopentenyl-PP and 2-F-geranyl-PP, analogues for the normal substrates. Competitive patterns were seen for farnesyl-PP and 2-F-geranyl-PP when geranyl-PP was varied, while noncompetitive patterns were found for all other combinations. A minor form of PP_i, $MgHPP_i^-$, is implicated as the species of PP_i in the magnesium-containing buffer which binds most tightly to the enzyme. This observation explains why *K*'s for PP_i calculated from the total concentration of PP_i are much larger than *K*'s for the organic pyrophosphates. The lack of regiospecificity in the binding of isopentenyl-PP, as evidenced by substrate inhibition patterns, introduces an element of ambiguity into mechanistic interpretations, and it is not possible to distinguish between ordered and random mechanisms on the basis of inhibition studies.

The prenyltransferase which catalyzes the sequential condensation of isopentenyl-PP[1] to dimethylallyl-PP and geranyl-PP (EC 2.5.1.1), as shown in Scheme 1, is a key building enzyme in the sterol biosynthetic pathway. Beginning in the late 1960s, Popjak and co-workers reported a series of kinetic studies with partially purified porcine liver enzyme (2–5) and concluded, primarily on the basis of product inhibition patterns, that the reaction involved an ordered sequential mechanism in which the allylic substrate adds before isopentenyl-PP and farnesyl-PP is released before PP_i. Recently homogeneous enzyme has been isolated from yeast (6), avian liver (7), and porcine liver (8) by Rilling and co-workers. Studies primarily with the more stable avian enzyme, suggest that the enzyme (M_r = 86,000) is a dimer with two identical subunits, each containing a single catalytic site that catalyzes both condensations (7). The catalytically active complex contains 2 molecules of magnesium (9, 10) in addition to the organic substrates. Binding studies (9–12) show that dimethylallyl-PP, geranyl-PP, and farnesyl-PP form a 1:1 complex with each subunit, presumably by binding to the allylic region of the active site. However, isopentenyl-PP and PP_i do not exhibit the same fidelity but form a 1:2 enzyme·substrate complex, 1 mol of which is displaced upon addition of an allylic pyrophosphate. The results for farnesyl-PP are particularly interesting since they suggest that the normal product of the enzymatic reaction prefers to bind to the allylic region of the active site rather than span the isopentenyl and allylic regions in the manner required for the enzyme·product complex immediately following catalysis. Additional verification for binding to the allylic region is found in the observation that farnesyl-PP participates in the 1′-4 condensation (1).

The kinetic analysis of Popjak and Holloway (2) is predicated on the assumption that farnesyl-PP and PP_i add in an orientation identical to that which is generated upon catalysis, and their inhibition patterns appear to confirm this assumption. In view of the potential conflict between the kinetic and binding experiments, we decided to examine the kinetic properties of crystalline avian liver prenyltransferase using recently developed dead-end inhibitors (13, 14). The experimental detail and a description of the results are presented in miniprint format.[2]

The nonlinear initial velocity pattern found when isopentenyl-PP was the varied substrate (Fig. 1) clearly reveals substrate inhibition by the homoallylic pyrophosphate. This phenomenon, first reported by Reed and Rilling (7), is consistent with binding measurements which indicate that 2 molecules of isopentenyl-PP are bound/catalytic site in the absence of geranyl-PP and that one is displaced upon addition of citronellyl pyrophosphate, an unreactive analogue for the C_{10} substrate (9). The competitive inhibition pattern seen for varied geranyl-PP at high concentrations of isopentenyl-PP (Fig. 2) indicates that the substrate inhibition results from addition to the allylic site. This result is not surprising in view of the topological resemblance of isopentenyl-PP to dimethylallyl-PP, the first allylic substrate for the enzyme. In contrast, there is no evidence for substrate inhibition by geranyl-PP.

The initial velocity patterns shown in Figs. 1 and 2 do not permit one to conclusively distinguish between a sequential

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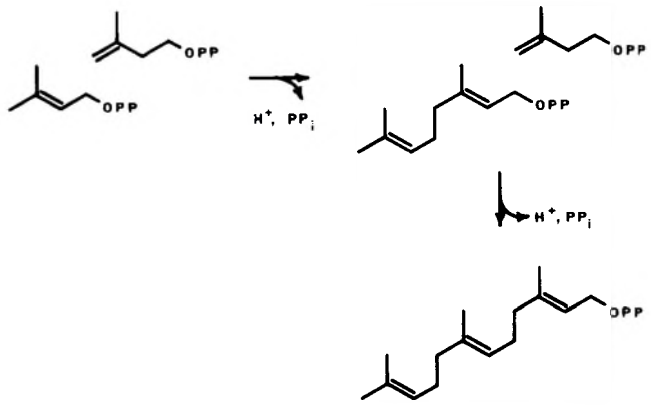
[1] The abbreviations used are: isopentenyl-PP, isopentenyl pyrophosphate; farnesyl-PP, farnesyl pyrophosphate; geranyl-PP, geranyl pyrophosphate; dimethylallyl-PP, dimethylallyl pyrophosphate; 2-F-geranyl-PP, 2-fluorogeranyl pyrophosphate; 2-F-isopentenyl-PP, 2-fluoroisopentenyl pyrophosphate; PP_i, inorganic pyrophosphate.

[2] The "Experimental Procedures" and "Results," including Figs. 1 through 10 and Tables II to IV, are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, Md. 20014. Request Document No. 79M-1BB, cite author(s), and include a check or money order for $1.65 per set of photocopies.



SCHEME 1. **Conversion of dimethylallyl-PP to farnesyl-PP by avian liver prenyltransferase.**

mechanism and a ping-pong mechanism with an enzyme-geranyl intermediate. A noncompetitive pattern is predicted for the linear portion of the curves in Fig. 1 by the former mechanism, and an uncompetitive pattern, by the latter. Although the slopes increase as the concentration of geranyl-PP is reduced from 1.0 to 0.1 μM, the change is only 25%. However, inhibition patterns with dead-end inhibitors 2-F-isopentenyl-PP (Figs. 7 and 8) and 2-F-geranyl-PP (Figs. 9 and 10) clearly eliminate the latter possibility. The ping-pong mechanism predicts an uncompetitive pattern for 2-F-geranyl-PP when isopentenyl-PP is the varied substrate, whereas a noncompetitive pattern was found. Evidence against a ping-pong mechanism was also obtained by incubating the enzyme with isopentenyl-PP and an excess of geranyl-PP in the presence of [^{32}P]PP_i (1). Recovered geranyl-PP was devoid of radioactivity under conditions where at least one part in 10^8 of a reverse reaction could have been detected. Thus, the 1′-4 coupling is irreversible, and if an enzyme-geranyl intermediate is formed, no partitioning is seen in the reverse direction. In view of the values for the dissociation constants of isopentenyl-PP and PP_i and the relative nucleophilicity of a carbon-carbon double bond and PP_i, a ping-pong sequence that is irreversible at the enzyme-geranyl stage is highly unlikely.

Holloway and Popjak (2) reported that farnesyl-PP gave noncompetitive patterns when isopentenyl-PP or geranyl-PP was varied, while PP_i gave a noncompetitive pattern when isopentenyl-PP was varied and a competitive pattern for geranyl-PP. Only an ordered bireactant mechanism in which geranyl-PP adds before isopentenyl-PP and farnesyl-PP dissociates before PP_i was considered to fit the inhibition patterns and the previously studied chemistry of the reaction (15). In contrast, at lower substrate concentrations with crystalline enzyme from avian liver, we found that PP_i gave a noncompetitive pattern when either substrate was varied, while farnesyl-PP is a noncompetitive inhibitor for variable isopentenyl-PP and competitive for geranyl-PP (Figs. 3 through 6). We have no explanation for the discrepancy. Preliminary experiments with the less stable enzyme from porcine liver (1, 13, 14) gave results that were in qualitative agreement with the avian system, and a change in mechanism for enzyme from the two different sources is not anticipated. The binding data from Rilling's laboratory (10–12) do suggest that farnesyl-PP binds to the allylic region of the active site, while PP_i lacks regiospecificity, and, thus, support our kinetic measurements.

Phosphate buffer used in previous experiments inhibits the reaction at the concentrations used in our assay, and as a result most of the kinetic constants listed in Table I are significantly lower than those previously reported (1, 10, 13, 14). The largest constants are those for PP_i, although the pyrophosphate moieties in the substrates are essential for optimal binding (1). This enigma can be explained by experiments which suggest that $MgHPP_i^-$, a minor form of PP_i in our buffer system, is a more potent inhibitor than the other PP_i species. It should be emphasized that $MgHPP_i^-$ is the hydrido analogue of the organophosphates, which exist almost entirely as the monometal salts in 1 mM magnesium (10). The inhibition constants for PP_i suggest a higher affinity for the allylic site, again in qualitative accord with competitive binding experiments.

When using product inhibition patterns to deduce a mechanism, one normally assumes that the products bind regiospecifically in orientations similar to those generated upon catalysis, especially for reversible reactions. This however, is clearly not the case for the irreversible 1′-4 condensation. Several lines of evidence, including binding studies (1, 9–12), condensation (1, 3) and hydrolysis (1, 12, 16) reactions where farnesyl-PP is an alternate substrate for the enzyme, and the competitive inhibition pattern shown in Fig. 6, all suggest that farnesyl-PP prefers to bind with its pyrophosphate moiety positioned in the allylic region of the catalytic site. This orientation is obviously much different than initially generated by 1′-4 condensation. In addition, the binding experiments of Saito and Rilling (12) and our kinetic work show that PP_i lacks regiospecificity.

Another observation to be considered is the difference between the Michaelis constant (0.10 μM) and the dissociation constant (2.5 μM) for isopentenyl-PP obtained by equilibrium measurements (9). Such differences often result from nonrapid equilibrium behavior, and a value of $V_1/K_M E_t$ of 4.7×10^{-6} M^{-1} s^{-1}, based on a molecular activity of 1.4 s^{-1} (1) for the enzyme, is in a range consistent with a steady state mechanism (17, 18).

The simplest case which incorporates the above mentioned features, including the binding characteristics of the substrates and inhibitors, is an ordered bireactant mechanism. Since the reaction is irreversible, the products can be treated as dead-end inhibitors, along with the fluorinated analogues. Thus, the three diagrams shown in Scheme 2, where *A* is geranyl-

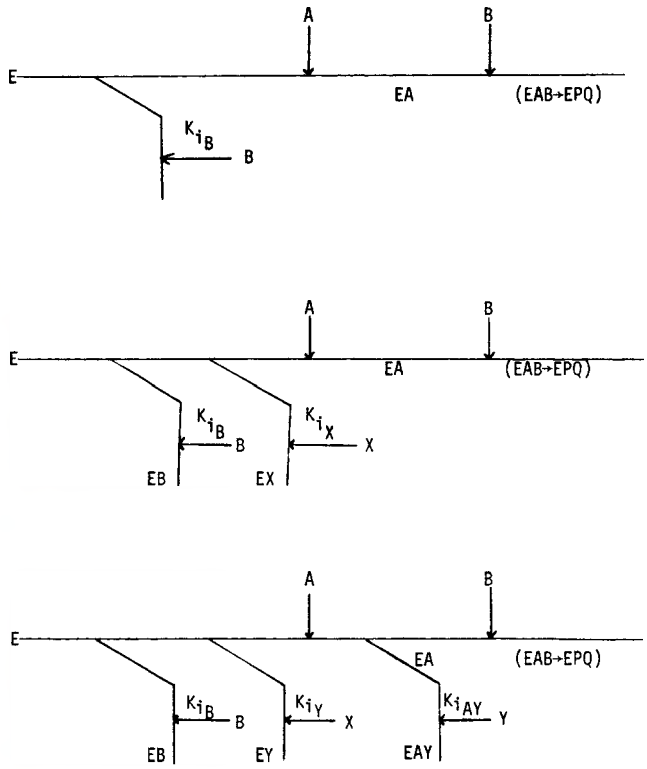
TABLE I

Kinetic constants for avian liver prenyltransferase

Fixed	Varied	Inhibitor	K	K_{is}	K_{ii}
			μM	μM	μM
Geranyl-PP	Isopentenyl-PP	2-F-isopentenyl-PP[a]	0.5 ± 0.004	0.46 ± 0.04	16 ± 3
Isopentenyl-PP	Geranyl-PP	2-F-isopentenyl-PP[a]	0.13 ± 0.006	3.7 ± 0.3	5.4 ± 0.3
Geranyl-PP	Isopentenyl-PP	2-F-geranyl-PP[a]	0.03 ± 0.001	1.1 ± 0.2	8.3 ± 0.9
Geranyl-PP	Isopentenyl-PP	PP_i[a]	0.05 ± 0.001	90 ± 100 (5 ± 5)	44 ± 7 (2.6 ± 0.4)
Isopentenyl-PP	Geranyl-PP	PP_i[a]	0.14 ± 0.002	12 ± 2 (0.7 ± 0.1)	90 ± 40 (5 ± 2)
Geranyl-PP	Isopentenyl-PP	Farnesyl-PP[a]	0.03 ± 0.003	0.7 ± 0.1	6.1 ± 0.4
Isopentenyl-PP	Geranyl-PP	Farnesyl-PP[b]	0.21 ± 0.02	0.86 ± 0.08	
Isopentenyl-PP	Geranyl-PP	2-F-geranyl-PP[b]	0.20 ± 0.02	0.89 ± 0.08	

[a] Noncompetitive.
[b] Competitive.

PP, *B* is isopentenyl-PP, *X* is farnesyl-PP or 2-F-geranyl-PP, and *Y* is PP_i or 2-F-isopentenyl-PP, suffice to illustrate the mechanism. In assigning Y to 2-F-isopentenyl-PP, it is assumed that this analogue for isopentenyl-PP shares the lack of regiospecificity found for the normal substrate. The kinetic equations (see the "Appendix" in the minprint) for the three cases presented in Scheme 2 predict initial velocity patterns identical to those that were found. Unfortunately, the affinity of 2-F-isopentenyl-PP for the allylic region of the active site introduces an inhibitor-dependent term into the slope of the equation for v^{-1} *versus* [geranyl-PP]$^{-1}$ at fixed concentrations of isopentenyl-PP (see Equation 11 in the miniprint). This addition transforms the uncompetitive profile expected for dead-end inhibition by 2-F-isopentenyl-PP in the ordered bireactant system into a noncompetitive pattern usually associated with a random bimolecular mechanism. Thus, one cannot distinguish between ordered and random mechanisms by inspection of inhibition patterns as previously claimed (2–5). Hot trap experiments are now underway to solve this dilemma.



SCHEME 2. **Schemes for ordered bireactant systems with substrate inhibition, with and without dead-end inhibitors.**

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Additional references are found on p. 9463.

SUPPLEMENTARY MATERIAL
TO
PRENYLTRANSFERASE. KINETIC STUDIES OF THE
1'-4 COUPLING REACTION WITH AVIAN LIVER ENZYME
F. MARK LASKOVICS, JAMES M. KRAFCIK, AND C. DALE POULTER

EXPERIMENTAL PROCEDURES AND RESULTS

Note: Schemes 1 and 2 and Table I appear in the parent paper.

Materials and Techniques. [1-^{3}H-^{4}C]-Isopentenyl-PP was purchased from Amersham/Searle, and sodium acid pyrophosphate was a gift from Stauffer Chemical Co. The organic pyrophosphates were prepared from the corresponding alcohols and purified by ion-exchange chromatography (1,14,19). The purities of the organopyrophosphates were determined by tlc (phosphate buffered silica gel H (E.M. Reagents); methanol: chloroform: water, 10:10:3), and concentrations of stock solutions were determined by analysis for phosphate (20). Concentrations of geranyl-PP (and [1-^{14}C]-isopentenyl-PP) in the 3 to 30 μM range were checked by incubation with excess [1-^{14}C]-isopentenyl-PP (or geranyl-PP) of known specific activity and enzyme.

Crystalline avian liver prenyltransferase (7) was provided by Professor H.C. Rilling.

Unless otherwise stated, kinetic runs were carried out in 0.5 mL total volumes of buffer (10 mM Pipes, 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.25 μM KN_3, pH 7.0 at 37°C) containing the specified amounts of substrates and enzyme. Solutions were prepared by adding aliquots of the enzyme to tubes containing the substrates cooled in ice, and the reactions were initiated by placing the cold tubes in a water-bath maintained at 37±0.5°C. After incubation for 10 min, the reaction was quenched with 0.1 mL of 25% HCl in methanol, and the samples were maintained at 37° for an additional 10 min to hydrolyze the allylic pyrophosphates. The aqueous solutions were extracted with 1.0 mL of ligroin (b.p. 90-120°C). Radioactivity in 0.5 mL portions of the extract was determined by liquid scintillation spectrometry (Searle Analytic 92) using 10 mL of 0.4% Omnifluor (New England Nuclear) in toluene. Each kinetic point is the average of at least five determinations, all of which agreed within 10%.

Preliminary indications of the inhibition patterns were obtained from double reciprocal plots using an unweighted least squares analysis. Additional refinement was obtained with modified versions of Cleland's programs (21). In all instances the "fits" for the patterns indicated in Table I were significantly better than alternatives as judged by significantly lower values for σ. In the closest case, the inhibition pattern for 2-F-isopentenyl-PP when isopentenyl-PP was varied, σ for the noncompetitive fit was half that of the competitive fit. Definitions of the computer-derived kinetic constants (K, K_{ii}, K_{is}) obtained by this procedure are given by Cleland (21).

Determination of K_M from the integrated Michaelis-Menten equation. While double reciprocal plots provide an easily interpreted, graphic representation of inhibition patterns, they often do not yield accurate estimates of Michaelis constants. This is particularly true in those cases where the slopes of the primary plots are nearly parallel. However, it is usually possible to construct a rate equation which can be integrated. With appropriate curve-fitting procedures, the integrated equation can often provide a more accurate estimate of kinetic constants (22), although the equations are not normally used to establish binding patterns (23,24). If a sequential mechanism is assumed for prenyltransferase, the Michaelis-Menten equation in the absence of products is:

$$dp/dt = v = \frac{V_i AB}{K_{ia}K_{M_B} + K_{M_B}A + K_{M_A}B + AB} \qquad (1)$$

Following the procedures of Cornish-Bowden (22), integration and rearrangement gives:

$$t = \frac{1}{V_i}\left[P + (K_{M_B} + \frac{K_{ia}K_{M_B}}{A_o - B_o})\ln\left(\frac{B_o}{B_o - P}\right) + (K_{M_A} + \frac{K_{M_B}K_{ia}}{B_o - A_o})\ln\left(\frac{A_o}{A_o - P}\right)\right] \qquad (2)$$

where A_o and B_o are the initial concentrations of geranyl-PP and isopentenyl-PP, respectively, at t=0, and P is the concentration of farnesyl-PP at time t. K_{ia}, K_{M_A}, and K_{M_B} are determined from the derivation of the appropriate steady-state expression. For time course measurements which are run under conditions where $A_o >> B_o$, equation 2 reduces to:

$$t = \frac{1}{V_i}\left[P + K_{M_B}\ln\left(\frac{B_o}{B_o - P}\right)\right] \qquad (3)$$

Time course measurements where the ratio of the initial concentration of geranyl-PP to that of isopentenyl-PP was at least 17.9:1 were fit to equation 3 using the nonlinear least squares program of Powell and Macdonald (25). The values obtained for K_{M_B} are listed in Table II. It should be emphasized that this constant is the one most difficult to obtain by a double reciprocal analysis.

Table II
Michaelis Constant for Isopentenyl-PP at 37°C

$[\text{Isopentenyl-PP}]_o$ μM	$[\text{Geranyl-PP}]_o$ μM	K_{M_B} μM
0.203	7.0	0.170±0.024
0.265	21.0	0.097±0.018
0.390	7.0	0.108±0.019
0.390	13.0	0.122±0.019
0.420	28.0	0.081±0.010
	Average	0.10±0.02

Initial velocity patterns with isopentenyl-PP and geranyl-PP. Initial velocity patterns for isopentenyl-PP and geranyl-PP are shown in Figures 1 and 2. For fixed concentrations of geranyl-PP ranging from 0.10 to 1.0 μM,

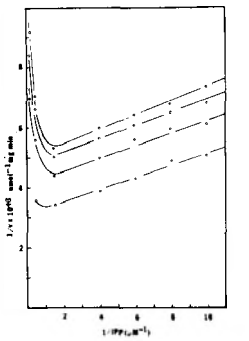

Figure 1: Initial velocity pattern with isopentenyl-PP as the varied substrate. Geranyl-PP concentrations: 0.10, 0.12, 0.18 and 1.0 μM. Enzyme concentration, 7.4 ng/ml.

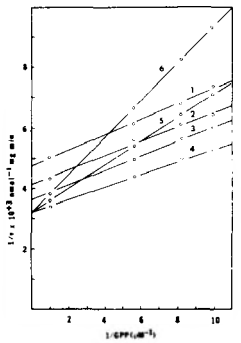

Figure 2: Initial velocity pattern with geranyl-PP as the varied substrate. Isopentenyl-PP concentrations: (1) 0.10, (2) 0.18, (3) 0.25, (4) 0.67, (5) 1.0 and (6) 2.0 μM. Enzyme concentration: 7.4 ng/ml.

the inverse rate varied linearly with the inverse of the concentration of isopentenyl-PP up to approximately 0.7 μM, at which point the lines curve sharply upward (Figure 1). When geranyl-PP is the varied substrate, linear patterns are obtained at all concentrations. For concentrations of isopentenyl-PP between 0.1 and 0.67 μM, the slopes are approximately equal; however, above 0.67 μM, the slopes increase rapidly (Figure 2). The profiles shown in Figures 1 and 2 are typical of those expected for substrate inhibition by isopentenyl-PP (26). Because of limitations associated with the assay and problems with the stability of the enzyme in dilute solutions, it was not possible to obtain accurate kinetic data for concentrations of isopentenyl-PP below ca 0.1 μM.

Inhibition Patterns. Inorganic pyrophosphate gave linear noncompetitive inhibition with isopentenyl-PP (Figure 3) or geranyl-PP (Figure 4) as the variable substrate. The inhibition constants measured for PP_i

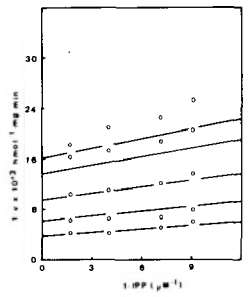

Figure 3: Reciprocal plots with isopentenyl-PP as the varied substrate and PP_i as inhibitor. The geranyl-PP concentration is 0.70 μM. Inorganic pyrophosphate concentrations: 0.0, 30, 70, 120 and 150 μM. Enzyme concentration, 11.4 ng/ml.

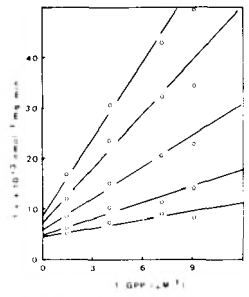

Figure 4: Reciprocal plots with geranyl-PP as varied substrate and PP_i as inhibitor. The isopentenyl-PP concentration is 0.60 μM. Inorganic pyrophosphate concentrations: 0.0, 10, 30, 60 and 90 μM. Enzyme concentrations, 11.4 ng/ml.

(Table I) are much higher than the Michaelis constants for isopentenyl-PP and geranyl-PP. However, at pH 7 and a magnesium ion concentrations of 1.0 mM, the bulk of PP_i exists as an equilibrium mixture of three magnesium complexes-$MgHPP_i^{-1}$, $MgPP_i^{-2}$, and Mg_2PP_i. Since Saito and Rilling (12) showed that magnesium ion is necessary for binding of PP_i to the enzyme, it is important to determine which of the three species is responsible for inhibition. The acid dissociation constants for pyrophosphoric acid and the stability constants for the magnesium-PP_i complexes are known (27,28); thus the relative percentages of the three complexes, as a function of pH and magnesium ion concentration, can be approximated (29) (Table III).

Table III
Relative proportions of magnesium-PP_i complexes present as a function of magnesium concentration and pH.

pH	$[Mg^{++}]$ mM	PP_i(free)[b]	Mole Fraction of Added PP_i[a] $MgHPP_i^-$	$MgPP_i^{-2}$	Mg_2PP_i
7.0	1.0	0.050	0.055	0.734	0.161
7.0	1.5	0.032	0.051	0.690	0.227
6.5	1.0	0.126	0.141	0.601	0.132

[a]Determined assuming an activity coefficient of 1.0. [b]PP_i not ligated to metal.

As the concentration of magnesium ion is increased from 1.0 to 1.5 mM at pH 7.0, the concentration of Mg_2PP_i increases by 41% while those of $MgHPP_i^{-1}$ and $MgPP_i^{-2}$ decrease by 7%. Similarly, as the pH of the solution is lowered from 7.0 to 6.5, the concentrations of $MgHPP_i^{-}$ triples while those of $MgPP_i^{-2}$ and Mg_2PP_i decrease by 18%. Thus, in principle it is possible to determine which of the three magnesium complexes inhibits the enzyme by suitable variations of pH and magnesium ion concentration. The results of kinetic experiments designed to determine which species inhibits the enzyme are summarized in Table IV. The only significant changes in reciprocal plots (slopes and intercepts) occurred when the pH was lowered from 7.0 to 6.5 and indicate better inhibition. Control runs in the absence of PP_i show no significant variation in V_1 for pH 6.5 and 7.0 at 1 mM Mg^{2+}, and only a slight stimulation as the concentration of

Table IV

Slopes and intercepts for Lineweaver-Burke plots as a function of $[MgCl_2]$ and pH[a]

pH	$[MgCl_2]$ mM	$[PP_i]$ μM	slope × 10^3 (mg^{-1} min $nmol^{-1}$)	Intercept × 10^3 (μM^{-1} mg min)
6.5	1.0	0	0.42±0.06	3.3±0.3
6.5	1.0	90	3.9±0.4	12.5±2.0
7.0	1.0	0	0.55±0.03	2.8±0.15
7.0	1.0	90	3.0±0.3	9.1±0.14
7.0	1.5	0	0.73±0.03	2.3±0.14
7.0	1.5	90	2.9±0.36	8.9±1.3

[a] [Isopentenyl-PP] = 0.67 μM; [Geranyl-PP] = 0.11, 0.30, 0.50 and 0.70 μM.

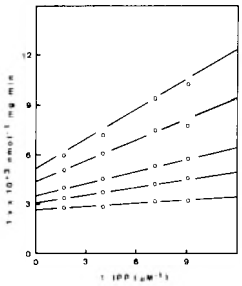

Figure 5: Reciprocal plots with isopentenyl-PP as varied substrate and farnesyl-PP as inhibitor. The geranyl-PP concentration is 0.70 μM. Farnesyl-PP concentrations: 0.0, 1.0, 2.0, 4.0 and 6.0 μM. Enzyme concentration, 5.6 ng/ml.

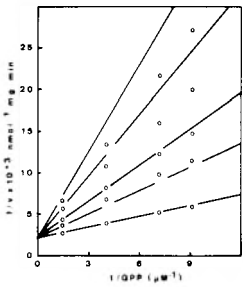

Figure 6: Reciprocal plots with geranyl-PP as varied substrate and farnesyl-PP as inhibitor. The isopentenyl-PP concentration is 0.60 μM. Farnesyl-PP concentrations: 0.0, 1.0, 2.0, 4.0, 6.0 μM. Enzyme concentration, 5.6 ng/ml.

Mg^{2+} is increased from 1 mM to 1.5 mM at pH 7.0. Thus, the $MgHPP_i^{-2}$ complex is a better inhibitor than the other two PP_i species. If one assumes that $MgHPP_i^{-}$ is the only form of PP_i which binds to the enzyme, the inhibition constant of PP_i is similar in value to the Michaelis constants of isopentenyl-PP and geranyl-PP. However, it should be noted that the percentages of PP_i given in Table III are derived from equilibrium constants determined at an ionic strength of 0.2 (28), while the ionic strength in our buffer system is ca. 0.01. Although the values listed in Table III can be used to predict trends, the inhibition constants for PP_i listed in parenthesis in Table I may contain a systematic error (30).

Farnesyl-PP gave linear noncompetitive inhibition when isopentenyl-PP was the varied substrate and linear competitive inhibition when geranyl-PP was varied (Figures 5 and 6). The range of concentrations over which farnesyl-PP can be studied is limited, probably by formation of micelles. Although we did not encounter any of these difficulties by working at low concentrations, they have been reported by others (31,32).

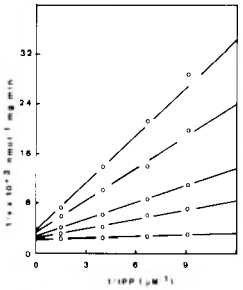

Figure 7: Reciprocal plots with isopentenyl-PP as varied substrate and 2-F-isopentenyl-PP as inhibitor. The geranyl-PP concentration is 0.70 μM. 2-F-Isopentenyl-PP concentrations: 0.0, 2.0, 4.0, 8.0 and 12.0 μM. Enzyme concentration, 11.4 ng/ml.

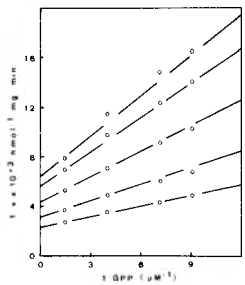

Figure 8: Reciprocal plots with geranyl-PP as varied substrate and 2-F-isopentenyl-PP as inhibitor. The isopentenyl-PP concentration is 0.67 μM. 2-F-Isopentenyl-PP concentrations: 0.0, 2.0, 5.0, 8.0 and 10.0 μM. Enzyme concentration, 11.4 ng/ml.

The 2-fluoro analogues of isopentenyl-PP and geranyl-PP are alternate substrates for the enzyme (1,13,14). However, the substitution of a hydrogen atom by fluorine retards the rate of the electrophilic condensations reactions to such an extent that insignificant amounts of the compounds are consumed when they are used in inhibition studies with the natural substrates. Thus, the fluorinated pyrophosphates function as dead-end inhibitors. 2-F-Isopentenyl-PP gave a linear pattern with isopentenyl-PP as the variable substrate (Figure 7) which, although the spread on the 1/v axis was small, gave a better fit to the equation for non-competitive inhibition than to that for competitive inhibition. The small spread in the 1/v intercepts probably reflects a minor contribution to the rate expression from binding of 2-F-isopentenyl-PP at the allylic site in accord with the substrate inhibition seen for isopentenyl-PP. A noncompetitive pattern was obtained when geranyl-PP was varied (Figure 8). Conversely, 2-F-geranyl-PP gave noncompetitive inhibtion when isopentenyl-

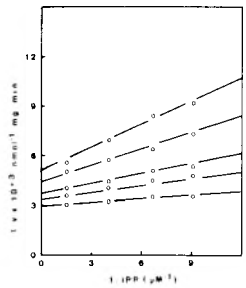

Figure 9: Reciprocal plots with isopentenyl-PP as varied substrate and 2-F-geranyl-PP as inhibitor. The geranyl-PP concentration is 1.0 μM. 2-F-geranyl-PP concentrations: 0.0, 1.0, 2.0, 4.0 and 6.0 μM. Enzyme concentration, 6.8 ng/ml.

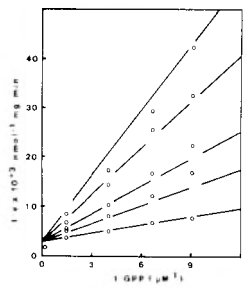

Figure 10: Reciprocal plots with geranyl-PP as varied substrate and 2-F-geranyl-PP as inhibitor. The isopentenyl-PP concentration is 0.67 μM. 2-F-geranyl-PP concentrations: 0.0, 1.0, 2.0, 4.0 and 6.0 μM. Enzyme concentration, 6.8 ng/ml.

PP was varied (Figure 9) and a competitive pattern with geranyl-PP as the variable substrate (Figure 10). The values of the inhibition constants for both fluorinated terpenes (Table I) are similar to those for the Michaelis constants of their complementary substrates, thus suggesting that substitution of a hydrogen at C(2) by fluorine does not significantly alter binding to the enzyme.

APPENDIX

Initial velocity equations for the three steady state ordered bireactant cases illustrated in Scheme 2 are presented below. Since the 1'-4 condensation is irreversible, the products are treated as dead-end inhibitors. Substrate inhibition in an ordered bireactant system has been treated by Segal (26) for the case where isopentenyl-PP (B) forms a dead-end enzyme-isopentenyl-PP complex, and definitions for the kinetic constants K_{ia}, K_{M_A}, and K_{M_B} are presented. The initial velocity equation:

$$\frac{v}{V_1} = \frac{AB}{K_{ia}K_{M_B} + \frac{K_{ia}K_{M_B}B}{K_{i_B}} + K_{M_A}B + \frac{K_{M_A}B^2}{K_{i_B}} + K_{M_B}A + AB} \quad (4)$$

yields for variable A:

$$\frac{1}{v} = \frac{1}{V_1}\left((1 + \frac{K_{M_B}}{B}) + [K_{M_A} + \frac{K_{ia}K_{M_B}}{K_{i_B}} + \frac{K_{ia}K_{M_B}}{B} + \frac{K_{M_A}B}{K_{i_B}}]\frac{1}{A}\right) \quad (5)$$

and for variable B

$$\frac{1}{v} = \frac{1}{V_1}\left(1 + \frac{K_{M_A}}{A} + \frac{K_{ia}K_{M_B}}{K_{i_B}A} + \frac{K_{M_A}B}{K_{i_B}A} + K_{M_B}(1 + \frac{K_{ia}}{A})\frac{1}{B}\right) \quad (6)$$

in reciprocal form. At higher concentrations of isopentenyl-PP the $\frac{K_{M_A}B}{K_{i_B}}$ and $\frac{K_{M_A}B}{K_{i_B}A}$ terms dominate the slope and intercept terms of equations 5 and 6, respectively.

For those cases where the added inhibitor X and substrate inhibitor B bind to the allylic (A) region of the active site (X = farnesyl-PP or 2-F-geranyl-PP), the initial velocity equation is:

$$\frac{v}{V_1} = \frac{AB}{K_{ia}K_{M_B} + \frac{K_{ia}K_{M_B}B}{K_{i_B}} + \frac{K_{ia}K_{M_B}X}{K_{i_X}} + \frac{K_{ia}K_{M_B}XB}{\alpha K_{i_B}K_{i_X}} + K_{M_A}B + \frac{K_{M_A}B^2}{K_{i_B}} + \frac{K_{M_A}XB}{K_{i_X}} + \frac{K_{M_A}B^2X}{\alpha K_{i_B}K_{i_X}} + K_{M_B}A + AB} \quad (7)$$

where α is an interaction factor between B and X. In reciprocal form for variable A:

$$\frac{1}{v} = \frac{1}{V_1}\left((1 + \frac{K_{M_B}}{B}) + [K_{M_A} + \frac{K_{ia}K_{M_B}}{K_{i_B}} + \frac{K_{ia}K_{M_B}}{B} + \frac{K_{M_A}B}{K_{i_B}} + \frac{X}{K_{i_X}}(K_{M_A} + \frac{K_{ia}K_{M_B}}{\alpha K_{i_B}}) + \frac{K_{ia}K_{M_B}}{B} + \frac{K_{M_A}B}{\alpha K_{i_B}}]\frac{1}{A}\right) \quad (8)$$

and for variable B:

$$\frac{1}{v} = \frac{1}{V_1}\left([1 + \frac{K_{M_A}}{A} + \frac{K_{ia}K_{M_B}}{K_{i_B}A} + \frac{K_{M_A}B}{K_{i_B}A} + \frac{X}{K_{i_X}}(\frac{K_{M_A}}{A} + \frac{K_{ia}K_{M_B}}{\alpha K_{i_B}A} + \frac{K_{M_A}B}{\alpha K_{i_B}A})] + K_{M_B}(1 + \frac{K_{ia}}{A} + \frac{K_{ia}X}{K_{i_X}A} + \frac{1}{B})\right) \quad (9)$$

When the inhibitor Y binds to both the allylic and isopentenyl regions (Y = PP_i and 2-F-isopentenyl-PP) of the active site, the initial velocity equation is:

$$\frac{v}{V_1} = \frac{AB}{K_{ia}K_{M_B}\frac{E}{K_{i_B}} + \frac{K_{ia}K_{M_B}Y}{K_{i_Y}} + \frac{K_{ia}K_{M_B}BY}{\alpha K_{i_B}K_{i_Y}} + K_{M_A}B + \frac{K_{M_A}B^2}{K_{i_B}} + \frac{K_{M_A}BY}{K_{i_Y}} + \frac{K_{M_A}B^2Y}{\alpha K_{i_B}K_{i_Y}} + K_{M_B}A + \frac{K_{M_B}AY}{K_{i_{AY}}} + AB} \quad (10)$$

In reciprocal form, for variable A:

$$\frac{1}{v} - \frac{1}{V_1}\left((1 + \frac{K_{M_B}}{B} + \frac{K_{M_B}Y}{K_{i_{AY}}B}) + [K_{M_A} + \frac{K_{ia}K_{M_B}}{K_{i_B}} + \frac{K_{ia}K_{M_B}}{B} + \frac{K_{M_A}B}{K_{i_B}} + \frac{Y}{K_{i_Y}} + (K_{M_A} + \frac{K_{ia}K_{M_B}}{\alpha K_{i_B}} + \frac{K_{ia}K_{M_B}}{B} + \frac{K_{M_A}B}{\alpha K_{i_B}})]\frac{1}{A}\right) \quad (11)$$

and for variable B:

$$\frac{1}{v} - \frac{1}{V_1}\left([1 + \frac{K_{M_A}}{A} + \frac{K_{ia}K_{M_B}}{K_{i_B}A} + \frac{K_{M_A}B}{K_{i_B}A} + \frac{Y}{K_{i_Y}}(\frac{K_{M_A}}{A} + \frac{K_{ia}K_{M_B}}{\alpha K_{i_B}A} + \frac{K_{M_A}B}{\alpha K_{i_B}A})] + K_{M_B}[1 + \frac{K_{ia}}{A} + Y(\frac{1}{K_{i_{AY}}} + \frac{K_{ia}}{K_{i_Y}A})]\frac{1}{B}\right) \quad (12)$$

Thus, assuming the binding properties shown in Scheme 2 for an ordered bireactant system, X only appears in the slope term (equation 8) and a competitive pattern is expected when A is varied; whereas, X appears in both slope and intercept terms (equation 9) and a noncompetitive plot is expected when B is varied. However, Y appears in the slope and intercept terms of equations 11 and 12, and noncompetitive patterns are expected when the concentration of either substrate is varied.

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Note: References 1-18 appear in the parent paper.

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