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The Significance of the Diphosphate Linkage Involved in the Substrates for Prenyltransferase. Geranyl- and Dimethylallyl Methylenediphosphonate as Artificial Substrates[1)]

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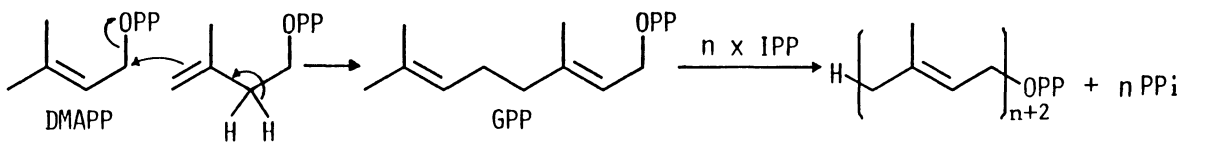
Dimethylallyl- and geranyl methylenediphosphonate are active as artificial substrates for farnesyl diphosphate synthase to give E,E-farnesyl diphosphate. By comparing the kinetic data obtained with the analogues and the natural substrates, the significance of the diphosphate moiety of the substrates of prenyltransferase is discussed.

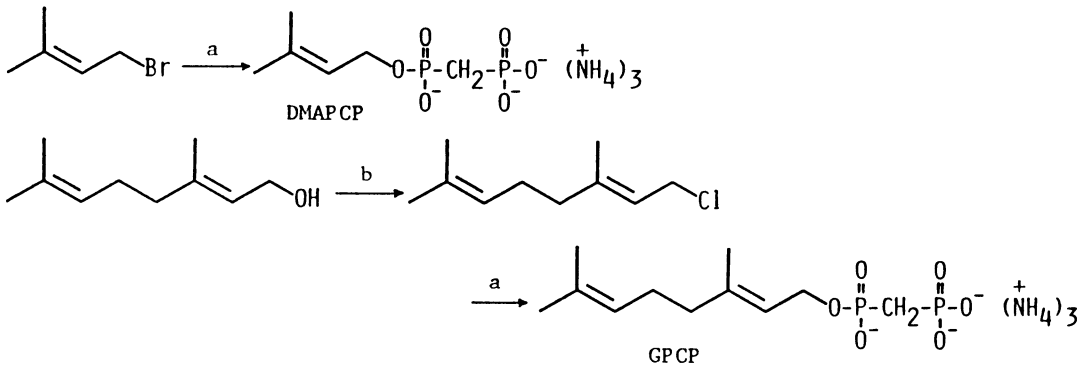
In biosynthetic pathways are often included enzymes which catalyze reversible reactions of bond formation coupled with the liberation of inorganic pyrophosphate (PPi). In general, PPi thus liberated is hydrolized by the action of intracellular pyrophosphatase to displace the equilibrium of reaction in favor of synthesis. Thus, the high energy P-O-P bond is ultimately utilized to drive the biosynthetic reaction.

The prenyltransfer reactions, however, proceed with the release of PPi in spite of the irreversibility of the reaction.[2)] This has led us to be interested in the significance of the diphosphate moiety of the substrates of prenyltransferase. In order to find a clue to understanding it, we first examined, as artificial substrates, methylenediphosphonate derivatives which are expected to be structurally analogous but without a high energy bond.



$OPP=OP_2O_6^{3-}$

Dimethylallyl methylenediphosphonate (DMAPCP) and geranyl methylenediphosphonate (GPCP), were synthesized essentially according to the method of Davisson et al.[3] by displacement of appropriate allylic halides with methylenediphosphonate (PCPi).



a) tris(tetra-n-butylammonium) hydrogen methylenediphosphonate
b) N-chlorosuccinimide , dimethyl sulfide

The first question was whether the analogues could act as substrates for prenyltransferase to condense with isopentenyl diphosphate (IPP). Both DMAPCP and GPCP were accepted as substrates for farnesyl diphosphate (FPP) synthase [EC 2.5.1.10] to give FPP, which was identified as E,E-farnesol after alkaline

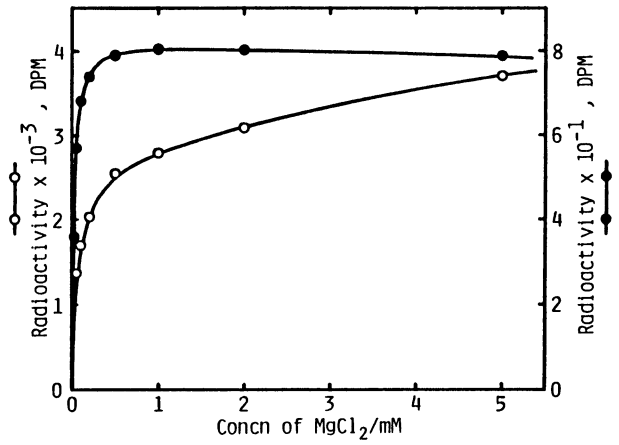


Fig. 1. Effect of Mg^{2+} concentration on the enzymatic activity. Enzymatic activity was measured at the indicated amount of $MgCl_2$ with GPP(o) or GPCP(●) and expressed as radioactivity in the hexane extracts as described in the legend to Table 1.

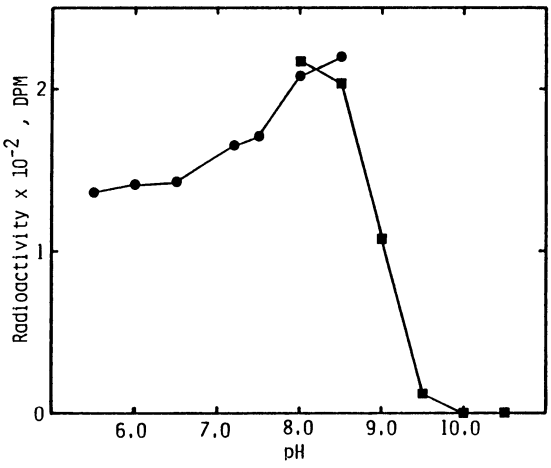


Fig. 2. Effect of pH on the enzymatic activity with GPCP. Enzymatic activity was measured at various pH conditions with 20 mM Tris-Maleate buffer (●) or Glycine-NaOH buffer (■) under the same procedure as described in the legend to Table 1.

phosphatase treatment of the product as usual. These are the first artificial substrates having a leaving group other than the diphosphoryl group.

The enzymatic activity with GPCP or DMAPCP reached a maximum at a lower metal ion concentration than for the natural substrates (Fig. 1). Moreover, the optimal pH of the reaction with GPCP was around 8.2 (Fig. 2), which was larger than that with GPP by 0.5. These phenomena are reflection of the stronger affinity for metal ions and the higher pKa of methylenediphosphonate (PCPi) than those of PPi.[4] On the other hand, the optimal pH with DMAPCP was around 7.4. This low value may be accounted for by the fact that the reaction consists of the consecutive reactions, namely DMAPCP to geranyl diphosphate (GPP) and GPP to FPP.

On the basis of the mechanism proposed by Poulter and Rilling showing that the cleavage of the C-O bond to generate an allylic cation is the rate determining step,[5-7] it was expected that this modification of the allylic substrates would retard the enzymatic reaction because PCPi, having higher pKa values, is a poorer leaving group than PPi.[8] Actually, GPCP was found to be hydrolyzed much slower than GPP, the rate constants (pH 7.7, 100 ^{o}C) being 5.93×10^{-7} S^{-1} and 8.97×10^{-4} S^{-1}, respectively. Kinetic parameters in the FPP synthase reaction of the analogues and of the natural substrates are summarized in Table 1. The V_{max} values of the analogues were only about 2% of those of the natural substrates but not smaller than that expected from the results of the solvolysis experiments. On the other hand, the Km values of the analogues were almost the

Table 1. Kinetic Parameters of the Analogues in the FPP Synthase Reaction

	DMAPP	DMAPCP	GPP	GPCP
Km/μM	3.5	11.4	8.1	2.6
V_{max}/nmol min^{-1} mg^{-1}	54.1	1.6	44.0	0.6

The assay mixture for the analogues contained in a final volume of 1.0 ml, 20 μmol Tris-HCl buffer (pH 8.2 for GPCP or 7.4 for DMAPCP), 5 μmol $MgCl_2$, 10 μmol 2-mercaptoethanol, 25 nmol [1-^{14}C]IPP (spec. act, 1 Ci/mol), 25 nmol DMAPCP or GPCP and 3.5 units of pig liver FPP synthase. The mixture was incubated at 37 ^{o}C for 1.5 h, and then made acidic by addition of HCl. The radioactive alcohols liberated by acid treatment were extracted with hexane and the radioactivity in the extracts was counted.[9] The assay for the natural substrates was the same as the above except that the pH of the buffer was 7.7.

same as those of the natural substrates, indicating that the analogues had a good affinity for the enzyme. These results indicate that the low reactivity of the analogues reflects only the poorer leaving nature and is not attributable to the absence of the high energy bond. Besides, dimethylallyl monophosphate (DMAP) and geranyl monophosphate (GP) are never accepted as substrates by this enzyme, in spite of the fact that the rate constant for hydrolysis of DMAP is much higher than that of DMAPCP.[8] Thus, it is concluded that the high energy bond involved in P-O-P of the allylic substrates is not essential for the enzymatic reaction, but the diphosphate structure is topologically important.

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