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Mechanism of the Prenyl-Transfer Reaction. Studies with (*E*)- and (*Z*)-3-Trifluoromethyl-2-buten-1-yl Pyrophosphate†

C. Dale Poulter*,‡ and Dennis M. Satterwhite

ABSTRACT: The prenyl-transfer reaction catalyzed by porcine farnesyl pyrophosphate synthetase has been studied using (*E*)- and (*Z*)-3-trifluoromethyl-2-buten-1-yl pyrophosphates as substrates and inhibitors. The rate of condensation between isopentenyl pyrophosphate (IPP) and the allylic fluoro analogues is drastically depressed relative to the normal catalytic rate observed with dimethylallyl pyrophosphate (DMAPP) or geranyl pyrophosphate (GPP). A similar depression is found in the rates of solvolysis for methanesulfonate derivatives of the fluoro analogues in aqueous actone under typical SN1 reaction conditions. Prolonged incubation of [^{14}C]IPP and (*E*)- or (*Z*)-CF_3-DMAPP with the enzyme, followed by treatment with alkaline phosphatase, gave a product that comigrated with geranylgeraniol on a polystyrene column. Both fluoro analogues showed mixed linear inhibition patterns with DMAPP or GPP as the variable substrate. We interpret these results in terms of an ionization–condensation–elimination mechanism for the prenyl-transfer reaction.

Prenyltransferase (EC 2.5.1.1) catalyzes the condensation between C_1 of an allylic pyrophosphate and C_4 of isopentenyl pyrophosphate (IPP),[1] yielding the five-carbon homologue of the allylic pyrophosphate. This is the basic polymerization reaction in the terpene biosynthetic pathway and leads to such diverse classes of natural products as sterols, dolichols, carotenoids, respiratory coenzymes, and a multitude of plant terpenoids. The mechanisms which have been proposed for the prenyl-transfer reaction can be grouped into two broad catagories (see Scheme I): those in which condensation is initiated by a heterolytic cleavage of the carbon–oxygen bond of the allylic pyrophosphate, with or without anchimeric assis-

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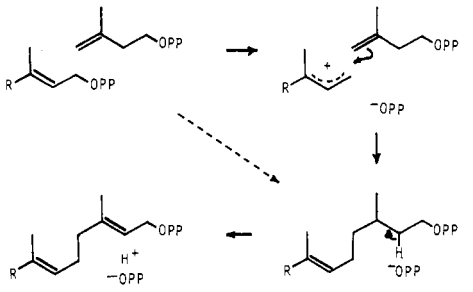
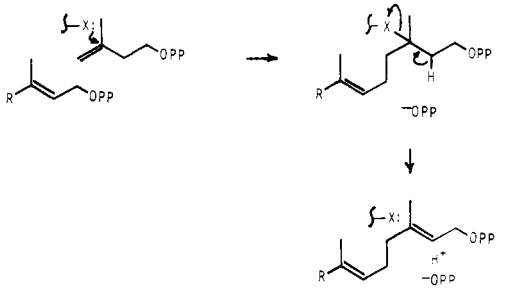
[1] Abbreviations used are: IPP, isopentenyl pyrophosphate; DMAPP, dimethylallyl pyrophosphate; CF_3-DMAPP, 3-trifluoromethyl-2-buten-1-yl pyrophosphate or trifluoromethyldimethylallyl pyrophosphate; GPP, geranyl pyrophosphate; FPP, farnesyl pyrophosphate; GGPP, geranylgeranyl pyrophosphate; NMR, nuclear magnetic resonance; UV, ultraviolet; IR, infrared; Me_4Si, tetramethylsilane; DSS, sodium 4,4-dimethyl-4-silapentanesulfonate; GLPC, gas–liquid phase chromatography.

SCHEME I

Ionization—Condensation—Elimination



Displacement—Elimination



tance from the double bond in IPP, yielding cationic intermediates (ionization–condensation–elimination) (Lynen et al., 1958; Rilling and Bloch, 1959; Cornforth and Popjak, 1959; Cornforth, 1968), and those in which condensation is initiated by attack of a nucleophilic moiety at the double bond of IPP with simultaneous formation of a bond between C_4 and C_1 and rupture of the $C_{1'}$–oxygen bond (an intermolecular S_N2' displacement–elimination) (Cornforth et al., 1966; Cornforth, 1968). Both mechanisms are logical on a chemical basis and are compatible with the stereochemistry of the prenyl-transfer reaction (Poulter and Rilling, 1976; Cornforth, 1968).

We reasoned that it might be possible to distinguish between the two mechanisms by selective substitution of hydrogen atoms in the allylic moiety with fluorine (Poulter et al., 1976). The strong electron-withdrawing effect of fluorine should retard the rate of ionization of the allylic substrate in an ionization–condensation–elimination sequence while having little effect on the rate of a displacement at $C_{1'}$. Thus, replacement of one of the terminal methyl groups in dimethylallyl pyrophosphate with the powerful electron-withdrawing trifluoromethyl moiety should drastically reduce the reactivity of the allylic substrate without introducing any large perturbations in the geometry of the substrate.

Materials and Methods

General. Boiling and melting points are uncorrected. NMR spectra were recorded on Varian A-60, EM-390, or XL-100-15 spectrometers using tetramethylsilane (Me_4Si) or sodium 4,4-dimethyl-4-silapentanesulfonate (DSS) as internal standards. Analytical gas chromatography was carried out on a Varian Model 1200 gas chromatograph with a flame ionization detector, using a 500 ft × 0.03 in. open tubular column coated with Carbowax 20 M. Microanalyses were performed by Chemalytics, Inc.

[1-^{14}C]Isopentenyl Pyrophosphate. [1-^{14}C]IPP was purchased from Amersham/Searle, and a portion was diluted to a specific activity of 10 μCi/μL with unlabeled material.

Dimethylallyl Pyrophosphate and Geranyl Pyrophosphate. The allylic pyrophosphates were prepared from the corresponding alcohols using the method of Cramer (Cramer and Weiman, 1960; Cornforth and Popjak, 1969), and purified by ion-exchange chromatography.

Ethyl (E)-3-Trifluoromethyl-2-butenoate. The ester was prepared from 1,1,1-trifluoroacetone (Columbia) and carbethoxymethylene triphenylphosphorane (Aldrich) by the procedure of Dull (Dull et al., 1967), bp 116–118 °C (650 mm); UV_{max} (pentane) 202 nm (ϵ 15 000); IR (neat) 3410, 2970, 1720, 1675, 1450, 1370, 1360, 1260, 1196, 1192, 1100, 1040, 1002, 932, 896, 813, and 789 cm^{-1}; NMR ($CDCl_3$) 1.30 (3, t, J = 7 Hz, CH_3 of ethyl group), 2.24 (3, d, J = 1.6 Hz, H at C_4), 4.21 (2, q, CH_2 of ethyl group), and 6.21 ppm (1, septet, H at C_2); mass spectrum (70 eV); *m/e* (rel intensity) 27 (14), 39 (23), 45 (13), 59 (14), 86 (24), 89 (21), 114 (17), 134 (15), 137 (100), 154 (30), 182 (9).

Anal. Calcd for $C_7H_9F_3O_2$: C, 46.16; H, 4.98. Found: C, 46.17; H, 5.22.

Ethyl (Z)-3-Trifluoromethyl-2-butenoate. A solution of 14.01 g (77 mmol) of ethyl (*E*)-3-trifluoromethyl-2-butenoate and 0.49 g (2.7 mmol) of benzophenone in 175 mL of dry benzene was irradiated through a Vycor filter with a 450-W Hanovia lamp. The reaction was followed by GLPC. When the *E/Z* ratio of esters reached 57/43, the reaction was stopped and the isomers were separated by spinning band distillation, yielding 2.73 g (20%) of the pure ester, bp 126 °C (650 mm): UV_{max} (pentane) 202 nm (ϵ 15 000); IR (neat) 3050, 1750, 1690, 1430, 1340, 1280, 1090, 1045, 925, 880, 840, 795, and 758 cm^{-1}; NMR ($CDCl_3$) 1.32 (3, t, J = 7 Hz, CH_3 of ethyl group), 2.00 (3, d, J = 1.6 Hz, H at C_4), 4.21 (2, q, J = 7 Hz, CH_2 of ethyl group), and 6.06 ppm (1, q, J = 1.6 Hz, H at C_2); mass spectrum (70 eV) *m/e* (rel intensity) 27 (12), 29 (21), 39 (14), 59 (11), 89 (18), 118 (22), 137 (100), 154 (15), 182 (0.1).

Anal. Calcd for $C_7H_9F_3O_2$: C, 46.16; H, 4.98. Found: C, 46.15; H, 5.01.

(E)-3-Trifluoromethyl-2-buten-1-ol. To a well-stirred slurry of 0.202 g (5.32 mmol) of lithium aluminum hydride (Alfa-Ventron) in 10 mL of anhydrous ether at −10 °C was added dropwise 1.24 g (6.82 mmol) of *E* ester in 5 mL of ether. The reduction was allowed to proceed for 12 min, after which 1.3 mL of saturated sodium chloride solution was added. Stirring was continued for 30 min, followed by filtration of the solids. The solids were washed with one 5-mL portion of ether and dried over 1 g of anhydrous magnesium sulfate. Solvent was removed at reduced pressure, followed by short-path distillation to afford 0.63 g (66%) of a colorless liquid: IR (neat) 3350, 2900, 1675, 1450, 1320, 1180, 1120, and 1020 cm^{-1}; NMR ($CDCl_3$) 1.80 (3, slightly broadened s, H at C_4), 2.50 (1, s, hydroxyl H), 4.25 (2, d of m, $J_{1,2}$ = 5.5 Hz, H at C_1), and 6.27 ppm (1, t of m, $J_{1,2}$ = 5.5 Hz, H at C_2); mass spectrum (70 eV) *m/e* (rel intensity) 31 (34), 39 (37), 41 (36), 43 (25), 51 (28), 53 (34), 65 (37), 66 (15), 69 (15), 71 (75), 73 (22), 72 (12), 77 (46), 91 (100), 92 (15), 105 (12), 120 (56), 140 (<0.1).

Anal. Calcd for $C_5H_7F_3O$: C, 42.86; H, 5.05. Found: C, 42.68; H, 5.22.

(Z)-3-Trifluoromethyl-2-buten-1-ol. By a procedure similar to that described for the *E* isomer, 0.300 g (1.65 mmol) of *Z* ester was reduced to give 0.162 g (70%) of the corresponding alcohol: IR (neat) 3400, 2900, 1680, 1460, 1320, 1170, 1120, and 1020 cm^{-1}; NMR ($CDCl_3$) 1.87 (3, d, J = 1.5 Hz, H at C_4), 4.32 (2, broadened d, $J_{1,2}$ = 6 Hz, H at C_1), 4.46 (1, s, hydroxyl H), and 5.83 ppm (1, broadened t, $J_{1,2}$ = 6 Hz, H at C_2); mass spectrum (70 eV) *m/e* (rel intensity) 31 (32), 39 (35), 41 (34), 43 (21), 51 (25), 53 (27), 64 (12), 65 (36), 69 (13), 71 (62), 72 (12), 73 (18), 75 (12), 77 (42), 91 (100), 92 (13), 105 (10), 120 (53), 140 (0.6).

Anal. Calcd for $C_5H_7F_3O$: C, 42.86; H, 5.05. Found: C, 43.03; H, 4.85.

(E)- and (Z)-Trifluoromethyl-2-buten-1-yl Pyrophosphate. In a representative synthesis, 0.140 g (1.0 mmol) of alcohol was dissolved in 0.9 g (6.2 mmol) of anhydrous trichloracetonitrile. Over 4 h, 0.67 g (2.26 mmol) of bis(triethylammonium) phosphate in 22 mL of anhydrous acetonitrile was added at room temperature, followed by stirring overnight. The resulting light yellow solution was poured into 50 mL of 0.1 N ammonium hydroxide in methanol, and the liquor filtered onto a previously prepared 1 × 15 cm ion-exchange column of formate Dowex AG1X8 (Bio-Rad) which had been washed three times with 30 mL of the same solvent. The column was washed with 50 mL of 10% aqueous methanol and eluted with a linear gradient of ammonium formate (recrystallized twice from methanol), 0.054 to 0.54 M, in 300 mL of 10% aqueous methanol.

Fractions (8 mL) were collected and spotted on Whatman chromatography paper. The phosphate containing fractions were visualized by spraying with a 0.5% solution of ferric chloride in 80% ethanol followed by 5% sulfosalicylic acid in 60% ethanol after the first spray had dried. The monophosphates appeared in fractions 11 to 15 and the pyrophosphates, between 19 and 25. Fractions 19 to 25 were combined and the solvent was removed under reduced pressure. The ammonium formate was removed by vacuum sublimation at 35–40 °C to afford 45 mg (13%) of an off-white powder. Thin-layer chromatograms on 5 × 20 cm phosphate buffered silica gel H plates in chloroform–methanol–water (10:10:3) failed to visualize in iodine vapor but gave white spots on a blue-violet field with a freshly prepared 1% solution of potassium permanganate in 95% acetone (R_f: monophosphate 0.581 (DMAP, 0.546); pyrophosphate 0.351 (DMAPP, 0.305)). Phosphate content of the material with an R_f of 0.35 was 86–88% of theoretical (Bartlett, 1959); NMR (D_2O, DSS internal standard): (*E*)-CF_3-DMAPP 1.92 (3, s, H at C_4), 4.65 (partially obscured by HDO at 4.7, H at C_1), and 6.25 ppm (1, broadened t, J = 6 Hz, H at C_2); (*Z*)-CF_3-DMAPP 1.78 (3, s, H at C_4), 4.58 (partially obscured by HDO at 4.7, H at C_1), and 6.24 ppm (1, broadened t, J = 7 Hz, H at C_2).

In separate experiments, 1 mg of (*E*)- and (*Z*)-CF_3-DMAPP was treated with 4 mg of calf mucosa alkaline phosphatase (Sigma, EC 3.1.3.1) in 3 mL of 50 mM lysine buffer which contained $MgCl_2$ (1 mM) and NaN_3 (1 mM), pH 10.4, for 16 h at 37 °C. The mixture was saturated with sodium chloride and extracted with ether. The extracts were shown to contain the appropriate alcohol by coinjection with authentic samples on the 500 ft × 0.03 in. open tubular column. No other peaks with retention times characteristic of C_5 alcohols were seen.

(E)- and (Z)-Trifluoromethyl-2-buten-1-yl Methanesulfonate. In a typical reaction, a mixture of 0.80 mmol of the appropriate alcohol, 1 mL of pentane, 1 mL of benzene, and 0.75 mmol of freshly distilled methanesulfonyl chloride was cooled to −10 °C. A white precipitate formed over a 5-min period following addition of 1.4 mmol of triethylamine. The mixture was stirred for a total of 15 min before the precipitate was removed by rapid filtration. Solvent was removed from the trifluoromethyl methanesulfonates at reduced pressure: NMR *E* isomer (CCl_4, Me_4Si internal standard) 1.88 (3, s, H at C_4), 2.96 (3, s, CH_3 at sulfur), 4.82 (2, broadened d, H at C_1, J = 7 Hz), and 6.22 ppm (1, broad t, H at C_2, J = 7 Hz); *Z* isomer (CCl_4, Me_4Si) 1.95 (3, s, H at C_4), 2.95 (3, s, CH_3 at sulfur), 4.86 (2, broadened d, H at C_1, J = 7 Hz), and 5.90 ppm (1, broadened t, H at C_2, J = 7 Hz).

Dimethylallyl Methanesulfonate. To a solution of 0.079 g (0.92 mmol) of dimethylallyl alcohol, 0.12 g (0.90 mmol) of methanesulfonyl chloride, 1 mL of pentane, and 1 mL of benzene (pentane and benzene were replaced by carbon tetrachloride in some preparations), which was cooled to −10 °C, was added 0.182 g (1.80 mmol) of dry triethylamine. After 15 min the white precipitate that formed was removed by rapid filtration, and the filtrate was used without further purification. The solution of methanesulfonate could be stored for several hours at −20 °C; however, at higher temperatures or during attempts to remove solvent, decomposition was rapid. NMR spectra at −35 °C showed that the desired derivative had been prepared: NMR (CCl_4, Me_4Si) 1.79 (6, broad s, CH_3's at C_3), 2.90 (3, s, CH_3 at sulfur), 4.65 (2, d, H at C_1, J = 7.5 Hz), and 5.39 ppm (1, t, H at C_2, J = 7.5 Hz).

Porcine Prenyltransferase. The enzyme was provided by Professor H. C. Rilling from a batch that was purified to homogeneity as judged by electrophoresis in natural and sodium dodecyl sulfate containing polyacrylamide gels. The enzyme was precipitated with ammonium sulfate and stored at 4 °C until used.

Assays. All incubations with prenyltransferase were run at 37 °C in 10 mM potassium phosphate, 1 mM magnesium chloride, 0.1 mM dithiothreitol, 1 μM sodium azide, pH 7.4. The acid-lability assay was used to determine the extent of the reaction (Reed and Rilling, 1975). Radioactivity was measured by liquid scintillation spectrometry in 10 mL of 0.4% Omnifluor (New England Nuclear) in toluene.

Solvolysis Studies. (a) Kinetics. Kinetic measurements were carried out conductometrically in a 10-mL (1-cm path between platinum electrodes) cell using a Radiometer CDM-3 conductivity meter. In a typical fast run, a solution of solvent and γ-collidine (86 μmol) was equilibrated at the appropriate temperature. A 20-μL portion of methanesulfonate (ca. 0.7 μmol) in pentane–benzene was injected into the cell, and the change in conductivity of the stirred solution was measured continuously. Good first-order kinetics were observed up to 9 half-lives. Rate constants were determined by a least-squares analysis. For kinetic runs at higher temperatures, solvent and γ-collidine were equilibrated in a volumetric flask. Approximately 2 μL of neat methanesulfonate was placed on the inner surface of the cell followed by addition of the warm solvent. The cell was immersed in the constant-temperature bath and a minimum of 5 min was allowed for equilibration before collection of data.

(b) Products. A solution of 35 μmol of dimethylallyl methanesulfonate and 0.3 mL of γ-collidine in 30 mL of 90% acetone–water was allowed to stand at 35 °C for 30 min. After dilution with an equal volume of water, the solution was extracted with 20 mL of diethyl ether, and the ether extract was washed in succession with three 15-mL portions of water, 75 mL of 1 N hydrochloric acid, 15 mL of saturated sodium bicarbonate, and 15 mL of saturated sodium chloride. The organic layer was dried over magnesium sulfate, and solvent was removed at reduced pressure to give 50–70 μL of a light yellow oil. Analysis by GLPC at 100 °C showed two components, dimethylallyl alcohol (41%) and 2-methyl-3-buten-2-ol (59%), as determined by coinjection with authentic samples (Aldrich).

For product studies with (*E*)- and (*Z*)-trifluoromethyl-2-buten-1-yl methanesulfonate, 0.042 g (0.192 mmol) of neat methanesulfonate was heated at 70 °C for 8 h in 10 mL of 50% acetone–water containing 50 μL of γ-collidine and 0.017 g (0.196 mmol) of 3-methylbutan-3-ol (internal standard). Both trifluoromethyl compounds gave their respective alcohols in 100% yield as the only detectible products (comparison by coinjection). The reactions were worked up as described for

the dimethylallyl derivative, products were separated from the internal standard by GLPC, and structures were reconfirmed by NMR spectroscopy.

Studies with Prenyltransferase. (a) Comparisons of Forward Velocities. The forward velocity of the prenyl-transfer reaction was determined by incubation of 1.7 μg of enzyme with 4 nmol of IPP (10 μCi/μmol), and 2 nmol of DMAPP in a total volume of 200 μL in each of five separate tubes. At 2-min intervals between 0 and 10 min, the reaction was stopped with methanolic HCl, and the tubes were assayed. Linear portions of the velocity curve were used to calculate the velocity of the reaction. A similar set of incubations was run with GPP.

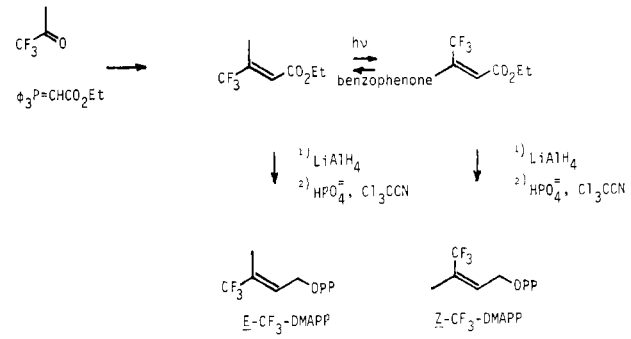
The very slow reaction found with (*E*)- and (*Z*)-CF_3-DMAPP required checks on the stability of enzyme and [^{14}C]IPP to prolonged incubation. Three sets of tubes were used in the determination of each point, two (a and b) contained a total volume of 200 μL and the third (c) contained 198 μL. Each tube in set a contained prenyltransferase (69 μg), [^{14}C]IPP (4 nmol), and (*E*)- or (*Z*)-CF_3DMAPP (2 nmol). The tubes in set b contained IPP and enzyme but no CF_3-DMAPP, while set c contained no enzyme and DMAPP in place of the fluoro analogues. Sets a and b were maintained at 37 °C and set c was kept at 4 °C. Tubes from each set were assayed at four time intervals. Just prior to addition of methanolic HCl to tubes in sets a and b, 2 μL of the contents of a was transferred to c. Tube c was allowed to stand for 10 min at 37 °C before acid was added. The progress of the condensation was determined using the acid-lability assay by subtracting background counts (tube b) from the total (tube a) at a given time interval. Tube c served as a control to ensure that the enzyme retained its catalytic activity during the four day incubation.

(b) Products. Farnesyl pyrophosphate synthetase (300 μg, SA 600), IPP (40 nmol, 61 mCi/mmol), and (*E*)-CF_3-DMAPP (8 nmol) in 200 μL total volume of buffer were incubated at 37 °C, pH 7.4, for 71 h. The mixture was heated to 60 °C for 5 min and cooled to 37 °C. Lysine buffer (10 μL, 1 M, pH 10.4) and 10 mg of calf mucosa alkaline phosphatase were added. The resulting mixture was incubated for 18 h. Geranylgeraniol (1 μL) was added to the tube before extraction with two 1-mL portions of Skelly B. The hydrocarbon extracts were dried over anhydrous sodium sulfate, and solvent was removed with a gentle stream of dry nitrogen. The residue was dissolved in 0.5 mL of 10:1 toluene:chloroform and chromatographed on a polystrene column, Bio-beads 5 × 2 (Bio-Rad), which cleanly separated C_5, C_{10}, C_{15}, and C_{20} terpene alcohols upon elution with toluene. The elution volume for geranylgeraniol was determined by spotting fractions on silica gel and developing with iodine. A similar run was carried out with the *Z* isomer. Each run was accompanied by a control which contained enzyme and IPP and served as a measure of background contamination.

In a similar experiment farnesyl pyrophosphate synthetase (14 μg, SA 500), IPP (8 nmol), and DMAPP (1 nmol) were incubated at 37 °C, pH 7.4, for 46 h. Geranylgeranyl pyrophosphate was added as a marker, and the mixture was extracted three times with 1-butanol (0.2 mL followed by two 0.1-mL portions). The butanol extracts were blown to dryness, and the residue was dissolved in 2 drops of 1:1 1-butanol:methanol. The solution was spotted on a silica gel H plate and developed with chloroform–methanol–water (5:5:1). The R_f of geranylgeranyl pyrophosphate was determined by iodine staining. The plate was scanned and slices were scraped and counted. A control tube without enzyme was run in parallel.

(c) Inhibition. Incubations were carried out for 10 min in the standard buffer at 37 °C. Fixed substrate concentration was 2 μM and variable substrate concentrations were 1, 2, 4, and 8 μM at inhibitor concentrations of 0, 50, 100, and 200 μM. All determinations were in duplicate. The data were analyzed on a Hewlett-Packard Model 9810A calculator-plotter. All double-reciprocal plots are computer drawn from the least-squares lines calculated at four concentrations of variable substrate. The error range reported for K_is and K_ms represents the average of the maximum and minimum values based on the standard deviation of the slopes and intercepts used for the calculations.

SCHEME II



Results

Synthesis of Fluoro Analogues. (*E*)- and (*Z*)-CF_3-DMAPP were prepared from 1,1,1-trifluoroacetone by the sequence of reactions shown in Scheme II. The Wittig condensation (Dull et al., 1967) gave a 97:3 mixture of *E* and *Z* isomers from which the pure *E* ester was obtained by spinning-band distillation. The mixture was enriched in the *Z* isomer by photosensitized isomerization of the C_2–C_3 double bond using benzophenone, and at photoequilibrium the *E*:*Z* ratio was 57:43. The *Z* ester was separated from its *E* isomer by a spinning-band distillation. Careful reduction of the esters with lithium aluminum hydride (McBee et al., 1954) followed by pyrophosphorylation of the resulting alcohols (Cramer and Weiman, 1960; Cornforth and Popjak, 1969) gave (*E*)- and (*Z*)-CF_3-DMAPP. The pyrophosphates were chromatographically pure on silica gel H with R_fs similar to those found for DMAPP, had the expected phosphate content, and gave the starting alcohols when treated with alkaline phosphatase.

The stereochemistry of the C_2–C_3 double bond was established by a nuclear Overhauser experiment. Solutions of the esters (ca. 25% in $CDCl_3$ containing 1% v/v tetramethylsilane) were degassed by four freeze-pump-thaw cycles and sealed under vacuum. Repeated integrations of the intensity of the protons at C_2 with and without irradiation at the resonance position of the protons for the methyl group at C_3 gave a 32 ±

3% enhancement for the isomer which boils at 126 °C and no enhancement (3 ± 3%) for the other isomer (bp 116–118 °C). Since nuclear Overhauser effects show a r^{-6} dependence on the distance (r) between the protons being observed and those being irradiated (Anet and Bourn, 1965), we assign the *Z* configuration to the higher boiling isomer, and the *E* configuration to the lower.

Solvolysis of (E)- and (Z)-CF_3-DMAPP. The effect on the rate of solvolysis by replacing a methyl group at C_3 in the dimethylallyl system with a trifluoromethyl moiety was de-

TABLE I: First-Order Rate Constants for Dimethylallyl Methanesulfonate and (*E*)- and (*Z*)-Trifluoromethyldimethylallyl Methanesulfonate.

Reactant	*T* (°C)	Acetone/H_2O (v/v)	*k* (s^{-1})
(*E*)-3-Trifluoromethyl-2-buten-1-yl methanesulfonate	20	92[a]	1×10^{-8}
	20	50[b]	1.55×10^{-6}
	60	50	$(1.28 \pm 0.03) \times 10^{-4}$
	70	50	$(3.06 \pm 0.20) \times 10^{-4}$
	80	50	$(7.98 \pm 0.55) \times 10^{-4}$
	80	60	$(3.48 \pm 0.17) \times 10^{-4}$
(*Z*)-3-Trifluoromethyl-2-buten-1-yl methanesulfonate	60	50	$(1.88 \pm 0.14) \times 10^{-4}$
3-Methyl-2-buten-1-yl methanesulfonate	0	92	$(3.82 \pm 0.11) \times 10^{-3}$
	20	92	$(2.99 \pm 0.12) \times 10^{-2}$

[a] Extrapolated from 50% acetone/water; $m = 0.60$, $Y = -2.25$. [b] Extrapolated from higher temperatures.

termined by comparing the first-order rate constant for hydrolysis of dimethylallyl methanesulfonate with those for (*E*)- and (*Z*)-3-trifluoromethyl-2-buten-1-yl methanesulfonate. All derivatives showed excellent first-order kinetic behavior over 4 half-lives (>90%) and rate constants for individual compounds are listed in Table I.

The trifluoromethyl compounds were so much less reactive than dimethylallyl methanesulfonate that it was not possible to compare rates at a common temperature and in a common solvent. Rate constants for the very reactive parent methanesulfonate were determined at 0 and 20 °C in 92% acetone/water, while those for the (*E*)-trifluoromethyldimethylallyl derivative were measured at three temperatures (60, 70, and 80 °C) in 50% acetone/water and in two solvents (50% and 60% acetone/water) at 80 °C. Temperature and solvent extrapolations were used to estimate the rate constant for hydrolysis of the fluorine containing compound at 20 °C in 92% acetone/water. First, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ were calculated for the hydrolysis reaction in 50% acetone/water, and activation parameters were used to calculate $k^{20°C}$(50% acetone/water). Next, the value of *m* for the methanesulfonate was determined from the rate constants in 50% and 60% acetone/water, and a form of the Winstein–Grunwald (Grunwald and Winstein, 1948; Fainberg and Winstein, 1956) equation (eq 1):

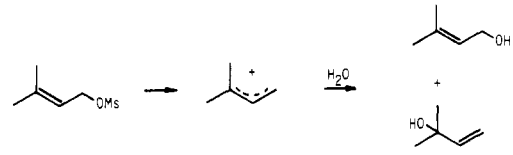
$$\log \frac{k_a}{k_b} = m(Y_a - Y_b) \qquad (1)$$

where k_a and k_b are rate constants in solvents a and b, Y_a and Y_b are measures of the ionizing power of the solvents and *m* is a constant characteristic of the reactant. The same equation was used to calculate $k^{20°C}$(92% acetone/water) from $k^{20°C}$(50% acetone/water). The difference between the first-order rate constant for dimethylallyl methanesulfonate ($k^{20°C}$(92% acetone/water) = 2.99×10^{-2} s^{-1}) and the estimated value for (*E*)-trifluoromethyldimethylallyl methanesulfonate ($k^{20°C}$(92% acetone/water) = 1×10^{-8} s^{-1}) is dramatic proof of the ability of a trifluoromethyl moiety to retard ionization in the allylic system. Although a double extrapolation was necessary to obtain $k^{20°C}$(92% acetone/water) for the trifluoromethyl analogue, the calculated rate constant should be within an order of magnitude of the true value. A complete kinetic study was not carried out for the (*Z*)-trifluoromethyl isomer; however, a comparison of the rate constants for the *E* and *Z* methanesulfonates at 60 °C in 50% aqueous acetone certainly suggests that the stereochemistry of the trifluoromethyl moiety changes the rate of the solvolysis reaction only slightly.

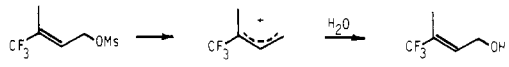
TABLE II: Activation Parameters and Solvent Constants.

Reactant	$\Delta H^{\ddagger}$ (kcal/mol)	$\Delta S^{\ddagger}$ (eu)	*m*
(*E*)-3-Trifluoromethyl-2-buten-1-yl methanesulfonate	20.7	−15	0.60
3-Methyl-2-buten-1-yl methanesulfonate	15.8	−12	-

Hydrolysis of dimethylallyl methanesulfonate yielded the two compounds 3-methyl-2-buten-1-ol (41%) and 2-methyl-



3-buten-2-ol (59%), which are the expected products from reaction at both ends of the intermediate allylic cation (Tidd, 1971). (*E*)- and (*Z*)-3-trifluoromethyl-2-buten-1-yl methanesulfonate gave the *E* and *Z* alcohols, respectively, as the



only product. Apparently the powerful electron-withdrawing effect of the trifluoromethyl group ($\sigma^{+} = 0.612$) (Okamoto et al., 1958) reduces delocalization of positive charge to C_3 rendering that center no longer sufficiently electrophilic to compete with C_1 in the reaction with water.

The dramatic retardation of the rate of hydrolysis when a methyl group is replaced by trifluoromethyl could be accompanied by a change in the mechanism for the reaction. Two lines of evidence indicate that this is not the case. A comparison of $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ (Table II) for hydrolysis of methyl and trifluoromethyl methanesulfonates shows that the slower rates observed for the trifluoromethyl derivative resulted from an increase in $\Delta H^{\ddagger}$ with essentially no change in $\Delta S^{\ddagger}$. Also, the value of *m* (0.60) found for hydrolysis of (*E*)-trifluoromethyldimethylallyl methanesulfonate is in the range expected for an SN1 solvolysis of an allylic reactant (Fry et al., 1970; Grunwald and Winstein, 1948).

Initial Velocity Measurements. Initial velocities for the prenyl-transfer reaction using the natural allylic substrates and trifluoromethyl analogues are listed in Table III. Two sets of data are presented representing measurements for DMAPP and GPP with two different batches of enzyme. One set compares the initial velocity of (*E*)-CF_3-DMAPP with that of GPP, while the other compares (*E*)- and (*Z*)-CF_3-DMAPP with DMAPP. The initial velocities for (*E*)- and (*Z*)-CF_3-DMAPP were *only* 10^{-6} to 10^{-7} those found for the normal allylic substrates. Because the fluoro analogues were so unreactive, it was not practical to carry out the full kinetic analysis necessary to determine V_{max}. Instead, forward velocities for the condensation reactions were measured at the same concentrations of IPP and allylic substrates. However, to obtain a detectible conversion with the fluoro analogues, the concentration of enzyme was increased by 40-fold, and the

TABLE III: Comparison of Forward Velocities with DMAPP and Fluoro Analogues.

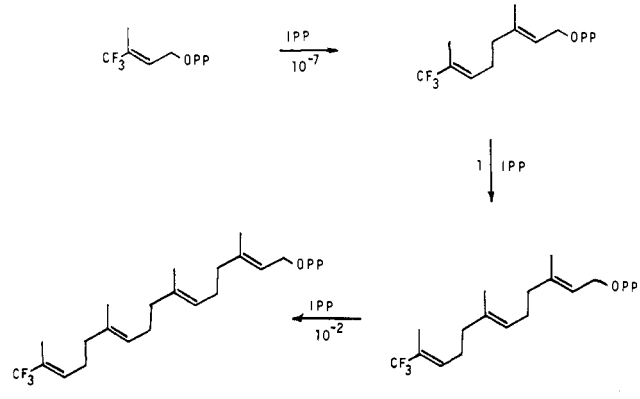
Allylic substrate	v^a (nmol min^{-1} mg^{-1})	Rel v	
		Uncorr[d]	Corr[e]
GPP	751 ± 34[b]	1	1
(*E*)-CF_3-DMAPP	(5.1 ± 1.1) × 10^{-4}[b]	7×10^{-7}	2×10^{-7}
DMAPP	472 ± 10[c]	1	1
(*E*)-CF_3-DMAPP	(3.9 ± 0.7) × 10^{-4}[c]	8×10^{-7}	3×10^{-7}
(*Z*)-CF_3-DMAPP	(4.9 ± 1) × 10^{-4}[c]	1×10^{-6}	3×10^{-7}

[a] Nanomoles of IPP consumed. [b] Prenyltransferase, batch a. [c] Prenyltransferase, batch b. [d] Assume 1:1 IPP:allylic substrate in condensed product. [e] Assume 3:1 IPP:allylic substrate in condensed product.

incubation time was increased to up to 430-fold. The different incubation conditions used for the analogues and the natural substrates introduce some uncertainty into the accuracy of the rates listed in Table III, but it is still clear that the trifluoromethyl substituent drastically retards the rate of the enzyme catalyzed condensation.

Products of the Prenyl-Transfer Reaction. Approximately 0.6 nmol of IPP was converted to acid-labile material during a 3-day incubation with the fluoro analogues, suggesting that condensation had occurred. This observation was supported by an analysis of products which involved treatment of the incubation mixture with alkaline phosphatase and chromatography of the resulting products on a polystyrene column which cleanly separately C_5, C_{10}, C_{15}, and C_{20} isoprene alcohols. The chromatographic data for (*E*)-CF_3-DMAPP are summarized in Figure 1. When a background correction for residual isopentenol and trace quantities of higher molecular weight impurities was applied, one major peak with a retention volume (maximum in fraction 51) very close to that of geranylgeraniol (maximum in fraction 52) and cleanly separated from farnesol (maximum in fraction 55), was seen. The same pattern was found for (*Z*)-CF_3-DMAPP. Thus, chromatographic data suggest that the major products from (*E*)- and (*Z*)-CF_3-DMAPP are the respective C_{20} terpenes, (*E*)- and (*Z*)-15-trifluoromethylgeranylgeranyl pyrophosphate.

Avian and porcine prenyltransferase are known to catalyze the condensation between FPP and IPP at a rate of approximately 10^{-2} that of the normal catalytic rate (Reed and Rilling, 1975). Since the initial products of the condensations between the trifluoromethyl analogues and IPP no longer have a trifluoromethyl moiety at C_3, most of the reactivity in the prenyl-transfer reaction should be restored, and subsequent condensations should proceed at rates close to those for the normal substrates. Thus, the slow step in the three step sequence, by perhaps as much as a factor of 10^5, is the initial condensation of IPP with the trifluoromethyl analogues. Under these conditions one would not expect to see any C_{15} products.



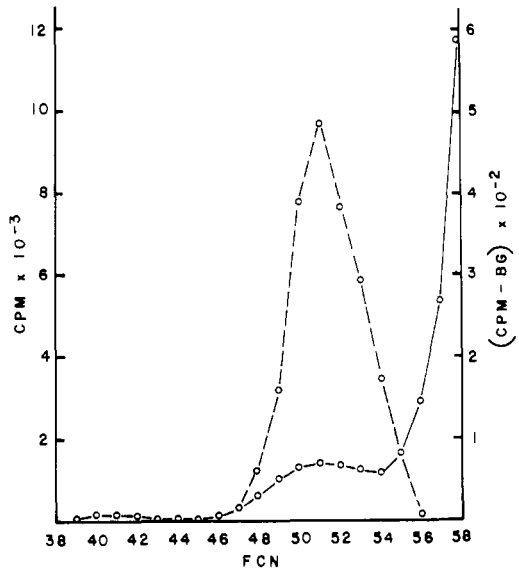


FIGURE 1: Elution profile on polystyrene for condensation with (*E*)-CF_3-DMAPP. (—) cpm; (- - -) cpm − background.

The accumulation of C_{20} material suggests that porcine prenyltransferase does not accept geranylgeranyl pyrophosphate as an allylic substrate for further condensation with IPP. We demonstrated the failure of GGPP to function as an allylic substrate by a prolonged incubation of DMAPP with an 8-fold molar excess of IPP. At the concentrations of substrates used in the experiment, prenyltransferase (35 ng/tube) had an initial velocity of 82 nmol mg^{-1} min^{-1} for consumption of IPP. For the product studies, prenyltransferase (14 μg/tube) was incubated with IPP and DMAPP for 1280 min. A thin-layer chromatogram of the mixture showed two major peaks in a radioscan, one of which comigrated with geranylgeranyl pyrophosphate (R_f 0.34) and another which migrated near the solvent front (R_f 0.91). The plate was sectioned and individual cuts were counted by liquid scintillation spectrometry. Again, two peaks with the appropriate R_fs were seen. Between R_f 0.34 and 0.91, a small peak was seen at R_f 0.62 (6% of peak at R_f 0.34) and the background counts were approximately 2% of those of the major peak. The peak at R_f 0.91 (32% of the major peak) is attributed to alcohols produced by hydrolysis of allylic pyrophosphates by the enzyme. We recently observed that procine farnesyl pyrophosphate synthetase hydrolyzes GPP at a rate up to approximately 10^{-2} that of the normal prenyltransfer reaction (Poulter and Rilling, 1976), and it is reasonable to assume that some hydrolysis would be seen under the forcing conditions used in these experiments.

Inhibition Studies. Inhibition constants for (*E*)- and (*Z*)-CF_3-DMAPP with FPP synthetase are listed in Table IV. The values were determined from double-reciprocal plots and the appropriate replots of slope and intercept. Initial velocities were determined from single point measurements in the linear portion of the velocity vs. time curve. The difficulties encountered with using single point assays for kinetic studies with this enzyme (Popjak et al., 1969b) are recognized. Lineweaver–Burk plots and slope and intercept replots were calculated by a least squares procedure and were drawn by a Hewlett-Packard Model 9810A calculator with a plotter. The

TABLE IV: Kinetic Constants for Porcine Farnesyl Pyrophosphate Synthetase.

Substrate					
Variable[a]	Fixed[b]	Inhibitor[c]	$K_{M_{IPP}}$ (μM)	$K_{M_{Allylic}}$ (μM)	K_i (μM)
IPP	GPP	(*E*)-CF_3-DMAPP	0.67 ± 0.1		46 ± 9
IPP	GPP	(*Z*)-CF_3-DMAPP	0.77 ± 0.1		61 ± 15
GPP	IPP	(*E*)-CF_3-DMAPP		0.85 ± 0.1	51 ± 8
GPP	IPP	(*Z*)-CF_3-DMAPP		0.92 ± 0.1	62 ± 3
DMAPP	IPP	(*E*)-CF_3-DMAPP		1.6 ± 0.9	23 ± 15
DMAPP	IPP	(*Z*)-CF_3-DMAPP		23 ± 0.8	29 ± 5

[a] 1, 2, 4, and 8 μM. [b] 2 μM. [c] 0, 50, 100, and 200 μM.

limits of error reported for the constants listed in the table are the average of the difference of the maximum and minimum values of the constants calculated from the standard deviations of the replot graphs. A typical Lineweaver–Burk plot for 2 μM IPP and variable GPP (and vice versa) at different concentrations of (*E*)-CF_3-DMAPP is shown in Figure 2, along with slope and intercept replots. Similar results were found with DMAPP as the allylic substrate and (or) (*Z*)-CF_3-DMAPP as the inhibitor.

Discussion

The main purpose of our work was to distinguish between an ionization–condensation–elimination mechanism and a condensation–elimination mechanism for the prenyl-transfer reaction. We believe that the bulk of the evidence supports the former mechanism. Certainly, the most compelling data are the dramatic reductions in solvolytic and enzymatic rates when a β-methyl substituent in the allylic substrate is replaced by a trifluoromethyl group. Two reactions were chosen as models to determine the magnitude of the rate effect one might expect for the methyl–trifluoromethyl interchange. For the SN2 model, a direct displacement of chlorine by iodide in anhydrous acetone, McBee and co-workers (McBee et al., 1962) found that the trifluoromethyl moiety was rate accelerating by a factor of 11. For the SN1 model, solvolysis of a methanesulfonate in aqueous acetone, we found that the dimethylallyl derivatives containing a trifluoromethyl group had first-order rate constants which were 3×10^{-7} those of the parent compound. Thus, the trifluoromethyl moiety slightly accelerates a SN2 reaction and severely depresses a SN1 reaction with a total difference in reactivities of ca. 10^{-8}!

Rate retardations found for the prenyl-transfer reaction could result from an inability of the enzyme to efficiently promote ionization of the fluorine-containing analogues, poor binding, or a combination of both. We find it difficult to attribute rate depressions of more than *six powers of ten* solely to poor binding in view of inhibition studies which give inhibition constants for the substrate analogues that are only 20 to 60 times smaller than the Michaelis constants of the normal substrates. The values we obtained for the Michaelis constants of IPP and GPP (see Table IV) are similar to those found by Reed and Rilling (1975) for the avian enzyme and are somewhat lower than the values reported by Popjak and co-workers, $K_{M_{IPP}} = 1.0$ μM and $K_{M_{GPP}} = 4.3$ μM, for an impure preparation of farnesyl pyrophosphate synthetase (Popjak et al., 1969a).

The Michaelis constants for DMAPP are slightly larger than those for GPP and in qualitative agreement with the equilibrium binding data for the avian enzyme where GPP is more tightly bound than DMAPP by approximately an order of magnitude (Reed and Rilling, 1976). Retardations of the enzyme catalyzed rates by ca. 3×10^{-7} are virtually the same as those found for the solvolytic reactions. Because of the enormous differences in the concentrations of prenyltransferase used in the enzymatic reactions, the differences in K_Ms for allylic substrates and analogues, and the temperature and solvent extrapolations necessary for comparing reactivities during solvolysis, such very close agreement must be viewed as fortuitous.

In view of the large number of alkyl-substituted allylic substrate analogues that have been successfully condensed with IPP by various prenyltransferases (Nishino et al., 1971, 1973; Ogura et al., 1970, 1972; Popjak et al., 1969c), we did not expect any steric problems with our fluoro analogues since the trifluoromethyl moiety is not much larger than the methyl group that it replaces (Edgell et al., 1957; DeCoen et al., 1967). However, K_is suggest that the fluoro analogues are bound less tightly than the natural substrates by as much as a factor of 10^2. It has been proposed that the active site of prenyltransferase has a polar region for the pyrophosphate moieties and a nonpolar region for the hydrocarbon portion of the substrates (Holloway and Popjak, 1967; Popjak et al., 1969b). Poorer binding of the fluorine-containing analogues could result from the trifluoromethyl moiety not binding as well in the lipophilic region because it is more polar than a methyl group rather than differences in size. Finally, K_is for DMAPP are approximately one-half those found for GPP in accord with a lower binding constant for the five carbon allylic substrate (Reed and Rilling, 1976).

The plots in Figure 1 indicate mixed linear inhibition of the normal substrates by (*E*)- and (*Z*)-CF_3-DMAPP. This observation was unexpected since the equilibrium binding studies (Reed and Rilling, 1975) with the avian enzyme indicate that DMAPP only binds to the allylic site, whereas the mixed linear pattern is more consistent with a scheme in which the allylic substrate analogues bind to the IPP and to the allylic site. If the allylic substrate is lengthened to a C_{10} pyrophosphate, binding to the IPP site should be lowered significantly, and in a preliminary study with 2-fluorogeranyl pyrophosphate we found competitive inhibition against GPP (Poulter et al., 1977). Apparently, introduction of the trifluoromethyl moiety reduces the selectivity of the analogue for the allylic site, and a mixed linear inhibition profile emerges.

Incubation of (*E*)- and (*Z*)-CF_3-DMAPP with [^{14}C]IPP and FPP synthetase gave a product which, after hydrolysis with alkaline phosphatase, had a retention volume very close to that of geranylgeraniol. In a separate experiment, prolonged incubation of IPP and DMAPP with FPP synthetase gave mostly C_{20} pyrophosphate. Since GGPP is not a substrate for FPP synthetase, and the catalytic rate of the C_{15} to C_{20} conversion is 10^{-2} that of the normal rate, one would expect to only isolate C_{20} material from the condensation of the C_5 fluoro analogues with IPP. Although we were not able to prepare enough C_{20} material for a mass spectrum, the steady, slow production of acid-labile material in the incubations with the fluoro analo-

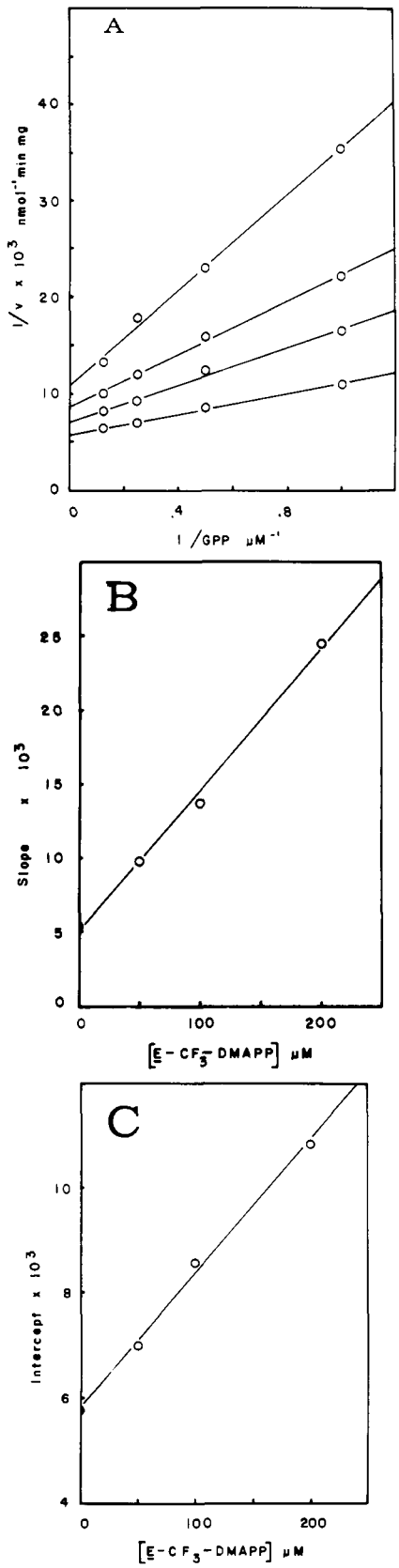


FIGURE 2: Double-reciprocal plot of initial velocity for condensation of IPP and GPP. Reactions were carried out at 37 °C in 200 μL of 10 mM potassium phosphate buffer, pH 7.4, 1 mM $MgCl_2$, 0.1 mM dithiothreitol, 1 μM NaN_3, and 2 μM IPP. (A) With 0, 50, 100, and 200 μM (*E*)-CF_3-DMAPP. (B) Slope vs. [I] replot. (C) Intercept vs. [I] replot.

gues and not in the control runs suggests that condensation occurred.

The data that we present argue strongly against a nucleophilic mechanism for the prenyl-transfer reaction. One could still envision participation by an X group in an ionization–condensation–elimination mechanism with a negatively charged moiety providing ion-pair stabilization (Cornforth, 1968). However, an X group is not needed to control stereochemistry during the condensation and, with the major impetus for its original inclusion in the mechanism removed, we feel that it is an unnecessary addition at this point. Rather, we suggest that the stereochemistry is principally determined by the orientation in which the enzyme binds its substrates (Poulter and Rilling, 1976).

In summary, we conclude that the prenyl-transfer reaction takes place by an ionization–condensation–elimination mechanism. Stereochemistry of carbon–carbon bond formation can be controlled by binding the substrates in an orientation where the *si* face of the C_3–C_4 double bond in IPP is positioned at the backside of C_1 in the allylic pyrophosphate. Once ionization of the allylic substrate has been triggered, presumably by removing negative charge from the P_1 region of the allylic pyrophosphate through tight coordination to magnesium or manganese (King and Rilling, 1977), condensation with IPP should be exothermic. If the pyrophosphate moieties of IPP and the allylic substrate are bound in the same general region of the active site, the pyrophosphate anion generated by ionization is in position to assist with elimination of the *pro-R* proton at C_2 in IPP, thus completing the enzymatic reaction (Poulter and Rilling, 1976; Popjak, 1971).

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Comparative Kinetics of Mg^{2+}-, Mn^{2+}-, Co^{2+}-, and Ni^{2+}-Activated Glyoxalase I. Evaluation of the Role of the Metal Ion†

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ABSTRACT: The disproportionation of the hemimercaptals of glutathione and α-ketoaldehydes into the corresponding thiol esters of glutathione and α-D-hydroxycarboxylic acids, catalyzed by rat erythrocyte glyoxalase I, requires a divalent metal ion. Mg^{2+}, Mn^{2+}, Co^{2+}, and Ni^{2+} were compared for their effects on a number of parameters: (1) V_{max} values using methylglyoxal as the α-ketoaldehyde agree within a factor of two for all four M^{2+}–glyoxalase I; (2) activation volumes, $\Delta V^{\ddagger}$, are similar (14 ± 2 cm^3/mol) regardless of which metal ion is present; (3) the stereospecific transfer of hydrogen from the hemimercaptal carbon to the α-ketone is insensitive to changes in the metal ion, giving 95 ± 2% D-lactate after hydrolysis of the thiol ester obtained from the disproportionation of the glutathione hemimercaptal of methylglyoxal; (4) deuterium isotope effects using phenylglyoxal and α-deuteriophenylglyoxal were observed on V_{max} for all four M^{2+}–glyoxalase I indicating that the breaking of the C–H bond of the hemimercaptal is rate determining. These results lead to the conclusion that the metal ion participates in the glyoxalase I reaction as a superacid which is able to polarize the α-ketone group to facilitate the disproportionation reaction. To support the proposal for a role for the metal ion at the active site of the enzyme, the approximate location of the metal ion was determined. The kinetics of inactivation of glyoxalase I by dansyl chloride indicate that the dansyl group modifies a residue at or near the active site. Dansylated apoglyoxalase I when titrated with Mn^{2+} shows extensive fluorescence quenching, indicating that the metal ion is binding close to the dansyl group.

The disproportionation of α-ketoaldehydes, such as methylglyoxal, into the corresponding α-hydroxycarboxylic acids proceeds by two enzyme-catalyzed reactions. The first reaction, catalyzed by glyoxalase I (*S*-lactoylglutathione methylglyoxal-lyase (isomerizing), EC 4.4.1.5), requires glutathione as cofactor and involves the conversion of an α-ketoaldehyde into a thiol ester of glutathione and an α-hydroxycarboxylic acid; the second reaction, catalyzed by glyoxalase II (*S*-2-hydroxyacylglutathione hydrolase, EC 3.1.2.6), involves the hydrolysis of the thiol ester to regenerate glutathione and liberate a free α-hydroxycarboxylic acid. There has been considerable interest in the mechanism of glyoxalase I especially regarding the involvement of a one- or a two-substrate pathway (Cliffe and Waley, 1961; Kermack and Matheson, 1957). Because glutathione adds to the α-ketoaldehyde rapidly and reversibly in a nonenzymic reaction, the determination of whether the hemimercaptal is the substrate for glyoxalase I or whether the α-ketoaldehyde and glutathione are the substrates has not been a simple problem. Our earlier studies on yeast glyoxalase I suggested that the one-substrate mechanism is the major pathway (Vander Jagt et al., 1975). In addition, yeast glyoxalase I shows very broad specificity for both aliphatic and aromatic α-ketoaldehydes with little sensitivity in its kinetic parameters V_{max} and K_m to variations in the substrate (Vander Jagt et al., 1972a). This is in contrast to the disproportionation of α-ketoaldehydes in alkaline solution, a model for glyoxalase I, where the polarity of the α-ketone group is a major factor in the rate of reaction (Vander Jagt et al., 1972b). For both the yeast glyoxalase I

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