Inhibition of liver prenyltransferase by citronellyl and geranyl phosphonate and phosphonylphosphate[1]

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Abstract Citronellyl- and geranylphosphonic acids and the corresponding phosphonylphosphates were made and tested as inhibitors of liver prenyltransferase. Kinetic analysis showed that citronellyl- and geranylphosphonylphosphate were powerful inhibitors of the enzyme, and that they were competitive inhibitors with geranyl diphosphate and noncompetitive inhibitors with isopentenyl diphosphate. Two inhibition constants, representing the equilibria $[E][I]/[EI] = K_5$ and $[ES_1][I]/[ES_1I] = K_9$, have been defined for the inhibitors. For citronellylphosphonylphosphate, the value of K_5 was 1.25 μM and K_9 was 3.30 μM; for geranylphosphonylphosphate, K_5 = 1.50 μM and K_9 = 1.60 μM. The phosphonates were very poor linear mixed noncompetitive inhibitors with respect to both substrates of the transferase. —**Popják, G., and C. Hadley.** Inhibition of liver prenyltransferase by citronellyl and geranyl phosphonate and phosphonylphosphate. *J. Lipid Res.* 1985. **26:** 1151–1159.

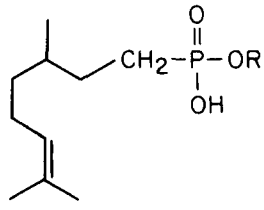
Supplementary key words citronellylphosphono-*O*-phosphonate • geranylphosphono-*O*-phosphonate

The need for pharmaceuticals that can lower plasma cholesterol levels of hypercholesterolemic individuals has directed much attention to the enzymes of cholesterol biosynthesis and to compounds that would inhibit these enzymes. Among the many inhibitors described (1–13), compactin (ML-236B) and mevinolin (1, 2) have been shown to lower plasma cholesterol concentrations in man and to be of therapeutic value in severe hypercholesterolemia associated with xanthomata (13). Other reported inhibitors have been used to elucidate the catalytic mechanisms of individual enzymes of sterol biosynthesis (5, 9–12). Studies with inhibitors of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase have revealed the basic features of the multifaceted regulation of that enzyme's activity and sterol production in vivo, and the vital role of that enzyme's product, mevalonate, in cell growth (14).

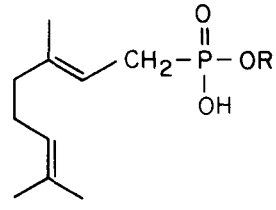
It was shown in our laboratory first that, contrary to earlier beliefs, mevalonate was not used by animals exclusively for polyprenyl synthesis, but that a portion of it was diverted to another metabolic pathway (15). It was postulated that the divergence of the metabolism of mevalonate from the polyprenyl synthetic pathway to the "shunt" pathway should occur after the formation of 3,3-dimethylallyl diphosphate (16). The existence of this shunt pathway, originally called the *trans*-methylglutaconate shunt, has been amply confirmed (for review see Landau and Brunengraber, 17), and the appearance of the carbon atoms of mevalonate in acetoacetate in a pattern as originally postulated (15, 16) has been unequivocally proven (18).

Given the existence of the *trans*-methylglutaconate shunt, which diverts carbon atoms of mevalonate from sterol biosynthesis, and the fact that this diversion must begin by the hydrolysis of 3,3-dimethylallyl diphosphate to the free alcohol, inhibition of liver prenyltransferase could be an effective means of reducing endogenous production of cholesterol since dimethylallyl diphosphate is one of the substrates of this enzyme. Inhibition of prenyltransferase would greatly enhance the chances of the hydrolysis of dimethylallyl diphosphate to the free alcohol.

We report now the preparation and kinetic characterization of four inhibitors of prenyltransferase: citronellyl- and geranylphosphonates and the corresponding phosphonylphosphates, compounds **1–4**.



1. R = H
3. R = $-PO_3H_2$



2. R = H
4. R = $-PO_3H_2$

Abbreviations: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A.

[1] Phosphonylphosphates could also be called phosphono-*O*-phosphonates according to the nomenclature recommended by IUPAC-IUB for phosphorus-containing compounds.

The phosphonylphosphates are, of course, analogues of the normal diphosphate substrates and products of the prenyltransferase reaction. The phosphonate analogues are chemically unreactive with the enzyme, and with phosphatases in general, since they lack a C-O-P functionality.

Pilot studies with pentyl- and decylphosphonates and phosphonylphosphates were published previously (7). These compounds behaved as linear, mixed noncompetitive inhibitors (19) of pig liver prenyltransferase relative to both isopentenyl and geranyl diphosphate. The phosphonylphosphates were more than ten times as potent inhibitors as the corresponding phosphonates (7) and thus mimicked the behavior of phosphate and diphosphate ester analogues of geranyl diphosphate that were the first reported inhibitors of liver prenyltransferase (4).

We show in this report that geranyl- and citronellylphosphonylphosphates are very effective inhibitors of purified pig liver prenyltransferase and that they act as competitive inhibitors with respect to geranyl diphosphate and noncompetitive inhibitors with isopentenyl diphosphate.

MATERIALS AND METHODS

General

A supply of the trilithium salt of geranyl diphosphate, made by a published procedure (20), was available in our laboratory from previous investigations. An ethanolic solution of the triammonium salt of [1-^{14}C]isopentenyl diphosphate (56 Ci/mol) was purchased from New England Nuclear, Boston, MA. Radioactivity was measured with a Searle Mark III, Model 6880, Liquid Scintillation System in 3a70B scintillation fluid (Research Products International, Elk Grove Village, IL). Thin-layer chromatography was carried out on 250 μm silica gel H plates with isopropanol–conc. ammonia–water 6:3:1 (by vol). Phosphorus-containing compounds were detected by spraying the plates with 10% perchloric acid, followed by heating at 110°C for 5 min, and then by spraying successively with 2.5% ammonium molybdate in 10% sulfuric acid and 10% aqueous ascorbic acid (21). The treated plates were placed in a humid jar for development of color. Proton NMR spectra were taken on the UCB 250 (University of California Berkeley), a 250 MHz FT instrument. Chemical shifts are reported in ppm downfield from an internal tetramethylsilane reference. Mass spectral data were obtained with an Atlas MS-12 spectrometer and at high resolution with a Consolidated 12-110B mass spectrometer.

Citronellyl- and geranylphosphonic acids (compounds 1 and 2)

The starting materials for these phosphonic acids were the corresponding diethyl esters, which were the generous gifts of Dr. N. F. Blau of the Veterans Administration Center, Witchita, KS. Their synthesis was described by Blau, Wang, and Buess (22). The phosphonic acids were generated from the diethyl esters by the method of McKenna et al. (23). For example, 9.5 ml (72 mmol) of trimethylsilyl bromide was cautiously added to 10.00 g (36.2 mmol) of stirred diethyl citronellylphosphonate which was under a nitrogen atmosphere and was cooled with an ice bath. After the reactants were combined, the mixture was stirred for 2 hr at ambient temperature; then 10 ml of methanol was added to decompose the excess silyl bromide. The methanol was removed with a rotary evaporator. The viscous residue was stirred overnight with 20 ml of water and then transferred to a separatory funnel with 30 ml of diethyl ether. The layers were separated and the aqueous phase (pH 1) was extracted with 20-ml portions of ether. The combined ether phases were extracted five times with 30-ml portions of 0.1 N aqueous ammonia. The aqueous extracts were combined and acidified with HCl to pH 1 and were extracted with ether (5 × 30 ml). The pH was adjusted before each extraction. The ether was evaporated from the combined organic layers leaving 8.7 g of yellow oil (theoretical yield 7.9 g) which upon TLC analysis and staining for phosphorus showed a single spot at an R_f value of 0.75.

Geranyl- and citronellylphosphonic acids crystallized readily from either hexane or acetone at 5°C. However, isolation of the intact crystals proved to be very difficult. From 6.2 g of crude citronellylphosphonic acid, only 0.8 g (3.6 mmol) of dry, stable crystals could be obtained (mp 112.5–113°C). The crystalline products were used for analysis by NMR and mass spectrometry and for kinetic studies with prenyltransferase. The crude products were converted to the phosphonomorpholidates without further purification.

The mass spectrum of citronellylphosphonic acid (1) at high resolution (see **Fig. 1**) gave a molecular ion at m/z 220.1227 (calc for $C_{10}H_{21}O_3P$: 220.12278), and also an ion, among others, at m/z 95.9980 (calc for $[CH_2 = P(OH)_3]^+$ 95.9976). The mass spectral analysis thus established the correct elemental composition of the substance and also that it was a phosphonic acid.

^{1}H-NMR in $CDCl_3$ showed resonances at δ values in ppm of 0.89 (d, 3H, J = 15 Hz; C-3 Me), 1.19 (m, 2H; C-2 methylene), 1.43 (m, 4H; C-1(?) + C-4(?) methylenes), 1.60 (s, 3H; terminal Me), 1.67 (s, 3H; terminal Me), 1.70 (partially hidden multiplet, 1H; C-3 methine), 1.97 (m, 2H; allylic methylene), 5.08 (5, 1H, J = 6 Hz; C-6 vinyl) and 9.55 (br s, 2H; phosphonic acid hydroxyls).

The mass spectrum of crystalline geranylphosphonic acid (2) (see **Fig. 2**) showed at high resolution a molecular ion of m/z 218.1067 (calc for $C_{10}H_{19}O_3P$: 218.1071) and an ion, as in the spectrum of citronellylphosphonic acid, of m/z 95.9980. These data established the elemental composition and the phosphonic acid nature of the substance.

^{1}H-NMR in $CDCl_3$ gave resonances at values in ppm

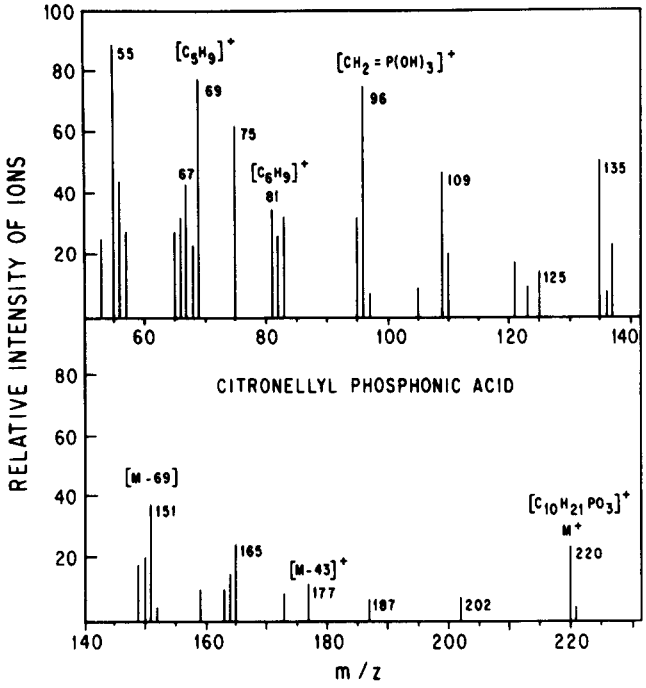


Fig. 1. Mass spectrum of citronellylphosphonic acid. Measurements at high resolution gave a mass for the molecular ion of 220.1227, and indicated that all fragment ions down from the molecular ion to the ion at m/z 96 (measured mass 95.9980; calculated for $[CH_2 = P(OH)_3]^+$ 95.9976) contained one atom of phosphorus. Measured values rarely deviated from values calculated for particular structures by more than 4 ppm.

of 1.60 (s, 3H; terminal methyl), 1.63 (d, 3H, J_{H-P} = 3 Hz; C-3 Me), 1.69 (s, 3H; terminal Me), 2.06 (br s, 4H; C-4 + C-5 allylic methylenes), 2.53 (dd, 2H, J_{H-H} = 10 Hz, J_{H-P} = 25 Hz; C-1 methylene), 5.08 (m, 1H; C-6 vinyl), 5.19 (d, 1H, J = 10 Hz; C-2 vinyl) and 9.25 (br s, 2H; phosphonic acid hydroxyls).

Citronellyl- and geranylphosphonylphosphates (compounds 3 and 4)

These phosphonylphosphates were prepared from the corresponding phosphonic acids via the phosphonomorpholidates as described by Donninger and Popják (24). The crude phosphonylphosphates were partially purified by chromatography on either Bio-Rad AG 1 × 8 anion exchange resin or Whatman DE-52 cellulose columns eluted with a 10-column volume linear gradient of 0.08 M - 1.0 M ammonium formate in methanol. However, pure phosphonylphosphates could be obtained from either the crude or chromatographed material by crystallization from 1.0 M ammonium formate in methanol at 12°C.

Citronellylphosphonylphosphate triammonium salt had an mp of 110–111°C and its ^{1}H-NMR in D_2O gave resonances at δ values in ppm of 0.69 (d, 3H, J = 6 Hz; C-3 Me), 0.96 (m, 2H; C-2 methylene), 1.10–1.35 (m, 4H; C-1 + C-4 methylenes), 1.43 (s, 3H; terminal Me), 1.49 (s, 3H; terminal Me), 1.52 (partially hidden multiplet, 1H; C-3 methine), 1.82 (m, 2H; C-5 allylic methylene), 5.05 (t, 1H, J = 8 Hz; C-6 vinyl).

Geranylphosphonylphosphate triammonium salt had an mp of 122°C (dec.) and its ^{1}H-NMR in D_2O gave resonances at δ values in ppm at 1.42 (s, 3H; terminal Me), 1.46 (d, 3H, J = 4 Hz; C-3 Me), 1.48 (s, 3H; terminal Me), 1.90 (m, 4H; C-4 + C-5 methylenes), 2.37 (dd, 2H, J_{H-H} = 7 Hz, J_{H-P} = 23 Hz; C-1 methylene), 5.02 (m, 1H; C-6 vinyl) and 5.09 (m, 1H; C-2 vinyl).

Prenyltransferase and its assay

The pig liver prenyltransferase (EC 2.5.1.1) used was isolated and purified by Dr. Graham Barnard as part of a previously published investigation (25) and was kept at 4°C as a precipitate in 60% saturated ammonium sulfate containing 10 mM 2-mercaptoethanol. This suspension of the enzyme contained 1.58 mg of protein per ml. The enzyme was assayed in the absence and presence of inhibitors by the radioactive assay procedure previously published (20). The 1-ml incubation mixtures contained 100 μM Tris-HCl buffer, pH 7.8, 5 mM $MgCl_2$, the unvaried substrate (either geranyl diphosphate or [^{14}C]isopentenyl diphosphate) and the inhibitor in concentrations

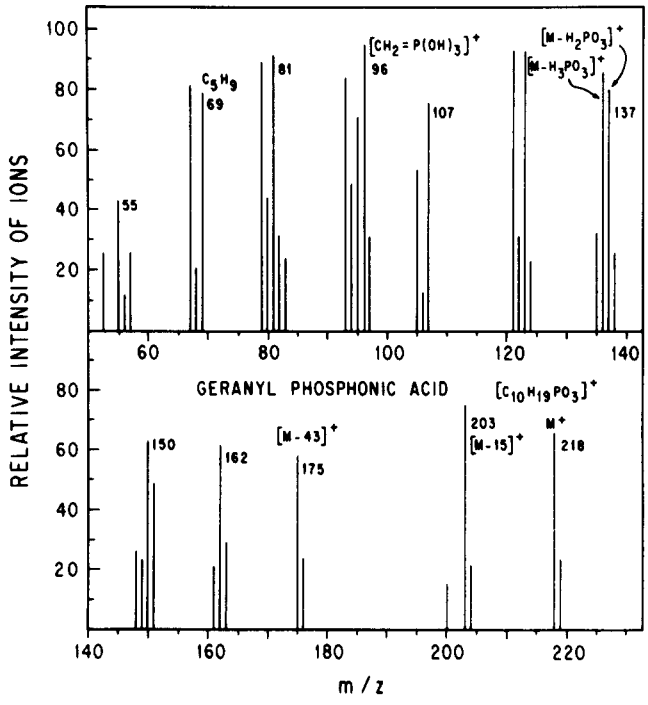


Fig. 2. Mass spectrum of geranylphosphonic acid. The measured mass of the molecular ion was 218.1067, within 2 ppm of the calculated value. The measured mass of the ion shown at m/z 96 was 95.9980, as in the spectrum of citronellylphosphonic acid.

shown in the tables. Each assay mixture was brought to 37°C, then 5 μl of the ammonium sulfate suspension of the enzyme (7.9 μg of protein) was added. Within 30 sec, the catalysis was initiated by the addition of the varied substrate in a few μl of slightly ammoniacal water. The reaction was terminated with 1 ml of 2 N HCl in 80% ethanol. One hundred μl of ethanol containing 1 mg each of farnesol and geraniol was added. After 1 hr at ambient temperature, the assay mixtures were made alkaline with 400 μl of 10% NaOH and were extracted three times with 1 ml of hexane. The combined extracts were diluted with 10 ml of scintillation cocktail for ^{14}C counting.

RESULTS AND DISCUSSION

The inhibitory properties of compounds **1–4** were studied with purified pig liver prenyltransferase in a series of eight kinetic experiments. The initial rates of [^{14}C]farnesyl diphosphate production were measured in the absence and presence of an inhibitor while the concentration of one substrate was held constant and that of the other was varied. Each inhibitor was evaluated with 1.0 μM geranyl diphosphate while the [^{14}C]isopentenyl diphosphate concentration was varied from 0.19 to 0.84 μM, and with 0.76 μM [^{14}C]isopentenyl diphosphate while the concentration of geranyl diphosphate was varied from 0.75 to 3.0 μM. Phosphonic acid concentrations of 200–800 μM were used. The effects of phosphonylphosphates were examined at 1.0, 2.0, and 5.0 μM. Each assay was carried out with 7.9 μg of protein and each assay was made in triplicate. When the concentration of geranyl diphosphate was fixed, assays were 1 min in length and when the concentration of isopentenyl diphosphate was constant, 3.0-min assays were carried out. Under these conditions conversion of substrate was always less than 20%, i.e., the measurements were those of initial velocities.

The initial reaction rates of prenyltransferase, measured in the absence and presence of citronellyl- and geranylphosphonylphosphates are shown in **Table 1**. These data

TABLE 1. Initial rates of prenyltransferase reaction in the absence and presence of citronellyl- and geranylphosphonylphosphate[a]

Varied Substrate (μM)	Inhibitor (μM) Citronellylphosphonylphosphate 0.0	1.0	2.0	5.0
		pmol/min		
Geranyl diphosphate				
0.75	42.5 ± 3.0	30.5 ± 1.3	25.1 ± 2.1	15.3 ± 0.8
1.0	49.6 ± 2.4	36.3 ± 1.5	29.9 ± 1.2	19.4 ± 0.2
1.5	53.0 ± 1.4	43.9 ± 1.5	36.2 ± 0.6	26.3 ± 1.2
3.0	63.5 ± 1.6	56.2 ± 1.2	52.6 ± 1.4	38.6 ± 2.0
Isopentenyl diphosphate				
0.19	24.5 ± 2.7	17.1 ± 0.8	15.3 ± 0.7	9.47 ± 0.4
0.25	29.1 ± 2.1	22.7 ± 0.8	18.6 ± 0.1	11.8 ± 0.2
0.38	37.6 ± 3.9	30.0 ± 1.4	24.5 ± 0.5	15.2 ± 0.9
0.76	55.9 ± 1.7	41.0 ± 0.9	36.1 ± 2.0	24.6 ± 0.7
	Geranylphosphonylphosphate			
	0.0	1.0	2.0	5.0
		pmol/min		
Geranyl diphosphate				
0.75	33.4 ± 1.2	26.6 ± 2.0	21.6 ± 1.3	14.0 ± 0.4
1.0	39.9 ± 1.6	31.0 ± 0.3	25.7 ± 0.7	17.1 ± 0.3
1.5	44.2 ± 0.9	37.4 ± 1.3	32.5 ± 0.4	23.2 ± 0.2
3.0	53.9 ± 1.1	48.5 ± 2.2	44.8 ± 1.0	33.5 ± 0.8
Isopentenyl diphosphate				
0.19	28.9 ± 3.6	17.7 ± 0.5	12.7 ± 0.1	7.22 ± 0.4
0.25	30.3 ± 0.8	21.4 ± 0.5	16.1 ± 0.5	8.99 ± 0.6
0.38	39.0 ± 0.4	26.6 ± 1.2	19.7 ± 0.9	12.0 ± 0.4
0.76	52.2 ± 1.1	38.7 ± 0.1	30.3 ± 0.9	–

[a] The data are the means of triplicate assays with standard deviation. When the concentration of geranyl diphosphate was varied, the concentration of isopentenyl diphosphate was constant at 0.76 μM; and when isopentenyl diphosphate was the varied substrate, the concentration of geranyl diphosphate was held at 1.0 μM.

were analyzed by Lineweaver-Burk plots shown in **Fig. 3** and **Fig. 4**. Both citronellylphosphonylphosphate (Fig. 3a) and geranylphosphonylphosphate (Fig. 4a) showed a typical pattern of competitive inhibition with geranyl diphosphate as these inhibitors changed only the slopes of the reciprocal plots without changing the V_{max} of the reactions when geranyl diphosphate was the varied substrate. On the other hand, with respect to isopentenyl diphosphate both substances behaved, within experimental error, as noncompetitive inhibitors (Figs. 3b and 4b).

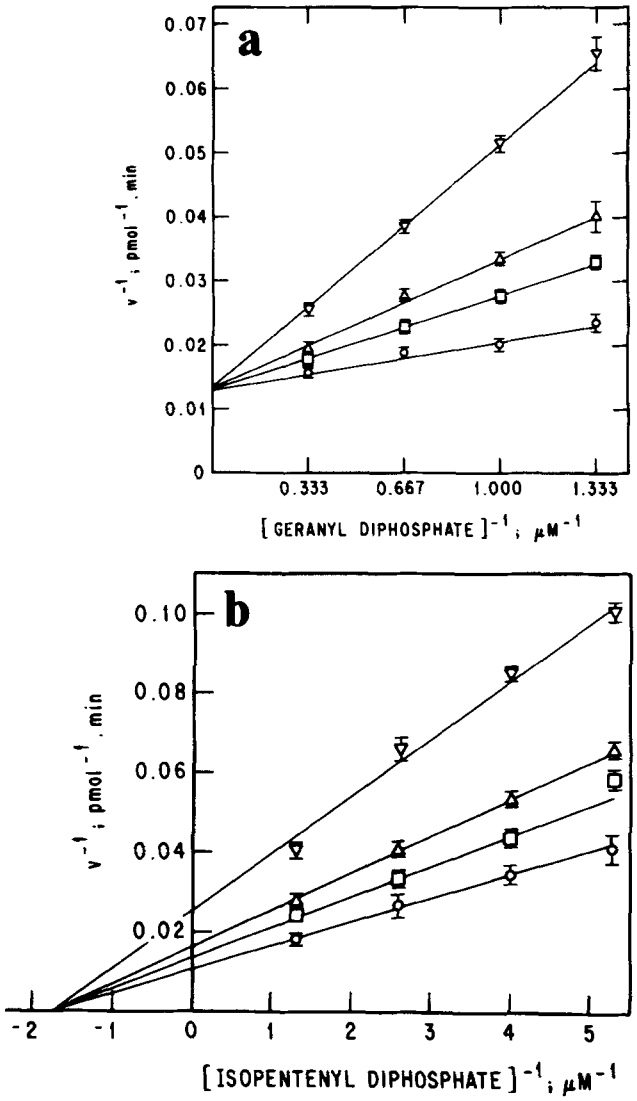


Fig. 3. Inhibition of prenyltransferase by citronellylphosphonylphosphate. The reciprocals of initial velocities, $1/v$, are plotted against the reciprocals of the concentration of the varied substrate: in (a) 1/[geranyl diphosphate]; and in (b) 1/[isopentenyl diphosphate]. The inhibitor concentrations (μM) were (○), 0.0; (□), 1.0; (△), 2.0; and (▽), 5.0. In experiments of (a) the concentration of isopentenyl diphosphate was fixed at 0.76 μM, and in (b) the concentration of geranyl diphosphate was constant at 1.0 μM.

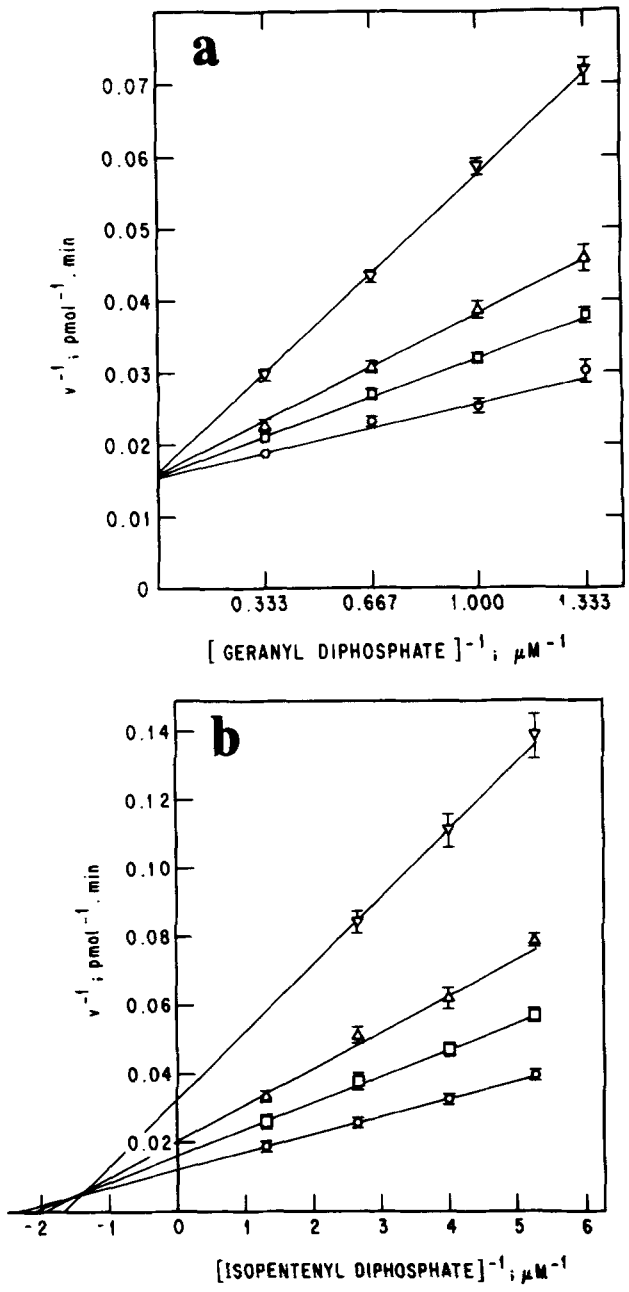


Fig. 4. Inhibition of prenyltransferase by geranylphosphonylphosphate. The reciprocals of initial velocities, $1/v$, are plotted against the reciprocals of the varied substrate as in Fig. 3. The inhibitor concentrations (μM) were (○), 0.0; (□), 1.0; (△), 2.0; and (▽), 5.0. In experiments of (a) the concentration of isopentenyl diphosphate was fixed at 0.76 μM; and in (b) the concentration of geranyl diphosphate was constant at 1.0 μM.

The basic kinetic data with citronellyl- and geranylphosphonate are shown in **Table 2**. Comparison of these data with those given in Table 1 reveals that the phosphonates are very poor inhibitors of the transferase requiring 100 to 200 times higher concentrations than the phosphonylphosphates to achieve degrees of inhibition comparable with those caused by the phosphonylphos-

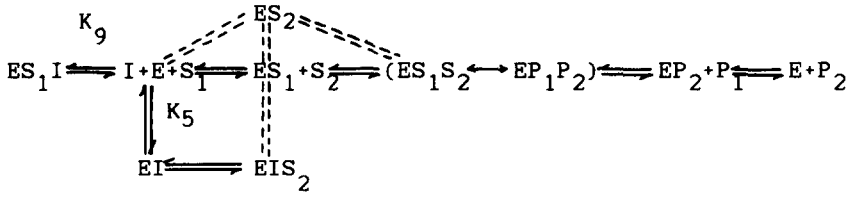
TABLE 2. Initial rates of prenyltransferase reaction in the absence and presence of citronellyl- and geranylphosphonate[a]

Varied Substrate (μM)	Inhibitor (μM) Citronellylphosphonate 0	200	300	600
		pmol/min		
Geranyl diphosphate				
0.75	–	29.8 ± 0.5	27.1 ± 0.4	21.6 ± 2.1
1.0	39.6 ± 1.0	35.7 ± 4.2	31.5 ± 1.3	24.7 ± 0.2
1.5	46.8 ± 2.7	39.4 ± 1.6	36.8 ± 1.1	28.6 ± 0.1
3.0	54.7 ± 1.0	46.6 ± 2.2	44.5 ± 1.2	36.3 ± 1.2
	0	250	400	800
Isopentenyl diphosphate				
0.20	37.3 ± 1.2	30.8 ± 0.6	26.7 ± 1.5	21.7 ± 0.6
0.26	45.3 ± 1.5	37.8 ± 0.5	33.2 ± 0.8	26.4 ± 1.2
0.40	58.7 ± 3.0	50.4 ± 1.4	47.1 ± 0.6	36.2 ± 0.2
0.76	83.6 ± 1.7	74.2 ± 1.3	66.3 ± 0.4	55.8 ± 1.0
	Geranylphosphonate			
	0	250	400	800
		pmol/min		
Geranyl diphosphate				
0.75	40.4 ± 2.0	27.7 ± 0.8	22.6 ± 1.3	13.9 ± 1.1
1.0	46.1 ± 1.1	30.8 ± 1.2	25.7 ± 0.5	17.0 ± 1.9
1.5	52.0 ± 1.0	36.3 ± 0.6	32.2 ± 0.8	21.0 ± 0.4
3.0	60.4 ± 0.1	45.8 ± 0.8	40.7 ± 0.9	28.6 ± 0.8
	0	250	400	800
Isopentenyl diphosphate				
0.21	40.3 ± 1.8	27.7 ± 1.6	21.8 ± 1.3	14.8 ± 1.0
0.27	52.5 ± 1.0	35.5 ± 2.6	28.3 ± 0.6	18.2 ± 0.7
0.42	67.1 ± 2.7	48.2 ± 2.1	37.8 ± 0.7	25.8 ± 1.7
0.84	99.6 ± 4.9	69.3 ± 5.5	63.1 ± 3.7	44.6 ± 0.0

[a] The data are the means of triplicate assays with standard deviation. When the concentration of geranyl diphosphate was varied, the concentration of isopentenyl diphosphate was constant at 0.76 μM; and when isopentenyl diphosphate was the varied substrate, the concentration of geranyl diphosphate was held at 1.0 μM.

phates. Further, analysis of the data by Lineweaver-Burk plots (not shown) demonstrated that the type of inhibition displayed by the phosphonates differed from that of the phosphonylphosphates in that they were mixed noncompetitive inhibitors of prenyltransferase as they increased the slope and the y intercept ($1/V_{max}$) of the plots and the apparent K_m values of the substrates when the concentration of either substrate was varied.

Corey and Volante (6) synthesized a number of methylenephosphonate and methylenephosphonylphosphate analogues of the substrates of prenyltransferase, among them geranylmethylenephosphonylphosphate, and found that the phosphonylphosphates powerfully inhibited the conversion of mevalonate into squalene in a 10,000 *g* supernatant of rat liver homogenates, but that the phosphonates were very poor inhibitors. However, Corey and Volante gave no quantitative information regarding the two types of inhibitors. At least a tenfold difference was found by Popják et al. (4) between the inhibitory powers of the diphosphate and monophosphate esters of octan-1-ol, tetrahydrogeraniol, and citronellol, the diphosphates being more powerful inhibitors of the transferase than the monophosphates. Similar experience was recorded by Parker et al. (7) with alkylphosphonates and alkylphosphonylphosphates. Holloway and Popják (20) expressed the view that the binding of substrate to prenyltransferase was primarily determined by the charges on the diphosphate group and secondarily by lipophilic forces. All experience so far with analogues of the substrates of prenyltransferase bears out this view and it apparently



Scheme 1. Model of reaction catalyzed by prenyltransferase and its inhibition (modified from ref. 4). S_1, geranyl diphosphate; S_2, isopentenyl diphosphate; P_1, farnesyl diphosphate; P_2, PP_i.

applies also to the phosphonate analogues. It might be recalled that such a simple substance as octyl diphosphate is a respectable inhibitor of prenyltransferase, with values of inhibition constants of 3.4 μM and 2.3 μM (4).

In their kinetic analysis of the reaction catalyzed by pig liver prenyltransferase, Holloway and Popják (20) concluded that an "ordered bi-bi" mechanism best described the reaction, as suggested in **Scheme 1**, in which E is the free enzyme, S_1 is geranyl diphosphate, the first substrate to bind to the enzyme, and S_2 is isopentenyl diphosphate, the second substrate that binds to the ES_1 complex. The same model was used by Popják et al. (4) and Parker et al. (7) for the analysis of inhibition of prenyltransferase by various substrate analogues. Both groups concluded that analogues of geranyl diphosphate interacted mainly with two forms of the enzyme: the free enzyme, E, and the ES_1 complex. Scrutiny of our data indicates that the citronellyl- and geranylphosphonylphosphates and the corresponding phosphonates behave similarly. Specifically, the phosphonylphosphates appear to be directed to the binding site of the allylic substrate and we can rule out an ordered mechanism with isopentenyl diphosphate binding to the enzyme first. Our data are consistent with an ordered mechanism catalyzed by the enzyme with geranyl diphosphate binding first. Thus the two inhibition constants, K_5 and K_9, according to notations used before (4), and which represent, respectively, the equilibria [E][I]/[EI] and $[ES_1][I]/[ES_1I]$, can be obtained by replotting the slopes of the Lineweaver-Burk lines against inhibitor concentrations. K_5 and K_9 are obtained directly by the extrapolation of the replots of the slopes of $1/v = f([1/S_1])$ and $1/v = f([1/S_2])$ lines, respectively, against inhibitor concentrations; the intercepts of these lines with the abscissa give the value of the two inhibition (equilibrium) constants with the minus sign. Such replots of the data obtained with citronellyl- and geranylphosphonylphosphate are shown in **Fig. 5** and **Fig. 6**, respectively, and those obtained with the corresponding phosphonates in **Fig. 7** and **Fig. 8**. The values of the inhibition constants deduced from these replots are presented in **Table 3** and emphasize the vast difference between the effectiveness of the phosphonates and phosphonylphosphates. The two phosphonylphosphates are the first competitive inhibitors of prenyltransferase reported with respect to geranyl diphosphate, their K_5 values rivalling the K_m value, 2.2 μM, of geranyl diphosphate (7). It is possible and probable that the geranylmethylenephosphonylphosphate, synthesized by Corey and Volante (6), might also have been a com-

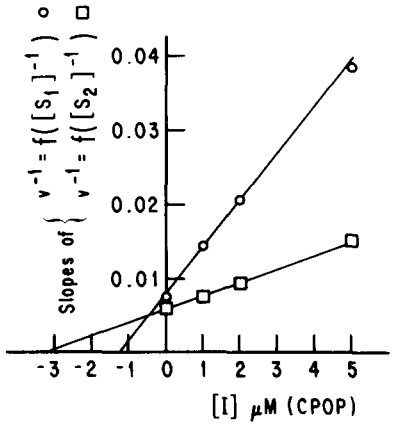


Fig. 5. The slopes of lines from Fig. 3a and 3b plotted against inhibitor concentrations. S_1, geranyl diphosphate; S_2, isopentenyl diphosphate. CPOP on the abscissa is citronellylphosphonylphosphate.

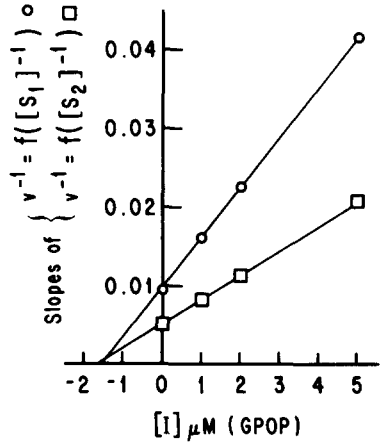


Fig. 6. Slopes of lines from Fig. 4a (○), and 4b (□) plotted against inhibitor concentrations. GPOP on the abscissa is geranylphosphonylphosphate.

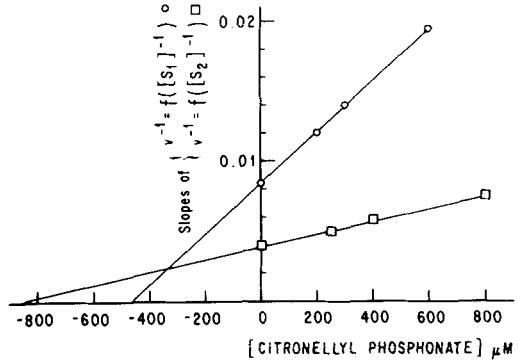


Fig. 7. The slopes of Lineweaver-Burk plots (not presented) from experiments with citronellylphosphonate plotted against inhibitor concentrations. Symbols: (○), lines from experiments when geranyl diphosphate was the varied substrate; (□), lines from experiments when the concentration of isopentenyl diphosphate was varied (cf. Table 2).

petitive inhibitor of prenyltransferase: at a concentration of 2 μM it inhibited by 50% the conversion of mevalonate into squalene in liver homogenates.

The mechanism of reaction catalyzed by prenyltransferase may seem to be purely ordered bi-bi only with the liver enzyme and at low substrate concentrations. In at least two other prenyltransferases, from human liver (25) and chicken liver (11), isopentenyl diphosphate at concentrations above 2 μM shows a strong inhibition of the reaction, giving a competitive pattern with geranyl diphosphate. The interactions of ES_2 and EIS_2 with ES_1, indicated by broken lines in Scheme 1, are meant to draw attention to this fact. Laskovics, Krafcik, and Poulter (11) have discussed in detail the mechanism of substrate inhibition of reaction in chicken liver prenyltransferase. In contrast to the behavior of isopentenyl diphosphate, geranyl diphosphate does not show substrate inhibition even when it is in large excess over isopentenyl diphosphate (25). In other words, isopentenyl diphosphate can bind to the site of the allylic substrate, but the allylic substrate cannot take the place of the homoallylic substrate.

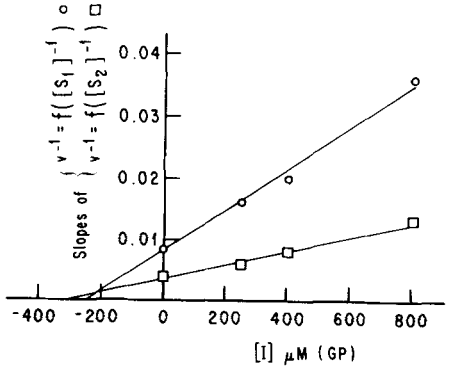


Fig. 8. Plots similar to those shown in Fig. 7 except that the inhibitor was geranylphosphonate. GP on the abscissa is geranylphosphonate.

TABLE 3. Inhibition constants of citronellyl- and geranylphosphonate and their phosphonylphosphates in the reaction catalyzed by pig liver prenyltransferase between geranyl diphosphate and isopentenyl diphosphate

Inhibitor	K_5	K_9
	μM	
Citronellylphosphonate	470.00	860.00
Citronellylphosphonylphosphate	1.25	3.30
Geranylphosphonate	250.00	330.00
Geranylphosphonylphosphate	1.50	1.60

We compared our data obtained with the two phosphonylphosphates with those of Popják et al. (4) obtained with the diphosphates of tetrahydrogeraniol and citronellol, which, structurally, are the closest analogues to our phosphonylphosphates. There is a curious difference between the kinetics of inhibition of the transferase by the two diphosphate esters and the phosphonylphosphates. Whereas the two phosphonylphosphates are noncompetitive inhibitors of the transferase with isopentenyl diphosphate, the two diphosphate esters were uncompetitive inhibitors with isopentenyl diphosphate: the Lineweaver-Burk plots of $1/v$ against $1/[S_2]$, at increasing concentrations of the esters, gave parallel lines. The kinetic analysis showed that the esters interacted with the free enzyme and the EP_2 complex, and not at all with the ES_1 complex. The difference is the more surprising as the overall length of citronellylphosphonylphosphate, in an extended conformation, is shorter by only about 1 Å than that of the citronellyl diphosphate. However, differences in the C-P-O and C-O-P bond angles may create conformational differences and account for the different behavior of the two types of molecules.

In spite of the high potencies of citronellyl- and geranylphosphonylphosphates as inhibitors of prenyltransferase, we do not think they could be effective in vivo, as a substantial barrier exists against cellular uptake of anionic molecules. Such a barrier might be overcome if the inhibitors could be linked to a substance for which a natural transport mechanism exists. Sheikh et al. (26) succeeded in linking 5-homomevalonophosphonate (5-carboxy-4-hydroxy-4-methylpentyl-1-phosphonic acid), an isosteric analogue of mevalonate 5-phosphate and an inhibitor of phosphomevalonate kinase (8), through its carboxyl group to the primary hydroxyl group of reduced cholic acid and showed that the complex, imitating the structure of taurocholic acid, was taken up by hepatocytes and that it inhibited cholesterol synthesis from mevalonate in vivo. It remains to be seen whether a similar carrier system for our phosphonylphosphates could be devised.

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