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Artificial Substrates for Prenyltransferase

By G. POPJÁK,* J. L. RABINOWITZ and J. M. BARON
Milstead Laboratory of Chemical Enzymology, 'Shell' Research Ltd., Broad Oak Road, Sittingbourne, Kent, and Department of Biological Chemistry, University of California at Los Angeles School of Medicine, Los Angeles, Calif. 90024, *U.S.A.*

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Four out of 16 new allylic pyrophosphates synthesized were found to be artificial substrates for liver prenyltransferase (EC 2.5.1.1). These were the *trans*- and the *cis*-3-ethyl-3-methylallyl, the 3,3-diethylallyl and the (mixture of *cis* and *trans*) 3-methyl-3-*n*-propylallyl pyrophosphates. The products synthesized from these substrates and isopentenyl pyrophosphate were the appropriate homo- and bishomo-farnesyl pyrophosphates. Substitution of 3,3-dimethylallyl pyrophosphate at C-2 with a methyl group destroyed its reactivity with the enzyme. Neither the unsubstituted allyl pyrophosphate nor the *cis*- or *trans*-3-methylallyl pyrophosphate could be condensed with isopentenyl pyrophosphate. Thus the simplest allylic substrate for prenyltransferase is 3,3-dimethylallyl pyrophosphate.

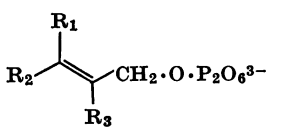
Prenyltransferase (EC 2.5.1.1) isolated from pig liver was found to catalyse the reactions not only between its natural substrates, 3,3-dimethylallyl pyrophosphate or geranyl pyrophosphate, and isopentenyl pyrophosphate, but also between 6,7-dihydrogeranyl pyrophosphate and isopentenyl pyrophosphate, the product of the reaction between the latter being 10,11-dihydrofarnesyl pyrophosphate (Popják, Holloway & Baron, 1969*a*). It was also shown that the natural substrates and also analogues of geranyl pyrophosphate were bound to the active sites of prenyltransferase mainly by forces between the enzyme and the pyrophosphate group of such substances and only secondarily through their alkyl residues (Holloway & Popják, 1967; Popják, Holloway, Bond & Roberts, 1969*b*). Thus it seemed probable that we might find further artificial substrates for prenyltransferase among analogues of 3,3-dimethylallyl pyrophosphate. We synthesized 16 allylic pyrophosphates and found among these four which were accepted by prenyltransferase as substrates. We report the results of experiments that establish the identity of the products synthesized by the enzyme from the four new allylic pyrophosphates plus isopentenyl pyrophosphate and also define the minimum structural features of an allyl pyrophosphate needed for an effective substrate of prenyltransferase.

MATERIALS AND METHODS

All the substances made and tested were analogues of 3,3-dimethylallyl pyrophosphate conforming to formula (I). Table 1 lists these substances according to the nature of the three substituents, R_1, R_2 and R_3; their synthesis is outlined in Scheme 1. Depending on the availability of suitable intermediates, these substances were made by one or more of the six steps shown in Scheme 1. In order to avoid unnecessary repetition, the methods common to their preparation will be described in detail only once and then referred to briefly when necessary.

Preparation of cis- *and* trans-*allylic esters by the Reformatsky reaction and by dehydration of 3-hydroxy esters* [*Scheme* 1; *steps* (1), (2) *and* (3)]. A solution of 1 mole of the appropriate ketone and 0·25 mole of ethyl 2-bromoacetate (or 2-bromopropionate) in 200 ml. of dry benzene was added during 1·5 hr. to 0·25 mole (16·4 g.) of zinc wool (activated with a crystal of iodine) in 200 ml. of dry benzene, the mixture being stirred continuously and heated to a refluxing temperature. The heating and stirring were continued for 3 hr. more; the mixture was then cooled and stirred for 1 hr. with 288 ml. of 6 M-H_2SO_4. The organic and aqueous layers were separated and the aqueous layer was extracted twice with benzene. The combined benzene extracts were washed successively with water, a saturated solution of $NaHCO_3$ and water, and then dried over anhydrous $MgSO_4$. The solvent and the excess of starting ketone were distilled off under vacuum (15 mm. Hg); the resulting 3-hydroxy ester (yields based on bromoacetate: 25–35%) was analysed by g.l.c.

The hydroxy esters were dehydrated to the mixture of the *cis*- and *trans*-allylic esters with $POCl_3$ and pyridine: 0·5 mole (76·8 g.) of $POCl_3$ was added to a solution of 0·1 mole of the 3-hydroxy esters in 300 ml. of pyridine cooled to 0°. After 15 hr. the mixture was heated on a boiling-water bath for 4 hr. and then poured over crushed ice. The resulting mixture



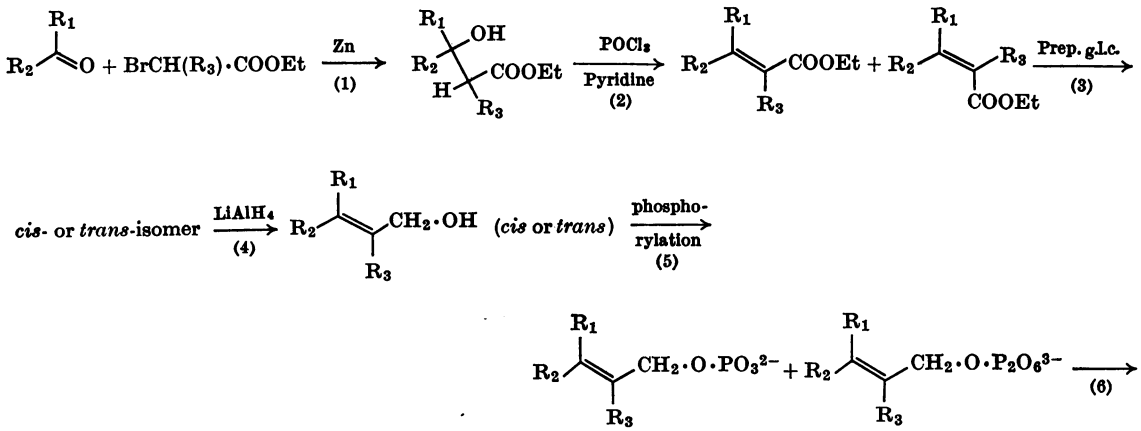
(I)

* Present address: Departments of Biological Chemistry and Psychiatry, University of California at Los Angeles School of Medicine, Los Angeles, Calif. 90024, U.S.A.

Table 1. *Analogues of 3,3-dimethylallyl pyrophosphate synthesized*

Compound no.	Substituents in formula (I) R_1	R_2	R_3	Starting compound for synthesis	Stages in synthesis*
1	H–	H–	H–	Prop-2-en-1-ol	(5), (6)
2	H–	H–	CH_3–	2-Methylprop-2-en-1-ol	(5), (6)
3	CH_3–	H–	H–	*cis*-But-2-en-1-ol	(5), (6)
4	H–	CH_3–	H–	*trans*-But-2-en-1-ol	(5), (6)
5	CH_3–	H–	CH_3–	*cis*-2-Methylbut-2-enoic (angelic) acid methyl ester	(4), (5), (6)
6	H–	CH_3–	CH_3–	*trans*-2-Methylbut-2-enoic (tiglic) acid methyl ester	(4), (5), (6)
7	CH_3–	CH_3–	CH_3–	Acetone + ethyl 2-bromopropionate	(1)–(6)
8	$CH_3 \cdot CH_2$–	CH_3–	H–	*cis*-Methylpent-2-enoic acid methyl ester	(4)–(6)
9	CH_3–	$CH_3 \cdot CH_2$–	H–	*trans*-3-Methylpent-2-enoic acid methyl ester	(4)–(6)
10	C_6H_5–	CH_3–	H–	Acetophenone + ethyl bromoacetate	(1)–(6)
11	CH_3–	C_6H_5–	H–		
12	$(CH_3)_3C$–	CH_3–	H–	3,3-Dimethylbutan-2-one (pinacolone) + ethyl bromoacetate	(1)–(6)
13	CH_3–	$(CH_3)_3C$–	H–		
14	$CH_3 \cdot CH_2$–	$CH_3 \cdot CH_2$–	H–	Pentan-3-one + ethyl bromoacetate	(1)–(6)
15	$CH_3 \cdot [CH_2]_2$–	CH_3–	H–	Pentan-2-one + ethyl bromoacetate	(1)–(6)
16	CH_3–	$CH_3 \cdot [CH_2]_2$–	H–		

* The numbers refer to the steps shown in Scheme 1.



Separation of mono- and pyro-phosphates by column chromatography

Scheme 1. Flow sheet of the synthesis of allyl pyrophosphates. Prep. g.l.c., preparative gas–liquid chromatography.

was extracted repeatedly with *n*-hexane. The combined hexane extracts were washed with 2 M-HCl and water and dried over $MgSO_4$. After the $MgSO_4$ had been filtered off the hexane was removed under vacuum in a rotary evaporator. The residue was examined by g.l.c. and was found to contain 40–45% of the allylic esters, the *trans*-isomer predominating (approx. 60% of the total). The two isomers of the allylic esters were purified and resolved, whenever possible, by preparative g.l.c.

Reduction of allylic esters to alcohols [*Scheme* 1; *step* (4)]. The esters were reduced with a twofold excess of $LiAlH_4$ in ether and the products worked up in the usual way. The allylic alcohols were purified by preparative g.l.c. before phosphorylation.

When a suitable $\alpha\beta$-unsaturated acid was available as a starting material, the acid was first converted into the methyl ester with a redistilled solution of diazomethane in ether, and the *cis*- and *trans*-isomers (if present) were

resolved by preparative g.l.c. before reduction to the corresponding alcohols.

All newly synthesized alcohols were identified by their i.r., n.m.r. and mass spectra.

Preparation of the allylic pyrophosphates [*Scheme* 1; *steps* (5) and (6)]. The allylic alcohols were phosphorylated by the method of Cramer & Böhm (1959) as modified by Popják, Cornforth, Cornforth, Ryhage & Goodman (1962) on scales ranging from 0·25 to 5mM. The products of phosphorylation were purified by chromatography on DEAE-cellulose columns (22cm. × 1·8cm.; Whatman DE-11, or BioRad Cellex D) equilibrated with aq. 80mM-ammonium formate, which was also the solvent used for the elution of the monophosphates. The pyrophosphates were eluted with the same solution also containing 0·1M-NH_3 (Popják *et al.* 1969*b*). The ammonium formate was removed from the fractions containing the pyrophosphate esters by the selective adsorption of the organophosphorus compounds on the polystyrene resin XAD-2 (Rohm and Haas Co., Philadelphia, Pa., U.S.A.) as described by Holloway & Popják (1967) and by Popják *et al.* (1969*b*). For one substance, 3-phenylbut-2-en-1-yl pyrophosphate, this technique was unsuitable because the XAD-2 resin adsorbed it irreversibly. This compound, after chromatography on the DEAE-cellulose column, was freed from ammonium formate by precipitation from the aqueous solution by the addition of acetone (see Holloway & Popják, 1967). The precipitated material, after sedimentation by centrifuging, was dissolved in aq. 0·01M-NH_3 and precipitated again with acetone; it was dissolved finally in aq. 0·01M-NH_3.

The purification and fractionation of the allylic mono- and pyro-phosphates was followed by t.l.c. on Eastman Kodak Co. Chromatoplates (type 6061) as described by Popják *et al.* (1969*b*) for various analogues of geranyl pyrophosphate.

The preparations of the allylic pyrophosphates were standardized by analysis for P as described by Goodman & Popják (1960) for farnesyl pyrophosphate. Their yields from the corresponding alcohols varied between 5 and 10%.

Sources of reagents. The [1-^{14}C]isopentenyl pyrophosphate (specific radioactivity 0·069μC/μmole) and the geranyl pyrophosphate were the same preparations as used in previous investigations (see, e.g., Popják *et al.* 1969*b*).

2-Methylallyl alcohol, crotyl alcohol, 3,3-dimethylbutan-2-one (pinacolone), pentan-2-one, pentan-3-one and ethyl 2-bromopropionate were supplied by Koch–Light Laboratories Ltd., Colnbrook, Bucks. Allyl alcohol and acetophenone were obtained from Hopkin and Williams Ltd., Chadwell Heath, Essex, and ethyl bromoacetate was from British Drug Houses Ltd., Poole, Dorset. The methyl ester of tiglic acid (2-methyl-*trans*-crotonic acid) and a specimen of 12-homofarnesol were gifts from Hoffmann–LaRoche, Basle, Switzerland; angelic acid (2-methyl-*cis*-crotonic acid) was supplied by K & K Laboratories Inc., Plainview, N.Y., U.S.A.

cis- *and* trans-*Crotyl alcohol* (cis- *and* trans-*but-2-en-1-ol*). The commercial preparation contained 26% of the *cis*- and 74% of the *trans*-isomer; these were separated by preparative g.l.c. According to all previous experience with g.l.c. of allylic alcohols and esters (e.g. Popják & Cornforth, 1960; Cornforth, Cornforth, Popják & Yengoyan, 1966) the *cis*-isomers have a shorter retention time (R_t) than the *trans*-isomers. Crotyl alcohol is the only allylic alcohol found so far not to obey this rule; its *trans*-isomer had a shorter R_t (14·5min. at 110°) than the *cis*-isomer (16·5min. at 110°). The identity of the two isomers, separated in the reverse order from that expected, was ascertained by their i.r. spectra and by the melting points of their 3,5-dinitrobenzoyl derivatives.

cis- *and* trans-2,3-*Dimethylallyl alcohol*. The *cis*-isomer was prepared from the methyl ester of commercial angelic acid, and one component only was shown by g.l.c. (R_t= 24min. at 102°). The *trans*-isomer was obtained by the reduction of the ethyl ester of tiglic acid; it had to be purified by preparative g.l.c. (R_t 25·5min. at 102°) as the preparation contained seven minor impurities.

2,3,3-*Trimethylallyl alcohol*. Ethyl 2,3-dimethyl-3-hydroxybutyrate was first synthesized in 34% yield by the Reformatsky reaction from 1·3moles of acetone and ethyl 2-bromopropionate and was dehydrated to a mixture of ethyl 2,3-dimethylbut-2-enoate (46%) and ethyl 2,3-dimethylbut-3-enoate (13%) by the method of Van Auken & Rinehart (1962). The isomeric esters were separated by preparative g.l.c. at a column temperature of 138° and gas pressure of 20lb./in.^{2}. The $\beta\gamma$-unsaturated ester had R_t 8min. and the $\alpha\beta$-unsaturated ester had R_t 13min. The ethyl 2,3-dimethylbut-2-enoate was then reduced with $LiAlH_4$ to the 2,3,3-trimethylallyl alcohol and was purified by g.l.c.; the final yield was 400mg. The whole specimen was phosphorylated.

cis- *and* trans-3-*Ethyl*-3-*methylallyl alcohol*. A specimen of 3-methylpent-2-enoic acid, containing the *cis*- and *trans*-isomers, remaining from the investigations of Cornforth *et al.* (1966), was methylated and the isomeric esters were resolved by g.l.c. at a column temperature of 82°. The *cis*- and *trans*-isomers had R_t values of 22 and 29min. respectively. The separated esters were then reduced to the corresponding alcohols. A 25mg. sample (0·25m-mole) of each alcohol was phosphorylated.

cis- *and* trans-3-*Methyl*-3-*phenylallyl alcohol*. A mixture of the isomeric ethyl 3-phenylcrotonates was prepared via the Reformatsky reaction starting from acetophenone (1mole) and ethyl bromoacetate. The intermediary hydroxy esters (yield 35%) were dehydrated to the mixed isomeric unsaturated esters (yield 40%), which were purified by g.l.c. at a column temperature of 190°, but the isomers could not be separated. The mixed esters (950mg.) were reduced with $LiAlH_4$ and gave 740mg. of the alcohols. The entire specimen was phosphorylated.

cis- *and* trans-3-tert.-*butyl*-3-*methylallyl alcohol* (3,4,4-*trimethylpent*-2-*en*-1-*ol*). A mixture of these alcohols was made as for the preceding specimen and on a similar scale from pinacolone and ethyl bromoacetate; the yield of the mixture of isomeric alcohols (180mg.) was much poorer than in the preceding synthesis. The whole specimen was phosphorylated.

3,3-*Diethylallyl alcohol*. This substance was made from pentan-3-one and ethyl bromoacetate on a 1molar scale by the Reformatsky reaction. The yield of the intermediary hydroxy ester (b.p. 92–96° at 11mm. Hg) was 25%. The dehydration of the hydroxy ester gave a preparation which showed a fast- and a slow-running component in the ratio of 2:3 on g.l.c. Small quantities of each of these were isolated; their i.r. spectra indicated that these were the $\beta\gamma$- and $\alpha\beta$-unsaturated esters respectively. In order to increase the yield of the allylic ester in the mixture the specimen was isomerized. To this end, 0·5g. of the mixture

of esters was added to a 0·5M solution of sodium ethoxide in ethanol and the mixture was stirred for 15hr. at room temperature. The sodium ethoxide was then destroyed by slow titration with dry ethanolic HCl at 0°. The mixture was then filtered and the solvent distilled off. The residue was distilled again and the fraction boiling between 85° and 87° at 18–20mm. Hg was collected. Examination of the distillate by g.l.c. again showed the same two components seen in the original product of dehydration, but their ratio changed to 1:4. The two fractions were separated by preparative g.l.c. at 105°; the $\beta\gamma$-unsaturated ester and the desired allylic ester had R_t values of 26 and 30min. respectively. The i.r. spectrum of the slower-moving component confirmed it to be the $\alpha\beta$-unsaturated ester ($\alpha\beta$-unsaturated ester stretching vibration at 1720cm.$^{-1}$ and *gem*-disubstituted alkene vibration at 1650cm.$^{-1}$).

The ethyl 3-ethylpent-2-enoate fraction (170mg.) gave, after reduction with $LiAlH_4$, 120mg. of the 3,3-diethylallyl alcohol. The whole specimen was phosphorylated.

cis- *and* trans-3-*Methyl*-3-n-*propylallyl alcohol*. The mixture of these alcohols was prepared also via the Reformatsky reaction starting with 1mole of pentan-2-one. The intermediary hydroxy ester (yield 27%; b.p. 87° at 8mm. Hg; n_D^{20} 1·4458) was dehydrated with $POCl_3$ and pyridine. The residue of the dehydration mixture was fractionated by preparative g.l.c. (column temperature 105°). The mixture of unresolved ethyl *cis*- and *trans*-3-methyl-3-*n*-propylacrylates was collected as a broad unresolved double peak with a mean retention time of 22min.; 156mg. were obtained. The i.r. spectrum of this specimen was very similar to that of the ethyl 3-ethylpent-2-enoate. The isomeric esters gave, after reduction with $LiAlH_4$, 100mg. of the mixture of the *cis*- and *trans*-3-methyl-3-*n*-propylallyl alcohols. The exact proportion of the two isomers in the mixture could not be determined. The whole specimen was phosphorylated.

Instrumental techniques. These, such as preparative and analytical g.l.c., gas–liquid radiochromatography, mass spectrometry, n.m.r. spectrometry and use of a liquid-scintillation spectrometer for the assay of ^{14}C, were fully described by Popják *et al.* (1969*a*). The i.r. spectra were taken with a Perkin–Elmer Infracord instrument on liquid films.

During preparative g.l.c. the pressure of carrier gas (He) was 25lb./in.^{2} unless otherwise specified; the temperatures at which the separations were made are given in the description of the preparation of each substance. The fractions collected were always examined on analytical g.l.c. columns and, when necessary, were rechromatographed until the preparation contained only one component. This is one reason for the poor yields of some of the allylic alcohols described.

Prenyltransferase (*EC* 2.5.1.1). This was prepared from pig liver as described by Holloway & Popják (1967). Two preparations were used: for most of the experiments the fraction designated G-200-1, free of isopentenyl pyrophosphate isomerase, was employed; in two instances (the large-scale experiments with 3-methyl-3-propylallyl pyrophosphate and 3,3-diethylallyl pyrophosphate) the FH^+ enzyme fraction, containing some isomerase as well as the transferase, was used.

Enzyme incubations. The synthesized allyl pyrophosphates were tested as potential substrates, in place of geranyl pyrophosphate or 3,3-dimethylallyl pyrophosphate, in the standard reaction mixture described for the assay of prenyltransferase (Holloway & Popják, 1967). The 1ml. mixtures contained: 100μmoles of tris–HCl buffer, pH7·8, 5μmoles of $MgCl_2$, 5μmoles, 50nmoles of [1-^{14}C]isopentenyl pyrophosphate, 5, 10, 20, 50, 100 or 200nmoles of synthetic allyl pyrophosphates and 50μg. of the G-200-1 enzyme. In addition one incubation mixture was set up with [1-^{14}C]isopentenyl pyrophosphate alone (as a check on the possible isomerase activity of the enzyme preparation), and another with[1-^{14}C]isopentenyl pyrophosphate (50nmoles) + geranyl pyrophosphate (50nmoles) to test the transferase activity of the enzyme preparation. After 5min. at 37°, the reactions were stopped by the addition of 1ml. of 2M-HCl in 80% (v/v) ethanol. Geraniol, nerolidol and farnesol (1μl. of each) were added to the mixture, which, after 30min. at 30°, was made alkaline with 10M-NaOH and extracted three times with light petroleum (b.p. 40–60°). The combined extracts were dried over $MgSO_4$ and made up to 5ml. with solvent; a sample of this was assayed for radioactivity. This is the same procedure as described previously for measuring the amount of farnesyl pyrophosphate synthesized from the natural substrates by prenyltransferase (Holloway & Popják, 1967).

Radioactivity in excess of that found in the control incubation mixture with [1-^{14}C]isopentenyl pyrophosphate alone was taken as an indication that an analogue allylic pyrophosphate had been acting as a substrate. Subsequently large-scale incubation mixtures were set up with such substrates at that concentration which gave the maximum yield of new product in the 1ml. incubation mixtures (see Table 2).

Large-scale incubation mixture for the identification of products synthesized from artificial substrates and [1-^{14}C]-*isopentenyl pyrophosphate*. When the standard assays

Table 2. *Data on incubations for identification of products synthesized by prenyltransferase from new allylic pyrophosphate substrates*

Substrate pyrophosphate	Amount of substrate (μmoles)	Amount of [1-^{14}C]-isopentenyl-pyrophosphate (μmoles)	Vol. of incubation mixture (ml.)	Percentage of ^{14}C found in allylic pyrophosphate products
trans-3-Ethyl-3-methylallyl	7·50	7·50	24·7	31·0
cis-3-Ethyl-3-methylallyl	2·64	2·88	17·0	22·4
3,3-Diethylallyl	5·50	4·60	11·1	26·5
cis- plus *trans*-3-Methyl-3-*n*-propyl	2·18	4·28	8·5	49·0

indicated that a new allylic pyrophosphate substrate had been found, large-scale reaction mixtures (8·5–24·7 ml.) were set up (see Table 2) and incubated for 3 hr. so that products might be obtained in amounts sufficient for characterization.

In these experiments the allylic pyrophosphates were not hydrolysed with acid but with alkaline phosphatase, exactly as described for the identification of 10,11-dihydrofarnesyl pyrophosphate synthesized by prenyltransferase from 6,7-dihydrogeranyl pyrophosphate and [^{14}C]isopentenyl pyrophosphate (Expt. *B*; Popják *et al.* 1969*a*). In the experiments made with the *cis*- and *trans*-3-ethyl-3-methylallyl pyrophosphate the ^{14}C-labelled alcohols liberated by the phosphatase were purified by t.l.c. as described by Popják *et al.* (1969*a*), and analysed both by gas–liquid radiochromatography and by mass spectrometry (see Figs. 1 and 2). In the experiments with 3,3-diethylallyl pyrophosphate and 3-methyl-3-*n*-propylallyl pyrophosphate the products were examined only by gas–liquid radiochromatography after addition of unlabelled linalool, geraniol, nerolidol and farnesol to the extracts from incubation mixtures.

RESULTS

The preliminary small-scale experiments indicated that only four out of the 16 synthetic allylic pyrophosphates were accepted by prenyltransferase as substrates. These were the *trans*- and *cis*-3-ethyl-3-methylallyl, 3,3-diethylallyl and 3-methyl-3-*n*-propylallyl pyrophosphates. To establish the nature of the products synthesized from these substances plus isopentenyl pyrophosphate, large-scale incubations were carried out (Table 2) in which the allylic pyrophosphates synthesized were first hydrolysed with intestinal alkaline phosphatase; the liberated alcohols were extracted and examined as described by Popják *et al.* (1969*a*).

Fig. 1 is the record of the gas–liquid radiochromatographic analysis of the alcohol obtained from the experiment with the *trans*-3-ethyl-3-methylallyl pyrophosphate and shows that more than 90% of the radioactivity in the extract was associated with a substance that had a retention volume (R_v) 1·32 times that of *trans–trans*-farnesol. An identical result was obtained with the *cis*-3-ethyl-3-methylallyl pyrophosphate, the R_v of the main radioactive component in the extract being also 1·32 relative to that of *trans–trans*-farnesol. The radioactive alcohols from these two experiments were purified on thin-layer plates as described for farnesol and for 10,11-dihydrofarnesol (Donninger & Popják, 1966; Popják *et al.* 1969*a*) and were examined by mass spectrometry. Both specimens had mol.wt. 236 and identical mass spectra. Fig. 2 is the mass spectrum of the alcohol obtained from the experiment with the *cis*-3-ethyl-3-methylallyl pyrophosphate, and shows unambiguously that the substance was homofarnesol (II). The primary-alcohol nature of the compound was indicated by the loss of water from the molecular ion ($[236-18]^+$) and by the presence of a relatively intense ion at *m/e* 31. That the one methylene group additional to those present in farnesol was at the ω-end of the molecule was shown by the loss of a C_4 unit from the dehydrated ion ($[218-57]^+$ and $[218-55]^+$, giving

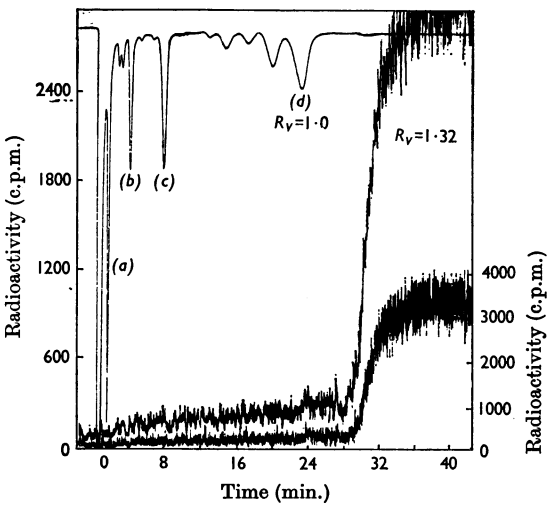


Fig. 1. Photograph of gas–liquid radiochromatogram of the product derived from the reaction between *trans*-3-ethyl-3-methylallyl pyrophosphate and [1-^{14}C]isopentenyl pyrophosphate. Unlabelled linalool (*a*), geraniol (*b*), nerolidol (*c*), *cis–trans*-farnesol and *trans–trans*-farnesol (*d*) were added to the extract obtained from the experiment. The radioactivity was recorded at two levels of sensitivity. The column temperature was 177°.

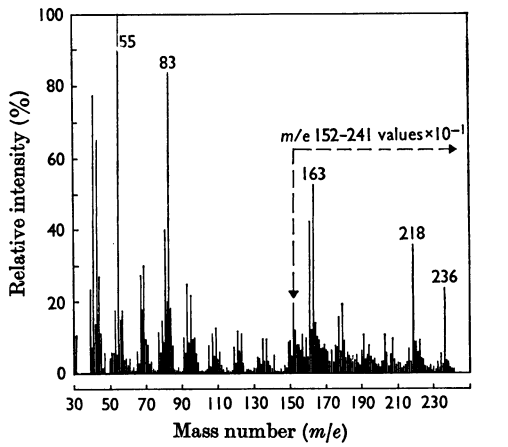


Fig. 2. Mass spectrum of the product synthesized by prenyltransferase from *cis*-3-ethyl-3-methylallyl pyrophosphate and [1-^{14}C]isopentenyl pyrophosphate. The relative intensity of the peaks above *m/e* 152 was multiplied by 10.

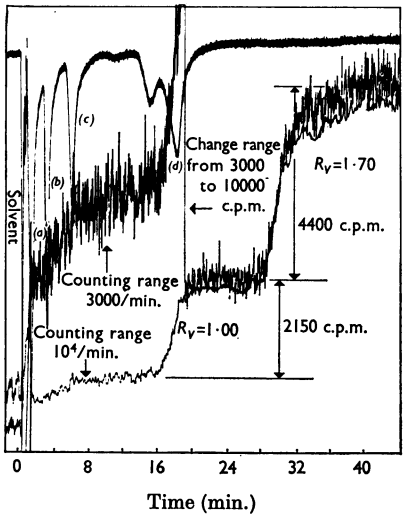


Fig. 3. Photograph of gas–liquid radiochromatogram of the products synthesized on incubation of prenyltransferase with 3-methyl-3-*n*-propylallyl pyrophosphate (*cis* + *trans*) and [1-^{14}C]isopentenylpyrophosphate as substrates. More than one-half of the total radioactivity in the specimen had R_v1·7 relative to *trans–trans*-farnesol. The presence of radioactive farnesol indicated that the enzyme preparation contained isopentenyl pyrophosphate isomerase as well as prenyltransferase. Unlabelled prenols were added to the specimen (see Fig. 1 for definition of symbols). The column temperature was 185°.

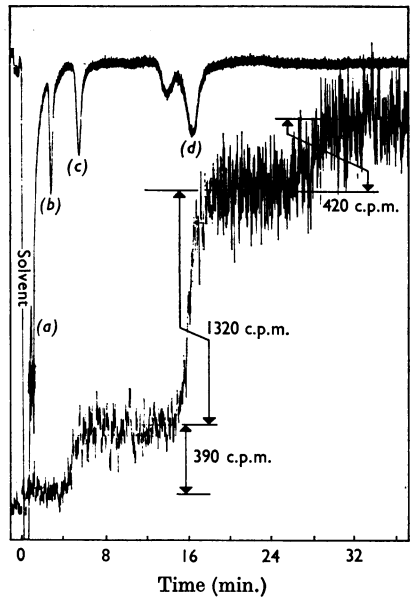
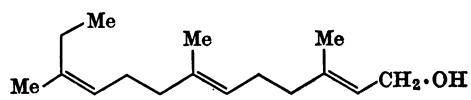


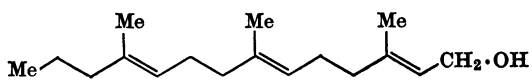
Fig. 4. Photograph of gas–liquid radiochromatogram of products synthesized in an incubation of prenyltransferase in which 3,3-diethylallyl pyrophosphate and [1-^{14}C]-isopentenyl pyrophosphate were the substrates. The first significant radioactive fraction (390 c.p.m.) preceded the emergence of nerolidol; its R_v relative to geraniol was 1·7. The R_v of the radioactive fraction that emerged after *trans–trans*-farnesol was 1·70 relative to farnesol. The presence of [^{14}C]farnesol in the specimen indicated that the enzyme preparation contained isopentenyl pyrophosphate isomerase as well as prenyltransferase. Unlabelled prenols were added to the specimen (abbreviations are defined in Fig. 1). The column temperature was 190°.

ions at *m/e* 161 and 163) and also by the loss of a C_6 unit from it (peaks at *m/e* 135 and 83). In the mass spectrum of farnesol the dehydrated ion is found at *m/e* 204, which loses characteristically 43 (C_3) and 69 (C_5) mass units, the base peak being at *m/e* 69. In the present spectrum the base peak was at *m/e* 55 and was accompanied by a peak of nearly equal intensity at *m/e* 83, the peak at *m/e* 69 being uncommonly small for a polyprenyl substance. An authentic specimen of 12-methylfarnesol (3,7,11-trimethyltrideca-2,6,10-trien-1-ol) gave a mass spectrum identical with that shown in Fig. 2.

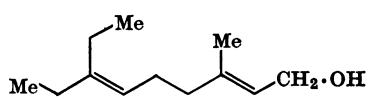
In the two further experiments, with 3-methyl-3-*n*-propylallyl pyrophosphate and 3,3-diethylallyl pyrophosphate, the enzyme preparation used contained also isopentenyl pyrophosphate isomerase. This became apparent from the analysis by gas–liquid radiochromatography of the alcohols extracted from the incubation mixtures (Figs. 3 and 4): significant amounts of farnesol were found in both. In the experiment with 3-methyl-3-*n*-propylallyl pyrophosphate (compounds 15+16) a little over one-half of the total radioactivity of the specimen was, however, accounted for by a fraction with R_v 1·70 relative to *trans–trans*-farnesol (Fig. 3). In the preparation derived from the experiment



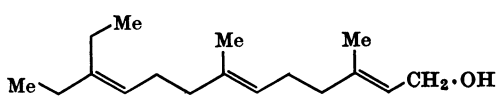
(II)



(III)



(IV)



(V)

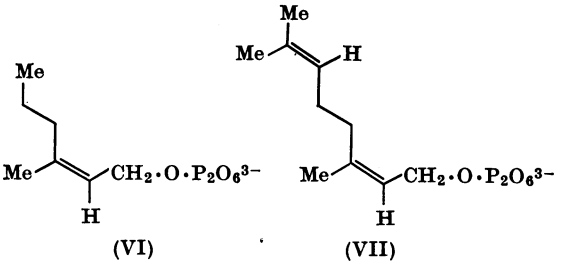
with 3,3-diethylallyl pyrophosphate (compound 14) there were two significant radioactive components in addition to farnesol: one of these had R_v 1·70 times that of geraniol and the other 1·70 times that of *trans–trans*-farnesol (Fig. 4). The identity of these products can be deduced from a consideration of their retention volumes and of the structure of the allylic pyrophosphates which were the condensing partners with isopentenyl pyrophosphate. Since the R_v of homofarnesol (II) relative to farnesol was 1·32, the predicted R_v of bis-homofarnesol is $1{\cdot}32^2 \simeq 1{\cdot}74$ relative to farnesol; similarly bis-homogeraniol should have a $R_v \simeq 1{\cdot}74$ relative to geraniol. These predicted values are sufficiently close to those observed to make us confident that compound (15+16) gave 3,7,11-trimethyltetradeca-2,6,10-trienol (III) and compound 14 to both 7-ethyl-3-methylnona-2,6-dienol (IV) and 11-ethyl-3,7-dimethyltrideca-2,6,10-trienol (V).

Detailed kinetic studies have not yet been made with the present artificial substrates. The phenomenon of substrate inhibition was, however, noted with both the *cis*- and the *trans*-3-ethyl-3-methylallyl pyrophosphates at concentrations higher than about 50 μM.

DISCUSSION

The results have defined the structural features of allyl pyrophosphates needed for a substrate of prenyltransferase. With reference to formula (I), the observations showed that R_3 cannot be other than a hydrogen atom and that C-3 must be disubstituted. This conclusion follows from our finding that neither the 2,3,3-trimethylallyl or the unsubstituted allyl pyrophosphate, nor the *cis*- or *trans*-3-methylallyl, nor the 2,2-dimethylallyl pyrophosphates were substrates. 3,3-Dimethylallyl pyrophosphate is thus the simplest allylic substrate for prenyltransferase. The requirements of the enzyme are not very stringent, however, with respect to the groups R_1 and R_2. Since previous investigation had shown that R_2 could be as large as $(CH_3)_2CH{\cdot}[CH_2]_3-$, as in 6,7-dihydrogeranyl pyrophosphate (Popják *et al.* 1969*a*) it was not surprising that an ethyl or *n*-propyl group was equally acceptable in that position.

The substituent R_1 can be at least as large as $CH_3{\cdot}CH_2-$, but it is uncertain whether the *n*-propyl group in this position would be compatible with reactivity with the enzyme or not. The specimen of the 3-methyl-3-*n*-propylallyl pyrophosphate, which gave rise to one of the bis-homofarnesyl pyrophosphates, contained both the *cis*- and *trans*-isomers because we could not separate them in the preparation of either the allylic ester or the alcohol, which were the starting materials for the synthesis of this pyrophosphate. It is uncertain, therefore,



whether the *cis*- or the *trans*-isomer or both had reacted on the enzyme with isopentenyl pyrophosphate. It seems nevertheless more likely that only the *trans*-isomer reacted because the *cis*-3-methyl-3-*n*-propylallyl pyrophosphate (VI) resembles structurally neryl (*cis*-geranyl) pyrophosphate (VII), which is not a substrate but an inhibitor of prenyltransferase (Popják *et al.* 1969*b*). The gas–liquid radiochromatogram shown in Fig. 3 indicated only a single component in the fraction with R_v 1·70 relative to *trans–trans*-farnesol.

Liver prenyltransferase has never been observed to synthesize from either 3,3-dimethylallyl pyrophosphate or geranyl pyrophosphate plus isopentenyl pyrophosphate a substance larger than farnesyl pyrophosphate. We supposed that the reason for this restriction was the inability of the enzyme to accommodate in the lipophilic area of the substrate-binding site an alkyl chain longer than that of geranyl pyrophosphate. Our results showed that even bis-homogeranyl pyrophosphate can be bound to the enzyme, but it remains to be seen whether *trans*-3-*n*-butyl-3-methylallyl or *trans*-3-methyl-3-*n*-pentylallyl pyrophosphates (which are likely to be substrates for prenyltransferase) would give rise to the synthesis of only 8-propyl- and 8-butylgeranyl pyrophosphates, or also of 12-propyl- and 12-butylfarnesyl pyrophosphates. If spatial restriction controls the chain length of the substance synthesized, then only the homologues of geranyl pyrophosphate should be synthesized. The accumulation of not only bishomofarnesyl but also of bishomogeranyl pyrophosphate in the experiment with 3,3-diethylallyl pyrophosphate (Fig. 4) might have been the expression of such a spatial restriction, for we have never seen the accumulation of geranyl pyrophosphate on incubation of prenyltransferase with 3,3-dimethylallyl pyrophosphate and isopentenyl pyrophosphate.

Holloway & Popják (1967) suggested that the substrates of prenyltransferase fit into a narrow groove in the protein. This idea is now supported by the fact that neither 3-*tert.*-butyl-3-methylallyl nor 3-methyl-3-phenylallyl pyrophosphate was either a substrate or an inhibitor of the enzyme, in

contrast with the reactivity of 3-methyl-3-propyl-allyl pyrophosphate.

J. L. R. was on leave of absence from the University of Pennsylvania, Veterans Administration Hospital, Philadelphia, Pa. 19104, U.S.A.

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