Mechanism of the Pyrophosphate Migration in the Enzymatic Cyclization of Geranyl and Linalyl Pyrophosphates to (+)- and (−)-Bornyl Pyrophosphates†

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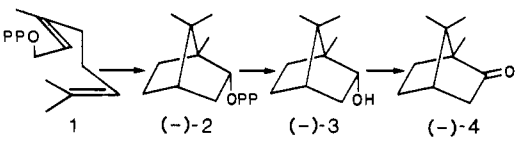
ABSTRACT: Soluble enzymes from sage (*Salvia officinalis*) and tansy (*Tanacetum vulgare*), which catalyze the cyclization of geranyl pyrophosphate and the presumptive intermediate linalyl pyrophosphate to the (+) and (−) enantiomers, respectively, of 2-bornyl pyrophosphate, were employed to evaluate mechanistic alternatives for the pyrophosphate migration in monoterpene cyclization reactions. Separate incubation of [1-$^3H_2$,α-^{32}P]- and [1-$^3H_2$,β-^{32}P]geranyl and (±)-linalyl pyrophosphates with partially purified preparations of each enantiomer-generating cyclase gave [^3H,^{32}P]bornyl pyrophosphates, which were selectively hydrolyzed to the corresponding bornyl phosphates. Measurement of ^3H:^{32}P ratios of these monophosphate esters established that the two ends of the pyrophosphate moiety retained their identifies in the cyclization of both precursors to both products and also indicated that there was no appreciable exchange with exogenous inorganic pyrophosphate in the reaction. Subsequent incubations of each cyclase with [8,9-^{14}C,1-^{18}O]geranyl pyrophosphate and with (1*E*)-(±)-[1-^3H,3-^{18}O]linalyl pyrophosphate gave the appropriate (+)- or (−)-bornyl pyrophosphates, which were hydrolyzed in situ to the corresponding borneols. Analysis of the derived benzoates by mass spectrometry demonstrated each of the product borneols to possess an ^{18}O enrichment essentially identical with that of the respective acyclic precursor. The absence of P_α–P_β interchange and the complete lack of positional ^{18}O isotope exchange of the pyrophosphate moiety are compatible with tight ion pairing of intermediates in the coupled isomerization–cyclization of geranyl pyrophosphate and establish a remarkably tight restriction on the motion of the transiently generated pyrophosphate anion with respect to its cationic terpenyl reaction partner.

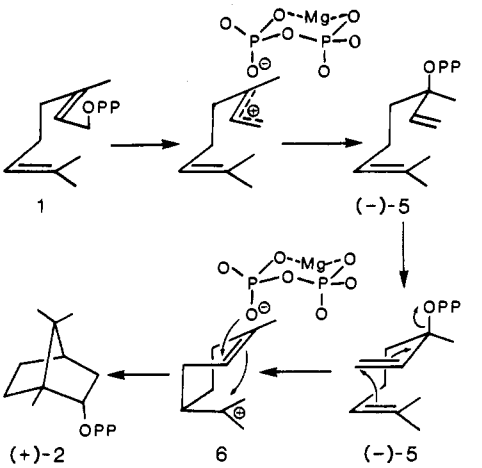
The biosynthesis of camphor (**4**) has been shown to involve the cyclization of geranyl pyrophosphate (**1**) to 2-bornyl pyrophosphate (**2**), which is hydrolyzed by a distinct pyrophosphatase to borneol (**3**) and subsequently oxidized to the bicyclic monoterpene ketone by an NAD-dependent dehydrogenase (Scheme I) (Croteau, 1984). Cyclases that catalyze the Mg^{2+}-dependent conversion of the common, achiral substrate to (+)-bornyl pyrophosphate and to (−)-bornyl pyrophosphate have been isolated from sage (*Salvia officinalis*) and from tansy (*Tanacetum vulgare*), respectively, and have been partially purified and characterized (Croteau & Karp, 1979a; Croteau & Shaskus, 1985). These enantiomer-generating enzymes are unique, thus far, among monoterpene cyclases in affording a pyrophosphate ester as the initial cyclic product, and they thus provide an unusual, direct opportunity to examine the role of the pyrophosphate moiety in monoterpene cyclization processes.

In the absence of competing phosphatases and pyrophosphatases, both geranyl pyrophosphate and its tertiary allylic isomer, linalyl pyrophosphate (**5**), are strongly preferred as substrates over the corresponding *cis*-allylic pyrophosphate neryl pyrophosphate, and all three acyclic precursors are cyclized without preliminary interconversion or formation of other detectable free intermediates (Croteau, 1984). On the basis of these and related studies with other monoterpene cyclases and sesquiterpene cyclases (Cane et al., 1981; Croteau & Cane, 1985; Croteau, 1985) and on the need to accommodate the topological impediment to the direct cyclization of geranyl pyrophosphate, a stereochemical model for the

Scheme I



Scheme II



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cyclization to bornyl pyrophosphate has been proposed (Scheme II). The basic elements of this multistep scheme are divalent metal ion assisted ionization with syn migration of the pyrophosphate to generate the bound tertiary allylic ester intermediate linalyl pyrophosphate (**5**), the initial cyclization of the cisoid, anti-endo conformer, and the subsequent electrophilic addition to the newly created cyclohexene double bond with capture of the resulting cation by the pyrophosphate anion to provide bornyl pyrophosphate (**2**) with the requisite stereochemistry. This broadly applicable proposal is entirely consistent with the results of numerous model studies of terpenoid cyclizations (Gotfredsen et al., 1977; Baxter et al., 1978; McCormick & Barton, 1978; Poulter & King, 1982a,b) and is strongly supported by a recent investigation of the stereochemistry at C1 of geranyl pyrophosphate in the enzymatic isomerization–cyclization reaction (Croteau et al., 1985).

During the course of the hypothetical transformation illustrated (Scheme II), the inorganic pyrophosphate moiety released by the ionization of the geranyl substrate may become more or less free of its cationic partner in subsequent steps, depending on the precise course of the reaction. For example, tumbling of the pyrophosphate anion would render the six nonbridged oxygens equivalent, whereas, at the other extreme, rigid binding of the Mg^{2+}–inorganic pyrophosphate complex could prevent scrambling of even the proximal pyrophosphate oxygen atoms. These and other mechanistic alternatives for the pyrophosphate migration, including ion pairing of intermediates as favored here, have been discussed (Cane, 1980; Cane et al., 1981). To probe the role of the pyrophosphate moiety in the reaction sequence and to explore the timing of bond-breaking and bond-making steps, we have prepared a series of specifically ^{18}O- and ^{32}P-labeled acylic precursors and examined their enzymatic conversion to the enantiomeric bornyl pyrophosphates. In this paper we detail preliminary experiments with ^{18}O- and ^{32}P-labeled geranyl pyrophosphate (Cane et al., 1982a), extend these studies with similarly labeled linalyl pyrophosphate to more precisely define the fate of the pyrophosphate in the transformation, and provide results that support an ion pair mechanism and imply very tight restriction on the motion of the transiently generated pyrophosphate anion during the coupled isomerization–cyclization reaction.

Materials and Methods

Labeled Substrates. [^{14}C]Methyl iodide (58 Ci/mol) was purchased from Amersham. [^{3}H]Lithium aluminum hydride (180 Ci/mol) was obtained from New England Nuclear Corp. [1-$^{3}H_2$]Geraniol[1] (150 Ci/mol), (±)-[1-$^{3}H_2$]linalool (150 Ci/mol), and (+)-[G-^{3}H]borneol and its mono- and pyrophosphate esters (all at 2 Ci/mol) were prepared as described previously (Croteau & Karp, 1976, 1977a,b). $H_2{}^{18}O$ (at 80, 95, and 99 atom %) was obtained from ICN-KOR Isotopes or Mound Laboratories. [^{32}P]Orthophosphoric acid (15 mCi, carrier free in H_2O from New England Nuclear Corp.) was diluted to a specific activity of 50 Ci/mol, and portions were converted to [^{32}P]bis(triethylammonium) hydrogen phosphate (Cornforth & Popják, 1969), to [^{32}P]bis(tri-*n*-butylammonium) hydrogen phosphate (Upper & West, 1967), and to [$^{32}P_2$]pyrophosphoric acid (Kornberg & Pricer, 1951), which was purified by paper chromatography (Saito & Rilling, 1979).

Each [1-$^{3}H_2$]terpenol was converted (at the 50–100-μmol scale) to a mixture of the corresponding phosphate and pyrophosphate esters by the Cramer & Böhm (1959) method essentially as described by Cornforth & Popják (1969) with unlabeled or ^{32}P-labeled bis(triethylammonium) hydrogen phosphate as the phosphorylating reagent. Crude reaction products were treated with inorganic pyrophosphatase (Del Campo et al., 1977), and the terpenyl monophosphate and pyrophosphate esters were separated from inorganic phosphate by ion-exchange chromatography on DEAE-cellulose[2] [2 × 20 cm, with a 10–50 mM $(NH_4)_2CO_3$ gradient, pH 9.0, 400 mL, 2.8-mL fractions] (Dugan et al.1968; Croteau & Karp, 1976). Fractions containing the respective monophosphate esters (18–26% yields from geraniol, 11–17% yields from linalool) and pyrophosphate esters (9–14% yields from geraniol, 5–7% yields from linalool) were separately pooled and lyophilized to remove $(NH_4)_2CO_3$, and each was subjected again to ion-exchange chromatography as above to remove all traces of inorganic phosphates from the respective terpenyl phosphate esters. The [1-$^{3}H_2$]terpenyl phosphates and [1-$^{3}H_2$,^{32}P]-terpenyl phosphates were converted to the corresponding pyrophosphates by the general procedure of Michelson (1964), with [^{32}P]bis(tri-*n*-butylammonium) hydrogen phosphate and the unlabeled salt, respectively, as the source of the terminal phosphate. In brief, the monophosphates [as the anhydrous mono(tri-*n*-butylammonium) salts] were converted to the terpenyl pyrophosphate via the intermediate formation of the P_β-diphenyl P_α-terpenyl pyrophosphate [with diphenyl chlorophosphate and tributylamine in dry dioxane–DMF (7:3)] essentially as described Michelson, 1964). However, the diphenyl intermediate itself was not isolated; rather, the reaction mixture was concentrated under vacuum to a syrup and extracted with ether, and the extract was concentrated and dried under vacuum. Following dissolution in dry dioxane, the mixture was treated directly with the appropriate labeled or unlabeled bis(tri-*n*-butylammonium) hydrogen phosphate in pyridine to effect the conversion to the pyrophosphate. This modification has been previously employed by Upper & West (1967) in the synthesis of geranylgeranyl pyrophosphate. The reaction mixture was partitioned between ether and 0.2 M NH_4OH, and following lyophilization of the aqueous phase, the terpenyl pyrophosphate was isolated (free of all radioactive contaminants) by ion-exchange chromatography as before. At the 10-μmol scale, yields of geranyl pyrophosphate were 13–18% on the basis of the monophosphate, whereas with linalyl pyrophosphate the corresponding yields were in the 5–9% range. The combination of the above procedures afforded [1-$^{3}H_2$]geranyl pyrophosphate, [1-$^{3}H_2$]linalyl pyrophosphate, and the six ^{3}H,^{32}P-labeled substrates. The latter were further diluted with the respective [1-^{3}H]terpenyl pyrophosphate (at 150 Ci/mol) to a ^{3}H:^{32}P ratio of ~10 (see Table I). Each substrate, following purification, adjustment of isotope ratio, and lyophilization, was dissolved in 10 mM NH_4HCO_3 (at ~1 mM) and frozen at −40 °C for later use.

To verify the position of ^{32}P-labeling in each of the substrates, 3-μCi (^{3}H) batches were hydrogenated over Pd in MeOH (Jacobson et al., 1957). Following workup, the tet-

[1] Geranyl pyrophosphate and linalyl pyrophosphate, unless otherwise designated, were labeled by reduction of an aldehyde with $NaB^{3}H_4$ and therefore contain ^{3}H at *either* of the C1 hydrogens (i.e., equal amounts of 1(*R*)- and 1(*S*)- or 1(*E*)- and 1(*Z*)-^{3}H). Thus, the substrate behaves as if both hydrogens at C1 are labeled, although no molecule of the substrate has *both* hydrogens replaced by ^{3}H. We designated this by the notation [1-$^{3}H_2$].

[2] Abbreviations: DEAE, *O*-(diethylaminoethyl); MES, 2-(*N*-morpholino)ethanesulfonic acid; THF, tetrahydrofuran; DMF, dimethylformamide; THP, tetrahydropyranyl; TLC, thin-layer chromatography; GLC–MS, gas–liquid chromatography–mass spectrometry; HPLC, high-pressure liquid chromatography; NMR, nuclear magnetic resonance.

rahydrogeranyl pyrophosphate was hydrolyzed to the monophosphate by treatment with 0.25 mL of 0.5 N HCl at 100 °C for 15 min (Tidd, 1971), and after lyophilization, the products were separated by TLC (silica gel H with 1-propanol–concentrated NH_4OH–H_2O, 6:3:1 v/v/v) (Cornforth & Popják, 1969). The monophosphate ester was located by radio scanning the plate (R_f 0.48–0.52), eluted from the gel with H_2O, and following lyophilization and dissolution in MeOH, an aliquot was counted (50–60% overall yield, see Table I). Tetrahydrolinalyl pyrophosphate was hydrolyzed by treatment for 1 h at 30 °C with 0.1 mL of 50 mM sodium acetate buffer, pH 6.0, containing 0.5 unit of potato apyrase (Sigma type VII) (Del Campo et al., 1977), and following lyophilization and TLC isolation of the monophosphate as above, an aliquot was counted (40–50% overall yield, see Table I).

[8,9-^{14}C,1-^{18}O]Geranyl pyrophosphate (375 mCi/mol, 67.9% ^{18}O atom; 14.2 mCi/mol, 68.2% ^{18}O atom) was prepared by mixing of appropriate proportions of [8,9-^{14}C]geraniol and [1-^{18}O]geraniol, conversion to the pyrophosphate ester by the method of Cramer & Böhm (1959), as adapted by Cornforth & Popják (1969), and purification on Dowex 1-X8 (200–400) with a linear gradient of methanolic ammonium formate as previously described (Cane et al., 1981). To prepare [8,9-^{14}C]geraniol, 20 μL (50 μmol) of 2.5 M *n*-butyllithium was added to a stirred suspension of 6.3 mg (17 μmol) of ethyltriphenylphosphonium bromide in 0.5 mL of THF at 0 °C under nitrogen. (The excess of *n*-butyllithium was required because of the small scale of the reaction. When metallation is carried out on a millimole scale, stoichiometric amounts of alkyllithium reagent are sufficient.) The mixture was stirred for 1 h, after which a solution of 0.5 mCi (10 μmol) of [^{14}C]methyl iodide in 1 mL of hexane was added, followed by 0.3 μL (5 μmol) of unlabeled methyl iodide in 10 μL of THF. The resulting white slurry was stirred for an additional hour at 0 °C and then reacted with 20 μL (50 μmol) of 2.5 M *n*-butyllithium for 1 h at 0 °C before addition of 3.6 mg (17 μmol) of geranyl tetrahydropyranyl ether trisnoraldehyde (Corey et al., 1969). The mixture was stirred for a further hour at 0 °C and then overnight at room temperature. The resulting pale yellow solution was poured into half-saturated NH_4Cl in 40% aqueous methanol and extracted 3 times with pentane. The combined extracts were washed with half-saturated NH_4Cl in 40% aqueous methanol, water, and saturated brine and then dried over sodium sulfate. After concentration under reduced pressure, the recovered [8,9-^{14}C]geranyl tetrahydropyranyl ether was dissolved in 0.5 mL of methanol to which was added 3 mg of *p*-toluenesulfonic acid. The solution was stirred for 2.5 h at room temperature to effect cleavage of the THP ether. After quenching in saturated $KHCO_3$ and extraction into pentane, the isolated [8,9-^{14}C]geraniol was purified by preparative layer chromatography on silica gel (R_f 0.27, with hexanes–ethyl acetate, 4:1 v/v).

The corresponding [1-^{18}O]geraniol was prepared by reaction of 25.2 mg (0.29 mmol) of [1,1-$^{18}O_2$]sodium acetate (Hutchinson & Makune, 1976) [84.1% ^{18}O atom, determined on the derived *p*-phenylphenacyl acetate as described by Cane et al. (1982b)] with 54.3 mg (0.25 mmol) of geranyl bromide and 3.6 mg (0.024 mmol) of sodium iodide in 1.0 mL of dry DMF under nitrogen at room temperature overnight. Addition of water and extraction with ether gave, after washing with water and saturated NaCl and drying over anhydrous sodium sulfate, geranyl acetate contaminated with ca. 25% geranyl formate resulting from reaction of geranyl bromide with the solvent DMF. The two products were readily separated by silica gel column chromatography (pentane–ether, 19:1 v/v). Methanolysis of the recovered [^{18}O]geranyl acetate (50 mg) in the presence of 165 mg of anhydrous K_2CO_3 for 30 min at room temperature gave, after routine extraction and concentration under reduced pressure, 40 mg of [1-^{18}O]geraniol, containing 73.5% ^{18}O atom as determined by mass spectrometric analysis of the *m/e* 154 (P) and 156 (P + 2) ions. After admixture with [8,9-^{14}C]geraniol, the combined samples were subjected to HPLC purification (μPorasil, 20% ether in hexanes) prior to conversion to the pyrophosphate ester as described above.

For the preparation of (1*E*)-(±)-[1-^{3}H,3-^{18}O]linalyl pyrophosphate, the tritiated linalool was obtained by stereospecific reduction of [1-^{3}H]-1,2-dehydrolinalool (Corey et al., 1967). Dehydrolinalool (40 mg, 0.26 mmol), prepared by reaction of ethynylmagnesium bromide with 6-methyl-5-hepten-2-one (Cane et al., 1981), was treated with 0.18 mL (0.38 mmol) of 2.1 M *n*-butyllithium on 0.5 mL of THF at 0 °C under nitrogen. The solution was stirred for 2 h at room temperature before addition of 10 μL of [^{3}H]water (10 mCi, 0.56 mmol). After an additional hour, the solution was poured into saturated NH_4Cl and extracted with ether. Drying and concentration of the extracts gave 35 mg (85%) of [1-^{3}H]-dehydrolinalool (4.03 Ci/mol, 9.3% radiochemical yield). The entire sample (35 mg, 0.23 mmol) was reacted with 44.6 mg (1.18 mmol) of lithium aluminum hydride and 126 mg (2.33 mmol) of sodium methoxide in 1.5 mL of THF, and the mixture was refluxed overnight. Excess reducing agent was consumed by successive addition of 0.03 mL of water, 0.03 mL of 15% methanol, and 0.09 mL of water. After addition of Celite, centrifugation, and washing of the precipitated solids with ether, the ethereal solution was shaken with saturated NaCl, dried, and concentrated. The residual oil was purified by flash column chromatography (Still et al., 1978) (pentane–ether, 19:1 v/v) to give 26 mg (63%) of (1*E*)-(±)-[1-^{3}H]linalool (1.35 Ci/mol).

The corresponding (±)-[3-^{18}O]linalool was prepared from [^{18}O]-6-methyl-5-hepten-2-one. The requisite labeled ketone was obtained by treatment of 1.0 g (7.3 mmol) of 6-methyl-5-hepten-2-one with 1.18 g (7.93 mmol) of freshly distilled triethyl orthoformate in 1.1 mL of ethanol in the presence of 2 μL of concentrated sulfuric acid for 18 h at room temperature. The reaction mixture was then heated at 60 °C for 4.5 h, quenched with ammonium hydroxide, and extracted with ether. Concentration of the dried extracts and distillation of the residue under reduced pressure gave 1.6 g (62%) of the diethyl ketal [bp (8 mm) 83–85 °C]. The ketal was hydrolyzed with 30 μL of [^{18}O]water (99% ^{18}O atom) and ca. 1 mg of *p*-toluenesulfonic acid in 10 mL of methylene chloride for 0.5 h at room temperature. Solid anhydrous Na_2CO_3 was added to the reaction mixture to neutralize the acid, the solid was removed by filtration, and the solvent was removed under vacuum to yield 87 mg (81%) of [^{18}O]-6-methyl-5-hepten-2-one. The labeled ketone (0.69 mmol) was dissolved in 3.0 mL of THF at 0 °C, and 0.7 mL (1.38 mmol) of 2.6 M vinylmagnesium bromide was added dropwise. After 15 min at 0 °C, the solution was stirred for 1 h at room temperature. Excess vinylmagnesium bromide was destroyed by addition of methanol followed by 50% saturated NH_4Cl. Extraction with ethyl ether, washing with saturated $KHCO_3$, and flash chromatography of the dried and concentrated extract (ethyl ether–hexanes, 1:9 v/v) gave 91 mg of [3-^{18}O]linalool (86%). The ^{18}O content of the alcohol was established by mass spectrometric analysis of the derived trimethylsilyl ether: 85.7 ± 0.3% ^{18}O atom. A portion of the (1*E*)-[1-^{3}H]linalool (11.7

mg, total activity 2.28×10^8 dpm) was mixed with 70 mg of [3-^{18}O]linalool, and the resulting mixture (0.193 Ci/mol ^{3}H) was converted to the corresponding (1*E*)-(±)-[1-^{3}H,3-^{18}O]-linalyl pyrophosphate by the method of Cramer & Böhm (1959). The pyrophosphate ester was purified by chromatography on DEAE-Sephadex A-25 (1 × 24 cm column, 18-mL bed volume, bicarbonate form) by elution with 60 mL of 0.1 M ammonium bicarbonate followed by 400 mL of a linear gradient of 0.10–0.25 M ammonium bicarbonate, collecting 4-mL fractions which were assayed for radioactivity. Fractions 14–24 (ionic strength 0.11 M) contained the monophosphate ester while fractions 46–57 (ionic strength 0.175 M) contained the desired pyrophosphate. Repeated lyophilization and addition of water gave 15.4 mg of (1*E*)-(±)-[1-^{3}H,3-^{18}O]linalyl pyrophosphate.

Preparation of Cyclases. The preparation, assay, and general properties of (+)-bornyl pyrophosphate cyclase (sage, *Salvia officinalis*) and (−)-bornyl pyrophosphate cyclase (tansy, *Tanacetum vulgare*) have been previously described (Croteau & Karp, 1979a; Croteau & Shaskus, 1985). For bulk enzyme incubations with ^{18}O-labeled substrates, in which maximum production of the cyclic product was desired and in which the presence of endogenous competing phosphatases/pyrophosphatases (Croteau & Karp, 1979b) was of no consequence, 105000*g* supernatants were prepared from whole leaf homogenates (10–15 g of tissue) and were concentrated to ~1 mg of protein/mL by ultrafiltration (Amicon PM30). The concentrates were next slurried (30 min at 0–4 °C) with an equal tissue weight of XAD-4 polystyrene resin to remove endogenous terpenoids (Loomis et al., 1979), filtered through glass wool, and then dialyzed before use against 5 mM sodium phosphate buffer, pH 6.2, containing 0.5 mM dithioerythritol, 1 mM sodium ascorbate, 10 mM $MgCl_2$, and 5% sucrose. Activity of these preparations was routinely assessed by radiochemical assay with [1-$^3H_2$]geranyl pyrophosphate as substrate.

For experiments with ^{3}H,^{32}P-labeled substrates, in which it was necessary to isolate the pyrophosphorylated end product, homogenates from mechanically stripped leaf epidermis were prepared (Croteau & Cane, 1985). Following centrifugation and concentration as before, these soluble preparations were fractionated by chromatography on a 2.5 × 120 cm column of Sephadex G-150 previously equilibrated and eluted with 15 mM MES buffer, pH 6.2, containing 0.5 mM dithioerythritol, 10 mM $MgCl_2$, and 5% sucrose (30 mL/h, 5-mL fractions). This extraction and purification procedure served to eliminate the bulk of the competing phosphatase/pyrophosphatase activity, and in the case of the sage epidermis preparation, it also removed the small amount of (−)-bornyl pyrophosphate cyclase activity having a lower molecular weight. Bornyl pyrophosphate cyclase was located by radiochemical assay (with [1-$^3H_2$]geranyl pyrophosphate), and the two or three column fractions containing the maximum activity were utilized as the enzyme source in subsequent experiments.

Experiments with ^{32}P-Labeled Precursors. The typical assay was run in a screw-capped vial in 5 mL of the MES buffer system, pH 6.2, containing dithioerythritol, $MgCl_2$, and sucrose as above, and ~500 μg of the partially purified protein. The reaction was initiated by the addition of substrate (to 20 μM) followed by incubation for 2 h at 30 °C. A sufficient number of such assays were run to ensure the production of a minimum of 0.25 μCi (^{3}H) of bornyl pyrophosphate. At the end of each incubation, ~10 μmol of (±)-bornyl pyrophosphate was added as carrier to each tube, followed by the addition of 0.5 mL of 10 N HCl and 2 mL of pentane with shaking at 30 °C for 30 min to effect the solvolysis of residual substrate (Cornforth & Popják, 1969; Tidd, 1971). The pentane solution was removed, and the opened tubes were then heated at 100 °C for 15 min to hydrolyze bornyl pyrophosphate to the monophosphate ester (Tidd, 1971). Upon cooling, the contents of each tube were centrifuged to remove denatured protein, the supernatants from like samples were combined, and the bornyl phosphate contained therein was extracted into H_2O-saturated 1-butanol (3 × 5 mL) (Croteau & Karp, 1979b). The butanol extract was treated with 0.5 mL of pyridine containing 5 mg of OsO_4 (the procedure converts olefinic compounds to the corresponding diols, and it was included here to reduce undefined ^{3}H background to a negligible level), and following stirring for 3 h at 30 °C and back-washing with 1 N HCl, the butanol extract was concentrated to dryness under vacuum. The dry residue was dissolved in a minimum volume of 2-propanol–NH_4OH (sp gravity 0.88)–H_2O (6:3:1 v/v/v) and applied to a 0.9 × 12 cm column of SilicAR CC-7 (Mallinckrodt) that was equilibrated and eluted with the same solvent. Bornyl phosphate, eluting in the 8–12-mL fraction, was collected and the organic solvent removed under vacuum followed by lyophilization. The resulting material was dissolved in 10 mM $(NH_4)_2CO_3$ and applied to a 0.9 × 17 cm DEAE-cellulose column developed with a 10–40 mM $(NH_4)_2CO_3$, pH 9.0, gradient (200 mL, 2-mL fractions). The bornyl phosphate, eluting in the 20–30 mM range, was collected, and two aliquots each comprising 25% of the sample were taken for determination of ^{3}H:^{32}P ratio (by lyophilization and dissolution in MeOH). The remaining half of the sample was lyophilized and then treated with 1 mL of a solution of 0.1 M sodium acetate, pH 5.0, containing 3 units of wheat germ acid phosphatase (Sigma type 1) in a screw-capped vial for 5 h at 30 °C. The ether-soluble products so liberated were diluted with 5 mg of (+)- or (−)-borneol, as appropriate, and separated by TLC [silica gel G with hexane–ethyl acetate (17:3 v/v)]. The purified borneol (R_f 0.2) was eluted from the gel with dry ether and an aliquot counted.

The above methods were developed with (+)-[G-^{3}H]bornyl phosphate and (+)-[G-^{3}H]bornyl pyrophosphate (both at 2 Ci/mol), and the complete assay procedure was tested with the biosynthetic products derived from [1-$^3H_2$,α,β-$^{32}P_2$]geranyl pyrophosphate. Additional experiments, in which the enzyme was incubated with [1-$^3H_2$]geranyl pyrophosphate (10 μM at 150 Ci/mol), (±)-[1-$^3H_2$]linalyl pyrophosphate (10 μM at 150 Ci/mol), or (+)-[G-^{3}H]bornyl pyrophosphate (10 μM at 2 Ci/mol) in the presence of a 20–50-fold excess of [$^{32}P_2$]inorganic pyrophosphate (50 Ci/mol), were carried out to determine if the pyrophosphate group of the substrate was the sole source of the pyrophosphate moiety of the product.

Experiments with ^{18}O-Labeled Precursors. For preparative-scale incubations of ^{18}O-labeled substrates with the (+)- and (−)-bornyl pyrophosphate cyclases, the dialyzed 105000*g* supernatants, prepared from whole leaf homogenates as described above, were employed. Aliquots (1.0 mL, containing about 1 mg of protein in the 5 mM phosphate buffer system, pH 6.2) were incubated in screw-capped vials at 30 °C for 3 h with 35 μM [8,9-^{14}C,1-^{18}O]geranyl pyrophosphate or (1*E*)-(±)-[1-^{3}H,3-^{18}O]linalyl pyrophosphate. At the end of the incubation, the chilled reaction mixtures were extracted with distilled pentane (2 × 1 mL) to recover the borneol and substrate-derived alcohol released from the corresponding pyrophosphates by endogenous phosphatases/pyrophosphatases (Croteau & Karp, 1979b). When linalyl pyrophosphate was employed as the substrate, the remaining aqueous phases were

flushed under a stream of N_2 to remove residual pentane and then treated with 1.0 mL of 0.1 M sodium acetate, pH 4.6, containing 2 units of apyrase and 3 units of acid phosphatase and incubated an additional 2 h at 30 °C to hydrolyze any residual pyrophosphate esters. The alcohols liberated were extracted with pentane, as before, and like samples from each cyclase/substrate combination were pooled and then concentrated by slow evaporative removal of pentane, using a Vigreaux condenser at 35 °C. A sufficient number of preparations, using each substrate and enzyme combination, were processed over a period of several weeks to ensure the production of a minimum of ~0.1 μmol of borneol in each case.

Product mixtures obtained from geranyl pyrophosphate were concentrated to ~1 mL, dried over anhydrous $MgSO_4$, and treated for 72 h at 30 °C with a 100-fold excess of benzoylating reagent consisting of benzoyl chloride–4-(dimethylamino)pyridine–pyridine (1:2.5:10 v/w/v). The reaction mixture was then extracted in sequence with 0.1 N HCl, H_2O, 0.1 N $NaHCO_3$, and H_2O, and the organic layer was dried over $MgSO_4$. This procedure for benzoylation at the microscale was developed with (+)-[G-^{3}H]borneol as standard. HPLC separation of bornyl benzoate (k' 3.3) and geranyl benzoate (k' 4.0) was achieved on μPorasil with hexane–ether (99.5:0.5 v/v; 1.3 mL/min). The purity and benzoate content of each HPLC fraction were measured directly by UV spectrophotometry at five previously calibrated wavelengths (220, 225, 230, 235, and 240 nm), while the ^{14}C content was determined on measured aliquots, thereby allowing the specific activity of each fraction to be calculated. Fractions of maximum specific activity from each run were combined in preparation for mass spectrometric analysis. The identity of the purified bornyl benzoate was further confirmed by 250-MHz ^{1}H NMR analysis of an 11-μg sample, which clearly showed signals at δ 5.05 (br d, J = 10 Hz, H-2, 1 H), 7.4 (m, aromatic H, 3 H), and 8.0 (d, J = 8 Hz, aromatic H, 2 H). Although additional signals were evident at the characteristic positions δ 2.5, 2.15, 1.75, 1.02, and 0.95, interference from residual hexane precluded integration of the upfield region of the spectrum.

The average specific activity of the purified [^{14}C]geranyl benzoate and the ^{18}O content determined by measurement of the molecular ion peaks of the benzoate or trimethylsilyl ether were essentially identical with those of the original geranyl pyrophosphate. The latter values were verified by hydrolysis of a sample with wheat germ acid phoshatase, purification of the resulting alcohol by HPLC (μPorasil; hexane–ethyl acetate, 17:3 v/v), and mass spectrometric analysis of the parent (P) (m/e 154) and P + 2 (m/e 156) ions. The specific activity of this sample was determined by analysis of the corresponding HPLC-purified geranyl benzoate as before.

Product mixtures obtained from linalyl pyrophosphate were concentrated to 1 mL, and an excess of OsO_4 in 0.1 mL of pyridine was added in order to convert the linalool contained therein to the corresponding pentaol and thereby eliminate possible interference in the subsequent GLC–MS analysis. Following overnight stirring at 30 °C, osmate esters were decomposed with aqueous $NaHSO_3$, and polar substances and inorganics were removed by repeated washing with brine. Products remaining in the organic layer were dried ($MgSO_4$) and then benzoylated as before. Radio GLC analysis of the crude OsO_4-treated, benzoylated products (5000 dpm aliquots) established the [^{3}H]bornyl benzoate content of each sample, while combined GLC–MS, with comparison of total ion current [and base peak at m/e 105 ($C_6H_5CO^+$)] to a calibration curve established with authentic (±)-bornyl benzoate, allowed the absolute quantity, and thereby the specific activity, of each benzoate ester to be determined. Full spectrum analysis was followed by GLC–MS analysis of another sample employing selected ion monitoring of m/e 258 (P) and m/e 260 (P + 2), with summation of six scans, from which the ^{18}O abundance was obtained.

Since recovery of the labeled borneol in these experiments was dependent upon enzymatic hydrolysis of the corresponding bornyl pyrophosphate, it was essential to confirm that hydrolysis of the phosphate ester linkage takes place with P–O bond cleavage. Accordingly, a control experiment was carried out in which (+)-[G-^{3}H]bornyl pyrophosphate (2 μmol at 2 Ci/mol) was incubated with the crude, highly concentrated sage leaf preparation under the usual conditions but in [^{18}O]water (72% ^{18}O atom by dilution). The [^{3}H]borneol liberated after 4 h (0.44 μol) was extracted, converted to the benzoate, and purified by HPLC as before in preparation for mass spectrometric analysis. In an additional control, a sample of (1E)-(±)-[1-^{3}H,3-^{18}O]linalyl pyrophosphate (0.5 μmol) was incubated with a mixture of acid phosphatase and apyrase, as above, and the linalool liberated (0.47 μol) was analyzed directly by combined GLC–MS and shown to contain 77 ± 1.8% ^{18}O atom by comparison of the parent (P) (m/e 154) and P + 2 (m/e 156) ions, and the P – CH_3 companions (m/e 139 and 141), to the corresponding peak intensities of an unlabeled reference standard measured under identical conditions. Comparison of total ion current to a calibration curve established with authentic (±)-linalool, in conjunction with aliquot counting, allowed verification of the specific activity of the sample (0.19 ± 0.01 Ci/mol).

Analytical Methods. TLC was done on 1.0-mm layers of silica gel G or H activated at 110 °C for 4 h, with the developing solvents indicated in the text. Components were located with a Berthold radioscanner or by being sprayed with an ethanolic solution of 2,7-dichlorofluorescein and visualization under UV light and were eluted as described in the text. Procedures for radio GLC analysis have been described (Croteau & Cane, 1985). Chromatography was performed in a 12 ft × 0.125 in. stainless steel column containing 15% SE-30 on 80/100-mesh Chromosorb WHP, with temperature programming from 80 to 200 °C at 10 °C/min at a 30 mL/min He flow rate.

Mass spectra of products derived from geranyl pyrophosphate were obtained at 70 eV on a Hitachi Perkin-Elmer RMU-6D mass spectrometer. The parent and daughter ions of interest were repetitively scanned at slow speed and the peak areas determined. Products derived from linalyl pyrophosphate were analyzed by GLC–MS (at 20 eV, with 2400-V accelerating voltage) on a Hewlett-Packard 5840A-5985B system with a 25-m fused silica column coated with OV-1 and programmed from 150 to 280 °C at 10 °C/min with a He flow rate of 1.5 mL/min. Full spectrum analysis confirmed the identity of each product and was followed by determination of ^{18}O abundance on identical samples with GLC–MS analysis in the selected ion monitoring mode. The base peak, P, and P + 2 pair and, generally, the corresponding P – CH_3 companions were analyzed by summation of a minimum of six (1.8-s) scans bracketing the appropriate retention time. The ratios of (P + 2)/[P + (P + 2)] were calculated, as were the corresponding ratios for the P – CH_3 companion ions. As a control for run to run comparison, the ratios [P + (P + 2)]/(base peak) were also determined. Isotope enrichments represent the average of several independent runs in all cases and are corrected for natural abundance background by comparison with spectra of unlabeled standards. Stated error

Table I: Conversion of $^3H,^{32}P$-Labeled Acyclic Precursors to (+)- and (−)-Bornyl Pyrophosphates and Determination of the $^3H:^{32}P$ Ratios of the Derived Monophosphates[a]

	$^3H:^{32}P$ ratio			
precursor	GPP or LPP	tetrahydro-GP or -LP[b]	(+)-BP[c]	(−)-BP[c]
[1-$^3H_2$,α,β-$^{32}P_2$]GPP	9.8	20.0	20.1	19.8
[1-$^3H_2$,α-^{32}P]GPP	9.4	9.4	9.5	9.6
[1-$^3H_2$,β-^{32}P]GPP	10.2	506	538	511
[1-$^3H_2$,α,β-$^{32}P_2$]LPP	10.1	20.1	20.6	20.4
[1-$^3H_2$,α-^{32}P]LPP	10.2	10.1	10.2	10.4
[1-$^3H_2$,β-^{32}P]LPP	10.4	515	519	495

[a] GPP refers to geranyl pyrophosphate, LPP refers to (±)-linalyl pyrophosphate, GP and LP refer to the respective monophosphates, and BP refers to bornyl phosphate. [b] For verification of labeling, each precursor was converted to the corresponding tetrahydro monophosphate derivative by hydrogenation over Pd and either acid or enzymatic hydrolysis as described under Materials and Methods. [c] A minimum of 3.5×10^5 dpm of ^3H was recovered in each product, of which 25% was counted to a probable error of less than 0.5%.

limits represent standard deviations.

Radioactivity in aqueous and organic liquid samples was assayed with either a Packard 3255 LS spectrometer in 15 mL of ScintiVerse (Fisher Scientific; ^3H efficiency, 44%; ^{14}C efficiency, 85%; ^{32}P efficiency, 98%) or a Packard 3330 LS spectrometer in 10 mL of toluene containing 0.72% BuPBD and 0.045% PBBO (^3H efficiency, 48%; ^{14}C efficiency, 65%). All samples were counted to a standard error of less than 1.5% and are quench and half-life corrected.

Results

The cyclization of geranyl pyrophosphate (**1**) to (+)- and (−)-bornyl pyrophosphates (**2**) is considered to involve a 1,3 migration of the pyrophosphate group to C3 to form the tertiary intermediate linalyl pyrophosphate (**5**) and ultimately a 1,2 shift to C2 of the bicyclic product in the terminating step of the reaction. To assess the degree of freedom of the pyrophosphate and terpenyl reaction partners in these transformations and the broader mechanistic implications in terpene cyclization processes, the conversion of a series of ^{18}O- and ^{32}P-labeled precursors to the enantiomeric bornyl pyrophosphates was examined.

Experiments with ^{18}O- and ^{32}P-Labeled Geranyl Pyrophosphate. Preliminary studies indicated the pyrophosphate moiety of geranyl pyrophosphate to be the essential source of the pyrophosphate ester function of the enzymatic product bornyl pyrophoshate. Thus, [1-$^3H_2$,α,β-$^{32}P_2$]geranyl pyrophosphate (20 μM) in the presence of a 20-fold excess of inorganic pyrophosphate was incubated with the (+)-bornyl pyrophosphate cyclase (from sage, *Salvia officinalis*) and with the (−)-bornyl pyrophosphate cyclase (from tansy, *Tanacetum vulgare*), both preparations of which had been freed of contaminating phosphatases/pyrophosphatases by gel-permeation chromatography on Sephadex G-150. The resulting bornyl pyrophosphates were selectively hydrolyzed to the corresponding bornyl phosphates (obtained in 2.8–3.5% overall yield from the acylic precursor), and these materials were purified by a combination of adsorption chromatography on silica and ion-exchange chromatography on DEAE-cellulose to afford the radiochemically pure products. In both cases the monophosphate bore a $^3H:^{32}P$ ratio nearly double that of the precursor, the precise values indicating that the transformation occurred with less than 2% of the cyclic product having incorporated phosphate from the exogenous source (Table I). Acid phosphatase hydrolysis of the monophosphate esters obtained above, followed by TLC separation of the liberated terpenols, indicated that greater than 95% of the ^3H-labeled material was borneol, confirming both the identity of the product and the suitability of the analytical methods.

More direct evidence for the minimal intervention of exogenous pyrophosphate in the cyclization was obtained when the partially purified (+)- and (−)-bornyl pyrophosphate cyclases were incubated with [1-$^3H_2$]geranyl pyrophosphate (10 μM at 150 Ci/mol) in the presence of a 50-fold excess of [$^{32}P_2$]pyrophosphate (500 μM at 50 Ci/mol). Conversion to, and isolation of, the monophosphates as before gave $^3H:^{32}P$ ratios of greater than 62, indicating that less than 5% of the cyclic product had incorporated exogenous inorganic pyrophosphate. That the incorporation of exogenous pyrophosphate, albeit minor, had occurred *during* the cyclization was suggested by the fact that when the cyclase from sage was incubated with (+)-[G-^3H]bornyl pyrophosphate (10 μM at 2 Ci/mol) in the presence of [$^{32}P_2$]pyrophosphate (500 μM at 50 Ci/mol) the reisolated product (as the pyrophosphate) did not contain detectable ^{32}P label.

Proof that the two ends of the pyrophosphate moiety retain their identities in the enzymatic cyclization of geranyl pyrophosphate to bornyl pyrophosphate was next obtained by specifically labeling each phosphorus atom of the acyclic precursor. For this purpose, [1-$^3H_2$,α-^{32}P]- and [1-$^3H_2$,β-^{32}P]geranyl pyrophosphate were prepared from the ^3H-labeled alcohol by sequential addition of phosphate groups, and following verification of the position of labeling (Table I), each substrate was separately incubated with the (+)- and (−)-bornyl pyrophosphate cyclases. The products of several incubations with each substrate (at 20 μM) were pooled to obtain, in each case, a minimum of 0.25 μCi (^3H) of bornyl pyrophosphate, which was hydrolyzed to the monophosphate and chromatographically purified as before. As summarized in Table I, both the (+)- and (−)-bornyl phosphates derived from [1-$^3H_2$,α-^{32}P]geranyl pyrophosphate exhibited essentially unchanged $^3H:^{32}P$ ratios, whereas the bornyl phosphates from the β-^{32}P-labeled precursor retained less than 2% of the original ^{32}P label.

With the lack of tumbling or end to end interchange of the pyrophosphate established in both cyclizations, it became critical to examine the fate of the C–O–P bridge oxygen of the precursor in these transformations. To this end, [8,9-^{14}C,1-^{18}O]geranyl pyrophosphate was prepared and converted to bornyl pyrophosphate by large-scale incubations with 105000*g* supernatants from whole leaf homogenates, in this instance taking advantage of the in situ hydrolysis of the product by endogenous phosphatases/pyrophosphatases. A control experiment confirmed that hydrolysis of bornyl pyrophosphate takes place, as expected (Fernley, 1971; Hollander, 1971), with P–O bond cleavage, thereby demonstrating that the carbinol oxygen of the recovered borneol corresponds to the ester oxygen of the pyrophosphate derivative. Thus, (+)-[G-^3H]bornyl pyrophosphate (2 μmol at 2 Ci/mol) was incubated with the crude enzyme preparation from sage under the usual conditions in $H_2{}^{18}O$ (72% atom), and the [^3H]borneol liberated (0.44 μmol) was extracted and converted to the benzoate. Analysis of the HPLC-purified product by mass spectrometry established the presence of less than 2% excess ^{18}O.

A total of 3.88 μmol of [8,9-^{14}C,1-^{18}O]geranyl pyrophosphate (357 mCi/mol, 67.9% ^{18}O) was processed over a period of several weeks by a series of incubations with (+)-bornyl pyrophosphate cyclase preparations from sage. The resulting borneol and geraniol, released from the corresponding pyrophosphates by endogenous phosphohydrolases, were ex-

Table II: Conversion of [8,9-^{14}C,1-^{18}O]Geranyl Pyrophosphate and (1*E*)-(±)-[1-^{3}H,3-^{18}O]Linalyl Pyrophosphate to (+)- and (−)-Bornyl Pyrophosphates and Determination of the ^{18}O Content of the Derived Benzoates

	substrate[b]		bornyl benzoate	
conversion[a]	mCi/mol	% ^{18}O	mCi/mol	% ^{18}O
GPP → (+)-BPP	357	67.9 ± 0.4	257 ± 14	72.0 ± 3.0[c]
GPP → (−)-BPP	14.2	68.2 ± 4.4	*d*	63.8 ± 2.8
LPP → (+)-BPP			58.4 ± 3.3	76.3 ± 5.4[c]
	193	77.0 ± 1.8		
LPP → (−)-BPP			75.7 ± 3.1	79.3 ± 4.1[c]

[a] GPP refers to geranyl pyrophosphate, LPP to (±)-linalyl pyrophosphate, and BPP to bornyl pyrophosphate. [b] Labeling was verified by enzymatic hydrolysis of the pyrophosphate ester and analysis of the resulting alcohol or a suitable derivative as described in the text. [c] ^{18}O enrichments are corrected for dilution with endogenous material on the basis of the measured decrease in ^{14}C or ^{3}H specific activity. [d] Greater than 11 mCi/mol; insufficient sample for accurate determination of specific activity.

tracted with pentane, and the concentrated extract was benzoylated. HPLC separation of the crude product mixture afforded 0.22 μmol of pure (+)-bornyl benzoate (at 257 mCi/mol), the identity of which was confirmed by 250-MHz ^{1}H NMR analysis of an 11-μg sample. Mass spectrometric measurement of the molecular ion peaks indicated the (+)-bornyl benzoate to contain 52.4 ± 1.4% ^{18}O, which, when adjusted for endogenous dilution that had lowered the ^{14}C specific activity of the recovered product, corresponded to a corrected ^{18}O abundance of 72 ± 3% (Table II). Since rotational equilibration of the three proximal oxygen atoms would have reduced the original ^{18}O enrichment of 67.9% to a value of 22.6% at C2 of borneol, it is clear that the isomerization–cyclization of [1-^{18}O]geranyl pyrophosphate involves essentially no positional oxygen isotope exchange.

Results obtained with the (+)-bornyl pyrophoshate cyclase from sage were again corroborated with the (−)-bornyl pyrophosphate cyclase from tansy, which converts the same precursor, geranyl pyrophosphate, to a product of identical structure but opposite enantiomeric configuration. Accordingly, a second sample of [8,9-^{14}C,1-^{18}O]geranyl pyrophosphate (14.2 mCi/mol, 68.2% ^{18}O) was processed with enzyme preparations from tansy leaves, and the terpenols so generated were benzoylated as before. HPLC purification of the mixture yielded 0.25 μmol of (−)-bornyl benzoate (and 0.75 μmol of recovered geranyl benzoates, which afforded an ^{18}O content of 67.9 ± 2.5% by MS analysis of the derived trimethylsilyl ether). As summarized in Table II, mass spectrometric determination on the purified (−)-bornyl benzoate indicated the product (−)-borneol to possess an ^{18}O enrichment essentially unchanged from that of the original [1-^{18}O]geranyl pyrophosphate precursor.

Experiments with ^{18}O- and ^{32}P-Labeled Linalyl Pyrophosphate. The above experiments clearly demonstrate that in the enzymatic conversion of geranyl pyrophosphate to either (+)- or (−)-bornyl pyrophosphate the original pyrophosphate ester oxygen of the precursor is the exclusive source of the pyrophosphate ester oxygen of the product. This unexpected absence of positional isotope exchange (Rose, 1979) among the nonbridging oxygen atoms of the α-phosphate group contrasts with the equilibration of the three proximal pyrophosphate oxygens observed in the closely related isomerization of farnesyl pyroposphate to nerolidyl pyrophosphate by an enzyme system from the fungus *Gibberella fujikuroi*, a result that has been interpreted in terms of an allylic cation–pyrophosphate anion pair (Cane et al., 1981). Although the present results imply very tight coupling of the pyrophosphate and terpenoid reaction partners within the enzyme active site, they do not distinguish between an initial [1,3]-sigmatropic rearrangement or tight ion pair in which rotational equilibrium about the P_α–OP_β bond is not achieved, a [3,3]-sigmatropic rearrangement involving the initial attachment of a nonbridge oxygen to C3 of the linalyl system and return of the formerly bridged ^{18}O atom to C2 of the bornyl system, or processes involving bonding of the β-phosphate group at the tertiary center.

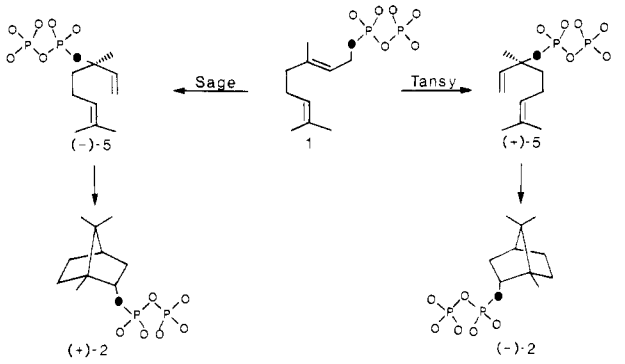
To examine these various possibilities with respect to the formation and subsequent cyclization of the tertiary intermediate, both α- and β-^{32}P-labeled and 3-^{18}O-labeled linalyl pyrophosphates were prepared. Samples of [1-$^{3}H_2$,α-^{32}P]- and [1-$^{3}H_2$,β-^{32}P]linalyl pyrophosphates were separately incubated with the partially purified cyclase preparations from sage and tansy, and the resulting bornyl pyrophosphate was hydrolyzed to bornyl phosphate and isolated as before. As described in Table I, both bornyl phosphates derived from [1-$^{3}H_2$,α-^{32}P]-linalyl pyrophosphate evidenced unchanged ^{3}H:^{32}P ratios, whereas the bornyl phosphates derived from the β-^{32}P-labeled precursor had lost essentially all of the original ^{32}P label. Direct involvement of the β-phosphate of geranyl pyrophosphate in the allylic rearrangement was thereby excluded.

Further details of the pyrophosphate migration were obtained by preparative-scale conversions of (1*E*)-(±)-[1-^{3}H,3-^{18}O]linalyl pyrophosphate to the enantiomeric bornyl pyrophosphates by the crude enzyme preparations from sage and tansy. A total of 2 μmol of this precursor was processed with each cyclase by a series of incubations, and the enzymatically hydrolyzed products so obtained were treated with OsO_4 to remove olefinic compounds and then benzoylated. Aliquot counting along with both radio GLC and full spectrum GLC–MS analysis established these samples to contain respectively 0.192 μmol of (+)-bornyl benzoate (67% of sample) and 0.088 μmol of (−)-bornyl benzoate (59% of sample), with the remainder comprised of 1,8-[^{3}H]cineole in each case. 1,8-Cineole cyclase (Croteau & Karp, 1977a) is the only other enzyme in these preparations capable of transforming the acyclic precursor to a saturated cyclic product. Comparison of total ion current and base-peak intensity to a calibration curve established with authentic (±)-bornyl benzoate allowed calculation of product specific activity and indicated that significant dilution with endogenous materal had occurred over the course of the numerous preparative incubations (Table II). Selected ion monitoring of the parent (*m/e* 258) and P + 2 (*m/e* 260) ions by combined GLC–MS and comparison with the corresponding ion intensities of an unlabeled reference standard of (±)-bornyl benzoate obtained under identical conditions established that the enzymatically derived (+)-bornyl benzoate contained 31.1% ^{18}O while the similarly derived (−)-bornyl benzoate contained 23.1% ^{18}O. These values, when corrected for dilution on the basis of the measured decrease in ^{3}H specific activity, yield a calculated ^{18}O enrichment of the carbinol oxygen atom of the benzoate esters virtually identical with that of the [3-^{18}O]linalyl pyrophosphate precursor (Table II). The alternative [3,3]-sigmatropic rearrangement was therefore eliminated.

Discussion

The summary of the results clearly indicates that it is solely the pyrophosphate ester oxygen of geranyl pyrophosphate that is involved in all the critical bonding processes in the coupled isomerization–cyclization leading to formation of both (+)- and (−)-bornyl pyrophosphates (Scheme III) and that the pyrophosphate moiety remains closely associated with its terpenyl partner throughout the course of the reaction. These

Scheme III



findings strongly support tight ion pairing in the transformation. Poulter has reported that [1-^{18}O]geranyl pyrophosphate reisolated from incubations with prenyl transferase (in the absence of isopentenyl pyrophosphate) had not undergone detectable scrambling, in spite of very strong evidence for the generation of the allylic cation at the enzyme active site (Mash et al., 1981). Little relative motion of the reaction partners is required in the ionization of geranyl pyrophosphate and its subsequent collapse observed in the case of prenyl transferase. The observed lack of positional isotope exchange in the case of bornyl pyrophosphate cyclase is, thus, all the more notable since the reaction seemingly involves a formal 1,3 followed by a 1,2 migration of the pyrophosphate, with the intervening generation of the transient α-terpinyl cation–pyrophosphate anion pair (**6**) in which the charge separation is at least 3 Å.

While an equilibrium between geranyl and linalyl pyrophosphate may exist at the active site of bornyl pyrophosphate cyclase, as presumed in the analogous sesquiterpenoid isomerization of farnesyl to nerolidyl pyrophosphate (Cane et al., 1981), the first cyclization step forming the new C1–C6 bond to generate the α-terpinyl cation (Scheme II, **6**) is most likely irreversible (reversibility of a terpene cyclization has never been reported). The absence of observable isotope scrambling in either (+)- or (−)-bornyl pyrophosphate implies that both the presumed initial isomerization step and the subsequent cyclization are fast compared to positional isotope exchange. However, no information is provided by this result as to the relative rates of isomerization and ring closure. Unpublished studies in which kinetic constants for geranyl pyrophosphate (**1**), (+)-linalyl pyrophosphate [(+)-**5**], and (−)-linalyl pyrophosphate [(−)-**5**] were compared for both cyclases suggest the isomerization to be the slower step in the coupled reaction sequence in both cases.

It is of interest to consider whether the restricted rotation of the anion, which results in exclusive recapture of the original ester oxygen atom by the terpenyl partner, reflects steric or ionic interaction between the enzyme and the pyrophosphate moiety, or the inherently strong electrostatic interaction between the Mg^{2+}–pyrophosphate complex and the paired carbocation. It seems highly unlikely that complexation of the pyrophosphate function with Mg^{2+} is sufficient, in itself, to account for the observation since, for example, scrambling of the terminal oxygens has been observed in ADP transiently generated in a number of enzymatic ATP-dependent reactions (Rose, 1979), in spite of the presumptive strong interaction with Mg^{2+} in each instance. Moreover, the aforementioned Mg^{2+}-dependent isomerization of farnesyl to nerolidyl pyrophosphate by the *G. fujikuroi* enzyme is accompanied by complete equilibration of the proximal phosphate oxygens (Cane et al., 1981). In directly comparing this isomerase to the bornyl pyrophosphate cyclases, we can assume the energetics of the ionization of the Mg^{2+}–pyrophosphate complex to be nearly identical and the corresponding terpenyl cation–pyrophosphate anion pairs to be of roughly equivalent energy. In the case of the isomerase, however, equilibration of the primary and tertiary allylic substrates may occur at the active site prior to product release, leading to an amplification of the positional isotope exchange accompanying a single turnover and thereby resulting in the observed scrambling of oxygen atoms attached to the proximal phosphate residue.

The present results obtained with the (+)- and (−)-bornyl pyrophosphate cyclases add to a growing body of evidence supporting the general involvement of ion-pair intermediates in the enzymatic transformations of allylic pyrophosphates (Cane, 1980; Cane et al., 1981; Mash et al., 1981) and imply this common mechanism for monoterpene cyclases, all of which are considered to catalyze the geranyl to linalyl pyrophosphate isomerization as the initial step of the coupled reaction sequence (Croteau, 1985). However, most monoterpene cyclases terminate the reaction by deprotonation of a carbocation to afford an olefin or carbocation capture by water rather than by the pyrophosphate anion. Thus, for example, neither (−)-*endo*-fenchol (Croteau et al., 1984) nor 1,8-cineole (unpublished) derived from [1-^{18}O]geranyl pyrophosphate in enzyme preparations from *Foeniculum vulgare* and *S. officinalis*, respectively, bears detectable ^{18}O label. These divergent results, implying water as the source of oxygen in the cyclic product, are nonetheless fully consistent with the unified stereochemical cyclization model (Croteau & Cane, 1985; Croteau, 1985) illustrated in Scheme II. Direct evidence for ion pairing in these and similar cases must await a means of dissecting the normally cryptic isomerization step from the coupled cyclization.

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Registry No. **1**, 763-10-0; (*E*)-[1-$^{3}H_2$]-**1**, 98945-14-3; (*E*)-[8,9-^{14}C,1-^{18}O]-**1**, 88888-00-0; (*E*)-[1-$^{3}H_2$,α,β-$^{32}P_2$]-**1**, 98945-24-5; (*E*)-[1-$^{3}H_2$,α-^{32}P]-**1**, 98945-25-6; (*E*)-[1-$^{3}H_2$,β-^{32}P]-**1**, 98945-26-7; **5**, 16789-26-7; (±)-[1-$^{3}H_2$]-**5**, 98945-15-4; (±)-[1-^{3}H,3-^{18}O]-**5** (*E*), 98945-18-7; (±)-[1-$^{3}H_2$,α,β-$^{32}P_2$]-**5**, 98945-27-8; (±)-[1-$^{3}H_2$,α-^{32}P]-**5**, 98945-28-9; (±)-[1-$^{3}H_2$,β-^{32}P]-**5**, 98945-29-0; (*E*)-$HOCH_2CH{=}C(CH_3)(CH_2)_2CH{=}C(^{14}CH_3)_2$, 98945-16-5; (*E*)-$H^{18}OCH_2CH{=}C(CH_3)(CH_2)_2CH{=}C(CH_3)_2$, 98945-17-6; (±)-$TC{\equiv}CC(OH)(CH_3)(CH_2)_2CH{=}C(CH_3)_2$, 98945-19-8; (±)-$THC{=}CHC(OH)(CH_3)(CH_2)_2CH{=}C(CH_3)_2$, 98945-20-1; (±)-$H_2C{=}CHC(^{18}OH)(CH_3)(CH_2)_2CH{=}C(CH_3)_2$, 98945-21-2; $Ph_3PEt^{+}Br^{-}$, 1530-32-1; (*E*)-$Me_2C{=}CH(CH_2)_2C(Me){=}CHCH_2Br$, 6138-90-5; $Me_2C{=}CH(CH_2)_2C(^{18}O)Me$, 98945-23-4; $Me_2C{=}CH(CH_2)_2C(O)Me$, 110-93-0; (*E*)-$(CHO)_2C{=}CH(CH_2)_2C(CHO){=}CHCH_2OTHP$, 98945-22-3; acyclic monoterpene pyrophosphate cyclase, 72840-91-6; (+)-bornyl pyrophosphate, 64822-87-3; (−)-bornyl pyrophosphate, 83247-84-1; geranyl pyrophosphate cyclase, 95829-20-2.

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Interaction of Adriamycin with Negatively Charged Model Membranes: Evidence of Two Types of Binding Sites†

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ABSTRACT: The interaction of the antitumor compound adriamycin with negatively charged unilamellar phospholipid vesicles was studied. The negative charges were provided by cardiolipin or phosphatidic acid. By analyzing the changes in the circular dichroism spectrum of adriamycin, we demonstrated the presence of two different spectral patterns corresponding to two different binding sites (I and II) on the vesicles. In site I, the amino sugar of adriamycin is bound to the ionized phosphate of either cardiolipin or phosphatidic acid, and the dihydroxyanthraquinone lies outside the bilayer. In site II, the amino sugar is still bound to the phosphate, but the dihydroxyanthraquinone moiety is embedded in the bilayer. This has been shown by measuring spectroscopically the binding of the aglycon part to an external probe and by measuring the susceptibility of bound adriamycin to reduction by NADH dehydrogenase.

The anthracycline antibiotic adriamycin (Adr) is an important antitumor agent with marked activity against a wide variety of human neoplasms (Blum & Carter, 1974; Lenaz & Page, 1976). From studies on the mechanism of action of anthracyclines in vivo, nuclear DNA has usually been considered the prime target for this antibiotic's antineoplastic action (Di Marco, 1975). However, recent studies have suggested that anthracyclines may be cytotoxic primarily as a result of mechanisms other than DNA binding (Duvernay et al., 1980): anthracyclines having a low affinity for DNA are still capable of inhibiting cell mitosis (Israel et al., 1975), and

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