Proc. Natl. Acad. Sci. USA
Vol. 84, pp. 4856–4859, July 1987
Biochemistry

Direct demonstration of the isomerization component of the monoterpene cyclase reaction using a cyclopropylcarbinyl pyrophosphate substrate analog

(isoprenoid biosynthesis/enzymatic ionization/squalene/phytoene)

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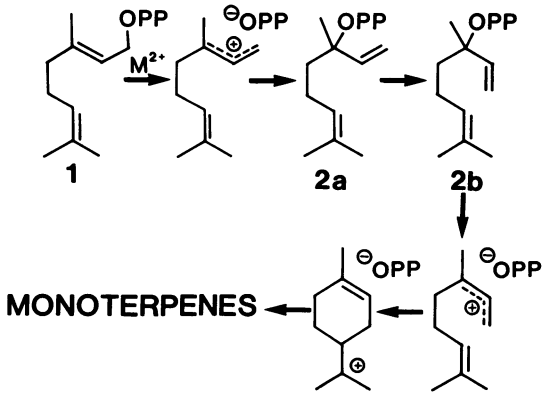
Communicated by Clarence A. Ryan, March 30, 1987 (received for review February 5, 1987)

ABSTRACT **The tightly coupled nature of the reaction sequence catalyzed by monoterpene cyclases has precluded direct observation of the topologically required isomerization step leading from geranyl pyrophosphate to the presumptive, enzyme-bound, tertiary allylic intermediate linalyl pyrophosphate, which ultimately cyclizes to the various monoterpene skeletons. By using a partially purified monoterpene cyclase preparation and 2,3-cyclopropylgeranyl pyrophosphate, a substrate analog designed to uncouple the reaction sequence, the production of the corresponding tertiary homoallylic pyrophosphate isomer was demonstrated. This provides direct evidence for the usually cryptic isomerase component of the overall catalytic cycle. A number of other related products generated by reaction of cyclase with the analog were also identified, the structures and proportions of which were consistent with the intermediacy in catalysis of a cyclopropylcarbinyl cation·pyrophosphate anion pair. Kinetic parameters for the analog were compared with those of the natural substrate geranyl pyrophosphate. The results presented confirm mechanistic similarities in the enzymatic ionization and subsequent transformation of allylic pyrophosphate and cyclopropylcarbinyl pyrophosphate intermediates of isoprenoid metabolism.**

Monoterpene cyclases catalyze the conversion of geranyl pyrophosphate (**1**), the ubiquitous C_{10} intermediate of the isoprenoid pathway, to a wide variety of monocyclic and bicyclic carbon skeletons. The crucial role of the cyclases in the origin of the different monoterpene classes has stimulated considerable interest in the details of these related enzymatic transformations, and a comprehensive proposal for the mechanism of this reaction type has been formulated (Scheme I) (1). All



Scheme I

monoterpene cyclases investigated to date are capable of overcoming the topological impediment to direct cyclization of geranyl pyrophosphate, imposed by the *trans* geometry of the C2—C3 double bond, by way of a preliminary isomerization step that occurs without the formation of detectable free intermediates. Thus, following productive binding (2), the coupled isomerization-cyclization sequence (Scheme I) is initiated by divalent metal ion-dependent ionization of geranyl pyrophosphate, with ensuing *syn* migration of the pyrophosphate moiety of the ion pair, to provide the bound tertiary intermediate linalyl pyrophosphate (**2a**). In this "ionization-isomerization" step, which removes the topological barrier to cyclization, the first formal chiral center is introduced at C3 [i.e., either (3*R*)- or (3*S*)-linalyl pyrophosphate is generated]. After rotation about the C2—C3 bond to afford the cisoid, *anti-endo* conformer (**2b**), linalyl pyrophosphate is itself ionized with C6—C1 ring closure to provide the corresponding monocyclic (4*R*)- or (4*S*)-α-terpinyl cation·pyrophosphate anion pair. These early mechanistic steps appear to be common to all monoterpene cyclizations, with subsequent steps involving termination of the reaction either by deprotonation or nucleophilic capture, or further electrophilic cyclization, hydride shift, or Wagner–Meerwein rearrangement before termination.

Although strong suggestive evidence has been accumulated for the intermediacy of linalyl pyrophosphate in cyclase catalysis (3–6), all previous efforts to observe directly this product of the mandatory isomerization step have failed, presumably because the highly reactive tertiary allylic system is generated at the same active site where the subsequent ionization and cyclization occur (i.e., in this tightly coupled sequence the binding and reaction of the intermediate generated at the active site are greatly favored over dissociation). To demonstrate the cryptic isomerization step of the normally coupled reaction sequence, a strategy was devised for employing a substrate analog that was competent to undergo the normal cyclase-catalyzed ionization-isomerization step but that would generate a corresponding tertiary pyrophosphate that was sufficiently unreactive toward the subsequent ionization-cyclization step to allow escape from the active site. In this paper, the preparation of (±)-[1-^{3}H]-2,3-cyclopropylgeranyl pyrophosphate (**3**), an analog intended to uncouple the reaction sequence, and the outcome of testing this analog with a partially purified cyclase preparation from sage (*Salvia officinalis*) are reported. The nature of the enzymatic products generated and the implications of these findings are described with regard to monoterpene cyclizations and, more generally, to the reactions of cyclopropylcarbinyl pyrophosphates in isoprenoid metabolism.

MATERIALS AND METHODS

Plant Materials, Substrates, and Reagents. Sage (*Salvia officinalis* L.) plants were grown from seed under conditions

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described previously (7), and rapidly expanding leaves of immature plants (3–6 weeks postgermination) were used in all experiments. [1-^{3}H]Geranyl pyrophosphate (53 Ci/mol; 1 Ci = 37 GBq) was prepared by literature procedures (7). [1-^{3}H]-2,3-Cyclopropylgeranyl pyrophosphate (132 Ci/mol) was obtained by pyrophosphorylation of [1-^{3}H]-2,3-cyclopropylgeraniol and purification of the material using published techniques (8). The radiolabeled alcohol was synthesized from 2,3-cyclopropylgeraniol (**6**) (9) by oxidation to the aldehyde using pyridinium chlorochromate (10) followed by reduction with NaB^3H_4 (New England Nuclear) (7), and it was shown to be free of radiolabeled contaminants and positional isomers by radio-GLC. Authentic standards of **5**, **7**, **8**, and **9** were prepared using established literature procedures (11, 12), and identities were confirmed by the combination of NMR, IR, and GLC/MS data.

Enzyme Preparation and Assay. The isolation from sage leaves, partial purification, and characterization of geranyl pyrophosphate:(+)-α-pinene{2,6,6-trimethylbicyclo[3.1.1]-hept-2-ene} cyclase and geranyl pyrophosphate:(+)-bornyl pyrophosphate {1,7,7-trimethylbicyclo[2.2.1]heptan-2-yl pyrophosphate(*endo*)} cyclase have been described (7, 13). These monoterpene cyclases share a number of common properties, including approximate molecular weight, and for convenience they were isolated together and were freed of phosphohydrolase, prenyl transferase, and all other competing activities (2).

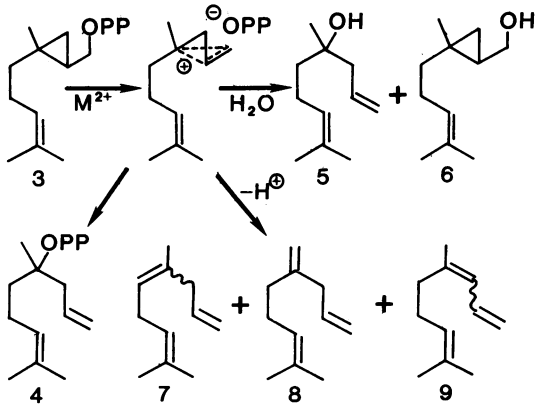
In a typical assay, a 1-ml aliquot of the partially purified preparation (0.1 mg protein/ml) was placed in a Teflon-sealed screw-capped vial (at 0–4°C), and the reaction was initiated by the sequential addition of 10 mM $MgCl_2$ and either the normal substrate or cyclopropyl analog followed by incubation with gentle swirling at 31°C for 1 hr. A published chromatographic procedure was used to isolate products derived from geranyl pyrophosphate (2), whereas a slightly modified version of the method was employed in examining products derived from the substrate analog. Thus, when using the cyclopropyl analog, 2 ml of ethyl ether was added to the reaction tube following incubation and the contents were vigorously mixed and then centrifuged to facilitate separation of the phases. Extraction was repeated with an additional 1 ml of ether, and the combined organic phases were passed through a short column of anhydrous $MgSO_4$. Total radioactivity in the ether extract was determined by aliquot counting, and the distribution of ether-soluble products was determined by means of conventional radio-GLC techniques following the addition of relevant carrier standards.

To test for the presence of phosphorylated products generated from the substrate analog, the aqueous phase remaining after ether extraction was adjusted to pH 2 with 10% aqueous formic acid and the mixture was agitated at 30°C for 16 hr. This treatment served to hydrolyze preferentially the cyclopropyl pyrophosphate (**3**) in the presence of homoallylic pyrophosphate **4** and thereby eliminated radiochemical cross-contamination in subsequent chromatographic steps [**3** is solvolyzed at least 20 times faster than **4** based on studies with corresponding *p*-nitrobenzoates (14–16), and under the reaction conditions employed here ≈65% of **3** originally present was hydrolyzed]. The treated sample was repeatedly extracted with ether and then frozen and lyophilized to remove formic acid and any residual, labeled volatile products. The lyophilized material was then dissolved in 1 ml of cold (0–4°C) 10 mM phosphate buffer (pH 6.1) containing 1% (wt/vol) sorbitol, to which 1 ml of 200 mM acetate buffer (pH 5) containing 1 unit each of wheat germ acid phosphatase and apyrase (Sigma) was added, and the sample was gently swirled at 30°C for 90 min to hydrolyze any pyrophosphate esters present to the corresponding alcohols. The sample was then extracted with ethyl ether (2 ml, then 1 ml) and the pooled organic extracts were passed through anhydrous $MgSO_4$. Authentic carrier standards were added, the extract was concentrated, and the alcohols contained therein were separated by TLC on silica gel [hexanes:ether, 2:1 (vol/vol)]. After visualization, the band corresponding to the homoallylic alcohol (**5**) (R_f = 0.35) was scraped from the plate and either eluted with ether (radio-GLC confirmed that only **5** was present) or assayed directly for determination of ^{3}H content. Boiled controls were included in all experiments, and all values reported have been corrected for nonenzymatic (solvolytic) background.

Analytical Methods. General procedures for TLC, radio-GLC, and liquid scintillation spectrometry have been described (2, 17, 18). Specific chromatographic conditions are provided in the text or in the figure legend. The counting efficiency for ^{3}H was 27%, and all samples were quench-corrected by internal standardization and were counted to a standard error of <1%.

RESULTS

Cyclase-Mediated Product Formation from Cyclopropylgeranyl Pyrophosphate. To determine whether monoterpene cyclases are capable of catalyzing the ionization of cyclopropylgeranyl pyrophosphate (**3**), in a manner analogous to the ionization-isomerization step of the normal reaction sequence with geranyl pyrophosphate (**1**) (1), a partially purified preparation of (+)-α-pinene cyclase and (+)-bornyl pyrophosphate cyclase [free of all other competing activities (2)] was incubated with a 20 μM concentration of the analog (**3**) under routine assay conditions (17). Based on the normal reaction sequence (1) (Scheme I), it was expected that the analog would give rise to the corresponding cyclopropylcarbinyl cation·pyrophosphate anion pair from which the isomerized product (**4**) and a series of structurally related compounds might be anticipated (Scheme II). Solvent extraction of the reaction mixture, followed by aliquot counting, indicated that ether-soluble, radiolabeled products were enzymatically generated at a rate (1.5% conversion) in excess of 10-fold that of nonenzymatic solvolysis under identical conditions (i.e., in boiled controls). Radio-GLC analysis of the ether-soluble products (Fig. 1) indicated that the trienes **7**, **8**, and **9** comprised 35% of the mixture, whereas alcohols **5** and **6** constituted the remaining 65%. Conversely, solvolytic products (obtained from control incubations) were comprised of 95% alcohols **5** and **6** in roughly equal amounts and 5% olefins of distribution similar to that generated enzymatically (data not shown). The homoallylic pyrophosphate (**4**) was also identified as a cyclase-derived product by enzymatic hydrolysis to the corresponding carbinol (**5**) and chromatographic purification. The rearranged product (**4**)



Scheme II

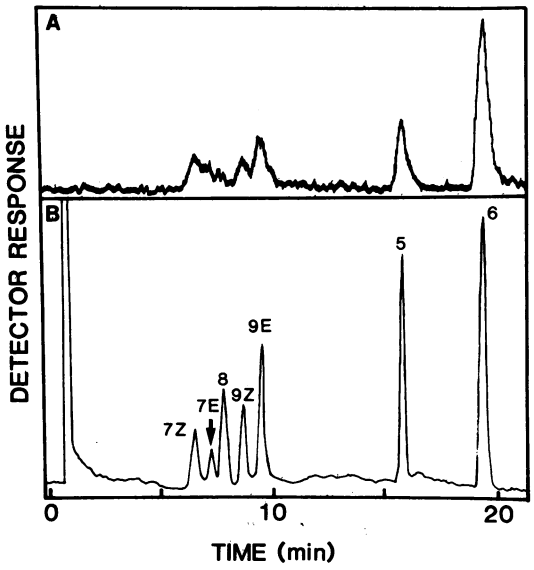


FIG. 1. Radio-GLC separation of the ether-soluble products generated from [1-^{3}H]-2,3-cyclopropylgeranyl pyrophosphate by a partially purified monoterpene cyclase preparation from *Salvia officinalis*. (*A*) Response of the radioactivity detector. (*B*) Response of the thermal conductivity detector to authentic internal standards of **5**, **6**, **7**, **8**, and **9**. *E* and *Z* denote olefin geometry. The chromatographic column employed was 3.7 m × 3 mm containing 10% Superox-20M on 80- to 100-mesh Chromasorb WHP (Gow-Mac Instruments) and was programed from 100°C (5-min hold) to 200°C at 10°C/min with He as carrier.

comprised about 10% of the total enzymatically generated material, and it was readily detected at levels some three times that of the apparent background observed in boiled controls.

Determination of Kinetic Parameters. The production of ether-soluble products by the cyclase preparation as a function of analog concentration in the range 1–40 μM exhibited saturation kinetics, and computer-assisted data analysis afforded a K_m value of about 18 μM for the cyclopropyl analog (Lineweaver–Burke = 17.7 μM, R^2 = 0.99; Eadie–Hofstee = 18.6 μM, R^2 = 0.96). This value is over five times the K_m values determined for the natural substrate geranyl pyrophosphate with (+)-α-pinene cyclase and (+)-bornyl pyrophosphate cyclase in similar preparations (2). Radio-GLC analysis indicated no significant alteration (±5%) in distribution of enzymatic products as a function of substrate concentration.

In the linear range of enzymatic activity with the cyclopropyl analog (1–10 μM), cyclase-catalyzed product formation (i.e., enzymatic ionization) was roughly 20 times the solvolytic background as determined with boiled controls. To assess the relative rate of enzyme-catalyzed ionization of cyclopropylgeranyl pyrophosphate versus enzyme-catalyzed cyclization of geranyl pyrophosphate, both substrates were examined at saturating levels (three times the respective K_m values) in parallel assays with the same enzyme preparation. The cyclopropyl analog was found to generate product at ≈6% of the rate observed with geranyl pyrophosphate. It is noteworthy that under the same assay conditions in boiled controls, the solvolytic conversion of the cyclopropyl analog was ≈14% of the rate of solvolysis of geranyl pyrophosphate.

DISCUSSION

It was expected that 2,3-cyclopropylgeranyl pyrophosphate (**3**) would bear sufficient stereoelectronic resemblance to the natural substrate (**1**) to be recognized by the cyclase (2), would be sufficiently reactive (in being "pseudo-allylic") to undergo initial ionization (19), and would, if isomerization occurred, give rise to the corresponding homoallylic, tertiary pyrophosphate (**4**), which, unlike the natural intermediate linalyl pyrophosphate (**2**), would be sufficiently unreactive in the second ionization step (14–16) (i.e., the cyclization) to permit release from the active site and detection. The expectation that the normal reaction sequence could be thus "uncoupled" was realized with the demonstration of the homoallylic pyrophosphate (**4**) as a significant component (10%) of the total enzymatic products generated from the cyclopropyl analog. Since the product isolation procedure involved selective acid hydrolysis of the residual substrate analog prior to phosphatase treatment and chromatographic separation, some loss of **4** would be expected and the observed level of this metabolite is very likely an underestimate. This result provides unambiguous, direct evidence for the discrete isomerization component of the monoterpene cyclase reaction sequence, a step for which only indirect evidence was previously available (3–6).

In addition to the isomerized product (**4**), a series of structurally related, ether-soluble products (Scheme II; **5**, **6**, **7**, **8**, and **9**) also arose from cyclopropylgeranyl pyrophosphate, presumably by deprotonation or capture by water of the initially generated cyclopropylcarbinyl cation. From the relative abundance of these enzymatic products (90% of total), it is clear that these processes compete favorably with the return of the paired pyrophosphate anion to afford **4**. It should be recalled that the formation of one of the normal cyclic products, bornyl pyrophosphate, also involves pyrophosphate return (5, 20). The reason why the isomerization of the analog is not more favorable in this case is presently unclear, although it may relate to cation-anion positioning effects arising from steric features of the cyclopropyl substituent. It is significant that the enzymatically generated ether-soluble materials differ from simple solvolysis products in the relatively high proportion of olefins, which indicates ionization in a nonpolar environment where deprotonation of the resulting cation competes favorably with nucleophile capture (15). The active sites of monoterpene cyclases are presumed to possess a hydrophobic pocket to shield the highly reactive carbocationic intermediates from water capture and thus prevent premature termination of the reaction sequence. This suggestion is also supported by earlier studies of product formation from the noncyclizable substrate analog 6,7-dihydrogeranyl pyrophosphate (2, 3), which cyclopropylgeranyl pyrophosphate resembles in general kinetic parameters.

Because a preparation containing two monoterpene cyclases [(+)-α-pinene cyclase and (+)-bornyl pyrophosphate cyclase] was used in this investigation, it is not possible to attribute specific products to a particular cyclase. The question is of little significance, however, since the activity observed was clearly enzymatic (20 times the solvolytic controls), the only enzymes present in the preparation, which were active with geranyl pyrophosphate, were the cyclases, and both enzymatic cyclizations under study must involve a similar, if not identical, isomerization step (1). The critical consideration is not which cyclase gives rise to which products, but rather that the products formed from the substrate analog must have arisen as a result of the action of one or the other cyclase.

In addition to the mechanistic implications of this work for the biosynthesis of regular monoterpenes, and of certain sesquiterpenes in which a similar isomerization step occurs (21, 22), the cyclase-catalyzed transformations of the cyclopropylcarbinyl pyrophosphate (**3**) are also relevant to other pathways of isoprenoid metabolism. The cyclopropylcarbinyl pyrophosphates presqualene pyrophosphate and

prephytoene pyrophosphate are essential intermediates in the biosynthesis of sterols (23) and carotenoids (24), respectively, whereas chrysanthemyl pyrophosphate, presumed to be synthesized by similar prenyl transfer, has been postulated as the key intermediate in the formation of irregular monoterpenes (25). Although the detailed mechanistic features of product formation from these cyclopropylcarbinyl pyrophosphates have not been unambiguously determined (23), and in spite of recent, alternate mechanistic rationales involving noncarbocationic processes (26, 27), the prevailing opinion favors ionization to form a cyclopropylcarbinyl cation·pyrophosphate anion pair that undergoes ring opening with concomitant proton loss (i.e., phytoene) or nucleophile capture (i.e., squalene and artemisyl alcohol). Recent experiments have documented a common electrophilic mechanism in prenyl transfer and cyclization reactions (28, 29). In view of the strong evidence for carbocationic processes in monoterpene cyclization reactions (1, 29, 30), the present report on the cyclase-catalyzed generation of the full spectrum of product types from a cyclopropylcarbinyl pyrophosphate now forges this common mechanistic link between three key reactions of isoprenoid metabolism: prenyl transfer and cyclization of allylic pyrophosphates, and ring opening of cyclopropylcarbinyl pyrophosphates.

This work was supported in part by National Institutes of Health Grant GM-31354 and is paper 7689, Project 0268, from the Agricultural Research Center at Washington State University.

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