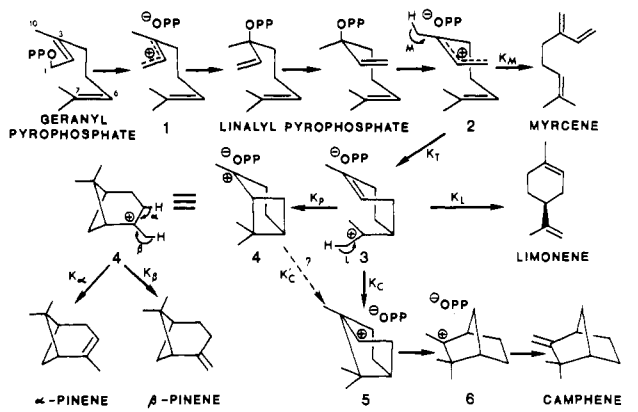
Isotopically Sensitive Branching in the Formation of Cyclic Monoterpenes: Proof That (−)-α-Pinene and (−)-β-Pinene Are Synthesized by the Same Monoterpene Cyclase via Deprotonation of a Common Intermediate†

Rodney B. Croteau* and Carl J. Wheeler
Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340
David E. Cane,* Roxanne Ebert, and Hyun-Joon Ha
Department of Chemistry, Brown University, Providence, Rhode Island 02912
Received February 19, 1987; Revised Manuscript Received April 17, 1987

ABSTRACT: To determine whether the bicyclic monoterpene olefins (−)-α-pinene and (−)-β-pinene arise biosynthetically from the same monoterpene cyclase by alternate deprotonations of a common carbocationic intermediate, the product distributions arising from the acyclic precursor [10-$^2H_3$,1-^3H]geranyl pyrophosphate were compared with those resulting from incubation of [1-^3H]geranyl pyrophosphate with (−)-pinene cyclase from *Salvia officinalis*. Alteration in proportions of the olefinic products generated by the partially purified pinene cyclase resulted from the suppression of the formation of (−)-β-pinene (C10 deprotonation) by a primary deuterium isotope effect with a compensating stimulation of the formation of (−)-α-pinene (C4 deprotonation). (−)-Pinene cyclase as well as (+)-pinene cyclase also exhibited a decrease in the proportion of the acyclic olefin myrcene generated from the deuteriated substrate, accompanied by a corresponding increase in the commitment to cyclized products. The observation of isotopically sensitive branching, in conjunction with quantitation of the magnitude of the secondary deuterium isotope effect on the overall rate of product formation by the (+)- and (−)-pinene cyclases as well as two other monoterpene cyclases from the same tissue, supports the biosynthetic origin of (−)-α-pinene and (−)-β-pinene by alternative deprotonations of a common enzymatic intermediate. A biogenetic scheme consistent with these results is presented, and alternate proposals for the origin of the pinenes are addressed.

Earlier work on pinene biosynthesis demonstrated that a soluble enzyme system from common sage (*Salvia officinalis*) catalyzed the divalent metal ion dependent cyclization of [1-^3H]geranyl pyrophosphate to (±)-[7-^3H]-α-pinene and (−)-[7-^3H]-β-pinene and to lesser amounts of other monoterpene olefins including the rearranged bicyclic product (±)-camphene, the monocyclic product (±)-limonene, and the acyclic compound myrcene (Gambliel & Croteau, 1982). The soluble preparation was fractionated by gel filtration to provide two regions of enzymatic activity termed geranyl pyrophosphate:pinene cyclase I (M_r ~96 000), which catalyzed the conversion of geranyl pyrophosphate to (+)-α-pinene and to smaller quantities of (+)-camphene, (+)-limonene, and myrcene, and geranyl pyrophosphate:pinene cyclase II (M_r ~55 000), which transformed the acyclic precursor to (−)-α-pinene and (−)-β-pinene, as well as to (−)-camphene, (−)-limonene, and myrcene (Gambliel & Croteau, 1984). Activities for the cyclization to the configurationally related (+)-olefins copurified without alteration in product distribution through four subsequent chromatographic steps and electrophoresis, as did the activities for the cyclization to the (−)-olefin series. Differential inhibition and inactivation studies provided additional evidence that a single enzyme generated the (+)-olefin product set and that the (−)-olefin series was also generated by a distinct, single enzyme species (Gambliel & Croteau, 1984).

Scheme I



Based on the above considerations and on related studies with other cyclases that produce bicyclic monoterpenes of the bornane (Croteau et al., 1986b) and fenchane (Satterwhite et al., 1985) type, a stereochemical scheme for the coupled isomerization and subsequent cyclization of geranyl pyrophosphate to the enantiomeric pinenes and related olefins has been proposed (Scheme I). The sequence, illustrated in Scheme I for the (−)-olefin series, is initiated by ionization of geranyl pyrophosphate, with suprafacial migration of the pyrophosphate moiety of the resulting ion pair (**1**) to afford the bound linalyl pyrophosphate intermediate. Rotation about the newly generated C2–C3 single bond to the cisoid conformer overcomes the original stereochemical impediment to the cyclization of geranyl pyrophosphate, while subsequent ionization of this tertiary allylic isomer allows C1–C6 cyclization of the anti-endo form or, alternatively, deprotonation of the resulting ion pair (**2**) to generate the acyclic olefin myrcene. Proton

† This research was supported by National Institutes of Health Grants GM-31354 (R.B.C.) and GM-30301 (D.E.C.). This is Scientific Paper No. 7693, Project 0268, from the Research Center, Washington State University College of Agriculture and Home Economics, Pullman, WA 99164. The Bruker WM-250 NMR spectrometer at Brown University was purchased with funds provided by the National Science Foundation and the Montedison Group of Milan.

0006-2960/87/0426-5383$01.50/0

loss from the monocyclic cation (**3**) may similarly afford limonene, whereas a second electrophilic cyclization gives rise to the bicyclic pinyl (**4**) or bornyl (**5**) cations. Wagner–Meerwein shift of the bornyl system generates the camphyl cation (**6**), which upon proton loss yields camphene. Unlike the camphyl system, the pinyl cation (**4**) may undergo deprotonation from either of two carbon atoms to provide isomeric olefinic products. If the proton is lost from the adjacent ring methylene (C4 of the substrate), α-pinene is formed, whereas proton loss from the adjacent methyl (C10 of the substrate) leads to β-pinene as the product. The biosynthetic transformations catalyzed by (+)-pinene cyclase (cyclase I) can be described by a mirror-image scheme, with the exception that only (+)-α-pinene is produced from the pinyl cation (**4**).

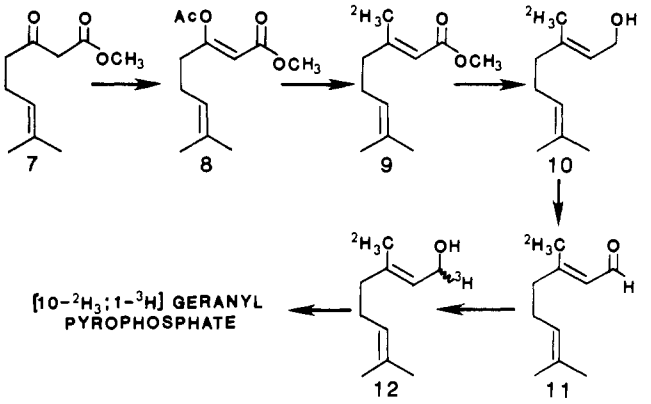
Application of the ionization–cyclization scheme to the origin of other monoterpene families has been described (Cane, 1980, 1985; Croteau, 1981, 1986a; Croteau & Cane, 1985), and salient features of the scheme have been investigated, including substrate binding (Wheeler & Croteau, 1987), ionization (Croteau, 1986b) and isomerization (Wheeler & Croteau, 1986a), regio- and stereochemistry of the transformations (Croteau et al., 1985a, 1986b; Satterwhite et al., 1985), and ion pairing of reaction intermediates (Croteau et al., 1985b, 1986a). On the basis of the enzymological evidence, the scheme as applied to the pinene cyclases posits a series of stereochemically related electrophilic cyclizations, rearrangements, and deprotonations arising from a common set of enzymatic intermediates. Although chemically induced carbocationic cascades to generate multiple terpenoids are well-known (Cramer & Rittersdorf, 1967; Haley et al., 1969; Brody & Gutsche, 1977; McCormick & Barton, 1978; Andersen et al., 1978; Bunton et al., 1979), such an *enzymatic* reaction system has scant precedent (Arigoni, 1975; Dorn et al., 1975). In this paper we present evidence, based on deuterium isotope effects observed in partially purified cell-free systems, which supports the proposal that a single enzyme gives rise to a common biosynthetic intermediate from which both (−)-α-pinene and (−)-β-pinene are derived by alternate deprotonations. Analysis of the data also affords new insights into the multistep biosynthetic transformations catalyzed by both (+)- and (−)-pinene cyclases.

Materials and Methods

Plant Materials and Reagents. Common sage (*Salvia officinalis* L.) plants were grown from commercially available seed under conditions described previously (Croteau & Karp, 1977, 1979; Gambliel & Croteau, 1984), and rapidly expanding leaves from the shoot apex of immature plants (3–6 weeks postgermination) were used in all experiments. All authentic monoterpene standards and solvents were purchased from Aldrich Chemical Co. and were purified prior to use.

Labeled Substrates. [1-^{3}H]Geranyl pyrophosphate (53 Ci/mol) was prepared and purified by literature procedures (Croteau & Karp, 1976). [10-$^2H_3$,1-^{3}H]Geranyl pyrophosphate was prepared as illustrated in Scheme II. Thus, the dianion of methyl acetoacetate (Weiler, 1970; Huckin & Weiler, 1974) was treated with dimethylallyl bromide at 0 °C to afford methyl 7-methyl-3-oxo-6-octenoate (**7**) in 60% yield after purification by silica gel flash column chromatography with hexane–ethyl acetate (11:1 v/v) (Still et al., 1978): ^{1}H NMR[1] δ 5.1 (m, 1 H), 3.75 (s, 3 H), 3.45 (s, 2 H), 2.6 (m, 2 H), 2.3 (m, 2 H), 1.68 (s, 3 H), 1.61 (s, 3 H). Methyl ester

Scheme II



7 was treated with excess isopropenyl acetate and a catalytic amount of *p*-toluenesulfonic acid at 110 °C for 12 h to afford the acetoxyenol ester **8** (Filler & Naqui, 1963; Casey & Marten, 1973) in 71% yield after flash column chromatographic purification as before: ^{1}H NMR δ 5.68 (s, 1 H), 5.12 (m, 1 H), 3.73 (s, 3 H), 2.18 (m, 7 H), 1.73 (s, 3 H), 1.64 (s, 3 H). Lithium iodide free [$^2H_3$]methyllithium was prepared as described (Aberhart & Lin, 1974) by treatment of [$^2H_3$]-methyl iodide (Aldrich, 99+ atom % ^{2}H) with *n*-butyllithium in hexane and redissolving the precipitated methyllithium in ether. The resultant ethereal solution [1.0 M, titrated according to Winkle et al. (1980)] was used to prepare [$^2H_6$]-dimethylcopper lithium by addition of 2 equiv to a slurry of anhydrous CuI in ethyl ether under an inert atmosphere at −78 °C, warming to 0 °C for 5 min, and then chilling to −78 °C. Addition of **8** to the chilled solution produced a mixture of geometrical isomers of **9** (10/1 *E*/*Z*) which, after separation by flash chromatography, afforded the desired *E* isomer in 47% yield (Casey & Marten, 1973; Casey et al., 1973): ^{1}H NMR δ 5.70 (s, 1 H), 5.10 (m, 1 H), 3.68 (s, 3 H), 2.17 (m, 4 H), 1.69 (s, 3 H), 1.61 (s, 3 H). Reduction of *E*-**9** with lithium ethoxyaluminum trihydride (Casey & Marten, 1973) at 0 °C gave trideuteriomethyl geraniol ([10-$^2H_3$]geraniol) (**10**) in 81% yield: ^{1}H NMR δ 5.42 (t, 1 H), 5.09 (m, 1 H), 4.15 (d, 2 H), 2.10 (m, 4 H), 1.69 (s, 3 H), 1.61 (s, 3 H). The corresponding aldehyde (**11**) was obtained by oxidation with activated MnO_2 (Aldrich Chemical Co.) in CCl_4 in 67% yield: ^{1}H NMR δ 9.98 (d, 1 H), 5.89 (m, 1 H), 5.10 (m, 1 H), 2.20 (m, 4 H), 1.69 (s, 3 H), 1.62 (s, 3 H). Reduction of an ethanolic solution of **11** by sequential addition of NaB^3H_4 (New England Nuclear, 25 mCi, 360 Ci/mol) followed by excess $NaBH_4$ provided, after separation of geometrical isomers by argentation TLC, a 45% chemical yield of [10-$^2H_3$,1-^{3}H]geraniol (**12**) (23% radiochemical yield, 32 Ci/mol) identical by ^{1}H NMR with **10**. The corresponding pyrophosphate ester was prepared and purified (20% yield) by standard procedures (Croteau & Karp, 1976).

Enzyme Preparation. Both pinene cyclase I [(+)-pinene cyclase] and pinene cyclase II [(−)-pinene cyclase], (+)-bornyl pyrophosphate cyclase, and cineole cyclase have been previously isolated from sage leaves, partially purified, and characterized (Gambliel & Croteau, 1984; Croteau & Karp, 1977, 1979). Briefly, sage leaves (10–13 g) were frozen in liquid N_2, ground to a fine powder with a mortar and pestle, and stirred into a cold slurry consisting of one-third the tissue weight of insoluble polyvinylpyrrolidone (Polyclar AT, GAF Corp.) in 5 mL/g 100 mM sodium phosphate buffer, pH 6.2, containing 5 mM dithiothreitol, 10 mM $Na_2S_2O_5$, 10 mM sodium ascorbate, 15 mM $MgCl_2$, and 20% (v/v) glycerol. The slurry was homogenized in portions with a Ten-Broeck

[1] Abbreviations: NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; GLC, gas–liquid chromatography.

homogenizer and then added to an equal tissue weight of washed and hydrated XAD-4 polystyrene resin (Rohm and Haas Corp.). After 10–20 min, the mixture was filtered through cheesecloth, and the filtrate was centrifuged at 27000*g* for 15 min (pellet discarded) and then at 140000*g* for 1 h. The supernatant was then concentrated to 3–5 mL by ultrafiltration (Amicon PM 30) and applied to a 2.5 × 110 cm Sephacryl S-200 column previously equilibrated and eluted with 10 mM sodium phosphate buffer, pH 6.1, containing 0.5 mM dithiothreitol, 1 mM sodium ascorbate, and 10% glycerol (v/v) while the effluent was monitored at 280 nm (6-mL fractions, 0.5 mL/min). Fractions were assayed for pinene cyclase activity (conversion of geranyl pyrophosphate to the pinenes and to lesser quantities of the coproducts camphene, limonene, and myrcene) as previously described (Gambliel & Croteau, 1984). Fractions containing (+)-pinene cyclase activity were pooled with the nearly coincident (+)-bornyl pyrophosphate cyclase, and those containing the (−)-pinene cyclase were similarly pooled with the nearly coincident 1,8-cineole cyclase. To eliminate any possibility of cross contamination of the pinene cyclases and to minimize contaminating (+)-sabinene cyclase (Karp & Croteau, 1982) in the (−)-pinene cyclase preparation, both pools were individually concentrated to 3–5 mL (Amicon PM-30) and rechromatographed on the Sephacryl S-200 column as before. The pooled fractions containing the (+)-pinene and (+)-bornyl pyrophosphate cyclases and the (−)-pinene and 1,8-cineole cyclases, were again separately pooled, and the glycerol in the buffer exchanged for sorbitol by repeated concentration and dilution with the above buffer containing 1% (w/v) sorbitol in lieu of glycerol. During this process the pH of the (−)-pinene cyclase preparation was also adjusted to the activity optimum of 7.1. Each cyclase preparation was then frozen with liquid N_2 in small aliquots, lyophilized (Wheeler & Croteau, 1986b), and stored under an argon atmosphere at −20 °C. (Such preparations can be stored for at least 6 months without change in activity.) The enzyme preparations were reconstituted with distilled water at 5 °C immediately before use, and several individual aliquots were combined to give a stock solution of ~0.1 mg of protein/mL.

Enzyme Assays. In a typical assay for monoterpene cyclases, a 1-mL aliquot of the stock solution of the partially purified preparation (~0.1 mg of protein) was added to a Teflon-sealed screw-cap vial (at 0–4 °C) and the reaction initiated by addition of 10 mM $MgCl_2$ and 20 μM geranyl pyrophosphate. Following incubation at 30 °C for 1 h with gentle swirling, the solution was chilled in ice and 2 mL of pentane added. Thorough mixing of the biphasic system was followed by centrifugation to facilitate separation of the phases, and the pentane layer was passed through a short column of silica gel (Mallinckrodt type 60A) overlaid with anhydrous $MgSO_4$. Extraction and elution were repeated with an additional 1 mL of pentane to provide a combined eluate containing only hydrocarbons, free of oxygenated terpenes, which either remained in the original solution or adsorbed to the silica. Total radioactivity in the hydrocarbon fraction was determined by aliquot counting, and product distribution was examined by radio-GLC following the addition of carrier standards.

The oxygenated monoterpenes were isolated by reextracting the original reaction mixture with 2 mL, then 1 mL, of diethyl ether, and this extract was also passed through the original silica column. This served to recover all the cineole and any borneol released from bornyl pyrophosphate should any trace levels of phosphohydrolase be present in the preparation. In order to obtain the majority of the bornane product present as bornyl pyrophosphate, the original aqueous reaction mixture was purged with a stream of N_2 for 1–2 min to remove any remaining organic solvents, and 1 mL of 200 mM sodium acetate buffer, pH 5.0, containing 1 unit each of wheat germ acid phosphatase and potato apyrase (both from Sigma Chemical Co.) was added. The mixture was incubated with gentle agitation for 75 min at 30 °C. After the mixture was cooled in ice, 2 mL of ether was added, and the biphasic mixture was vigorously shaken and then centrifuged to separate phases. The organic layer was dried by passage through a short column of $MgSO_4$ and the entire extraction repeated with an additional 1-mL portion of ether. All of the ether extracts were combined to provide a sample containing cineole and borneol (which represented all of the bornyl pyrophosphate produced). To facilitate the TLC separation of these products from unsaturated monoterpenols present in the combined extract, excess OsO_4 in pyridine was added and the sample stirred overnight. To the sample was then added 3 mL of saturated aqueous $NaHSO_3$ (followed by stirring for 3 h at room temperature) to decompose osmate esters, thereby completing the conversion of olefinic compounds to the corresponding diols and drastically reducing their mobility in the subsequent TLC step. The organic phase and an additional 1-mL ether extract of the aqueous phase were combined, dried over anhydrous $MgSO_4$, and diluted with ~5 mg each of unlabeled (±)-borneol and cineole as carriers, and the sample was concentrated under vacuum (Savant Speed Vac) and separated by TLC on silica gel G with hexanes–ether (2:1 v/v). The developed plate was sprayed with an ethanolic solution of 2,7-dichlorofluorescein and visualized under long-wave UV light. The gel bands corresponding to borneol (R_f = 0.4) and 1,8-cineole (R_f = 0.6) were either eluted with ether (both products were radiochemically pure by radio-GLC) or scraped directly into scintillation vials and, following the addition of scintillation cocktail, the amounts of ^3H-labeled cineole and borneol present were determined directly. Boiled controls were included in each experiment, and in all cases nonenzymatic product formation was negligible.

Analytical Methods. TLC was done on 1.0-mm layers of silica gel G (EM Laboratories Inc.), activated at 100 °C for 3 h, with the developing solvents indicated elsewhere in the text. Column chromatography was done with silica gel 60A special (100–200 mesh; Mallinckrodt). Eluting solvents are described elsewhere in the text.

Procedures for radio-GLC have been described (Croteau & Cane, 1985). The chromatographic column used was 12 ft × 0.125 in. o.d. stainless steel containing 15% Silar 10C on 80/100 mesh Chromosorb WHP and was run isothermally at 75 °C with 45 mL/min He flow. Radioactivity in organic liquid samples and in TLC isolates was determined with a Packard 3255 liquid scintillation spectrometer in a counting solution of 30% ethanol in toluene containing 0.4% (w/v) Omnifluor (New England Nuclear, efficiency for ^3H = 27%). All samples were quench corrected by the internal standard method and counted to a standard error of less than 1%. ^1H NMR spectra were obtained by using a Bruker WM-250 spectrometer, employing $CDCl_3$ as solvent and tetramethylsilane as internal standard.

Results and Discussion

The reactions catalyzed by a typical monoterpene cyclase involve a complex series of isomerization and intramolecular electrophilic bond-forming events, frequently accompanied by molecular rearrangements and hydride shifts and terminated either by loss of a proton or by capture by an external nu-

cleophile such as water or the pyrophosphate counterion. Unfortunately, these fascinating transformations ordinarily cannot be observed directly since none of the intermediates between the acyclic precursor geranyl pyrophosphate and the ultimate cyclization product is released from the enzyme surface to the surrounding medium. Both linalyl pyrophosphate and ionic species generated in the course of the isomerization–cyclization sequence are tightly sequestered by the cyclase, although there is no evidence that any of the relevant intermediates are covalently attached to the enzyme. Several strategies employing modified substrates that can alternatively act as cyclase inhibitors or undergo partial reactions catalyzed by the cyclases have been exploited to decipher the normally cryptic events of the cyclization process (Croteau et al., 1985b, 1986a,b; Satterwhite et al., 1985; Wheeler & Croteau, 1985a, 1987). Nonetheless, much of the evidence for the sequence of bond-making and bond-breaking events remains indirect and relies heavily on analogy to well-studied nonenzymatic model reactions.

The availability of a pair of enzymes, (+)- and (−)-pinene cyclase, each catalyzing the formation of an apparent mixture of monoterpene products, presents an unusually rich opportunity to penetrate the normally obscure sequence of events by which individual monoterpenes are generated. Since all of the observed olefinic products derived from geranyl pyrophosphate presumably represent the quenching of individual intermediates at various stages of the multistep isomerization–cyclization–rearrangement process, it becomes possible, at least in principle, to clarify the sequence of biosynthetic reactions and to analyze the factors influencing the partitioning of the various carbocationic intermediates. The possibility of any such analysis, of course, depends on the validity of the assumption that the formation of all observed products is mediated by a common enzyme species rather than being the consequence of the combined action of a family of closely related enzymes. While there is no question that the (+) and (−) series of olefinic products are generated by readily separable and therefore distinct cyclases, the evidence that the individual product mixtures consisting of α- (and β)-pinene, camphene, myrcene, and limonene are generated by a single enzyme, while strong, has been largely circumstantial, and the available data (Gambliel & Croteau, 1984) do not rigorously exclude the action of a group of closely related cyclases.

In order to confirm that the product mixtures resulting from incubation of geranyl pyrophosphate with (−)-pinene cyclase are in fact the consequence of the action of a single enzyme species, we chose to take advantage of induced kinetic isotope effects (Samuelson & Carpenter, 1981) on the observed product ratios resulting from reaction with an isotopically labeled substrate. Examination of the mechanistic hypothesis outlined in Scheme I reveals that formation of the various acyclic, monocyclic, and bicyclic products involves several stages at which each of the successively formed intermediates may partition either between product formation and generation of the next intermediate or into at least two new intermediates. Thus the allylic cation:pyrophosphate anion pair **2** resulting from ionization of (3*S*)-linalyl pyrophosphate can either generate the corresponding (4*S*)-α-terpinyl cation (**3**) or be quenched by loss of a C3-methyl proton to yield myrcene. [The latter product may also arise by quenching of the transoid allylic cation (**1**) linking geranyl and linalyl pyrophosphate.] Similarly, the (4*S*)-α-terpinyl cation can itself partition into up to three products: simple deprotonation leads to formation of (4*S*)-limonene, whereas competing intramolecular electrophilic attack on the alternate positions of the cyclohexene double bond generates the corresponding pinyl (bicyclo[3.1.1]heptane) (**4**) and bornyl (bicyclo[2.2.1]heptane) (**5**) cations. The latter cation must itself undergo an additional Wagner–Meerwein rearrangement to **6** before losing a proton from the original C3 methyl to produce (−)-camphene. Loss of a proton from the C3 methyl in the pinyl cation results in formation of β-pinene, whereas deprotonation at C4 of **4** generates the corresponding α-pinene isomer. It will also be noted that the bornyl skeleton need not arise by direct cyclization of the α-terpinyl cation (**3**) but may instead be formed by chemically well-precedented rearrangement of the pinyl cation itself. It is therefore clear that by judicious choice of isotopically labeled substrates it should be possible to perturb the normal proportions of products by selectively influencing the partitioning of the various cationic intermediates. The underlying phenomenon, known as isotopically sensitive branching (Jones et al., 1986) or, less precisely, metabolic switching (Harada et al., 1984), has been used effectively to probe the generation of product mixtures in cytochrome P-450 catalyzed oxidations (Jones et al., 1986; Harada et al., 1984; White et al., 1986), the effect of mechanism-based inhibitors on RTEM β-lactamase (Brenner et al., 1981), and the formation of mixtures of the isomeric sesquiterpenes longifolene and sativene (Arigoni, 1975; Dorn et al., 1975). Such an approach not only provides an unambiguous test of the operation of a single enzyme species but, by proper analysis of the data, allows a quantitative evaluation of the intrinsic isotope effects associated with the isotopically sensitive steps.

The required sample of [10-$^2H_3$]geraniol was readily prepared by treatment of the (*Z*)-acetoxyenol ester **8** with perdeuteriodimethylcopper lithium and reduction of the resulting methyl [10-$^2H_3$]geranoate (**9**) with lithium ethoxyaluminum trihydride (Scheme II). Oxidation of **10** with MnO_2 to geranial (**11**) and reduction with NaB^3H_4 afforded [10-$^2H_3$,1-^{3}H]geraniol, which was converted to the corresponding pyrophosphate ester by standard methods. The latter substrate, at saturating concentrations (8–10 K_m), was incubated with partially purified preparations of the (+)- and (−)-pinene cyclases in parallel with reference incubations of [1-^{3}H]geranyl pyrophosphate. The effect on overall rate of product formation was also assessed for two other cyclases from *S. officinalis*.

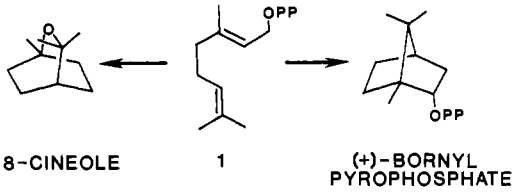
Previous studies have indicated that all monoterpene cyclases share a common initial ionization step, involving generation of the paired cation:pyrophosphate anion **1**, which appears to determine the overall rate of monoterpene production (Satterwhite et al., 1985; Croteau, 1986b). Nonenzymatic solvolytic studies with C4- and C3-methyl-deuteriated geranyl chlorides (Bunton et al., 1972) and related compounds (Brody & Gutsche, 1977) have indicated that a perdeuteriomethyl group can suppress the rate of ionization to the allylic cation. The precise magnitude of such secondary isotope effects on any solvolytic rate will reflect the angular dependence of β-deuterium destabilization of neighboring cations (Sunko et al., 1977) as well as transition-state electron demand (Liu & Wu, 1986), with observed $k(CH_3)/k(CD_3)$ values ranging from 1.09 to 2.13 (Fisher et al., 1975). For enzymatic reactions, such secondary isotope effects are likely to be further attenuated if the ionization step involved in isomerization–cyclization is not uniquely rate determining. It was therefore of some interest that a spectrum of rate suppressions was observed with different monoterpene cyclases isolated from sage. Thus 1,8-cineole cyclase exhibited a 23% reduction in the rate of product formation when [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate was utilized as substrate, while (+)-bornyl pyrophosphate cyclase suffered a 6% rate decrease. Since the formation of

Table I: Product Distributions and Rates of Monoterpene Olefin Formation with (+)- and (−)-Pinene Cyclases[a]

enzyme	substrate	product distribution (%) α-pinene	β-pinene	camphene	sabinene	myrcene	limonene	velocity (total olefins) [nmol/(mg·h)]
(−)-pinene cyclase II	[1-^{3}H]GPP[b]	26	21	28	9	9	8	16
	[10-$^2H_3$,1-^{3}H]GPP	38	13	31	7	4	8	12
(+)-pinene cyclase I	[1-^{3}H]GPP	37		31		10	22	16
	[10-$^2H_3$,1-^{3}H]GPP	42		32		4	22	14

[a] Total monoterpene olefin production [nmol/(mg of protein·h)] was determined by standard assay of aliquots of the same enzyme preparation at a saturating substrate concentration of 20 μM [i.e., (8–10)K_m], distribution of olefinic products was determined by radio gas–liquid chromatographic analysis (see Figure 1), and the experiment was run in quadruplicate with essentially identical results. [b] GPP refers to geranyl pyrophosphate.

Scheme III



neither product involves breaking of a C–H bond (Scheme III), the observed rate decreases must be the result of secondary isotope effects, presumably on the common step involving ionization of the acyclic precursor. Similarly, incubation of [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate with (+)-pinene cyclase resulted in a 13% reduction in total olefin formation, while the (−)-pinene cyclase showed a net 25% decrease in the rate of formation of enzymatically derived olefins. Since saturating concentrations of geranyl pyrophosphate were employed and rates of net product formation from deuteriated and nondeuteriated substrates were compared, the observed rate suppressions represent effects on V_{max} rather than V_{max}/K_m.

Of even greater interest were the distributions of total olefins generated by the (+)- and (−)-pinene cyclases, as determined by radio-GLC analysis of the reaction products from deuteriated and nondeuteriated geranyl pyrophosphate (Figure 1 and Table I). For both cyclases, a noticeable decrease was observed in the proportion of myrcene obtained from the reaction with deuteriated substrate (from 9–10% to 4%), reflecting a primary isotope effect on deprotonation of acyclic cations **2** or **1** and resulting in a suppression of the rate of myrcene formation with a consequent increase in the net commitment to cyclization. Thus, although the *rate constant* k_T does not change, the steady-state concentration of the linalyl cation **2** increases as a result of the decrease in k_M, the rate constant for formation of myrcene, with a consequent enhancement in the rate of cyclization. The most dramatic effect on the product distribution could be seen in the relative proportions of (−)-α-pinene and (−)-β-pinene generated by (−)-pinene cyclase. Whereas nondeuteriated geranyl pyrophosphate gave a ratio of (−)-α-pinene to (−)-β-pinene of 26:21, the corresponding ratio from incubation with deuteriated geranyl pyrophosphate increased to 38:13, representing an enhanced partitioning of the pinyl cation to (−)-α-pinene due to the suppression in (−)-β-pinene formation in response to the primary kinetic isotope effect on the methyl deprotonation, k_β^D. When the net 25% decrease in the overall rate of total olefin formation previously noted is taken into account, it can be seen that, whereas (−)-β-pinene production decreased in both relative proportion and absolute amount [from 3.4 to 1.6 nmol/(mg·h)] when [10-$^2H_3$]geranyl pyrophosphate was utilized as the cyclization substrate, *under saturating conditions both the relative proportion and absolute rate of α-pinene formation were enhanced* [from 4.2 to 4.6 nmol/(mg·h)], in spite of the reduction in the rate of total olefin formation. Moreover, the change in product ratios of (−)-α- and (−)-β-

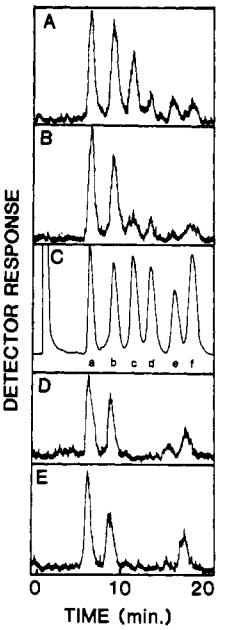


FIGURE 1: Radio gas–liquid chromatographic separation of monoterpene olefins derived from geranyl pyrophosphate with the pinene cyclases: (A) products of (−)-pinene cyclase (cyclase II) generated from [1-^{3}H]geranyl pyrophosphate; (B) products of the same enzyme generated from [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate; (C) elution profile of the authentic monoterpene olefin standards α-pinene (a), camphene (b), β-pinene (c), sabinene (d), myrcene (e), and limonene (f); (D) products of (+)-pinene cyclase (cyclase I) generated from [1-^{3}H]geranyl pyrophosphate; (E) products of the same enzyme generated from [10-$^2H_3$,1-^{3}H]geranyl pyrophosphate. See Materials and Methods for conditions of the chromatographic analysis and other experimental details.

pinene can be used to calculate the isotope effect on the methyl deprotonation, k_β^H/k_β^D. Thus, from the relationships $k_\beta^H/k_\alpha^H = 21/26$ and $k_\beta^D/k_\alpha^H = 13/38$, the apparent k_β^H/k_β^D can be calculated to be 2.4. If movement of the enzyme base from C10 to C4 is rapid compared to both k_β and k_α, the calculated value of 2.4 represents the intrinsic isotope effect on k_β. If, on the other hand, equilibration between the two states leading to each product is slow compared to the individual deprotonation rates, the intrinsic isotope effect will be attenuated and 2.4 represents a lower limit for k_β^H/k_β^D. Interestingly, Pascal has recently reported the results of an investigation utilizing natural abundance ^{2}H NMR spectrometry to analyze commercially available samples of (−)-α- and (−)-β-pinene derived from *Pinus* species (Pascal et al., 1986). Although without knowledge of the number of enzyme species mediating pinene formation in *Pinus*, mechanistic deductions must be tentative, the measured isotopic abundances at key sites in each pinene isomer were used to argue that both compounds arise by partitioning of a common intermediate and that $k_\beta^H/k_\beta^D = 2.1$. The observed isotope effect may be compared with a $k^H/k^D = 1$, which has been inferred for the side-chain methyl deprotonation leading to the generation of the $\Delta^{24(28)}$ inter-

mediate of ergosterol biosynthesis (Arigoni, 1978) and corresponding intramolecular isotope effects of $k^H/k^D = 4.25$ and 5.59, respectively, which have been measured for the methyl deprotonation that generates the exomethylene group of copalyl pyrophosphate and for the terminating deprotonation to form the exomethylene of kaurene (R. M. Coates, H. Brunner, and S. Inoue, personal communication). Needless to say, the observation of isotopically sensitive branching in the formation of (−)-α-pinene and (−)-β-pinene unambiguously confirms that both products are generated from a *common* intermediate, that is, by a *single enzyme*.

In contrast to the marked effect on the proportion of (−)-α-pinene to (−)-β-pinene produced by incubation of [10-$^2H_3$]geranyl pyrophosphate with (−)-pinene cyclase, there was no effect on the relative proportions of (−)-camphene to *total* pinenes, which corresponded to 28:47 = 0.6 and 31:51 = 0.6, for nondeuteriated and deuteriated precursor, respectively. The latter observation establishes that the branching to the bornyl cation (**5**) occurs prior to the isotopically sensitive step, thereby implying that **5** is formed directly from the (4*S*)-α-terpinyl cation (**3**) rather than by rearrangement of a common pinyl cation (**4**). The latter results would appear to lay to rest any concerns that the direct formation of the bicyclo[2.2.1]heptane ring system in this and related systems is somehow forbidden on the basis of a perceived violation of Baldwin's empirical rules for ring closure (Baldwin, 1976; Baldwin et al., 1976). It should also be noted that, although the terminating deprotonation of the camphyl cation (**6**) is likely to be itself subject to a microscopic isotope effect, this will not be translated into an effect on product distribution, provided that the formation of **5** and/or **6** is irreversible. The small increase in the measured proportion of (+)-α-pinene to (+)-camphene when deuteriated geranyl pyrophosphate was incubated with (+)-pinene cyclase is almost certainly not sufficient to warrant the inference that the formation of **5** is reversible. Finally, it should be noted that the proportions of (+)-sabinene and (+)- and (−)-limonene remained essentially invariant (Table I). The former is the product of minor contaminating sabinene cyclase activity, and the partitioning to, and the terminating deprotonations of, the latter products would be expected to be minimally perturbed by the isotopic substitution.

The results described here, as well as earlier studies with the pinene cyclases from *S. officinalis* (Gambliel & Croteau, 1984), invalidate a proposal that α-pinene arises *via* isomerization of β-pinene, or vice versa (Banthorpe et al., 1984). Evidence for this discrete isomerization step was based on in vivo studies in *Pinus* species, and it is possible that such fundamentally different routes to the same monoterpene occur in different species. Several lines of evidence argue against this interpretation, however, including the well-documented interspecies mechanistic similarities evidenced by other cyclases affording the same monoterpene product (Croteau et al., 1985a,b), in vivo studies that support a mechanistically similar partitioning phenomenon in the biosynthesis of the sesquiterpenes sativene and longifolene (Arigoni, 1975; Dorn et al., 1975), and the recent evidence for a common biosynthetic origin of the (−)-pinenes from *Pinus* based on natural abundance ^2H NMR spectrometry (Pascal et al., 1986).

ACKNOWLEDGMENTS

We thank Greg Wichelns for raising the plants and Bonnie Frazier for typing the manuscript.

REFERENCES

Aberhart, D. J., & Lin, L. J. (1974) *J. Chem. Soc., Perkin Trans. 1*, 2320–2326.
Andersen, N. H., Ohta, Y., & Syrdal, D. D. (1978) *Bioorg. Chem. 2*, 1–37.
Arigoni, D. (1975) *Pure Appl. Chem. 41*, 219–245.
Arigoni, D. (1978) *Ciba Found. Symp. 60*, 243–261.
Baldwin, J. E. (1976) *J. Chem. Soc., Chem. Commun.*, 734–736.
Baldwin, J. E., Cutting, J., Dupont, W., Kruse, L., Silberman, L., & Thomas, R. C. (1976) *J. Chem. Soc., Chem. Commun.*, 736–738.
Banthorpe, D. V., Ekundayo, O., & Njar, V. C. O. (1984) *Phytochemistry 23*, 291–294.
Brenner, D. G., Knowles, J. R., & Rihs, G. (1981) *Biochemistry 20*, 3680–3687.
Brody, E. P., & Gutsche, C. D. (1977) *Tetrahedron 33*, 723–729.
Bunton, C. A., Leresche, J.-P., & Hachey, D. (1972) *Tetrahedron Lett.*, 2431–2434.
Bunton, C. A., Cori, O., Hachey, D., & Leresche, J.-P. (1979) *J. Org. Chem. 44*, 3238–3244.
Cane, D. E. (1980) *Tetrahedron 36*, 1109–1159.
Cane, D. E. (1985) *Acc. Chem. Res. 18*, 220–226.
Casey, C. P., & Marten, D. F. (1973) *Synth. Commun. 3*, 321–324.
Casey, C. P., Marten, D. F., & Boggs, R. A. (1973) *Tetrahedron Lett.*, 2071–2074.
Cramer, F., & Rittersdorf, W. (1967) *Tetrahedron 23*, 3015–3022.
Croteau, R. (1981) in *Biosynthesis of Isoprenoid Compounds* (Porter, J. W., & Spurgeon, S. L., Eds.) Vol. 1, pp 225–282, Wiley, New York.
Croteau, R. (1986a) in *Biogeneration of Aromas* (Parliment, T., & Croteau, R., Eds.) ACS Symp. Ser. No. 317, pp 134–156, American Chemical Society, Washington, DC.
Croteau, R. (1986b) *Arch. Biochem. Biophys. 251*, 777–782.
Croteau, R., & Karp, F. (1976) *Arch. Biochem. Biophys. 176*, 734–746.
Croteau, R., & Karp, F. (1977) *Arch. Biochem. Biophys. 179*, 257–265.
Croteau, R., & Karp, F. (1979) *Arch. Biochem. Biophys. 198*, 523–532.
Croteau, R., & Cane, D. E., (1985) *Methods Enzymol. 110*, 352–405.
Croteau, R., Felton, N. M., & Wheeler, C. J. (1985a) *J. Biol. Chem. 260*, 5956–5962.
Croteau, R., Shaskus, J. J., Renstrøm, B., Felton, N. M., Cane, D. E., Saito, A., & Chang, C. (1985b) *Biochemistry 24*, 7077–7086.
Croteau, R., Wheeler, C. J., Aksela, R., & Oehlschlager, A. C. (1986a) *J. Biol. Chem. 261*, 7257–7263.
Croteau, R., Satterwhite, D. M., Cane, D. E., & Chang, C. C. (1986b) *J. Biol. Chem. 261*, 13438–13445.
Dorn, F., Bernasconi, P., & Arigoni, D. (1975) *Chimia 29*, 24–25.
Filler, R., & Naqui, S. M. (1963) *Tetrahedron 19*, 879–889.
Fisher, R. D., Seib, R. C., Shiner, V. J., Jr., Szele, I., Tomič, M., & Sunko, D. E. (1975) *J. Am. Chem. Soc. 97*, 2408–2413.
Gambliel, H., & Croteau, R. (1982) *J. Biol. Chem. 257*, 2335–2342.
Gambliel, H., & Croteau, R. (1984) *J. Biol. Chem. 259*, 740–748.
Gelb, M. H., Heimbock, D. C., Mälkönen, P., & Sligar, S. G. (1982) *Biochemistry 21*, 370–377.
Haley, R. C., Miller, J. A., & Wood, H. C. S. (1969) *J. Chem. Soc. C*, 264–266.

Harada, N., Miwa, G. T., Walsh, J. S., & Lu, A. Y. H. (1984) *J. Biol. Chem. 259* 3005–3010.
Huckin, S. N., & Weiler, L. (1974) *J. Am. Chem. Soc. 96*, 1082–1087.
Jones, J. P., Korzekwa, K. R., Rettie, A. E., & Trager, W. F. (1986) *J. Am. Chem. Soc. 108*, 7074–7078.
Karp, F., & Croteau, R. (1982) *Arch. Biochem. Biophys. 216*, 616–624.
Kluger, R., & Brandl, M. (1986) *J. Org. Chem. 51*, 3964–3968.
Liu, K.-T., & Wu, Y. W. (1986) *Tetrahedron Lett.*, 3623–3626.
McCormick, J. P., & Barton, D. L. (1978) *Tetrahedron 34*, 325–330.
Pascal, R. A., Jr., Baum, M. W., Wagner, C. K., Rodgers, L. R., & Huang, D.-S. (1986) *J. Am. Chem. Soc. 108*, 6477–6482.
Samuelson, A. G., & Carpenter, B. K. (1981) *J. Chem. Soc., Chem. Commun.*, 354–356.
Satterwhite, D. M., Wheeler, C. J., & Croteau, R. (1985) *J. Biol. Chem. 260*, 13901–13908.
Still, W. C., Kahn, M., & Mitra, A. (1978) *J. Org. Chem. 43*, 2923–2925.
Sunko, D. E., Szele, I., & Hehre, W. J. (1977) *J. Am. Chem. Soc. 99*, 5000–5005.
Weiler, L. (1970) *J. Am. Chem. Soc. 92*, 6702–6704.
Wheeler, C. J., & Croteau, R. (1986a) *Arch. Biochem. Biophys. 246*, 733–742.
Wheeler, C. J., & Croteau, R. (1986b) *Arch. Biochem. Biophys. 248*, 429–434.
Wheeler, C. J., & Croteau, R. (1987) *J. Biol. Chem. 262*, 8213–8219.
Winkle, M. B., Lansinger, J. M., & Ronald, R. C. (1980) *J. Chem. Soc., Chem. Commun.*, 87–88.

Spontaneous Fusion of Phosphatidylcholine Small Unilamellar Vesicles in the Fluid Phase†

Barry R. Lentz,* Tamra J. Carpenter,‡ and Dennis R. Alford§
Department of Biochemistry and Nutrition, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27514

Received September 18, 1986; Revised Manuscript Received January 20, 1987

ABSTRACT: Using a high-sensitivity differential scanning microcalorimeter capable of performing cooling scans, we have examined the phase behavior of small unilamellar vesicles (SUV) as a function of time of storage above their order–disorder phase transition. Vesicles composed of dipalmitoylphosphatidylcholine (DPPC) and dimyristoylphosphatidylcholine (DMPC) were examined. Cooling scans on fresh (5–7-h postsonication) samples revealed broad, relatively simple heat capacity peaks (peak temperatures: 19.9 °C for DMPC, 37.8 °C for DPPC) free of high-temperature spikes or shoulders. Subsequent heating scans displayed a sharp peak characteristic of previously described fusion products formed below the phase transition. SUV samples stored for 1 or more days above their phase transition displayed a moderately broad, high-temperature shoulder (23.8 °C for DMPC and 40.2 °C for DPPC) in the cooling profile. For DMPC, the enthalpy associated with this peak increased in a first-order fashion with time. Hydrolysis products were not detected until 12–20 days of storage. Both the rate and extent of shoulder appearance increased with temperature (k = 0.0017 h^{-1}, fraction of total enthalpy = 0.1 at 36 °C; k = 0.0037 h^{-1}, fraction = 0.2 at 42 °C). Freeze–fracture electron micrographs confirmed that an intermediate-sized vesicle population (diameters 400–500 Å) appeared in SUV samples stored above their phase transition. Also, the trapped volume of DMPC SUV increased from 0.26 μL/μmol after 17 h of storage to 0.54 μL/μmol after storage for 16 days at 36 °C. We ruled out the possibility that esterolytic hydrolysis products might be responsible for inducing membrane fusion by performing experiments with ditetradecylphosphorylcholine, an ether lipid analogue of DMPC. Comparable results were obtained (k = 0.0047 h^{-1}, fraction = 0.25 at 41.5 °C). Our results demonstrate that at least a subpopulation of a normal SUV preparation is thermodynamically unstable even above the SUV phase transition.

Sonicated small unilamellar vesicles (SUV)[1] and large multilamellar or large unilamellar vesicles (LMV or LUV) are popular systems for modeling the properties of the phospholipid bilayer portions of biological membranes. However, use of SUV has been limited to modeling the effects of local bilayer curvature on biomembrane properties (Sackmann et al., 1984; Gruner, 1985). This is because SUV experience molecular packing constraints resulting from their high curvature (Sheetz & Chan, 1972; Huang & Mason, 1978). The packing inhomogeneities caused by high curvature are believed to account for the unique physical properties of SUV. Among these are the asymmetric distribution of certain phospholipid species between the inner and outer leaflets of the bilayer

† Supported by USPHS Grant GM 32707.
* Address correspondence to this author.
‡ Present address: Operations Research Analyst, RCA Staff Center, Princeton, NJ 08540.
§ Present address: Department of Pharmacy and Pharmaceutical Chemistry, School of Pharmacy, University of California at San Francisco, San Francisco, CA 94143.

[1] Abbreviations: SUV, small unilamellar vesicles(s); LMV, large multilamellar vesicle(s); LUV, large unilamellar vesicle(s); DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DTPC, 1,2-ditetradecyl-3-*sn*-phosphorylcholine; HPLC, high-performance liquid chromatography.

0006-2960/87/0426-5389$01.50/0