Biosynthesis of (±)-α-Pinene and (−)-β-Pinene from Geranyl Pyrophosphate by a Soluble Enzyme System from Sage (Salvia officinalis)*

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The volatile oil of immature sage (Salvia officinalis) leaves was shown to contain (±)-α-pinene and (−)-β-pinene as major constituents, and soluble enzyme preparations from this tissue catalyzed the divergent cation-dependent conversion of the acyclic C10 precursor geranyl pyrophosphate to α-pinene and β-pinene and to lesser amounts of other monoterpene olefins. The identities of the principal biosynthetic products as α-pinene and β-pinene were confirmed by chromatographic analysis and by the synthesis of crystalline derivatives. The biosynthetic pinenes derived from [1-3H]geranyl pyrophosphate were stereospecifically converted to camphor, and exchange of the α-hydrogens of this ketone established that all the tritium of the original pinene nucleus was located on the methylene bridge carbon (C-7), thus demonstrating specific conversion of the acyclic precursor. Stereospecific conversion of the biosynthetic pinenes to fenchone oxime, followed by resolution of the corresponding diastereomeric (−)-methylxoxymethyl ethers, confirmed the stereochemistry of the original products as (±)-(−)-[7-3H]α-pinene and (−)-(−)[7-7-H]β-pinene. The possibility of isomerization of β-pinene to α-pinene was eliminated, as was the involvement of other free intermediates, thus indicating direct cyclization of geranyl pyrophosphate to the mixture of pinene isomers. Neryl pyrophosphate and linalool pyrophosphate could also function as acyclic precursors of the pinenes and of the other monoterpene olefins, but only geranyl pyrophosphate afforded a product distribution comparable to that formed in vivo. Preliminary examination of the cell-free system suggested the presence of more than one pinene cyclase activity.

α-Pinene1 and β-pinene (Fig. 1) are among the most widely

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1 The abbreviations and trivial names used are: α-pinene, 2,6,6-trimethylbicyclo[3.1.1]hept-2-ene; GPP, pyrophosphate ester of geraniol (3,7-dimethylocta-2,6-dien-1-ol); NPP, pyrophosphate ester of nerol (3,7-dimethylocta-2,6-dien-1-ol); LPP, pyrophosphate ester of linalool (3,7-dimethyl-1,6-dien-3-ol); β-pinene, 4,6-dimethyl-2-methylenecyclo[3.1.1]heptane; camphene, 2,2-dimethyl-3-methyl-2-methylenecyclo[2.2.2]heptane; terpinolene, 1-methyl-4-isopropylidene-1-cyclohexene; limonene, 1-methyl-4-isopropylidene-1-cyclohexene; distributed monoterpenes in the plant kingdom and are the major constituents of the various turpentes (1). Both optical antipodes of α-pinene are natural products, and they may co-occur with either isomer predominant (1–3). By contrast, β-pinene almost always occurs as the optically pure (−)-[1S: 5S] isomer (1), the (−)-(1R,5S) antipode apparently being of rather limited distribution (2). Ruticka et al. (4) proposed an ionic scheme for the formation of the pinenes (Fig. 2, Scheme I) involving cyclization of the neryl cation to a monocyclic α-terpinyl intermediate which undergoes internal Markownikoff addition to a vinyl cation that is subsequently stabilized by two variants of proton loss. On the basis of recent in vivo time course studies with Pinus pinaster (5), the acyclic trienes cis-ocimene and myrcene were suggested as precursors of α- and β-pinene, respectively (Fig. 2, Scheme II), reviving earlier radical-type cyclization schemes for the construction of the pinenes (6, 7). Finally, the favorable isomerization of the exocyclic to the endocyclic isomer (7, 8) raises the possibility of a single cyclization of an acyclic precursor to β-pinene, followed by enzymic (or nonenzymic) conversion to α-pinene (Fig. 2, Scheme III). The labeling pattern of α-pinene derived from exogenous [2-14C]mevalonate in Pinus attenuata (9) is consistent with any of the above biogenetic schemes and no experimental evidence to distinguish among the alternatives is available. While the origin of the pinenes is thus uncertain, the results of chemotaxonomic studies (10) and in vivo tracer studies (11) suggest that the various isomers probably arise via distinct routes involving variants both in the stereochemistry of ring formation and in the generation of the double bond in either the endocyclic or exocyclic position. The fact that positionally isomeric and erantiomeric pinenes co-occur, with the combination (±)-α-pinene and (−)-β-pinene being particularly common (12, 13), further implies that such multiple routes are operative in the same organism.

Crude cell-free extracts from Citrus limonum (14), Pinus radiata (15), and common sage (Salvia officinalis) (16, 17) convert the acyclic C10 precursors GPP and NPP to products chromatographically coincident with α-pinene and β-pinene. However, the identities of the biosynthetic products were not confirmed, and, although both tritium atoms from [1-3H]U-14C JGPW were shown to be retained in the pinenes in the latter study (17), the labeling pattern and the stereochemistry of the products were not verified. Furthermore, the possible isomery of

myrcene, 3-methylethyl-1,6-octadiene; ocmene, 3,7-di-methyl-1,3(cis-or trans)-6-octadiene; α-terpineol, 1-methyl-4(2-hydroxyisopropyl)-1-cyclohexene; borneol, 1,7,7-trimethylbicyclo[2.2.1]heptan-2-ol(endo); 1,8-cineole, 1,3,3-trimethyl-2-oxabicyclo[2.2.2]octane; MRS, 2-N-morpholinoethanesulfonic acid; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; GLC, gas-liquid chromatography; TLC, thin layer chromatography.
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The essential oil of common sage contains  $\alpha$-pinene (thought to be mainly the (+)-isomer (2)) and $\beta$-pinene (presumed to be the (−)-isomer) as the major terpene hydrocarbons, and sage leaf extracts possess the requisite biosynthetic capability (16, 17). This system, which has been utilized for studies on the biosynthesis of p-methane and bornane monoterpenes (18, 19), therefore provided the potential opportunity to examine in detail the biosynthesis of isomeric pinenes. In this communication, we report the conversion of $[1-^3H]$GPP, and other acyclic precursors, to (+)-[7-^3H]$\alpha$-pinene, (−)-[7-^3H]$\alpha$-pinene, and (−)-[7-^3H]$\beta$-pinene by a soluble enzyme preparation from sage leaves and describe some of the basic characteristics of this novel biosynthetic system.

EXPERIMENTAL PROCEDURES

Plants—Sage plants (S. officinalis L.) were grown from seed purchased from George Ball, Pacific Inc. (Sunnyvale, CA) in peat moss/perlite/sand (1:1:1) under long day conditions (16 h; 1800 foot-candles). Rapidly expanding leaves (2–3 cm in length) from the shoot apex of immature plants (3–5 weeks postgermination) were collected and washed with distilled water before use.

Substrates and Reagents—The preparation of [1-^3H]GPP (300 Ci/mmol) and [1-^3H]NPP (900 Ci/mmol) has been described (16, 19). (±)-[1-^3H]Linalool was obtained by mesylation of [1-^3H]geraniol in triethylamine (20) followed by solvolysis in water. The product was purified by TLC (Silia Gel G containing 8% AgNO$_3$, solvent system A) and its identity confirmed by radio GLC. [1-^3H]LPP (200 Ci/mol) was prepared from the alcohol by modification of the Cramer and Bohm method (21, 22) and was purified by ion-exchange chromatography as described previously (16). The purity of this product was verified as before (16) by analysis of the products of alkaline phosphatase-arylsulfatase hydrolysis and acid hydrolysis. (+)-$\alpha$-Pinene, (−)-$\alpha$-pinene, and (−)-$\beta$-pinene were obtained from Aldrich Chemical Co., cis- and trans-pinanol were from PCR Research Chemicals Inc., (±)-limonene was from ICN Pharmaceuticals, terpinolene was from Haarmann and Reimer GmbH, Holzminden, West Germany, and the ocimenes were from International Flavors and Fragrances. All other monoterpenes were purchased from Pfaltz and Bauer, Inc. and were purified by distillation or chromatography before use. For isotopic dilution experiments, the monoterpenes were suspended in water with the aid of Tween 20 (40 $\mu$g/ml of terpene) and sonication (needle probe of a Biosonik III at full power). Insoluble polystyrylpyrrolidone (Polyclar AT, GAP Corp.) and Amberlite XAD-4 polystyrene resin (Robim and Haas Corp.) were purified by standard procedures for use as adsorbents (23, 24). All other biochemicals and reagents were obtained from Sigma or Aldrich.

**Enzyme Preparation**—Sage leaves (2–3 g) were homogenized in a Ten-Broek homogenizer containing a slurry of an equivalent leaf weight of insoluble polystyrylpyrrolidone in cold 75 mM MES-5 mM sodium phosphate buffer, pH 6.5, containing 20% glycerol, 5 mM dithioerythritol, 10 mM Na$_2$SO$_4$, 10 mM sodium ascorbate, 15 mM MgCl$_2$. The homogenate was then slurried with an equal tissue weight of hydrated XAD-4 polystyrene resin and passed through four layers of cheesecloth. Centrifugation at 27,000 × g for 20 min (pellet discarded), then at 105,000 × g for 1 h, provided the soluble supernatant that, after dialysis to assay conditions, was used as the enzyme source.

**Enzyme Assays**—A typical reaction mixture contained 0.2 to 0.5 mg of protein in a total volume of 1 ml of 10 mM MES-5 mM sodium phosphate buffer or 10 mM Tricine-5 mM sodium phosphate buffer, pH 6.5, containing 0.5 mM dithioerythritol, 15 mM MgCl$_2$ (or 1 mM MnCl$_2$) in a Teflon-sealed screw cap vial. In some experiments, 1 mM sodium ascorbate and 1 mM Na$_2$SO$_4$ were included in the assay medium, and the buffer contained 10% glycerol. The reaction was started by the addition of up to 10 μM (10 nmol) of either of the three acyclic precursors ([1-^3H]GPP, [1-^3H]NPP, or (±)[1-^3H]LPP), and 1 ml of pentane was carefully overlaid to trap volatile monoterpenes. The addition of the pentane layer improves the recovery of monoterpenes olefins by as much as 30% and therefore was used routinely. After incubation for 1 h at 28 °C, the reaction was quenched by vigorous mixing, and the pentane layer was removed. The aqueous phase was re-extracted with an additional 1 ml of pentane, and the combined extract was eluted through a column (0.5 × 3 cm) of silica acid (Mallinkrodt, SilicAR CC-7) in a Pasteur pipette, followed by washing with another 0.5 ml of pentane. The hydrocarbon fraction thus obtained is free of oxygenated products which remain adsorbed on the siliceous acid. Internal standards (5 mg each of (±)-$\alpha$-pinene, (−)-$\beta$-pinene, (±)-camphene, myrcene, (±)-limonene, and terpinolene) were then added, and the total volume was adjusted to 3.5 ml. A 1-ml aliquot was removed for determination of tritium, and the...
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remainder was concentrated under a stream of nitrogen at 0°C for analysis by radio GLC or TLC (Silica Gel G containing 12% AgNO3, solvent system B).

The oxygenated monoterpene products were recovered by re-extracting the original aqueous layer of the incubation mixture with ether (1.5 ml), followed by elution of this extract through the original pentane-washed silicic acid column. Internal standards were added to the eluent 1-4 mg each of 1,8-cineole, (+)-limonene, (±)-borneol, geraniol, and nerol and the volume was adjusted to 3.5 ml, as for the hydrocarbon assay. The tritium content was determined on a 1-ml aliquot, and the remainder was concentrated as before for radio GLC and TLC (Silica Gel G containing 8% AgNO3, solvent system E). TLC-purified products to be analyzed further were eluted from the silica gel with ether.

Examination of the incubation mixture for phosphorylated products was carried out by adjusting the previously extracted aqueous phase to pH 5.0 with 0.1 M sodium acetate buffer, followed by incubation with 2 units of wheat germ acid phosphatase (Sigma) for 1 h at 28°C. Alcohols thus liberated were extracted with ether (2 x 1.5 ml) and the extract was dried by passage through a column (0.5 x 3 cm) of anhydrous Na2SO4. Nerol, geraniol, and (±)-limonene (~3 mg each) were added, and the extract was brought to a volume of 3.5 ml with ether. An aliquot was taken for tritium determination and the remaining sample was concentrated for radio GLC and TLC analysis as described above for oxygenated monoterpene products.

Appropriate boiled controls were included in each experiment, and in all cases nonenzymic formation of the terpenes of interest was negligible. Two-week-old cultures, enriched in (+)-a-pinene, showed no effect of this detergent on pineene biosynthesis from GPP. The recovery of labeled products by the above assay procedures was routinely ≥ 95%, as determined with authentic [1-3H]limonene as standard. Protein was routinely estimated by the dye binding assay (Bio-Rad Laboratories).

Purification and Characterization of Monoterpene Olefins from Sage Oil—Young sage leaves (0.4 kg) were exhaustively extracted with pentane and an aliquot was analyzed by packed column and capillary GLC. The extract was concentrated and subjected to vacuum distillation (75°C at 10 mm Hg), and the distillate was re-examined by GLC. No qualitative or quantitative differences in monoterpene composition were noted between the native and distilled oil. The oil was diluted with pentane and passed through a column (2 x 50 cm) of silicic acid (Mallinkrodt, SilicaAR CC-7) to remove oxygenated terpenes. The hydrocarbon fraction was recovered, and the individual olefins of interest were isolated by repeated preparative argentation TLC (Silica Gel G impregnated with 8% AgNO3, solvent system B). Olefin samples for the determination of optical rotation were of chemical purity greater than 96% as determined by GLC. For the examination of the influence of steam distillation on sage terpenes, alkaline hydrolysis of the extracted olefins, as well as the pure olefins, were distilled from 0.1 M HCl, 0.1 M KOH, or water, and the distillates were examined by GLC.

The labeled biosynthetic olefins, obtained from the hydrocarbon fraction of large scale enzyme preparations, were purified for product identification by TLC (Silica Gel G containing 12% AgNO3, solvent system C). The products were visualized on the chromatogram by fluorescence quenching of 2,7-dichlorofluorescein and were recovered from the scraped gel by pentane extraction.

Chemical Conversions—The stereospecific isomerization of β-pinene to α-pinene was carried out by modification of the procedure of Rudakov and Shestaeva (25) (Fig. 4). Two mmol of purified [1-3H]β-pinene (1.88 mCi/mol) were transferred to an ampoule with 0.2 mmol of benzoic acid and 0.1 mmol of hydroquinone (as antioxidant), and the ampoule was sealed under N2 and heated at 120°C for 15 h. The cooled reaction mixture was taken up in pentane, washed with saturated sodium bicarbonate and ether, dried over activated charcoal (Aktivrich decolorizing charcoal), and eluted through a silicic acid column (1 x 6 cm) with pentane. The [1-3H]α-pinene obtained in this fashion (>85% yield) was freed from residual starting material by preparative argentation TLC (Silica Gel G containing 8% AgNO3, solvent system C).

The oxidation of α-pinene to carvone was performed by a variation of the method of Sam and Simmons (26) (Fig. 6). Two mmol of (±)-[1-3H]α-pinene (0.51 mCi/mol) were dissolved in 15 ml of dry benzene containing 5.5 mmol of K2MoO4 and 1 mmol of dicyclohexano-18-crown-6. The reaction mixture was stirred vigorously with coarse sand for 5 h at 20°C and then acidified. MoO3 formed in the reaction was converted to the soluble sulfate with the minimum quantity of Na2SO4, and the organic products were extracted with ether which was back-washed with brine. Extraction of the ether layer with 0.1 m KOH afforded the crude pinonic acid, which was recovered from the aqueous phase by phase separation and re-extraction with ether. This material was taken to dryness under vacuum, and the residue was subjected to TLC (Silica Gel G, solvent system D) to afford (±)-cis-pinonic acid (Rf = 0.3) which was recrystallized from hot water (Fisher Johns; melting point, 104°C).

The hydroxylation of α-pinene to the rearranged products bornyl acetate and fenchol was accomplished by the technique described by Williams and Whittaker (27) (Fig. 4). Two mmol of [1-14C]α-pinene (1.55 mCi/mol) or [1-3H]β-pinene (3.56 mCi/mol) were dissolved in acetonitrile-water (19:1, v/v) containing 0.075 M H2SO4 and heated at 75°C in a sealed tube for 12 h. After cooling and neutralization with Na2CO3, the reaction mixture was diluted with pentane and the pentane layer was repeatedly washed with brine. The pentane extract was concentrated and subjected to TLC (Silica Gel G, solvent system E) to afford [1-3H]endofenchol (Rf = 0.51; yield, 8-10%) and [3H]bornyl (Rf = 0.41; yield, 9-12%). Two-phase CrO3 oxidation (28) of the alcohols provided the respective ketones fenchone and camphor in nearly quantitative yield. The ketones were purified by TLC as above and their purities were verified to be greater than 95% by GLC.

The stereospecificity of the rearrangements was examined in separate reactions in which the optical purities of reactants and products were determined. It was thus demonstrated that the conversion to endo-fenchol proceeds without racemization, while the conversion to bornyl occurs with up to 25% racemization [(+) -α-pinene, [α]+ = 46.5° (neat) +52.4°, Ref. 29], affords (+)-fenchone, [α]+ = 38.7° (124 in hexane) and (+)-camphor, [α]+ = 45.0° (167 in hexane), while (−)-β-pinene, [α] − = 39.6° (6.4 in hexane) and (−)-camphor, [α] − = 50.5° (6.7 in hexane) (Fig. 4). The optical rotations of the fenchone and camphor thus obtained were compared to reference standards of known purity and to literature values (2). To determine the stereochemical composition of the biosynthetic [3H]-labeled pinenes, the stereospecifically derived fenchones were derivatized to the crystalline oximes and then converted to the diastereomeric (−)-menthoxyethyl ethers (Fig. 4) which were separated by TLC as described previously (30).

Chromatography and Determination of Radioactivity—TLC was performed on plates of Silica Gel G or Silica Gel G impregnated with AgNO3 (8% to 15%), which had been activated at 110°C for 4 h. The solvent systems employed were: (A) hexane/ethyl acetate (73, 27, v/v); (B) ethanolic solution of 2,7-dichlorofluorescein and water (IM, v/v); (C) hexane/ether/acetic acid (25:25:5, v/v/v); and (D) hexane/ether/acetic acid (55:35:5, v/v/v), (E) hexane/ethyl acetate (82:18, v/v). After development, chromatograms were sprayed with a 0.2% ethanolic solution of 2,7-dichlorofluorescein and viewed under UV light to locate the appropriate components. Analytical capillary GLC was performed with a Perkin-Elmer Sigma 3B chromatograph on a 25-meter fused silicon column coated with Carbowax 20M at 50°C (0.75 mm outer diameter stainless steel (hydrocarbons) and the packed column GLC was performed with a Packard 420 chromatograph. The columns were: 25% Carbosieve 4000 on 80/100 mesh Gas-Chrom Q, 3.4 x 3 mm outer diameter stainless steel (other hydrocarbons) and the packed column GLC was performed with a Packard 420 chromatograph. The columns were: 25% Carbosieve 4000 on 80/100 mesh Gas-Chrom Q, 3.4 x 3 mm outer diameter stainless steel. Other chromatographic conditions are described in the text. Radio GLC was performed with a Varian chromatograph attached to a Model 7357 Nuclear Chicago radioactivity monitor calibrated externally with [3H]lucane. Quantitation of data was by integration of radio and mass peaks of the recorder output.

Radioactivity in liquid samples and thin layer fractions was determined in a counting solution (15 ml) consisting of 0.3% (w/v) Omnifluor (New England Nuclear) dissolved in 30% ethanol in toluene with a Packard 3250 LS spectrometer. The counting efficiency for tritium was 75% and all assays were conducted with a standard deviation of less than 3%.

RESULTS AND DISCUSSION

Monoterpene Composition of S. officinalis—Previous analyses of the monoterpene s of S. officinalis have been carried out on the steam-distilled oil, and have indicated significant variation in α- and β-pinene content (16) and in optical purity of α-pinene (31). As β-pinene is known to isomerize to α-pinene under a variety of conditions (7, 8, 25), the results of these analyses were initially suspect. Samples of sage leaves, of the age used for the following biosynthetic studies, were therefore thoroughly extracted with pentane and the extracts

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were analyzed directly by capillary and packed column GLC, revealing a monoterpene olefin fraction consisting of α-pinene (27-28%), camphene (19-21%), β-pinene (38-44%), myrcene (3-5%), limonene (4-6%), sabinene (<3%), terpinolene (<1%), and α-thujene (<1%) (Fig. 3). Vacuum distillation of the pentane extract (GLC analysis indicated no change in the olefin fraction which comprised ~33% of the volatile oil), followed by column chromatography and repetitive argenta-
tion TLC, afforded pure α-pinene and β-pinene. Determination of optical rotation revealed the α-pinene to consist of 70% of the (+)-(1R:5R)-isomer and 30% of the (−)-(1S:5S)-isomer ([α]D° 21.7° (6.4 in CHCl3); ([α]D° 52.4° in Ref. 29)), and the β-pinene to consist of the essentially pure (−)-(1S:5S)-isomer ([α]D° 20.9° (5.5 in CHCl3); ([α]D° 22.7° in Ref. 29)). These analytical values are assumed to provide an accurate assessment of the quantity and optical purity of the pinenes in their native state.

To examine the possibility of artifact formation, aliquots of the pentane extract and vacuum-distilled oil were steam disti-
Fined under acidic, neutral, and basic conditions, and the steam distillate was examined by GLC. Under no circum-
stances did the content of α-pinene and β-pinene vary from its original composition in the undistilled extract. Furthermore, when pure α-pinene and β-pinene of known optical purity were separately subjected to all of the chromatographic and distillation procedures described, no significant interconversion (<1%) or change in optical rotation was observed. Thus, previous analyses may be accepted, and the observed variation in α-pinene and β-pinene content and optical purity may be attributed to developmental variation of the tissue, rather than to artifact formation during isolation and analysis.

Conversion of Acyclic Precursors to Pinenes by a Cell-free Preparation—Recent studies (12) have shown the general isoprenoid intermediate GPP to be an efficient precursor of cyclic monoterpenes, but in this study no cyclic monoterpenes were formed. We have shown that the (+)-(1R:5R)-GPP isomer (Fig. 3A) was converted to α-pinene (Fig. 3B), whereas the (−)-(1S:5S)-isomer (Fig. 3C) gave rise to P-pinene (Fig. 3D). The configuration of this acyclic compound prevents simple C-1-C-6 cyclization. Therefore, to directly examine the biosyn-
thesis of pinene monoterpenes, aliquots of a dialyzed 105,000 × g supernatant, prepared from a homogenate of immature sage leaves, were incubated with [1-3H]GPP in the presence of saturating levels of MgCl2 (15 mM) or MnCl2 (1 mM). The acyclic compounds [1-3H]NPP and [1-3H]LPP were also tested as possible precursors, as they have been traditionally regarded on stereochemical grounds as more likely precursors of cyclic monoterpenes than GPP (32-34). The hydrocarbon fraction isolated from each incubation mixture of acyclic pre-
cursor and cation was radioactive (3-5% conversion of sub-
strate was observed), and when subjected to radio GLC analysis was shown to contain several monoterpenes (Fig. 3, A–F). No labeled hydrocarbons were detected in the absence of cation or when the complete mixtures were incubated with boiled enzyme. Particulate fractions, prepared by differential centrifugation, were essentially inactive in the biosynthesis of monoterpenes olefins, consistent with preliminary observations (16).

With [1-3H]GPP as the acyclic precursor and Mg2+ as the cation, α-pinene and β-pinene were the major hydrocarbon products of the soluble enzyme preparation, with lesser amounts of camphene, myrcene, and limonene also formed (Fig. 3A). The distribution of the biosynthetic products was virtually identical with that of the monoterpenes olefin fraction isolated from sage leaves at the same stage of development. Substitution of Mn2+ for Mg2+ in the reaction mixture sub-
stantially altered the yield and distribution of olefins derived from GPP, affording 33% lower conversion and β-pinene as the major product (Fig. 3D). [1-3H]NPP in the presence of either Mg2+ or Mn2+ also gave rise to the pinenes, but in this instance inordinately large quantities of limonene and significant levels of terpinolene were also formed (Fig. 3, B and E). Limonene and terpinolene are minor constituents of the hydro-
carbon fraction from the oil of sage. The constituents were identified as α-pinene (a), camphene (b), β-pinene (c), myrcene (d), limonene (e), and terpinolene (h). α-Thujene and sabine are minor components located, respectively, on the leading edge of the α-pinene peak and the trailing edge of the β-pinene peak. The arrows at f and g indicate the positions of cis- and trans-ocimene, respectively, which were not detected as components of the hydrocarbon fraction. The top tracings (A–F) are the responses of the radioactivity monitor attached to the gas-liquid chromatograph. The chromatographic column (Carbowax 4000) was held at 80 °C with an argon flow rate of 120 cm3/min.

Fig. 3. Radio gas-liquid chromatograms of the labeled hydrocarbon fractions. Radioactive products were generated by incubation of a 105,000 × g supernatant obtained from a homogenate of sage leaves with [1-3H]GPP and 15 mM MgCl2, 7-8% conversion to olefins (A); [1-3H]NPP and 15 mM MgCl2, 3-4% conversion to olefins (B); [1-3H]LPP and 15 mM MgCl2, 14-15% conversion to olefins (C); [1-3H]GPP and 1 mM MnCl2, 4-5% conversion to olefins (D); [1-3H]NPP and 1 mM MnCl2, 4-5% conversion to olefins (E); or [1-3H]LPP and 1 mM MnCl2, 6-7% conversion to olefins (F). Each reaction mixture containing 500 µg of protein and the cation indicated, 10 µM of the appropriate acyclic precursor, 1 mM dithioerythritol, 1 mM sodium ascorbate, and 1 mM Na2S,03, in a total volume of 1 ml of 80 mM Tricine-25 mM sodium phosphate buffer, pH 6.5, containing 10% glycerol, was incubated for 1 h at 28 °C. The bottom tracing is the flame ionization detector response obtained from the co-injected hydrocarbon fraction from the oil of sage. The constituents were identified as α-pinene (a), camphene (b), β-pinene (c), myrcene (d), limonene (e), and terpinolene (h). α-Thujene and sabine are minor components located, respectively, on the leading edge of the α-pinene peak and the trailing edge of the β-pinene peak. The arrows at f and g indicate the positions of cis- and trans-ocimene, respectively, which were not detected as components of the hydrocarbon fraction. The top tracings (A–F) are the responses of the radioactivity monitor attached to the gas-liquid chromatograph. The chromatographic column (Carbowax 4000) was held at 80 °C with an argon flow rate of 120 cm3/min.
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enantiomer is likely to be active in cyclization, the other might conceivably give rise to acyclic products.

In view of the qualitative and quantitative similarity of the olefin distribution pattern produced in vitro with GPP as substrate (in the presence of Mg²⁺), it seems reasonable to suggest that the natural precursor of monoterpane olefins in sage is GPP. This suggestion, although based on circumstantial evidence in the present case, is supported by recent studies with other types of monoterpane cyclases which also implicate GPP as a key acyclic precursor.

Radiochromatographic analysis of the oxygenated monoterpane fraction obtained from the incubation mixtures revealed, in all instances, various levels of 1,8-cineole (Mn⁴⁺-dependent) and borneol (Mg²⁺-dependent), two monoterpenes whose biosynthetic origins have been previously examined. Also present in these fractions was a sizeable quantity of alcohol (equivalent to 15–30% of the substrate) liberated by the hydrolysis of the corresponding pyrophosphate by competing phosphatases. Detailed examination of the oxygenated products derived from [1⁻²H]GPP for the presence of [¹H]nerol or [¹H]linalool revealed no detectable levels of these compounds. No [¹H]geraniol or [¹H]linalool appeared to be formed from [1⁻²H]LPP. Additionally, examination of the aqueous products remaining after incubation (by acid phosphatase hydrolysis to the corresponding terpenols) provided no evidence for the interconversion of the three pyrophosphorylated acyclic precursors.

Product Identification and Localization of Tritium—Because [1⁻²H]GPP yielded a radioactive hydrocarbon fraction nearly identical in composition with that produced in vivo, the products derived from this acyclic precursor were examined further. To confirm the identity of the biosynthetic olefins, which were tentatively identified by radio GLC, these products, obtained from a large scale incubation, were first purified by argentation TLC. The [¹H]α-pinene so obtained was diluted with (+)-α-pinene (specific activity ~0.61 mCi/mol) and oxidized with KMnO₄ to yield (+)-cis-pinonic acid (Fig. 4), which was recrystallized to a constant specific activity of 0.41 mCi/mmol (melting point, 104 °C; 104 °C in Ref. 37), thereby confirming the identification of the original product. [¹H]β-Pinene was diluted with (-)-β-pinene and stereospecifically isomerized (25) to (-)-α-pinene (Fig. 4). This material was diluted with an equivalent amount of (+)-α-pinene (specific activity ~0.56 mCi/mol) and oxidized with KMnO₄ as before. Recrystallization from water afforded (+)-cis-pinonic acid with constant specific activity of 0.40 mCi/mmol (melting point, 104 °C), thus verifying the identification of the original product as β-pinene. Corroboration of these results was provided by the nearly quantitative hydroboration-oxidation (38) of biosynthetic [¹H]α-pinene, and [¹H]α-pinene isomerized from biosynthetic [¹H]β-pinene, to [¹H]isopinocamphor.

To determine the location of the tritium in α-pinene derived from [1⁻²H]GPP, the purified biosynthetic product was diluted with (±)-α-pinene and treated with acetaldehyde (27) to provide the rearranged monoterpenol borneol (Fig. 4). The pinane to bornane skeletal rearrangement proceeds with varying degrees of racemization, which under the carefully controlled conditions was verified at approximately 20% (i.e. (±)-α-pinene affords 81% (+)-borneol and 19% (−)-borneol). Oxidation of borneol to camphor (28), followed by vigorous exchange of both exo- and endo-α-hydrogens (18, 39), reduced and

![Diagram of synthetic procedures](http://www.jbc.org/)

FIG. 4. Outline of synthetic procedures. Synthetic routes for product identification, determination of the location of tritium, and analysis of the stereochemical composition of biosynthetic [¹H]α-pinene and [¹H]β-pinenes are provided. See “Experimental Procedures” for details. The asterisk at C-7 of the pinane nucleus denotes the position of tritium derived from [1⁻²H]GPP. Racemization of borneol is assumed to proceed by a series of 1,3-hydride shifts or through the symmetrical tricyclic intermediate. DMF. N,N-dimethyl formamide.
the specific activity of the derived camphor by 76%, from 0.037 to 0.009 mCi/mol (i.e. the tritium of the racemized product, predictably, was not exchanged). Similar treatment of [3H]α-pinene derived from [3H]β-pinene afforded bornalene as before (racemization verified at up to 20%), and this product was oxidized to camphor and subjected to the exchange procedure. The specific activity of the recovered camphor was reduced from 0.078 to 0.019 mCi/mol (i.e., 75% loss of 3H). These findings, when corrected for racemization, demonstrate that essentially all of the tritium of the biosynthetic α-pinene and β-pinene was specifically located on the methylene bridge carbon (C-7), indicating direct derivation from [1-3H]GPP and a consistency with the labeling pattern observed in earlier in vivo studies with [2-14C]mevalonate as precursor (9).

[3H]Camphene derived from [1-3H]GPP was purified by argentation TLC and subjected to KMnO4-NaI04 oxidation (40). The major radioactive product obtained was chromatographically coincident (radio GLC and TLC) with camphene, confirming the identity of the original product. Hydrogenation of limonene, obtained from incubations with [1-3H]NPP, yielded a single radioactive product chromatographically coincident with cis-p-menthane as expected (16). The identities of the other olefins were verified by radio GLC on several columns of differing selectivity (Carbowax 4000 and SE-30).

Stereochemistry of the Biosynthetic Pinenes—To establish the stereochemical composition of the [7-3H]α-pinene derived from [1-3H]GPP by the cell-free preparation, the labeled product was diluted with (±)-α-pinene and treated with acidic aqueous acetone as before, and the derived (±)-endo-fenchol was isolated. This product was oxidized to fenchone and the crystalline oxime was prepared. Treatment of this material with (−)-menthyl chloromethyl ether afforded the diastereomeric O-(−)-menthoxymethyl ethers of (±)-fenchone oxime (Fig. 4) in 90% yield (30). The stereospecificity of the transformation of α-pinene to enanto-fenchol as well as the retention of absolute configuration of the derived fenchone nucleus throughout subsequent derivatization has been previously documented (30) and was independently verified here with (+)-β-pinene of known optical purity. TLC with (+)-β-pinene as an asymmetric developing solvent (30) provided unambiguous separation of the diastereomers (Fig. 5A) and clearly demonstrated the enzymatic formation of both (+)-[7-3H]α-pinene and (−)-[7-3H]α-pinene in nearly 2:5:1 ratio.

The purified [7-3H]α-pinene derived from [7-3H]β-pinene was diluted with unlabeled material and converted to enanto-fenchol as before. Subsequent steps (Fig. 4) followed by TLC separation of the diastereomers firmly established that essentially all of the radioactivity (>95%) of the mixture was associated with the diastereomer derived from (−)-β-pinene (Fig. 5B). These results thus demonstrate that the cell-free synthesis of α-pinene and β-pinene from GPP reflects not only the chemical composition, but also the stereochemical composition, of the pinenes synthesized in vivo.

Absence of (−)-β-Pinene to (−)-α-Pinene Isomerization—The possible in vitro isomerization of (−)-β-pinene was investigated by analyzing the products obtained on incubation of the complete enzyme system with 0.75 μM (−)-[7-3H]β-pinene (biosynthetically prepared at 300 Ci/mol and assayed without pentane overlay). No [3H]α-pinene was observed on radio GLC analysis of the isolated olefinic products by a procedure which was capable of detecting <1% conversion. Additionally, incubation of the enzyme preparation with [1-3H]GPP for 6 h, with sequential product analysis (radio GLC) at 30-min intervals, showed that the ratio of α-pinene to β-pinene produced remained constant throughout. Finally, (−)-β-pinene, when included in the incubation mixture at levels 100-fold higher than [1-3H]GPP, had no effect on the rate of α-pinene formation. From all the evidence, it appears safe to conclude that no significant β-pinene to α-pinene isomerization occurs under operational conditions of the enzyme assay.

Search for Free Intermediates—To investigate the previously suggested intermediacy of myrcene and cis-ocimene in the formation of the pinenes (5) (Fig. 2, Scheme II), individual isotopic dilution experiments were performed in the presence of a 100-fold excess (relative to [1-3H]GPP) of unlabeled myrcene, cis-ocimene, and trans-ocimene, as well as (±)-camphene and (±)-limonene. Under no circumstances was the conversion of [1-3H]GPP into [7-3H]α-pinene or [7-3H]β-pinene altered relative to the controls. Furthermore, no radioactivity above the normal control levels was found to be associated with the putative intermediates. Similar isotopic dilution experiments in the presence of 100-fold excess of the unlabeled monoterpenes (±)-α-terpinuel, (±)-cis-pinanol, and (±)-trans-pinanol, which could conceivably be regarded as possible intermediates, did result in decreased pinene formation (up to 45%). However, the relatively high levels (1 mM) of these alcohols required to reduce product formation, as well as the inability of these presumptive intermediates to trap detectable radioactivity, suggest that neither oicinens nor myrcene, nor any of the other monoterpenes tested, are free intermediates in the biosynthesis of pinenes from GPP.

Properties of the Enzyme System—A preliminary investigation of the properties of the soluble enzyme system was undertaken to assess optimal operating conditions, and to investigate the likelihood of multiple pinene biosynthetic enzymes. Under the conditions of the assay, the rate of conversion of GPP to α-pinene and β-pinene was constant for up to 1 h at the 0.5 mg/ml protein level, and all subsequent measurements of enzyme activity were carried out within this
linear range. Dialysis of the preparation against the assay buffer, to remove divalent cations present in the extraction medium, reduced α- and β-pinene cyclase activity to negligible levels. However, activity could be fully restored by re-addition of 15 mM MgCl₂ thus demonstrating the absolute requirement for a divalent cation as with other monoterpene cyclases (12) (the Cl⁻ anion was without effect). MnCl₂ (1 mM) was about 85% as effective as MgCl₂ (15 mM) in restoring β-pinene cyclase activity and roughly 50% as effective as MgCl₂ in restoring α-pinene cyclase activity under these saturating conditions. The observed preference for Mg²⁺ is in contrast to the cation requirement for α- and β-pinene synthesis by C. limonum extracts, in which Mn²⁺ is reportedly preferred (14).

In 50 mM Tricine-25 mM sodium phosphate buffer, β-pinene synthetase activity exhibited a distinct pH optimum near 7.2 (Fig. 6). By contrast, the pH curve for α-pinene synthetase activity was bimodal with optima near 6.0 and 7.2 (Fig. 6), suggesting the presence of two α-pinene cyclase activities. p-Hydroxymercuribenzoate severely inhibited the conversion of GPP to both pinenes, but β-pinene cyclase activity was far more sensitive to the thiol-directed reagent than was α-pinene cyclase (Fig. 7). All monoterpene cyclases thus far examined are inhibited by thiol-directed reagents (12). Thermal inactivation studies, at temperatures ranging from 45 to 56 °C, revealed that β-pinene cyclase activity was significantly more labile than α-pinene cyclase activity (Fig. 7), providing a further distinction between the enzymes involved in the formation of these positional isomers.

The enzyme system described here is like other monoterpene cyclases in general properties and in the ability to utilize the acyclic precursors GPP, NPP, and LPP with varying degrees of efficiency (12). A notable feature of the present work was the preliminary finding that, of the three possible acyclic precursors examined, only GPP afforded a distribution of monoterpene olefin products which paralleled that found in vivo. NPP and LPP both yielded, as major products, olefins which either are minor constituents or are not detected in the olefin fraction. Until the preparation is fractionated into its constituent activities and the competing phosphatases are removed, the qualitative and quantitative differences in product formation observed with the various acyclic precursors and cations cannot readily be clarified. The tentative results do suggest, however, that GPP is the likely precursor of pinenes and other cyclic olefins in vivo. The fact that GPP was transformed to such cyclic products without detectable conversion to the other acyclic precursors also argues against the need for isomerization to free NPP or LPP as a prelude to cyclization (13) but does not rule out the intermediacy of the bound equivalents of these compounds.

Earlier chemotaxonomic studies (10) and in vivo tracer studies (11) imply the existence of multiple pathways to the pinenes in other species, and in the present case the distribution of isomeric constituents would seem to necessitate the involvement of at least two pinene cyclases. The preliminary characterization of the relatively crude system described here, which appears to be the first report on the cell-free biosynthesis of enantiomeric terpenes, does strongly suggest the presence of multiple pinene cyclases. Detailed studies on the number and nature of the GPP:pinene cyclases of sage are now underway.

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Biosynthesis of Isomeric Pinenes from Geranyl Pyrophosphate

REFERENCES

Biosynthesis of (+/-)-alpha-pinene and (-)-beta-pinene from geranyl pyrophosphate by a soluble enzyme system from sage (Salvia officinalis).

H Gambriel and R Croteau


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