The conversion of geranyl pyrophosphate to (-)-endo-fenchol is considered to proceed by the initial isomerization of the substrate to (-)-(3R)-linalyl pyrophosphate and the subsequent cyclization of this bound intermediate. Incubation of (1R)-[2-14C,1-3H]- and (1S)-[2-14C,1-3H]geranyl pyrophosphate with a preparation of (-)-endo-fenchol cyclase (synthase) from common fennel (Foeniculum vulgare) gave labeled product of unchanged 3H:14C ratio in both cases, and each was dehydrated to a mixture of α- and β-fenchene which were oxidized to the corresponding α- and β-fenchocamphorones, again without change in isotope ratio. The location of the tritium label was deduced in each case by stereoselective, base-catalyzed exchange of the exo-α-hydrogen of the derived ketone. The findings indicated that the configuration at C1 of the substrate was retained in the enzymatic transformation to (-)-endo-fenchol which is entirely consistent with the syn-isomerization of geranyl pyrophosphate to (3R)-linalyl pyrophosphate and cyclization of the latter via the anti-endo-conformer. These absolute stereochemical elements of the reaction sequence were confirmed by the enzymatic conversion of (3R)-1Z-[1-3H]linalyl pyrophosphate to (-)-endo-fenchol and by the location of the tritium in the derived fenchocamphorones as before. The summation of the results fully defines the overall stereochemistry of the coupled isomerization and cyclization of geranyl pyrophosphate to (-)-endo-fenchol.

Geranyl pyrophosphate:(-)-endo-fenchol cyclase from fennel (Foeniculum vulgare M.) catalyzes the Mn²⁺-dependent cyclization of (1RS)-[1-3H]geranyl pyrophosphate, via rearrangement of a pinyl intermediate, to (-)-(1S)-endo-[7-3H]fenchol (1) which in this species is subsequently oxidized to (+)-(1S)-fenchone (2). The enzyme exhibits a marked preference for geranyl pyrophosphate over the cis-isomer, neryl pyrophosphate, as the substrate, in spite of the topological impediment to direct cyclization of the former, and the reaction occurs without loss of ³H from C1 of the substrate (ruling out redox interconversion of the acyclic precursors) and without formation of detectable free intermediates (3, 4). Since the trans-double bond at C2 of geranyl pyrophosphate prevents the direct cyclization of this precursor, it is clear that the cyclase is capable of converting the substrate to a bound intermediate competent to cyclize and of catalyzing the cyclization reaction itself. Based on the above considerations, related work with other enzymes which produce bicyclic monoterpenes (5, 6), the results of other relevant biogenetic studies, and the conclusions drawn from model reactions, a general scheme for the isomerization and subsequent cyclization of geranyl pyrophosphate has been proposed (7). The key elements of this basic scheme as applied to the origin of (-)-endo-fenchol (Fig. 1) are ionization with syn-migration of the pyrophosphate to afford the bound tertiary allylic isomer, linalyl pyrophosphate, rotation about the newly formed C2-C3 single bond to the cisoid-conformer, and a second ionization with cyclization of the anti-endo form. Subsequent internal electrophilic attack on the cyclohexene double bond of the monocyclic, α-pinanyl intermediate generates the pinyl skeleton, which upon rearrangement and backside capture of the resulting cation by water produces (-)-endo-fenchol (8).

This stereochemical model predicts the intermediacy of (3R)-linalyl pyrophosphate in the reaction, a prediction recently confirmed by direct testing of this tertiary isomer, the result of which also indicated the isomerization step to be rate-limiting in the coupled isomerization-cyclization catalyzed by this enzyme (4). A related, central prediction of the model is the net retention of configuration at C1 of geranyl pyrophosphate in the transformation to endo-fenchol as a direct consequence of the syn-isomerization-transoid to cisoid rotation-anti-cyclization sequence (Fig. 1). We have now examined the stereochemical alteration at C1 of both geranyl pyrophosphate and (3R)-linalyl pyrophosphate in the enzymatic conversion to (-)-endo-fenchol. The results obtained are fully consistent with stereochemical prediction and provide strong evidence to support the general syn-isomerization-anti-cyclization model for monoterpene biosynthesis.

**EXPERIMENTAL PROCEDURES**

**RESULTS AND DISCUSSION**

The proposed stereochemical model for the cyclization of geranyl pyrophosphate to (-)-endo-fenchol predicts that the configuration at C1 of the substrate will be retained in the transformation (Fig. 2). To examine the stereochemical fate of C1 of the acyclic precursor, (1R)-[2-14C,1-3H]geranyl pyrophosphate (³H:14C = 10.2 ± 0.1) and (1S)-[2-14C,1-3H]geranyl pyrophosphate (³H:14C = 10.1 ± 0.1) were prepared by combination of enzymatic and chemical methods (5). Each of

---

*This investigation was supported in part by Grant GM 31354 from the National Institutes of Health, and by Project 0268 from the Washington State University Agricultural Research Center. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence and reprint requests should be addressed.

---

1 The "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.
Stereochemistry of Cyclization to (−)-endo-Fenchol

These substrates, as well as (1RS)-[2-14C,1-3H]geranyl pyrophosphate (H:14C = 10.2 ± 0.1) as a control, was then enzymatically converted to (−)-(1S)-endo-fenchol by partially purified cyclase preparations from F. vulgare (fennel) fruits (schizocarps). This source of the enzyme, and the purification procedure used (ion exchange and gel permeation chromatography), gave the fenchol cyclase (M, ~ 60,000) essentially free of competing phosphohydrolases.

Following extraction of the reaction mixtures, pooling of samples from like assays, and the addition of a small quantity of authentic carrier, the fenchol was isolated by TLC after OsO4 treatment to remove unsaturated compounds (i.e. cyclic monoterpenes olefins and geraniol liberated by residual phosphohydrolases). The (−)-endo-fenchol derived enzymatically from each substrate was first examined for yield by aliquot counting, then diluted to a specific activity of approximately 0.25 μCi of 1H/mmol, and 10% of each sample was oxidized with pyridinium chlorochromate (9) to (+)-(1S)-fenchone which was converted to the crystalline semicarbazone (10) (all melting points within 182.5–184 °C; 182–183 °C in Ref. 11). The H:14C ratios of each fenchol sample and its respective fenchone semicarbazone were identical, within experimental error, with that of the corresponding geranyl pyrophosphate starting material (i.e. H:14C = 10.2 ± 0.3). These results confirm earlier observations that hydrogen from C1 of the acyclic precursor is not lost in the conversion to this and other cyclic monoterpenes (4, 12).

A sufficient number of incubations were carried out with each cyclase preparation to ensure the production of at least 0.3 μCi of H of (−)-endo-fenchol from each substrate. Each (−)-endo-fenchol sample (1 mmol) was dehydrated with KHSO4 (13) to give, via Wagner-Meerwein rearrangement, (−)-(1S)-α-fenchene (~15%) and, via 2,6-hydride shift and similar rearrangement, (+)-(1R)-β-fenchene (~20%) (Fig. 2) along with a series of related endocyclic olefins (15, 14). The α-fenchene and β-fenchene were separated and oxidized with RuO4 (15) to yield (+)-(1R)-α-fenchocamphorone (~13% overall yield from fenchol) and (+)-(1R)-β-fenchocamphorone (~18% overall yield from fenchol), respectively (Fig. 2). Both ketones gave satisfactory melting points (α m.p. = 110 °C; β m.p. = 63–64 °C) corresponding to literature values (11), and 20% of each was converted to the corresponding semicarbazone (melting point ranges again consistent with literature values (11)) for determination of H:14C ratio (α-fenchocamphorone = 10.1 ± 0.3; β-fenchocamphorone = 10.1 ± 0.3). Such verification that the H:14C ratios were unchanged from the corresponding starting material was crucial since the transformation to fenchocamphorones allows advantage to be taken of the selectivity of exo-α-hydrogen exchange of these bicyclo[2.2.1]heptanes (16–19) in locating the tritium.

Individual exchange runs with each sample of α- and β-fenchocamphorone (0.04 μCi of 1H per run at minimum) derived from 1R- and 1S-labeled geranyl pyrophosphate, and from the 1RS-labeled precursor as control, were carried out in 0.5 M NaOH/dioxane at 50 °C, and the tritium loss (relative to the 14C-labeled internal standard) at corresponding time points for like samples was averaged. At least two independent runs were made for each substrate-product combination, and the H:14C ratios for replicate time points did not vary by more than ±10% of the mean for any determination. The exchange curves for α-fenchocamphorone (via fenchol cyclase) derived from (1R)-[14C,1-3H]-, (1S)-[14C,1-3H]- and (1R)-[14C,1-3H]geranyl pyrophosphate are shown in Fig. 3A. The loss of tritium from α-fenchocamphorone derived from (1S)-[14C,1-3H]geranyl pyrophosphate was far more rapid than that from the product derived from (1R)-[14C,1-3H]geranyl pyrophosphate, indicating that the 1-pro-R-hydrogen of the acyclic precursor gives rise predominantly to the exo-α-hydrogen of the derived α-fenchocamphorone (and that the 1-pro-R hydrogen gives rise to the endo-α-hydrogen) as predicted from the stereochemical cyclization scheme (Fig. 2). Comparison of exchange rates from the linear portion of the curves (0–6 min) for the product derived from (1S)- and (1R)-labeled precursors, respectively, gave a ratio in excess of 15 which agrees well with predictions based upon the relative exo-versus endo-exchange rates observed for the α-hydrogens of similar bridge-substituted bicyclo[2.2.1]heptanes (16–19).

The exchange curves for β-fenchocamphorone (via fenchol cyclase) derived from (1R)-[14C,1-3H]-, (1S)-[14C,1-3H]-, and (1R)-[14C,1-3H] geranyl pyrophosphate are illustrated in Fig. 3B. In this instance, the rate of tritium loss from β-
Stereochemistry of Cyclization to (-)-endo-Fenchol

from C1 of geranyl pyrophosphate is retained in the enzymatic cyclization scheme. At the end, conversion to (-)-endo-fenchol, an observation entirely consistent with the proposed cyclization scheme. Comparisons of exchange rates from the linear portion of the curves for the α-hydrogens of related fenchocamphorones, it is clear the relative exo- versus endo-exchange rates previously noted for a-fenchocamphorone and in com-

fenchocamphorone derived from (1R)-[1-14C,1-3H]geranyl pyrophosphate was very much faster than that from the product generated in the control incubations with the racemic [14C,1-3H]-labeled precursor. Thus, the 1-pro-R-hydrogen of a-fenchocamphorone and the exo-α-hydrogen of α-fenchocamphorone and theexo-α-hydrogen of β-fenchocamphorone (Fig. 4).

To examine this possibility, (3R)-1Z-[1-3H]linalyl pyrophosphate was enzymatically converted to (+)-(1S)-endo-fenchol by the partially purified cyclase preparation. The favorable kinetics for the conversion of this substrate permitted in excess of 0.45 μCi of fenchol to be easily obtained, and following TLC purification, as before, this product was diluted with authentic carrier to about 0.25 μCi/mmol. One-sixth of the sample was oxidized to (+)-(1S)-fenchone, and the semicarbazone was prepared and crystallized to constant melting point and specific activity (0.24 ± 0.01 μCi/mmol). The remaining sample was dehydrated with KHSO₄ to afford

---

**Figure 3.** Location of tritium by exchange of the α-hydrogens of α- and β-[3H]fenchocamphorone. The exchange curves illustrated are for α-fenchocamphorone derived from (1R)-[1-3H], (1R5)-[1-3H], and (1S)-[1-3H]geranyl pyrophosphate (1R-α, 1RS-α, and 1S-α, respectively, of panel A), for β-fenchocamphorones derived from (1S)-[1-3H], (1R5)-[1-3H], and (1R)-[1-3H]geranyl pyrophosphate (1S-β, 1RS-β, and 1R-β, respectively, of panel B), and for α- and β-
fenchocamphorone derived from (3R)-1Z-[1-3H]linalyl pyrophosphate (1Z-α and 1Z-β, respectively, of panel C) by the (-)-endo-fenchol cyclase from fennel. The preparation of the fenchocamphorones and the details of the base-catalyzed exchange procedure are provided under "Experimental Procedures."

---

**Figure 4.** Stereochemistry at C1 of geranyl pyrophosphate and (3R)-linalyl pyrophosphate in the enzymatic transformation to (-)-endo-fenchol and in the conversion of the product to fenchocamphorones via the corresponding fenchenes. The circled R designates the 1-pro-R hydrogen and the circled S the 1-pro-S hydrogen of geranyl pyrophosphate (GPP). The circled E designates the E hydrogen and the circled Z the Z hydrogen of linalyl pyrophosphate (LPP). OPP indicates the pyrophosphate moiety.
α- and β-fenchene which were converted to α-fenchocamphorone and β-fenchocamphorone, respectively, as before. One-fifth of each ketone was then converted to the corresponding semicarbazone which was recrystallized to a constant specific activity essentially the same as that of the starting material ($\alpha = 0.24 \pm 0.02 \mu$Ci/mm; $\beta = 0.23 \pm 0.02 \mu$Ci/mm), and the remainder of each was subjected to base-catalyzed exchange of the $\alpha$-hydrogens as before to provide the exchange curves illustrated in Fig. 3C. The very rapid loss of label from $\beta$-fenchocamphorone in this instance indicated positioning of the original 1-2H label of (3R)-linalyl pyrophosphate in the exo-$\alpha$-position of the derived ketone, whereas the very slow rate of exchange of label in the case of α-fenchocamphorone placed the original 1-Z-2H label in the endo-$\alpha$-position in this case. These predicted results serve to confirm the syn-stereochemistry in the isomerization of geranyl pyrophosphate to (3R)-linalyl pyrophosphate and are fully consistent with the net retention of configuration at C1 of the geranyl precursor in the coupled transformation to (-)-(1S)-endo-fenchol.

As in the earlier experiments with C1-labeled geranyl precursors, the residual fenchocamphorones were subjected to exhaustive base-catalyzed exchange and thereby shown to have undergone a reduction in specific activity (determined as the semicarbazone) to less than 0.01 µCi/mm, confirming that essentially all of the original tritium label was located at the $\alpha$-position of the derived ketones.

The results presented here, in addition to confirming the syn-isomerization and anti-cyclization (which increasing evidence suggests may be the universally preferred stereochemistry in related mono-, sesqui-, and diterpene cyclizations (5, 23–27)), establish the helical conformation of the reacting geranyl precursor and the anti-endo configuration of the cyclizing tertiary intermediate, and they rule out all other possible conformers for this coupled reaction sequence. When taken together with the previously described configurational imperative for (3R)-linalyl pyrophosphate (4), the summation of these studies unequivocally establishes all of the absolute stereochemical elements in the isomerization and cyclization of geranyl pyrophosphate to (-)-endo-fenchol, and thus supports the general model for this complex reaction type (7, 28).

Acknowledgments—We thank Greg Wichelns for raising the plants and Nancy Madsen for typing the manuscript.

REFERENCES
Stereochemistry of Cyclization to (−)-endo-Fenchol

Stereocentrics of the enzymatic cyclization of geranyl pyrophosphate to (−)-endo-fenchol by Rodney Croteau, D. Michael Satterwhite, Carl J. Wheeler and N. Mark Feltan

EXPERIMENTAL PROCEEDINGS

Substances and Standards - The preparation of (−)-1-acetyl, 1-
[1-14C]phelleraic pyrophosphate (1H-1C = 19.02), 1H-12C-1-[3-14C]geranyl pyrophosphate (1H-1C = 19.12) and 1H-12C-1-[3-14C]-
[3-14C]geranyl pyrophosphate (1H-1C = 19.11), by methods and pyrophosphorylation of the corresponding isomers ([1-14C]Dimonc) and [1-14C]fenchol ([4-14C]alcohol), and the procedures used to verify the stereoechemical composition of these substances, have been described (1). 1H-12C-1-[3-14C]geranyl pyrophosphate ([C]-CNP1) was prepared via labeled metathesis of the corresponding alcohol ([4-14C] and pyrophosphorylation, and the substrate verified by procedures previously described (1). The source of (−)-citrilenone, (−)-17β-3-methyl-2-fenchol, and (−)-17β-3-methyl-2-fenchol specified as the phtalate have been described(1).

Preparation of Enzyme System - The preparation, assay, and general properties of geranyl pyrophosphate dimethyl acetal dehydrogenase from fennel leaves (1) were described previously (1). The procedure involving HCl/NaCIO precipitation, action chromatography and thin layer chromatography, used for bulk preparation of the enzyme from fruit of this species (4), was employed here since these preparations provided the lowest phosphate activity in assay conditions.

Enzymic Conversion of Fennel Pyrophosphate to Acetylgeraniol - For the enzymic synthesis of (−)-acetylgeraniol, 1H-12C-1-[3-14C]geranyl pyrophosphate, pH 7.0, containing a racemic mixture of dicarboxylic sodium acetate, was incubated in bicarbonate buffer (pH 8.3) using a 100 ml, 5 ml reaction volume with 20% of the enzyme preparation (1) at 30°C for 14 h. Aliquots were removed at intervals, and the enantiomeric composition of the product was determined by direct radioactivity analysis using a 1H-12C-1-geranyl pyrophosphate (1) as a reference standard. The procedure involved the use of brass weighing points, action chromatography and thin layer chromatography, used for bulk preparation of the enzyme from fruit of this species (4), was employed here since these preparations provided the lowest phosphate activity in assay conditions.

Conversion of Fennel Fenchol to (−)-Acetylgeraniol - The enzymatic acetylation of 1H-[6-14C]fenchol, 1H-12C-1-[3-14C]geranyl pyrophosphate (1H-1C = 19.11), and appropriate [1-14C]dimethyl acetal dehydrogenase from fennel leaves was described previously (1). The properties of the enzyme preparation (1) were used in the present study, and aliquots were removed at intervals, and the enantiomeric composition of the product was determined by direct radioactivity analysis using a 1H-12C-1-geranyl pyrophosphate (1) as a reference standard. The procedure involved the use of brass weighing points, action chromatography and thin layer chromatography, used for bulk preparation of the enzyme from fruit of this species (4), was employed here since these preparations provided the lowest phosphate activity in assay conditions.

Synthesis of (−)-[1-14C]geranyl pyrophosphate - A 1H-12C-1-[3-14C]geranyl pyrophosphate (1H-1C = 19.11), was used as a reference compound for the determination of enantiomeric purity of products synthesized from (−)-fenchol. The preparation of (−)-[1-14C]geranyl pyrophosphate (1H-1C = 19.11), was described previously (1). The procedure involved the use of brass weighing points, action chromatography and thin layer chromatography, used for bulk preparation of the enzyme from fruit of this species (4), was employed here since these preparations provided the lowest phosphate activity in assay conditions.

Enzymic Conversion of Fennel Fenchol to (−)-Acetylgeraniol - The enzymatic acetylation of 1H-[6-14C]fenchol, 1H-12C-1-[3-14C]geranyl pyrophosphate (1H-1C = 19.11), and appropriate [1-14C]dimethyl acetal dehydrogenase from fennel leaves was described previously (1). The properties of the enzyme preparation (1) were used in the present study, and aliquots were removed at intervals, and the enantiomeric composition of the product was determined by direct radioactivity analysis using a 1H-12C-1-geranyl pyrophosphate (1) as a reference standard. The procedure involved the use of brass weighing points, action chromatography and thin layer chromatography, used for bulk preparation of the enzyme from fruit of this species (4), was employed here since these preparations provided the lowest phosphate activity in assay conditions.

Synthesis of (−)-[1-14C]geranyl pyrophosphate - A 1H-12C-1-[3-14C]geranyl pyrophosphate (1H-1C = 19.11), was used as a reference compound for the determination of enantiomeric purity of products synthesized from (−)-fenchol. The preparation of (−)-[1-14C]geranyl pyrophosphate (1H-1C = 19.11), was described previously (1). The procedure involved the use of brass weighing points, action chromatography and thin layer chromatography, used for bulk preparation of the enzyme from fruit of this species (4), was employed here since these preparations provided the lowest phosphate activity in assay conditions.