Biosynthesis of Monoterpenes

ENANTIOSELECTIVITY IN THE ENZYMATIC CYCLIZATION OF (+)- AND (−)-PINENE AND (+)- AND (−)-CAMPHENE*

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Rodney Croteau§, D. Michael Satterwhite¶, David E. Cane¶, and Conway C. Chang¶

From the §Institute of Biological Chemistry, Washington State University, Pullman, Washington 99164-6340 and the ¶Department of Chemistry, Brown University, Providence, Rhode Island 02912

Cyclase I from Salvia officinalis leaf catalyzes the conversion of geranyl pyrophosphate to the stereochemically related bicyclic monoterpenes (+)-α-pinene and (+)-camphene and to lesser quantities of monocyclic and acyclic olefins, whereas cyclase II from this plant tissue converts the same acyclic precursor to (−)-α-pinene, (−)-β-pinene and (−)-camphene as well as to lesser amounts of monocyclics and acyclics. These antipodal cyclizations are considered to proceed by the initial isomerization of the substrate to the respective bound tertiary allylic intermediates (−)-(3R)- and (+)-(3S)-linalyl pyrophosphate. [(3R)-8,9-14C,(3RS)-1E-3H]linalyl pyrophosphate ([H]:14C = 5.14) was tested as a substrate with both cyclases to determine the configuration of the cyclizing intermediate. This substrate with cyclase I yielded α-pinene and camphene with [H]:14C ratios of 3.1 and 4.2, respectively, indicating preferential, but not exclusive, utilization of the (3R)-enantiomer. With cyclase II, the doubly labeled substrate gave bicyclic olefins with [H]:14C ratios of from 13 to 20, indicating preferential, but not exclusive, utilization of the (3S)-enantiomer in this case. [(3R)- and (3S)-1Z-3H]linalyl pyrophosphate were separately compared to the achiral precursors [1-3H]geranyl pyrophosphate and [1-3H]neral pyrophosphate (cis-isomer) as substrates for the cyclizations. With cyclase I, geranyl, neryl, and (3R)-α-linalyl pyrophosphate gave rise exclusively to (+)-α-pinene and (+)-camphene, whereas (3S)-linalyl pyrophosphate produced, at relatively low rates, the (−)-isomers. With cyclase II, geranyl, neryl, and (3S)-α-linalyl pyrophosphate yielded exclusively the (−)-isomer series, whereas (3R)-linalyl pyrophosphate afforded the (−)-isomers at low rates. These results are entirely consistent with the predicted stereochemistries and additionally revealed the unusual ability of these enzymes to catalyze antipodal cyclizations when presented with the unnatural linalyl enantiomer.

Previous studies on pinene biosynthesis in soluble enzyme preparations from common sage (Salvia officinalis) have demonstrated the presence of two separable activities that catalyze the cyclization of geranyl pyrophosphate (1) to antipodal sets of monoterpane olefins (1, 2). Thus, geranyl pyrophosphate:pinene cyclase I (M, 96,000) was shown to transform geranyl pyrophosphate to (+)-α-pinene ((+)-10) and to smaller quantities of (+)-camphene ((+)-13), (+)-limonene ((+)-6), myrcene (5) and terpinene (7), whereas geranyl pyrophosphate:pinene cyclase II (M, 55,000) was shown to convert the acyclic precursor to (−)-α-pinene ((−)-10) and (−)-β-pinene ((−)-11) as well as to lesser quantities of (−)-camphene ((−)-13), (−)-limonene ((−)-8), myrcene (5), and terpinene (7). Activities for the cyclization to the configurationally related (+)-olefins copurified without alteration in product distribution through five chromatographic fractionation steps and electrophoresis, as did the activities for the cyclization to the (−)-olefin series (2). Differential inhibition and inactivation studies (2), as well as a recent investigation of deuterium isotope effects on these enzymatic cyclizations (3), have indicated that a single enzyme is responsible for generating the (+)-olefin product set and that the (−)-olefin series is also generated by a distinct, single enzyme species.

A general stereochemical model for the coupled isomerization and subsequent cyclization of geranyl pyrophosphate to bicyclic monoterpenes has been proposed (4-7) that is based primarily on studies of the origin of bornane and fenchane derivatives (8-10). This scheme, as applied to the pinene cyclases (Scheme I), posits the initial ionization of geranyl pyrophosphate (1), with suprafacial migration of the pyrophosphate moiety of the resulting ion pair (2), to afford the bound linalyl pyrophosphate intermediate (3). Rotation about the newly generated C2-C3 single bond to the cisoid conformer overcomes the original stereochmmical 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 (4) to generate the acyclic olefin myrcene (5) (which additionally could be generated from 2). Two modes of proton loss from the monocyclic α-terpinyl cation (14) may similarly afford limonene (6) or terpineol (7), whereas a second electrophilic cyclization gives rise to the bicyclic pinyl (8) or bornyl (9) cations. Deprotonation from the adjacent ring methylene of the pinyl cation yields α-pinene (10), whereas in the case of pinene cyclase II alternative deprotonation from the adjacent methyl is allowed, permitting formation of (−)-β-pinene (11). Wagner-Meerwein shift of the bornyl system (9) generates the camphyl cation (12) which upon proton loss provides camphene (13). As can be seen, the biosynthetic transformations catalyzed by (+)-pinene cyclase (cyclase I) and (−)-pinene cyclase (cyclase II) are described by exactly mirror-image schemes, with the exception that β-pinene ((−)-11) is produced only by the (−)-pinene cyclase.

A central feature of the stereochemical model is the pre
predicted intermediacy of (−)-(3R)-linalyl pyrophosphate, or its ion-paired equivalent, in the construction of the (+)-olefin series, and the intermediacy of the antipodal (+)-(3S)-isomer in the corresponding enantiomeric cyclizations to the (−)-pinenes and related olefins. This prediction is based upon the observed preference for anti-endo cyclization of linalyl derivatives in solution (11) and by related monoterpene cyclases (8–10) and is fully consistent with the labeling patterns of the pinenes derived from [1-3H]geranyl pyrophosphate which indicate that the antipodes are derived via enantiomeric cyclizations involving antipodal linalyl and α-terpinyl intermediates rather than by way of a hydride shift or other rearrangement from a common cyclic progenitor (1, 2). It has been observed that most monoterpene cyclases, including the pinene cyclases, can readily utilize racemic linalyl pyrophosphate as an alternate acyclic precursor without preliminary interconversion or formation of other detectable free intermediates (2, 9, 13–15). Thus, linalyl pyrophosphate, at minimum, must resemble the corresponding bound intermediate actually formed at the active site (12), and this feature allows the absolute configurations of the respective bound intermediates to be readily probed. This stereochemical problem was approached by examining enantioselectivity in the utilization of [(3R)-8,9-14C,(3RS)-1E-3H]linalyl pyrophosphate to each of the antipodal sets of products. In all cases, product formation was compared with that obtained from geranyl pyrophosphate, which is presumed to be the natural substrate for these enzymes since only this acyclic precursor gives rise to a mixture of olefinic products corresponding to that produced in vivo by sage leaves (1, 2). The results obtained are fully consistent with the stereochemical predictions and establish the configuration of the respective cyclizing intermediates. Additionally, the results indicate that exogenous linalyl pyrophosphate does not precisely mimic the bound intermediates that arise by isomerization of geranyl pyrophosphate at the cyclase active sites.

EXPERIMENTAL PROCEDURES

RESULTS AND DISCUSSION

Conversion of [(3R)-8,9-14C,(3RS)-1E-3H]Linalyl Pyrophosphate to Cyclic Olefins As in earlier studies to determine enantiomeric selectivity in the conversion of linalyl pyrophosphate to bornane monoterpenes (10), initial experiments were directed toward examining the conversion of [(3R)-8,9-14C,(3RS)-1E-3H]linalyl pyrophosphate (−74% R, 3H;14C = 5.14) to the stereochemically related monoterpene olefin sets produced by (+)-pinene cyclase (cyclase I) and (−)-pinene cyclase (cyclase II). Preparative scale incubations with partially purified preparations at optimum assay conditions in the presence of 15 mM MgCl₂ and saturating concentrations (20 μM) of the doubly labeled substrate gave rise with both cyclases to labeled olefinic products (8–11% conversion over the course of three experiments after minor correction for

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.
nonenzymatic background), which were analyzed by radio-
GLC (3H-detection) following the addition of appropriate
racemic carrier standards. The corrected product distributions
generated from linalyl pyrophosphate were somewhat differ-
ent than those produced with geranyl pyrophosphate, the
natural substrate for these enzymes. Thus, for cyclase I the
(±)-linalyl precursor gave rise to 53% α-pinene, 7% cam-
phene, 10% myrcene, 14% limonene, and 10% terpinolene,
with about 5% of label in olefins tentatively identified as cis-
and trans-ocimenes, whereas with saturating concentrations
of geranyl pyrophosphate, 49% α-pinene, 30% camphene, 6%
myrcene, 10% limonene, and 5% terpinolene were produced
with negligible levels of ocimenes. For cyclase II with
geranyl pyrophosphate, 49% α-pinene, 30% camphene, 6%
limonene, and 7% terpinolene, with about 12% of the
total label associated with the ocimenes. With geranyl pyro-
phosphate as substrate, the olefin products constituted 28% α-
pinene, 24% camphene, 35% β-pinene, 6% myrcene, 5%
limonene, and 2% terpinolene, with negligible (<1%) uniden-
tified materials. Cyclase II preparations also produced sabi-
nene from linalyl and geranyl pyrophosphate (9–12% of the
olefin fraction); however, this olefin is the product of a con-
taminating sabine cyclase activity in the preparation and
will not be considered further. The most notable features of
the product distribution afforded with linalyl pyrophosphate
as substrate are the relatively low proportion of camphene
in the case of cyclase I and the relatively high proportion of
acrylic (myrcene and ocimenes) and monocyclic (limonene
and terpinolene) olefins produced by both cyclases compared
with those product distributions generated from geranyl py-
rophosphate. A similar anomalous distribution of enzymatic
reaction products (relative to that obtained with geranyl pyro-
phosphate) had also been observed earlier when crude
extracts containing both cyclases were incubated with racemic
[1-3H]linalyl pyrophosphate (1).

Individual olefins of the mixtures derived from [14C,3H]
linalyl pyrophosphate with each cyclase were isolated by
ergentation TLC, and the purity of each product was verified
by radio-GLC. The 3H:14C ratio was determined with the
respective pure olefin and, in most cases, as a crystalline
derivative, and the entire experiment was run in triplicate
using three individual enzyme preparations. The α-pinene
(∼25 nCi of 3H) isolated from incubations with cyclase I (+-
pinene cyclase) was analyzed as cis-pinonic acid and gave a
3H:14C ratio of 3.12 ± 0.27. Since exclusive use of (3R)-linalyl
pyrophosphate would result in product with 3H:14C = 2.57 (no
selectivity would yield 3H:14C = 5.14), a product ratio of 3.12
indicates preferential, although not entirely specific, utiliza-
tion of the (3R)-enantiomer, which is consistent with the
predicted conversion of the (3R)-linalyl pyrophosphate
intermediate to (+)-(1R:5R)-α-pinene (Scheme I) (2–7). Cam-
phene, although produced in abnormally low amounts in these
incubations, was isolated (∼11 nCi of 3H) and gave (as cam-
phenilone oxime) a 3H:14C ratio of 4.2 ± 0.5. This result
indicates significant, but clearly not exclusive, preference for
the utilization of (3R)-linalyl pyrophosphate by cyclase I,
again consistent with the predicted intermediate for this
cyclization sequence. Limonene (3H:14C = 5.2 ± 0.3), terpin-
olene (3H:14C = 5.1 ± 0.5), and myrcene (3H:14C = 4.5 ± 0.4)
gave ratios indicating little or no selectivity, which was un-
anticipated based on the stereochemical model. The origin
of these enzymatically generated acrylic and monocyclic olefins
will be described in a separate communication.

With regard to cyclase II ((−)-pinene cyclase), the β-pinene
(∼20 nCi of 3H) from preparative incubations with the
[14C,3H]linalyl pyrophosphate precursor gave (as napinone
oxide) a 3H:14C ratio of 20.2 ± 1.67, which is fully consistent
with the predicted preferential use of the (3S)-enantiomer as
the intermediate in this cyclization to (−)-(1S:5S)-β-pinene.
Initial studies with α-pinene (as cis-pinonic acid) and cam-
phene (as camphenilone oxime) isolated from preparative
incubations with the partially purified cyclase II (∼16 nCi of
3H each) indicated 3H:14C ratios somewhat lower than that
anticipated from the result with the coproduct β-pinene; how-
ever, it was subsequently discovered that these preparations
were contaminated with cyclase I (∼5–12% depending on the
amount of protein separated). When corrected for the con-
tribution of contaminating cyclase I, the α-pinene produced
by cyclase II gave a 3H:14C ratio in excess of 13, and the camphene
gave a 3H:14C ratio in excess of 16, confirming the preferential,
but not exclusive, utilization of the (3S)-enantiomer as pre-
dicted from the model. The acrylic and monocyclic olefins
produced by cyclase II from this precursor, as in the case of
cyclase I, gave 3H:14C ratios indicating little enantiomeraselection
(for limonene, 3H:14C = 5.6 ± 0.4; for terpinolene, 3H:14C =
6.1 ± 0.5; for myrcene, 3H:14C = 8.5 ± 0.5).

The summation of the results with [(3R)-8,9-14C,(3RS)-1E-
3H]linalyl pyrophosphate as precursor demonstrates that the
bicyclic olefins α-pinene and camphene are synthesized by
cyclase I with preferential utilization of (3R)-linalyl pyro-
phosphate as predicted from the stereochemical model. Simi-
larly, (3S)-linalyl pyrophosphate is preferentially utilized by
cyclase II for the cyclization to α-pinene, β-pinene, and cam-
phene. In no case, however, was the exclusive utilization of
(3R)- or (3S)-linalyl pyrophosphate observed for the biosyn-
thesis of bicyclic monoterpenes by either cyclase, and it was
clear that the "unnatural" enantiomer participated to a readily
detectable degree in each of the cyclizations. A similar con-
clusion has been drawn from studies on the conversion of linalyl
pyrophosphate to (+)-bornyl pyrophosphate and (−)-endose-
fenchol, that is, the relevant cyclases are enantioselective but
are capable of cyclizing the unnatural antipode of the normal
linalyl pyrophosphate intermediate (9, 10).

Kinetic Comparison of Acyclic Precursors and Determination of
Product Stereochemistries—To define in greater detail the
close of the conclusions drawn from analysis of the olefins derived
from [3R]-[8,9-14C,(3RS)-1E-3H]linalyl pyrophosphate, (3R)-[1Z-
3H]- and (3S)-[1Z-3H]linalyl pyrophosphate (9) were sepa-
ately tested as precursors with each enzyme and compared
to the natural substrate [1-3H]geranyl pyrophosphate and to
the cis, primary allyl cycloalkene [1-3H]neral pyrophosphate.
These acyclic precursors have been shown to be mutually
competitive inhibitors indicating that they are all converted
to olefinic products at a common active site of the respective
cyclase. (3S)-[3-1H]α-Terpinyl pyrophosphate, (RS)-[G-3H]
bornyl pyrophosphate, and (RS)-[3-1H]cis-2-pinyl pyrophos-
phate, the pyrophosphate ester analogs of predicted cyclic,
cationic intermediates of the enzymatic reaction cascade lead-
ing to bicyclic monoterpenes (see 14, 9, and 8 of Scheme I),
were also screened as potential precursors as part of this
investigation. Although each was a modest inhibitor of the
cyclization of geranyl pyrophosphate (I50 > 15 μM), none was
converted to bicyclic olefins at a detectable level (α-terpinyl
pyrophosphate did give rise to limonene and terpinolene),
confirming related studies which showed that such analogs
were unable to intercalate the cyclization cascade (16, 17).

The rate of olefin formation by both (+)-pinene cyclase and

1 The abbreviations used are: GLC, gas-liquid chromatography;
TLC, thin-layer chromatography; MBS, 2-(N-morpholinio)ethane-
sulfonic acid.
Cyclase I with geranyl pyrophosphate and details of the analysis are provided under "Experimental Procedures." Assay conditions detector response obtained from co-injected standards of α-pinene linalyl pyrophosphate pyrophosphate ranyl pyrophosphate (II-NPP), (3R)-linalyl pyrophosphate (II-(3R)-LPP) and (3S)-linalyl pyrophosphate (II-(3S)-LPP). The smooth bottom tracing is the thermal conductivity detector response obtained from co-injected standards of α-pinene (1), camphene (2), β-pinene (3), sabine (4), myrcene (5), limonene (6), cis-ocimene (7), trans-ocimene (8), and terpinolene (9). The top tracings indicate the corresponding responses of the radioactivity monitor attached to the gas-liquid chromatograph. Assay conditions and details of the analysis are provided under "Experimental Procedures."

In comparing relative velocities for bicyclic olefin formation by cyclase I from sage, it was clear that at saturation, (3R)-linalyl pyrophosphate was most rapidly converted to α-pinene, followed by geranyl pyrophosphate, which was about 25% as efficient, and then neryl pyrophosphate and (3S)-linalyl pyrophosphate. The calculated $K_m$ values for each substrate varied within a relatively narrow range from 2.2 µM for geranyl pyrophosphate to about 6 µM for neryl pyrophosphate, so that the catalytic efficiency parameters ($V_{max}/K_m$) roughly paralleled the differences based on $V_{max}$ alone. Although the values for $K_m$ and $V_{max}$ must be regarded as approximations (within ±15%) because of the very cumbersome assays required, it was obvious that (3R)-linalyl pyrophosphate was the preferred substrate for α-pinene formation by cyclase I, followed by geranyl pyrophosphate, (3S)-linalyl pyrophosphate, and neryl pyrophosphate in the order of enzyme preference.

Notable in the case of camphene formation by cyclase I was the relatively low amount of this product generated from precursors other than geranyl pyrophosphate, and the correspondingly higher absolute levels of acyclic and monocyclic products obtained from these alternate substrates (Fig. 1). Based on either $V_{max}$ or $V_{max}/K_m$, it was clear that geranyl pyrophosphate was the preferred substrate for camphene formation by this enzyme, followed in decreasing order by (3R)-linalyl pyrophosphate, (3S)-linalyl pyrophosphate, and neryl pyrophosphate. The unfavorable enzymatic conversion of neryl pyrophosphate to bicyclic products, such as α-pinene and camphene, has been observed previously with this and other cyclases and is discussed in greater detail elsewhere (1, 16, 18, 19). Geranyl pyrophosphate has also been previously compared to racemic [1-3H]linalyl pyrophosphate as a precursor of olefins with this enzyme (1) and, although not readily interpretable at the time, the earlier results obtained with the racemate (and with the double-labeled precursor as well) are not inconsistent with the kinetic parameters determined for the individual enantiomers.

A most significant feature to emerge from these studies with (3R)-linalyl pyrophosphate (and also with the racemate) is that α-pinene is efficiently produced relative to geranyl pyrophosphate, whereas camphene is formed in relatively low amounts, and acyclic and monocyclic olefins (myrcene, ocimenes, limonene, and terpinolene) are enzymatically generated in anomalously high levels. These results indicate that exogenous linalyl pyrophosphate does not precisely mimic the linalyl intermediate which arises by the isomerization of geranyl pyrophosphate at the cyclase active site. Since the pyrophosphate moiety of the substrate is the primary determinant of binding (20), it seems safe to assume that this functional grouping is bound in the same orientation regardless of the acyclic precursor. It therefore follows that the alterations in product distribution that result with the different precursors are likely due to differences in positioning effects between the fixed pyrophosphate and remaining chain.

Fig. 1. Radio gas-liquid chromatograms of the labeled olefin fractions. Radioactive olefins were generated by incubating cyclase I with geranyl pyrophosphate (I-GPP), neryl pyrophosphate (I-NPP), (3R)-linalyl pyrophosphate (I-(3R)-LPP), and (3S)-linalyl pyrophosphate (I-(3S)-LPP), and by incubating cyclase II with geranyl pyrophosphate (II-GPP), neryl pyrophosphate (II-NPP), (3R)-linalyl pyrophosphate (II-(3R)-LPP) and (3S)-linalyl pyrophosphate (II-(3S)-LPP). The smooth bottom tracing is the thermal conductivity detector response obtained from co-injected standards of α-pinene (1), camphene (2), β-pinene (3), sabine (4), myrcene (5), limonene (6), cis-ocimene (7), trans-ocimene (8), and terpinolene (9). The top tracings indicate the corresponding responses of the radioactivity monitor attached to the gas-liquid chromatograph. Assay conditions and details of the analysis are provided under "Experimental Procedures."
and probably reflect the inability to achieve the “optimum” conformation of the olefinic domains of the substrate prior to ionization. It should be noted that the cyclization of geranyl pyrophosphate requires a relatively slow ionization-isomerization step (21, 22) before cyclization can occur, whereas both neryl and linalyl precursors can cyclize directly thereby providing a rationale for temporal differences in achieving optimal orientation (i.e. overlap, backside shielding, etc. (20)) after initial binding occurs. It seems clear that such alteration in the mode(s) of binding of the linalyl precursor must reflect differences in both acyclic and monocyclic intermediates carried through (or abortively terminated in) the cationic reaction cascade, since product formation under these circumstances favors acyclics and monocyclics and allows generation of the pinyl system (α-pinene), but disfavors production of the bornyl and/or camphyl system which gives rise to camphene. Acyclics, for example, could arise from transoid orientations which are incapable of cyclization, monospecifics from cisoid, exo-conformations which are incapable of bicyclication, and the pinyl system could be relatively preferred by positioning of the paired pyrophosphate anion over the more highly substituted double bond position of the α-terpinyl intermediate or the proximity of the cationic isopropyl center to the alternate double bond position of this monocyclic intermediate. The relative positioning of enzyme base(s) involved in deprotonation of the various cationic intermediates of the reaction sequence could also be influenced by conformational differences in the terpenoid chain which are achieved prior to the ionization-cyclization step (22), and this too could be expected to influence product outcome.

To examine the stereochemistry of the bicyclic products derived from the various acyclic precursors, large scale incubations with cyclase I were carried out at saturating concentrations of each substrate. The 2H-labeled olefin fractions thus obtained were diluted with the appropriate racemic carriers, the individual products were separated by argentation TLC, and the radiochemical purity of each was verified by preparative incubations with optically pure starting materials (Fig. 2). By such analysis it was shown that geranyl pyrophosphate gave rise exclusively to α-pinene, confirming previous results (2), as did both neryl pyrophosphate and (3R)-linalyl pyrophosphate (Fig. 2, α-c). (3S)-Linalyl pyrophosphate, on the other hand, gave essentially only α-pinene (Fig. 2d), as suspected from the earlier work (the trace level of α-pinene detected no doubt arises from the few percent of (3R)-linalyl pyrophosphate that are present in the (3S)-linalyl pyrophosphate preparation).

TABLE I

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<thead>
<tr>
<th>Substrate specificity of (+)- and (−)-pinene cyclases</th>
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<tr>
<td>Enzyme</td>
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<tr>
<td>(+)-Pinene</td>
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<tr>
<td>α-Pinene</td>
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<tr>
<td>Camphene</td>
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<td>Total olefins</td>
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<tr>
<td>β-Pinene</td>
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<tr>
<td>Camphene</td>
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<td>Total olefins</td>
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In comparing relative velocities (or V<sub>m/K<sub>m</sub></sub>) for pinene cyclase II from sage, it was obvious that (3S)-linalyl pyrophosphate was the most efficient precursor of the bicyclic olefins α-pinene, β-pinene, and camphene and that geranyl pyrophosphate was roughly 25% as efficient as a substrate (Table I). In this case the relative proportions of α- and β-pinene and camphene produced were similar with geranyl pyrophosphate and (3S)-linalyl pyrophosphate, but analysis of total product distributions (Fig. 1) indicated a much higher ratio of acyclics and monocyclics to bicyclics with the (3S)-linalyl precursor than with the natural substrate. Neryl pyrophosphate and (3R)-linalyl pyrophosphate were relatively inefficient substrates for bicyclic olefin synthesis by cyclase II (Table I).

Product stereochemistry was again determined following preparative incubations with each labeled substrate and TLC
isolation of each olefin after dilution with racemic carrier. α-Pinene was stereospecifically isomerized to α-pinene and chromatographically resolved as the ketals of isopinocamphone as before. Geranyl pyrophosphate and (3S)-linalyl pyrophosphate were shown by such analysis to give rise to essentially only (−)-β-pinene (Fig. 2, i and f). The small amount of (+)-β-pinene detected with geranyl pyrophosphate as substrate was verified in several independent experiments and lead to a search for this metabolite in sage leaf oil. From 0–8% of this previously undetected isomer was observed in the course of numerous chromatographic resolution analyses of the β-pinene isolated from the leaf oil of plants drawn from several seed sources and grown under a variety of environmental conditions. The origin of this unusual minor metabolite (most naturally occurring β-pinene is the (−)-(1S,5S)-isomer (23)) is presently uncertain. Although neryl pyrophosphate and (3R)-linalyl pyrophosphate were rather poor precursors of β-pinene, sufficient sample was accumulated for chromatographic resolution to demonstrate that the neryl substrate yielded (−)-β-pinene and that the (3R)-linalyl substrate yielded the antipodal (+)-isomer (Fig. 2, j and k). In the case of α-pinene generated from the various acyclic precursors with cyclase II, chromatographic resolution of the derived isopinocamphone ketals showed (data not illustrated) that geranyl, neryl, and (3S)-linalyl pyrophosphates gave rise to only (−)-α-pinene, whereas (3R)-linalyl pyrophosphate yielded readily detectable levels of (+)-α-pinene as the singular product. Camphene produced by cyclase II was chromatographically resolved as the ketal of camphenilone as before, and geranyl, neryl, and (3S)-linalyl pyrophosphates were shown to produce exclusively (−)-camphene (Fig. 2, m, n, and p). Sufficient product derived from (3R)-linalyl pyrophosphate was accumulated to show that the only detectable product from the unnatural enantiomer was (+)-camphene (Fig. 2, o).

The results of this investigation have confirmed the configuration of the respective cyclizing linalyl pyrophosphate intermediates which were predicted on the basis of the absolute configuration of the (+)- and (−)-olefin products and the assumption of an anti-endo transition state (4–7) as illustrated in Scheme I (i.e. (3R)-linalyl pyrophosphate gives rise to (+)-olefins; (3S)-linalyl pyrophosphate gives rise to (−)-olefins). These results are in full agreement with related studies on the stereochemistry of cyclization to bornane and fenchane monoterpenes (8–10) and are consistent with the observed anti-stereochemistry of similar ring-generating allylic displacements in the sesquiterpene and diterpene series (24–27). The preference for the indicated linalyl pyrophosphate enantiomer among the various acyclic precursors of these enzymatic bicyclizations is generally clear from comparison of either $V_{rel}$ or the catalytic efficiency parameter ($V_{rel}/K_{cat}$) (Table I), with camphene from cyclase I being the notable exception. In the cases examined here, the cyclization of the appropriate enantiomer of linalyl pyrophosphate is α-pinene (left) and (−)- and (+)-camphene (right). The upper panels indicate radioactivity recorded by the monitor attached to the chromatograph and represent the derivatives of α-pinene, β-pinene, and camphene obtained from cyclase I and cyclase II with various acyclic precursors as coded in the legend to Fig. 1 and described below.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Cyclase I</th>
<th>Cyclase II</th>
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approximately four times faster than the coupled isomerization-cyclization of geranyl pyrophosphate to the same product. These, and similar results with other cyclases (9, 10), have been taken as a rough indication of the relative rates of these normally coupled reaction steps. In all cases thus far examined, with the exception of camphene biosynthesis by cyclase I, the rate of cyclization of the normally cryptic linalyl intermediate is considerably faster than the corresponding isomerization-cyclization of the natural geranyl precursor.

Although both cyclase I and cyclase II were confirmed to carry out the stereospecific bicyclization of the achiral substrates, geranyl and neryl pyrophosphates, to the respective (+)- or (-)-olefin series, neither enzyme was enantiospecific in utilizing (3R)- or (3S)-linalyl pyrophosphate since the rate of cyclization of the stereochromically inappropriate linalyl enantiomer (while generally slow) was readily detected with the dual-labeled and optically pure substrate. The anomalous, yet stereospecific, cyclizations of the unnatural linalyl intermediates to the antipodal olefins from the pinene cyclases from sage are very unusual, but preceded by two other examples. The (+)-endo-fenchol cyclase from Foeniculum vulgare catalyzes the specific conversion of geranyl, neryl, and (3R)-linalyl pyrophosphate to (+)-endo-fenchol, as well as, at a slower rate, the transformation of (3S)-linalyl pyrophosphate to (+)-endo-fenchol and related cyclo products (9). And the (+)-bornyl pyrophosphate cyclase from sage catalyzes the conversion of geranyl, neryl, and (3R)-linalyl pyrophosphate to (+)-bornyl pyrophosphate exclusively, yet is also capable of transforming (3S)-linalyl pyrophosphate to (+)-bornyl pyrophosphate (10). The olefin cyclases from sage, in fact, exhibit a higher degree of enantioselectivity do either of the above examples. Of the limited number of monoterpene cyclases examined thus far with regard to enantiomer preference, only the (+)-bornyl pyrophosphate cyclase from Tanacetum vulgare is enantiospecific with regard to linalyl pyrophosphate (10), suggesting that the lack of complete selectivity, along with the ability to catalyze the antipodal cyclization, may be a common, albeit unusual, property of this enzyme type.

That the cyclases are capable of ionizing the unnatural enantiomer of linalyl pyrophosphate is not in itself surprising since even the disfavored substrate neryl pyrophosphate gives rise to product, and both the (+)-pinene cyclase and the (+)-bornyl pyrophosphate cyclase have been shown to catalyze the solvolysis of a variety of allyl pyrophosphates (20, 21). That stereochemical control of bicyclic product formation is maintained in the aberrant cyclizations of precursors unlikely to be encountered in vivo, however, most unusual, yet still entirely consistent with the general stereochemical model for the electrophilic cyclization of geranyl pyrophosphate, via syn-isomerization to linalyl pyrophosphate in the cisoid, anti-endo form, to the cyclohexanoid family of monoterpenes. The remarkable capability of catalyzing the antipodal cyclization of the unnatural isomer rests upon the inability of the cyclase to distinguish between the linalyl enantiomers, which are effectively isosteric when folded in the anti-endo conformation (10), and upon the ability to accommodate the mirror image transformations, which are in fact remarkably similar in geometry when viewed along the accidental mirror plane defined by C-2, C-3, C-7, and C-8 of these substrates. These concepts have been more fully developed previously in the context of borneane and fenchane cyclizations (5, 7, 9, 10). It must be borne in mind that such anomalous antipodal cyclizations are inefficient, and in a real sense artificial, since linalyl pyrophosphate has never been observed as a free intermediate in the cyclization of geranyl pyrophosphate (6, 7, 12); nor is free linalyl pyrophosphate presumed to be generated in sage by any other means. The cyclases therefore encounter linalyl pyrophosphate only as a consequence of the binding and isomerization of geranyl pyrophosphate. As this process is tightly coupled and the intermediate generated is not free to equilibrate with the medium in the time frame of the reaction, the response to the individual enantiomers of linalyl pyrophosphate in solution is enzymatically irrelevant, except as a probe of the rate and stereochemistry of cyclization when effectively decoupled from the required, but normally cryptic, isomerization step.

The general picture which emerges from these and related studies is one in which the isomerization-cyclization of geranyl pyrophosphate is stereospecific (the enantiomer formed depending on the initial, chiral folding of the precursor), as are the respective cyclizations of the linalyl pyrophosphate enantiomers. With the achiral substrate, stereospecificity is induced at the ionization-isomerization step in that once the chirality of the bound tertiary intermediate is established from the appropriate helical conformer of the geranyl substrate, the stereochemical outcome of the subsequent cyclizations is fixed and thereafter determined relative to the absolute configuration at the C-3 tertiary center. Thus, although linalyl pyrophosphate is the first explicitly chiral intermediate of the reaction scheme, the overall stereochemical outcome is determined by the helical conformation of geranyl pyrophosphate achieved on initial binding. If only a single enantiomeric conformation of geranyl pyrophosphate can be bound and isomerized by the cyclase, then the absolute stereochemical control of the reaction leading from this substrate is a natural consequence. That exogenous linalyl pyrophosphate does not produce a product set identical in composition to that produced by geranyl pyrophosphate with the pinene cyclases indicates that the linalyl precursor does not mimic exactly the bound intermediate that arises from isomerization of geranyl pyrophosphate, and this observation suggests that exogenous linalyl pyrophosphate is bound and ionized in conformations in addition to the cisoid, anti-endo form which is presumed to give rise to bicyclic olefins. To address this specific point, and to examine the origin of acyclic and monocyclic olefins in general, will require the stereochemical resolution of the limonene product derived from each acyclic precursor. This work is underway.

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REFERENCES
Stereochemistry of Cyclization to Monoterpene Olefins

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Supplemental Material To:

BIOSYNTHESIS OF MONOTERPENES.

Stereospecificity and Stereoselectivity in the Enzymic Cyclization of (+)- and (-) Linalyl Pyrophosphate to (+)- and (-)-Pinenes and (+)- and (-)-Caryophyllene

by

University of California

by

David E. Cane and Rodney C. Chang

EXPERIMENTAL PROCEDURES

Plant Material, Substrates and Standards - Sagu maclea officinalis (L.) plants were grown from seed under standard conditions (18), and rapidly expanding leaves of immature plants (3-6 weeks post germination) were used in all experiments. The preparation and purification of each of the following substrates have been described: (+)-[2H6]linalyl pyrophosphate (10.1 Ci/mol) (6); [1-3H]linalyl pyrophosphate (40.6 Ci/mol) (26); [1-14C]linalyl pyrophosphate (0.92 Ci/mol) (11). The following substrates, all prepared in our laboratory, were obtained from suppliers described previously (25): (+)-3-11111inalyl pyrophosphate (86 Ci/mol) (29, 38); [2-14C]linalyl pyrophosphate (90 Ci/mol) (9); [1-14C]linalyl pyrophosphate (3.3 Ci/mol) (8); [2-1H]linalyl pyrophosphate (66 Ci/mol) (29); [9-1H]linalyl pyrophosphate (14 Ci/mol) (31); (E)-[3H]-linalyl pyrophosphate (78 Ci/mol) (92); and (Z)-[1-3H]linalyl pyrophosphate (16 Ci/mol) (16).

(++)-Pinenes was prepared from (-)-pinenes by hydroboration with 9-boradicyclo[3.3.1]nonane (9-BBN, Aldrich) by a modification of the procedure of Maland and associates (33), and dehydroboration of the 3-3-hydrate=3-3-hexene adduct. (---)-Pinenes (25 mg/ml) and 55 ml of 0.3% N-300 in TSP were refluxed under a nitrogen atmosphere for 1 h. The TSP was removed under vacuum, 40 ml of ethylene glycol added to the flask, and reflux continued for 30 h. Upon cooling to 30°C, 30% acetic acid was added, and the mixture was stirred at 40-50°C for 2 h. The cooled mixture was extracted with pentane (3 x 45 ml) following the addition of water, and the extract was washed with brine and then dried over anhydrous MgSO4. Removal of solvent under reduced pressure afforded a 45% yield of (++)-pinenes, which was purified from residual 3-hydroxy-pinenes by TLC as needed. Other monoterpene olefins and sesquiterpenes were prepared as described previously (11, 28), or were obtained from Alltech, Fluka, Pfaltz and Bauer or I.C.R.

Enzyme Preparation - (+)-Pinenes cyclase (cyclase I) and (-)-linalyl pyrophosphate cyclase (cyclase I) have been previously isolated from sage leaves, partially purified and characterized (26, 27), and the published isolation procedure (21) was employed to provide the 14.000g supernatant used as the enzyme source (23). The preparation was concentrated to 3.5 ml by ultrafiltration (Amicon M-100) and applied to a 2.5 x 320 cm Sepharose 6-200 column previously equilibrated and eluted with 50 ml H2O (15).

morpholino-salmine sulfonic acid)-3 5 potassium phosphate buffer, pH 6.5, containing 0.5 M dichloroacetic acid, 15 mM MgCl2, and 10% (v/v) glycerol. This procedure provided complete separation of cyclase I (M, = 100,000) from cyclase II (M, = 55,000) and removed most of the phosphohydrolase activity from cyclase I. More than 80% of this competing activity from cyclase II. Early attempts to separate larger-scale preparations (+) 15 g tissues) by this technique, using a buffer system of lower tonicity (200 M KOH - 1 M phosphate), gave cyclase I contaminated with variable amounts of cyclase I activity (5-12%), and so this procedure was abandoned. Cyclases I and II were located by a standard assay for the formation of monoterpene olefins (see below) and the appropriate column fractions containing each cyclase were separately pooled, concentrated by ultrafiltration, and dialyzed to assay conditions. intervening column fractions, which were subsequently shown to contain primarily a distinct (+)-l-homom cycle activity (32), were discarded.

Enzyme Assays - The assay for cyclase I was run in an 1 ml of 20 mM MES - 5 mM potassium phosphate buffer, pH 6.5, containing 0.5 M dichloroacetic acid, 15 mM MgCl2, and generally, 10% (v/v) glycerol. The buffer system was the same for the assay of cyclase II except that the pH was adjusted to the optimum of 12.1. The assay was initiated by the addition of the allylic pyrophosphate substrate and the mixture in a sealed tube were incubated with gentle swirling for 1 h at 30°C. For estimating kinetic constants, assays were run under linear conditions in duplicate over a substrate concentration range of 0.3 to 40 M. Preparative scale incubations were run with 1 ml of the enzyme preparation and saturating substrate concentrations, and the assay time was extended to 3 h. Inhibitors (fluoro, vanadate, and molybdate) were not employed to suppress phosphatase activity, since the levels required to inhibit the relatively non-selective phosphatases (9, 33) also inhibited the cyclases. Following incubation in all cases, 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 (Mallinkrodt type 60A) overfilled with anhydrous MgSO4. Extraction and elution were repeated with an additional 2 ml pentane to achieve a dried eluant containing only hydrocarbons, free of oxygenated terpenes which either remained in the original solution or reformed in the pentane layer.
were crystallized following acidification. The combined organic phases were dried with 

\[ \text{camphene} \] in pentane, and the filtrate was evaporated to dryness. The resulting ketones were purified by TLC and analyzed directly by radio-GC. Controls run with optically pure standards and racemic standards confirmed that each reaction sequence proceeded without racemization and without a significant degree of enantioselectivity.

Analytical Procedures - TLC was done on silica gel 60 plates each of which was covered with 20% by weight AgNO₃. The products were dissolved in 1% aq. AgNO₃ and were spotted onto the silica gel plate. The plates were developed with diethyl ether and chromatography was accomplished by using helium as the carrier gas. The radioactivity was monitored by a Packard 3320A dual-wavelength detector using a carbon sample as the reference gas. The radioactivity was determined with a Packard 4650 liquid scintillation spectrometer in a counting solution of 20% ethanol in toluene containing 0.1% (w/v) Omnifluor (New England Nuclear) efficiency for 74° = 77%, efficiency for 34° = 85%. All samples were counted to a standard error of 2%.

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Products isolated from the hydrocarbon mixtures by argentation-TLC or silica gel containing 11% AgNO₃ with 1% aq. AgNO₃ as a developing solvent. For olefins generated from "[..."