Biosynthesis of Monoterpenes

STEREOCHEMICAL IMPLICATIONS OF ACYCLIC AND MONOCYCLIC OLEFIN FORMATION BY (+)- AND (-)-PINENE CYCLASES FROM SAGE

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(+)-Pinene cyclase from sage (Salvia officinalis) catalyzes the isomerization and cyclization of geranyl pyrophosphate to (+)-α-pinene and (+)-camphene, and to lesser amounts of (+)-limonene, myrcene, and terpinolene, whereas (-)-pinene cyclase from this tissue catalyzes the conversion of the acyclic precursor to (-)-α-pinene, (-)-β-pinene, and (-)-camphene, and to lesser quantities of (-)-limonene, myrcene, and terpinolene. The bicyclic products of these enzymes (pinene and camphene) are derived via the cyclization of the cisoid,anti-end0-conformers of the bound, tertiary allylic intermediates (3R)-linalyl pyrophosphate (+)-pinene cyclase) and (3S)-linalyl pyrophosphate (-)-pinene cyclase). When challenged with either enantiomer of linalyl pyrophosphate or with neryl pyrophosphate (cis-isomer of geranyl pyrophosphate) as substrate, both pinene cyclases synthesize disproportionately high levels of acyclic olefins (myrcene and ocimene) and monocyclic olefins (limonene and terpinolene), compared with the product mixtures generated from the natural geranyl precursor. Resolution of the limonene derived from linalyl pyrophosphate and neryl pyrophosphate demonstrated that this monocyclic olefin was formed via conformational foldings in addition to the cisoid,anti-end0-pattern. These results indicate that the alternate substrates are ionized by the cyclases prior to their achieving the optimum orientation for bicyclization. In the case of geranyl pyrophosphate, a preassociation mechanism is suggested in which optimum folding of the terpenyl chain precedes the initial ionization step.

Geranyl pyrophosphate:pinene cyclase I (M₀ ~ 96,000) from common sage (Salvia officinalis) catalyzes the conversion of geranyl pyrophosphate (1) to (+)-α-pinene ((+)-10) and to smaller quantities of (+)-camphene ((+)-13), (+)-limonene ((+)-6), myrcene (5), and terpinolene (7), whereas geranyl pyrophosphate:pinene cyclase II (M₀ ~ 55,000) from this species transforms the acyclic precursor to (-)-α-pinene ((-)-10) and (-)-β-pinene ((-)-11), as well as to lesser quantities of (-)-camphene ((-)-13), (-)-limonene ((-)-6), myrcene (5), and terpinolene (7). Co-purification of the activities and differential inhibition and inactivation studies (1), as well as investigation of deuterium isotope effects on these enzymatic cyclizations (2), 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. As a class, the terpenoid cyclases are unusual in their collective ability to synthesize antipodal products from geranyl pyrophosphate, and they are perhaps unique in the ability of individual cyclases to synthesize multiple products from a single precursor.

A general stereochemical model for the coupled isomerization and subsequent cyclization of geranyl pyrophosphate to bicyclic monoterpenes has been proposed (3). 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 stereochemical impediment to the cyclization of geranyl pyrophosphate, while subsequent ionization of this enzyme-bound tertiary allylic isomer allows C1-C6 cyclization of the anti-end0-conformer to 14 (3-5) or, alternatively, deprotonation of the resulting ion-pair (4) to the acyclic olefin myrcene (5). Myrcene might also be generated directly from 2. Two modes of proton loss from the monocyclic α-terpinyl cation (14) may similarly afford limonene (6) or terpinolene (7), whereas a second electrophilic cyclization leads to the bicyclic pinyl (8) or bornyl (9) cations. The pinyl cation (8) may undergo deprotonation of the adjacent ring methylene to yield α-pinene (10). In the case of pinene cyclase II, alternative deprotonation from the adjacent methyl is allowed to permit 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 (cycIase I) and (-)-pinene cyclase (cycIase II) are described by exactly mirror image pathways, with the exception that β-pinene ((-)-11) is produced only by the (-)-pinene cyclase.

A central feature of the stereochemical model is the predicted intermediacy of (-)-(3R)-linalyl pyrophosphate 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. Since the pinene cyclases, like other monoterpene cyclases, can utilize linalyl pyrophosphate (and neryl pyrophosphate (16), the cis-isomer of the geranyl substrate) as alternate precursors at the same active site (3, 6), these stereochemical predictions were tested by examining the conversion of [(3R)-8,9-14C,(3RS)-1E,3H]linalyl pyrophosphate to the configurationally related monoterpene olefin sets produced by (+)-pinene cyclase and (-)-pinene cyclase, and by directly evalu-

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the sole chiral product of the "abnormal" terpene mixtures. This novel possibility could not be adequately addressed until the stereochemical model. Moreover, when the cyclases were challenged with the individual linalyl enantiomers or with neryl pyrophosphate, abnormally high proportions of these acyclic and monocyclic olefins were produced compared to the product set characteristic of this species that is generated from the natural substrate geranyl pyrophosphate (1, 6, 7).

The alterations in product distribution that result with the different precursors were suggested to arise via conformational differences of the olefinic domains of the various substrates achieved on binding prior to ionization (7). However, this novel possibility could not be adequately addressed until the olefinic product of the "abnormal" terpene mixtures (limonene) could be resolved at the radiochemical scale. A technique for this purpose was recently developed, which depends upon the stereospecific conversion of limonene to carvone and radio-GLC of diasteromeric ketals derived from (-)-(2R,3R)-butanediol (8). In this paper we describe the use of this method to address the stereochemical implications of acyclic and monocyclic olefin formation by the pinene cyclases from sage. From the outcome of the aberrant reactions of neryl and linalyl pyrophosphate, an important mechanistic feature of the normal isomerization-cyclization of geranyl pyrophosphate is revealed.

The abbreviations used are: GLC, gas-liquid chromatography; MES, 4-morpholineethanesulfonic acid.

### EXPERIMENTAL PROCEDURES

**RESULTS**

Conversion of Acyclic Precursors to Monoterpene Olefins—initial experiments were directed toward examining the conversion of 1-3H-labeled geranyl, neryl, (3R)-linalyl, and (3S)-linalyl pyrophosphates to the related monoterpene olefin sets produced by (+)-pinene cyclase (cyclase I) and (-)-pinene cyclase (cyclase II). The response of each partially purified enzyme to increasing concentrations of each substrate was determined under linear assay conditions in the presence of 15 mM MgCl₂. In each case, product formation was evaluated by a combination of aliquot counting of the olefin fraction prepared by column chromatography and separation of individual olefins by radio-GLC (H detection) following the addition of appropriate racemic carrier standards. The generation of each olefin could be monitored in this way, and it was shown that the product distributions (following minor correction for nonenzymatic background) did not detectably vary for a given substrate as a function of concentration. Product distributions observed at saturating concentrations of each substrate are provided in Table I. Double-reciprocal plots for the production of each olefin from each acyclic precursor by each cyclase were linear, from which the corresponding \( K_m \) values were determined and shown to be the same, within experimental error, for all olefins with a given precursor-cyclase pair (Table I). It is interesting to note that the relative binding affinities of the four acyclic precursors are quite similar, all of the \( K_m \) values differ by less than a factor of three for (+)-pinene cyclase and by less than a factor of two for (-)-pinene cyclase.

In comparing product formation at saturating concentrations of each substrate (i.e., a measure of relative velocities) with each cyclase (Table I), it is clear that the olefin distributions derived from neryl and linalyl pyrophosphate differ markedly from that generated by the natural substrate, geranyl pyrophosphate, which affords an olefin product distribution typical of that found in sage oil (1, 6). Most notable is the abundant production of acyclic and monocyclic olefins relative to bicyclics from neryl and linalyl pyrophosphate, and the formation of cis- and trans-ocimenes (cis- and trans-15) from both linalyl enantiomers but from neither the neryl nor geranyl precursor. The favorable production of acyclics and monocyclics from the neryl and linalyl precursors is suggestive of the solvolytic decomposition of these compounds (9-12); yet, control experiments eliminated the possibility of any significant contribution from nonenzymatic processes to product formation.

The inefficient conversion of neryl pyrophosphae (16) to bicyclic products, such as \( \alpha \)-pinene and camphene, and the predominant formation of limonene, has been observed previously with these and other cyclases (6, 13). Geranyl pyrophosphate has also been previously compared to (3RS)-[1-3H]linalyl pyrophosphate and to [3R]-15C, [3S]-15-E-[3H]-linalyl pyrophosphate as precursors of olefins with these enzymes (6, 7). Although these earlier results were not interpretable at the time, the unusual product distribution generated from the racemate (6), and the limited enantiospecificity observed in the formation of monocyclic and acyclic olefins from the double-labeled precursor (7), can now be rationalized on the basis of the kinetic parameters determined for the individual linalyl antipodes. The utilization of both linalyl...
enantiomers for the synthesis of acyclics and monocycles by both pinene cyclases is unusual but not surprising, since these enzymes, and other monoterpene cyclases, are not enantiospecific with regard to the utilization of the linalyl antipodes in the formation of bicyclic products (7, 9, 10). However, such enzymatic bicyclizations of the linalyl enantiomers are in all cases completely stereospecific, although the efficiencies of utilization of the two enantiomers differ markedly depending on the individual cyclase, with the “unnatural” enantiomer being of lower overall efficiency (7, 13).

**Resolution of Biosynthetic Limonene**—Preliminary observations of the aberrant enzymatic formation of acyclic and monocyclic olefins from the neryl and the antipodal linalyl precursors prompted us to suggest that these unusual results were due to differences in positioning effects between the pyrophosphate ester function and the remaining terpenyl chain that were achieved on binding of these alternate substrates to the cyclase active site prior to ionization (7). To approach such conformational questions required the resolution of the enzymatically generated limonene, the only chiral olefin of the “abnormal” product set; such a technique was recently developed (8).

To examine the stereochemistry of the limonene derived from the various acyclic precursors, large scale incubations with each pinene cyclase were carried out at saturating concentrations of each substrate. The $^3$H-labeled olefin fractions thus obtained were diluted with racemic carriers, the ($\pm$)-limonene from each was isolated by argentation TLC, and the radiochemical purity of this product was verified to be $>97\%$ in each case by radio-GLC. The amount of $^3$H-limonene obtained depended on the precursor and enzyme combination, and ranged from 0.064 pCi in the case of geranyl pyrophosphate with ($\pm$)-pinene cyclase, to 0.54 pCi in the case of (3S)-linalyl pyrophosphate with this enzyme. Limonene was stereospecifically converted to carvone, via the nitrosochloride, and thence transformed to a mixture of diastereomeric ketals with (–)-(R)-limonene (–)-(R)-carvone ketal) and (–)-(S)-limonene ((+)-(S)-carvone ketal) were chromatographically well resolved, and the elution order and stereospecificity of the chemical reaction sequence were confirmed by control studies with optically pure starting materials (Fig. 1). By this analysis it was shown that (+)-pinene cyclase with geranyl pyrophosphate gave rise almost exclusively to (+)-limonene (Fig. 1a), confirming previous results obtained by more tedious radiochemical fractional crystallization techniques (1). Similarly, the (–)-pinene cyclase yielded essentially only (–)-limonene (96%) when incubated with geranyl pyrophosphate (Fig. 1f), confirming earlier results with this enzyme (1).

In the case of (+)-pinene cyclase with the (3R)-linalyl precursor, the predominant product was (+)-limonene as expected from the normal cisoid,anti-endo-cyclization of this enantiomer; however, about 13% of the product was (–)-limonene (Fig. 1b), which was greatly in excess of the amount that might be expected from antipodal cyclization of the few percent of (3S)-linalyl pyrophosphate containing the (3R)-substrate (7). With the (–)-pinene cyclase, a very similar result was obtained (i.e. at least 15% of the product was (–)-limonene (Fig. 1g) that could not arise from the anti-endo-conformation of the (3R)-precursor leading to bicyclic products. With (3S)-linalyl pyrophosphate as substrate, the (+)-pinene cyclase generated primarily (–)-limonene (Fig. 1c). Since the enzyme is capable of the relatively inefficient anti-endo-cyclization of this precursor to optically pure bicyclic products of the same configuration at C4 (e.g. (–)-a-pinene and (–)- camphene (7)), this observation was not surprising; yet, here again, over 10% of the monocyclic product was the opposite (+)-a-antipode. With the (–)-pinene cyclase, this unusual effect was even more pronounced, in that nearly 40% of the limonene product was the (–)-isomer (Fig. 1h). (It must be re-emphasized that this enzyme specifically converts (3S)- linalyl pyrophosphate to bicyclic olefins related to (–)-limonene (i.e. to optically pure (–)-a-pinene, (–)-8-pinene, and (–)-camphene (7)).)

Although neryl pyrophosphate (16) was a relatively poor

### Table I

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>GPP</th>
<th>NPP</th>
<th>(3R)-LPP</th>
<th>(3S)-LPP</th>
</tr>
</thead>
<tbody>
<tr>
<td>(+)-Pinene cyclase (I)</td>
<td>[2.1 ± 0.2 μM]</td>
<td>[6.0 ± 0.5 μM]</td>
<td>[4.0 ± 0.4 μM]</td>
<td>[4.9 ± 0.5 μM]</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>49</td>
<td>17</td>
<td>187</td>
<td>17</td>
</tr>
<tr>
<td>Camphene</td>
<td>30</td>
<td>11</td>
<td>20</td>
<td>16</td>
</tr>
<tr>
<td>Linalene</td>
<td>10</td>
<td>75</td>
<td>32</td>
<td>62</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>5</td>
<td>10</td>
<td>21</td>
<td>51</td>
</tr>
<tr>
<td>Myrcene</td>
<td>6</td>
<td>7</td>
<td>31</td>
<td>21</td>
</tr>
<tr>
<td>Ocimene (cis)</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>Ocimene (trans)</td>
<td>ND</td>
<td>ND</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Total olefins</td>
<td>100</td>
<td>120</td>
<td>305</td>
<td>182</td>
</tr>
<tr>
<td>(+)-Pinene cyclase (II)</td>
<td>[3.0 ± 0.3 μM]</td>
<td>[2.1 ± 0.5 μM]</td>
<td>[3.5 ± 0.5 μM]</td>
<td>[3.5 ± 0.2 μM]</td>
</tr>
<tr>
<td>α-Pinene</td>
<td>28</td>
<td>6</td>
<td>17</td>
<td>122</td>
</tr>
<tr>
<td>β-Pinene</td>
<td>35</td>
<td>6</td>
<td>9</td>
<td>147</td>
</tr>
<tr>
<td>Camphene</td>
<td>24</td>
<td>4</td>
<td>3</td>
<td>97</td>
</tr>
<tr>
<td>Linalene</td>
<td>5</td>
<td>13</td>
<td>28</td>
<td>90</td>
</tr>
<tr>
<td>Terpinolene</td>
<td>2</td>
<td>3</td>
<td>9</td>
<td>49</td>
</tr>
<tr>
<td>Myrcene</td>
<td>6</td>
<td>3</td>
<td>23</td>
<td>188</td>
</tr>
<tr>
<td>Ocimene (cis)</td>
<td>ND</td>
<td>ND</td>
<td>3</td>
<td>26</td>
</tr>
<tr>
<td>Ocimene (trans)</td>
<td>ND</td>
<td>ND</td>
<td>13</td>
<td>72</td>
</tr>
<tr>
<td>Total olefins</td>
<td>100</td>
<td>35</td>
<td>105</td>
<td>791</td>
</tr>
</tbody>
</table>
Acyclic and Monocyclic Olefin Formation by Pinene Cyclases

FIG. 1. Radio gas-liquid chromatographic separation of the diastereomeric (2R,3R)-butanediol ketals of carvone derived from biosynthetic limonene. The isolation of limonene, the preparation of the ketals, and the conditions for the analysis are provided under "Experimental Procedures." The smooth separation of the ketals, and the conditions for the analysis are provided (Scheme I). The isolation of limonene, the preparation of the ketals, and the conditions for the analysis are provided (Scheme I).

Substrate compared with geranyl pyrophosphate for the production of optically pure bicyclic olefins by the pinene cyclases (7), this precursor was efficiently converted to limonene (Table I). Preparative scale incubations provided a minimum of 0.25 μCi of purified limonene in each case, and the product was resolved as the diastereomeric ketals derived from (−)- and (+)-limonene. The upper traces indicate radioactivity recorded by the monitor attached to the chromatograph and represent the derivatives of limonene obtained from cyclase I with geranyl pyrophosphate (a), (3R)-linalyl pyrophosphate (b), (3S)-linalyl pyrophosphate (c), and neryl pyrophosphate (d), and from cyclase II with geranyl pyrophosphate (e), (3R)-linalyl pyrophosphate (f), (3S)-linalyl pyrophosphate (g), (3S)-linalyl pyrophosphate (h), and neryl pyrophosphate (i).

DISCUSSION

In explaining these stereochemical results, as well as the anomalously high levels of acyclic and monocyclic olefins generated by the pinene cyclases from linalyl and neryl pyrophosphates, it seems safe to assume that the pyrophosphate moiety is bound in a similar orientation, regardless of the acyclic precursor, since this functional grouping is the primary determinant of substrate binding (15, 16). It follows that the alterations in product distribution that result with the different precursors are due to differences in the positioning of the remaining chain relative to the fixed pyrophosphate, and probably reflect a lower probability of achieving "optimum" conformation (for bicyclization) of the olefinic domains of the neryl and linalyl substrates prior to ionization. It should be noted that the transformation of geranyl pyrophosphate, for stereochemical reasons, requires a relatively slow ionization-isomerization sequence (3, 7, 17) before cyclization can occur, whereas both neryl and linalyl precursors can cyclize directly with the possibility of π participation by the remote double bond in the initial pyrophosphate displacement (18–21).

These significant stereochemical differences could, in part, account for temporal differences between the geranyl and the neryl and linalyl precursors in achieving optimal orientation (i.e., π overlap, backside shielding, etc. (15)) before initial ionization occurs. It also seems necessary to assume that all cyclization processes occur by anti-allylic displacement based on chemical model studies (4), and because this appears to be imperative for this enzymatic reaction type in the monoterpene (22-24) and higher terpene series (25-28); direct syn-cyclizations are obviously precluded for the neryl substrate (29).

In examining the potential for olefin formation from the possible conformational extremes of linalyl pyrophosphate (Scheme II), it is immediately apparent that only acyclic products (myrcene (5), cis- and trans-ocimene (15)) could arise by ionization of transoid orientations which are incapable of cyclization. Both acyclics and monocyclics (limonene (6), terpinolene (7)) could arise from cisoid,exo-conformations which are incapable of bicyclization with these enzymes (the absolute configuration of the relevant bicyclic olefins dictates an endo-intermediate). With the cisoid,exo-conformer of the (3S)-linalyl enantiomer, anti-cyclization will afford (+)-limonene, which is observed as the minor product from this antipode with both cyclases (Fig. 1, c and h). Conversely, anti-cyclization of the cisoid,exo-orientation of the (3R)-linalyl form will yield (−)-limonene which is observed as the minor metabolite from this antipode with both enzymes (Fig. 1, b and g). Acyclics, monocyclics, and bicyclics (pinene and camphene) can be generated from the cisoid,endo-conformers of the linalyl intermediates, and anti-cyclization of the (3S)-isomer has been shown to give rise exclusively to the (+)-

Scheme II
olefin series, whereas anti-cyclization of the (3S)-isomer has been shown to yield exclusively the (--)-olefin series, as expected (Scheme I) (7, 24).

The precise formation of acyclic products from the linalyl precursors must remain conjecture, since there is presently no simple means of probing their conformational origin. Nevertheless, it seems likely that the ocimeses arise from transoid-orientations of the linalyl precursors because the cisoid-substrate, neryl pyrophosphate, does not afford detectable levels of these anomalous products (Table I).

Since the enzymatic reaction products generated from neryl pyrophosphate differ significantly in composition from those produced by the linalyl precursors, it seems likely that olefin formation from the neryl substrate is largely direct and does not involve preliminary isomerization to a linalyl intermediate. The origin of abnormal olefinic products from neryl pyrophosphate therefore appears simpler to rationalize, in that the cisoid-configuration is fixed and, as noted above, syn-displacements are precluded. Thus, cyclase I, on binding the pyrophosphate moiety of neryl pyrophosphate (16) in a manner analogous to that of geranyl pyrophosphate, could produce (+)-limonene (as well as (+)-bicyclic olefins, terpinolene, and myrcene) from the anti-endo-folding, and (-)-limonene (as well as terpinolene and myrcene) from the anti-exo-form (Scheme III). Similarly, cyclase II, on binding the appropriate helical conformer of neryl pyrophosphate, could produce (--)-limonene (as well as (--)-bicyclic olefins, terpinolene, and myrcene) from the anti-endo-conformer, and (+)-limonene (as well as terpinolene and myrcene) from the anti-exo-folding (Scheme III). The extended conformation is thought to contribute to product formation in solvolytic displacements of neryl derivatives (20, 30, 31). Since both cyclases can cyclize both enantiomers of linalyl pyrophosphate to generate the corresponding enantiomeric bicyclic products (7), it is possible that both enzymes could bind and cyclize both enantiomeric helical conformers of neryl pyrophosphate. In view of the fact that each cyclase appears to convert only a single helical conformer of geranyl pyrophosphate (13, 23, 24), and that the hydrophobic/hydrophilic profiles of the two helical foldings of neryl pyrophosphate differ markedly (unlike the linalyl pyrophosphate enantiomers which are nearly isosteric in cisoid, anti-endo-form (5, 13)), this alternative possibility seems unlikely. Anti-exo-cyclization thus remains the more reasonable explanation for the aberrant formation of limonene (and terpinolene) from the neryl substrate.

The general conclusion that conformational differences underlie the origin of the abnormal olefins is strongly supported by the results with (+)- and (--)-limonene, which indicate that substrate (intermediate) orientations other than the cisoid,anti-endo-form (which yields bicycles) are catalytically productive in the formation of monocyclic (and probably acyclic) products by these cyclases. It is also clear from these results, and from earlier work on the origin of bicyclic products from the linalyl enantiomers (7), that exogenous linalyl pyrophosphate does not precisely mimic the linalyl intermediate that arises by the isomerization of geranyl pyrophosphate at the cyclase active site. However, it is important to note that, of the alternate precursors, the product mixture generated from the predicted linalyl intermediate (3S)-isomer for (+)-pinene cyclase, (3R)-isomer for (--)-pinene cyclase) most closely resembles that afforded by the natural substrate geranyl pyrophosphate. As implied above, the reason for the lack of close correspondence in product formation between geranyl pyrophosphate and the appropriate linalyl enantiomer is premature ionization of the tertiary allylic isomer before optimum orientation (for bicyclization) is achieved. The importance of the olefinic domains of geranyl pyrophosphate (particularly the isopropylidene groupings) in orienting the substrate has been described (15). Considering both the present results and previous conclusions regarding the stereochemistry of the bicyclization reactions (7, 13, 14, 22–24), the summation of evidence suggests a preassociation mechanism (32) for this reaction type (3, 5, 15, 26) in which the isopropylidene residue of geranyl pyrophosphate is aligned in endo-fashion prior to ionization. Similar conformational rigidity cannot be enforced in binding at the proximal allylic pyrophosphate moiety, since the movement attending the normal pyrophosphate migration and transoid-to-cisoid rotation must be accommodated. However, fixing the position of the distal C6-C7 double bond relative to the plane of the pyrophosphate migration (and the C2-C3 rotation axis) is sufficient to ensure stereoechemical fidelity in the construction from geranyl pyrophosphate of cyclic products which bear the same configuration at C4 (of the cyclohexenoid ring). The formation of acyclic olefins cannot be similarly constrained by binding conformation because all orientations of the ge-
ranyl and linalyl precursors (Schemes I and II) would appear to allow the formation of these products, and the transoid-conformations would yield exclusively acyclics. In cisoid, anti-conformations, the production of cyclic olefins would thus depend upon the rate of capture of the acyclic cation by the C6-C7 double bond relative to the rate of deprotonation of the isomerization-cyclization reaction. The responses of the cyclases to precursors other than geranyl pyrophosphate are thus of biosynthetic significance only as they reveal mechanistic details of the relative positioning of the chain with respect to active site conformers other than the cisoid, anti-endo-orientation make insignificant contribution to product formation. The relative balance of cyclics to acyclics produced by the other precursors (Table 64:36 for linalyl pyrophosphate with cyclase 15314) have revealed the remarkable versatility of these cyclases in exhibiting little difference in values for the various pyrophosphate ester substrates, in lacking strict anti-isospecificity with regard to the utilization of linalyl anipodes when the cyclization is decoupled from the normal isomerization step, and in yielding what can only be regarded as abnormal acyclic and monocyclic products when presented with the alternate substrates which seemingly are allowed to react in several fundamentally different conformations. However, it should be emphasized that these cyclases do not encounter free neryl and linalyl derivatives in vivo (3), and they engage the latter in bound form only in the course of the reaction itself in which regio- and stereochemistry of product outcome are presumably fixed on initial binding of the natural geranyl substrate from which only enantiomerically pure products are formed. The responses of the cyclases to precursors other than geranyl pyrophosphate are thus of biosynthetic significance only as they reveal mechanistic details of the isomerization-cyclization reaction.

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REFERENCES

EXPERIMENTAL PROCEDURES

Plant Materials, Substrates and Standards - Sage (Salvia officinalis L.) plants were grown from seed under standardized conditions [2,3] and rapidly expanding leaves of mature plants were used for the experiments. Preparation and purification of each of the following substrates has been described: 1) (-)-limonene, phytolacca chia (p.m.) (21); 2) (-)-limonene pyrophosphate (10.5 mg/ml) (16); 3) (-)-limonene phytol (24 mg/ml) (16); and 4) (-)-limonene pyrophosphate (20.5 mg/ml) (14). The (+)-steroidal auxin, 2.5 ml, by ultrafiltration (Amicon PM 30) and applied to a 2.5 x 120 cm Sephacryl S-200 column previously equilibrated and eluted with 50 mM MES (2-<br>morpholinoethanesulfonic acid)-5 mM potassium phosphate buffer, pH 6.5, containing 0.5 mM dithioerythritol, 15 mM MgCl2, and 10% glycerol. This procedure provided complete separation of the two fractions which contained a distinct (±)-stereoisomeric activity. Each purity was determined with a gas chromatograph equipped with a thermal conductivity detector and operated at 230°C with the split ratio 1:10. The elution was monitored with a flame ionization detector at 270°C with the split ratio 1:20. The separation was confirmed by radio-gas chromatography. Precautions were taken to avoid contamination of the reaction mixture by internal standards. The reaction mixture was then adjusted to pH 6.5 with concentrated HCl and extracted with ethyl acetate. The extracts were washed with water, dried over sodium sulfate, and concentrated to dryness in a vacuum. The residue was chromatographed on silica gel (1000 mg) and eluted with ethyl acetate-hexane (9:1) to give the product. The product was then purified by preparative thin-layer chromatography (TLC) on silica gel (Merck silica gel 60 F-254) using the solvent system chloroform-methanol (9:1). The product was then eluted from the column and concentrated to dryness in a vacuum. The residue was then dissolved in a small amount of chloroform and applied to a column of silica gel (1000 mg) using the solvent system chloroform. The product was then eluted from the column and concentrated to dryness in a vacuum. The residue was then dissolved in a small amount of chloroform and applied to a column of silica gel (1000 mg) using the solvent system chloroform.