Pure & Appl. Chem., Vol. 61, No. 3, pp. 493–496, 1989.
Printed in Great Britain.

Stereochemical studies of natural products biosynthesis

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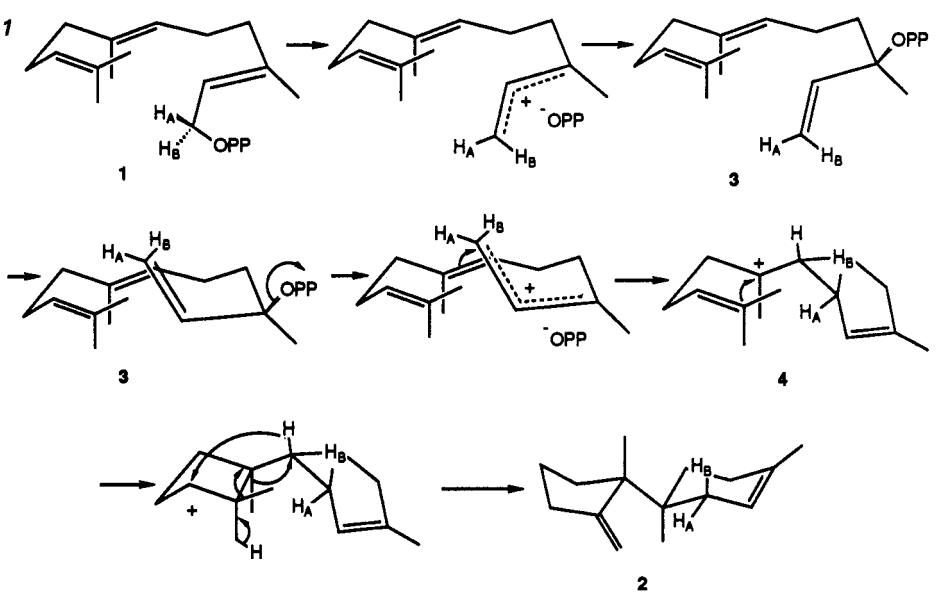
Abstract - Trichodiene synthetase, an enzyme isolated from the fungus *Trichothecium roseum*, catalyzes the cyclization of farnesyl pyrophosphate (**1**) to trichodiene (**2**), the parent hydrocarbon of the trichothecane family of fungal metabolites. Studies of the cyclization support a mechanism whereby the *trans*-primary allylic pyrophosphate (**1**) is initially isomerized to enzyme-bound (3*R*)-nerolidyl pyrophosphate (**3**), the corresponding tertiary allylic isomer, by way of an allylic cation - pyrophosphate anion pair. Rotation about the newly formed 2,3-single bond allows **3** to adopt the *anti-endo* conformation which undergoes cyclization to the bisabolyl cation **4** upon reionization to the *allylic* cation. Further intramolecular electrophilic cyclization followed by a hydride shift, a pair of methyl migrations, and deprotonation generates trichodiene. In related studies of the recently isolated bergamotene synthetase, we have found that a cell-free extract of *Pseudeurotium ovalis* will catalyze the conversion of farnesyl pyrophosphate to β-*trans* -bergamotene (**5**), a cyclization which is proposed to take place through the intermediacy of (3*S*)-nerolidyl pyrophosphate and the corresponding (4*S*)-bisabolyl cation **4**.

The study of terpenoid biosynthesis has increasingly shifted in recent years to investigations of the fascinating enzymes which catalyze the conversion of the universal acyclic precursors farnesyl pyrophosphate (**1**) and geranyl pyrophosphate to sesquiterpenes and monoterpenes, respectively (ref. 1-3). The relative handful of these cyclases which have been investigated, out of what is believed to be a total of some 200, have all proven to be operationally soluble, moderately lipophilic, proteins of molecular weight 40,000-100,000, requiring no cofactors other than a divalent metal, usually Mg^{++} and occasionally Mn^{++}. The focus of most work to date has been on the mechanisms of the cyclization reactions themselves. One of the best studied cyclases is trichodiene synthetase, an enzyme isolated from a variety of fungi which catalyzes the cyclization of *trans,trans*-farnesyl pyrophosphate to trichodiene (**2**), the parent sesquiterpene hydrocarbon of the trichothecane family of fungal metabolites (ref. 4,5). This enzyme has recently been purified to homogeneity from *Fusarium sporotrichioides* by Hohn who has shown that the protein is in fact a dimer composed of two identical subunits M_r 45,000 and established that the K_m for farnesyl pyrophosphate is 25 nM (ref. 6).

Scheme 1

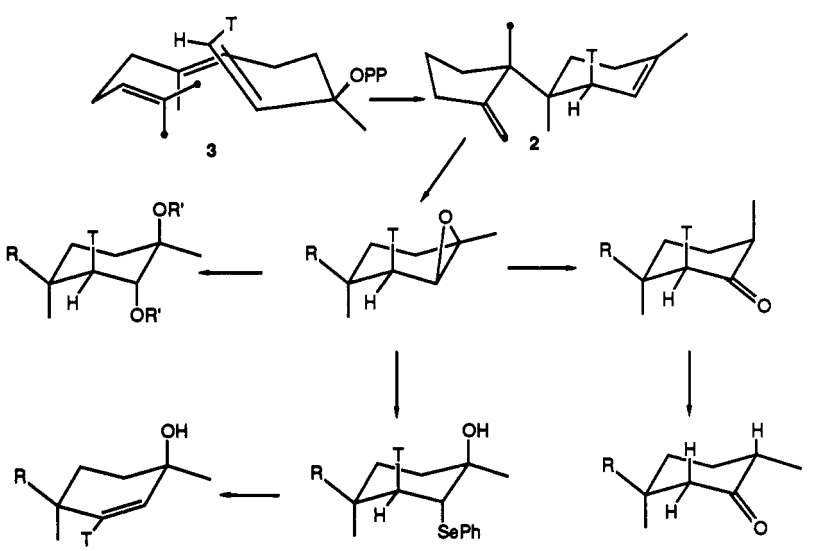


Cyclization of farnesyl pyrophosphate (**1**) to trichodiene (**2**).

In our own laboratory we have shown that trichodiene synthetase, isolated from *Trichothecium roseum*, catalyzes the cyclization of farnesyl pyrophosphate without loss of either of the original hydrogen atoms at C-1 of the substrate (ref. 4) and with net retention of configuration at this center (ref. 5). Any explanation for these results must also account for the isomerization of the *trans*-2,3-double bond of the precursor which is geometrically incapable of cyclizing directly to a six-membered ring product containing a necessarily *cis* double bond. We have proposed a cyclization mechanism, illustrated in Scheme 1, involving initial rearrangement of farnesyl pyrophosphate (**1**) to the correpsonding tertiary allylic isomer, nerolidyl pyrophosphate (**3**). The original geometrical constraint is no longer present in the latter intermediate, which can adopt a conformation capable of further cyclization to the bisabolyl cation (**4**) by rotation about the newly formed 2,3-single bond. A series of well established hydride shifts and methyl migrations can then lead to the formation of the product trichodiene. Although the proposed intermediacy of nerolidyl pyrophosphate is consistent with a considerable body of model reactions (ref. 7), as well as the well established role of the analogous tertiary allylic pyrophosphate, linalyl pyrophosphate, in monoterpene cyclizations (ref. 3), direct evidence implicating **3** in the formation of any cyclized terpene has been lacking.

To establish the viability of nerolidyl pyrophosphate as a substrate for trichodiene synthetase, we prepared (1*Z*)-[1-^{3}H, 12,13-^{14}C]nerolidyl pyrophosphate which was incubated with trichodiene synthetase (ref. 8,9). The resulting trichodiene was subjected to the degradation sequence summarized in Scheme 2 to establish the position and stereochemistry of labeling. The demonstration that the tritium label was confined to the 11β position confirmed that nerolidyl pyrophosphate could serve as a substrate for trichodiene synthetase and established that the cyclization had taken place on the 1-*re* face of the vinyl double bond. The absolute configuration of the nerolidyl pyrophosphate enantiomer which undergoes cyclization was determined by carrying out a competitive incubation of (3*S*)-[1-^{3}H]nerolidyl pyrophosphate and (3*RS*)-[12,13-^{14}C]nerolidyl pyrophosphate. The finding that the resulting trichodiene was labeled exclusively with ^{14}C established that trichodiene synthetase utilizes exclusively (3*R*)-**3**. These results are completely consistent with the proposed anti-endo conformation for the cyclization of nerolidyl pyrophosphate illustrated in Scheme 1. Taken together with the earlier demonstration that cyclization of farnesyl pyrophosphate to trichodiene takes place with net retention of configuration at C-1 of the precursor, it can be inferred that the initial allylic rearrangement of **1** to **3** is a suprafacial process (ref. 10).

Scheme 2



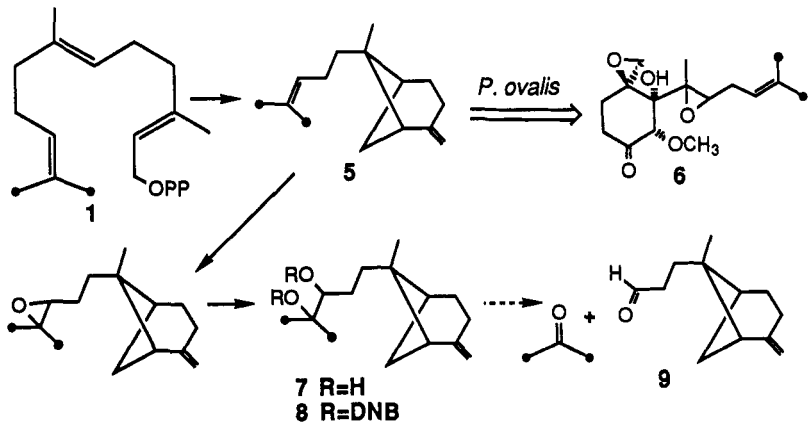
Conversion of (3*R*)-nerolidyl pyrophosphate (3) to trichodiene and degradation.

To demonstrate that nerolidyl pyrophosphate is an *intermediate* of the conversion of farnesyl pyrophosphate to trichodiene, we carried out a competition experiment between [1-^{3}H]-**1** and [12,13-^{14}C]-**3**. Comparison of the ^{3}H/^{14}C ratio of the resulting trichodiene with that of unreacted farnesyl and nerolidyl pyrophosphate established that nerolidyl pyrophospahte is in fact an enzyme-bound intermediate with an apparent V_{max}/K_m 1.5 - 2.0 times the apparent V_{max}/K_m for farnesyl pyrophosphate. The latter experiments ruled out alternative mechanistic explanations involving either release of **3** from the active site or preliminary conversion of nerolidyl pyrophosphate to farnesyl pyrophosphate followed by conversion of **1** to trichodiene by an unspecified mechanism.

From the foregoing experiments, the picture which has emerged is that of trichodiene synthetase as an *isomerase - cyclase* which catalyzes each part reaction by ionization of the substrate to an allylic cation - pyrophosphate anion pair. Thus ionization of farnesyl pyrophosphate generates a transoid ion pair which can only revert to starting

material or collapse to the tertiary allylic isomer, thereby effecting the rearrangement to nerolidyl pyrophosphate. This isomerization necessarily takes place with net suprafacial stereochemistry. Following rotation about the 2,3-single bond of **3** to the anti-endo conformer, a second ionization generates the corresponding cisoid ion pair. In the latter case, the allylic cation is directly juxtaposed over the 6,7-double bond which is therefore ideally positioned to intercept the ion pair by backside attack, resulting in generation of the bisabolyl cation **4**.

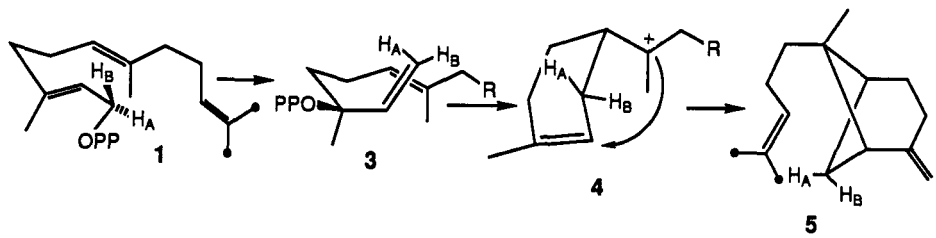
Scheme 3



Conversion of farnesyl pyrophosphate to bergamotene (**5**).

To test the generality of this cyclization mechanism, we have begun to explore a second sesquiterpene cyclase. We have recently found that a cell-free extract of the fungus *Pseudeurotium ovalis* can cyclize farnesyl pyrophosphate to a sesquiterpene hydrocarbon which is chromatographically identical with β-*trans* -bergamotene (**5**). In earlier work we had already isolated **5** from mycelial extracts of *P. ovalis* (ref. 11) and demonstrated that [12,13-^{13}C]-**5** could serve as a precursor of the immunosuppresant antibiotic ovalicin (**6**) (ref. 12). To confirm the specificity of the enzyme reaction we have prepared [12,13-^{14}C]farnesyl pyrophosphate and incubated this substrate with a crude *P. ovalis* extract. The derived labeled bergamotene was diluted with inactive carrier and converted to the corresponding diol (**7**) by selective epoxidation of the side chain and hydrolysis with aqueous perchloric acid. A portion of the diol was converted to the corresponding bis(dinitrobenzoate) (**8**) which was recrystallized to constant activity while the remainer was treated with periodate to generate the inactive trisnoraldehyde **9** and acetone, trapped as the derived semicarbazone and bearing the expected ^{14}C activity. These results are readily interpreted in terms of an isomerization - cylization mechanism, illustrated in Scheme 4, in which farnesyl pyrophosphate (**1**) is initially rearranged to (3*S*)-nerolidyl pyrophosphate (**3**) which undergoes ionization and cyclization to the (4*S*)-bisabolyl cation (**4**), enantiomeric to the corresponding intermediate of trichodiene biosynthesis. Further cyclization of **4** and loss of a methyl proton will generate the characteristic bicyclo[3.1.1]heptane skeleton of β-*trans* -bergamotene. Further work to confirm the proposed intermediacy of nerolidyl pyrophosphate in this cyclization is now in progress.

Scheme 4



Cyclization of farnesyl pyrophosphate to bergamotene (**5**).

Acknowledgement

This work was supported by grants from the National Institutes of Health, GM22172 and GM30301.

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